

Inorganic nitrogen fertilizers induce changes in ammonium assimilation and gas exchange in *Camellia sinensis* L.

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Received: 20.11.2013 • Accepted: 16.07.2014 • Published Online: 02.01.2015 • Printed: 30.01.2015

Abstract: *Camellia sinensis* L. is one of the most economically important crops in the world today. The leaves of this plant are harvested for tea production on a massive scale through a process that requires a large amount of inorganic nitrogen (N). However, knowledge of how inorganic nitrogen regulates the growth physiology of *C. sinensis* L. remains limited. In order to investigate its effects on gas exchange, N assimilation, and the efficiency of N use in *C. sinensis* L., we carried out experiments with 4 cultivars (WENZ, YS, ZN139, and LJ43) under 4 different treatments: 1) a control treatment, 2) a treatment fed only with NO_3^- -N, 3) a treatment fed only with NH_4^+ -N, and 4) a treatment fed with a mixture of NO_3^- -N and NH_4^+ -N in a 1:1 ratio. We found that the application of N, especially in a mixture, encouraged higher foliar N content per unit area (N_f), which in turn promoted glutamine synthetase (GS) activity and glutamate synthase (GOGAT) activity. Our mixture-fed treatment increased N_f , GS, and GOGAT activity by 26.18%, 110.2%, and 92.21%, respectively, for the WENZ cultivar. The mixed-fed treatment also had the highest net photosynthetic (Pn), stomatal conductance (Gs), and transpiration (Tr) rates, while the NH_4^+ -fed treatment had the highest intercellular CO_2 content (Ci). The mixture-fed and NO_3^- -fed applications caused significant increases in photosynthetic N-use efficiency (PNUE), but the NH_4^+ -fed application decreased PNUE and water-use efficiency (WUE). Furthermore, we observed that differences among cultivars for some of these parameters were significant due to different N solutions. We found that the YS and LJ43 cultivars showed no significant response to different N applications (excluding control) in key enzyme activity and Pn, respectively, but this was not the case for most of the other parameters. Our study indicates that the mixture-fed application promotes increased foliar N content and ammonium assimilation, which in turn increases photosynthesis rates, suggesting that the mixed-feeding of plants provides optimal conditions to promote the growth of *C. sinensis* L. for tea cultivation.

Key words: *Camellia sinensis* L., glutamine synthetase/glutamate synthase activity, inorganic nitrogen forms, photosynthesis, photosynthetic N-use efficiency, water-use efficiency

1. Introduction

Nitrogen (N), which is mainly used as nitrate nitrogen (NO_3^- -N) and ammonium nitrogen (NH_4^+ -N) in agriculture, is essential for plant growth and development. Different forms of inorganic N exert various effects on plant growth, physiology, and N-use efficiency. There is an increasing body of evidence to suggest that inorganic N affects ammonium assimilation (Duan et al., 2003), photosynthetic physiology (Cramer and Lewis, 1993; Zhou et al., 2011), plant growth (Alpha et al., 2009; Zhou et al., 2011), root structure (Domenicano, et al. 2011), plant affinity to nitrate or ammonium nitrogen (Du and Peng, 2010), and even N or water use efficiency (Guo et al., 2002; Jampeetong and Brix, 2009). GS and GOGAT enzymes, which are critical for ammonium assimilation

and essential for the successful optimization of inorganic N use (Lea and Mifflin, 1974; Cren and Hirel, 1999), are sensitive to exogenous nutrients (Lam et al., 1996; Oliveira et al., 2002), light (Duan et al., 2003; Liu et al., 2004), and carbohydrates (Cren and Hirel, 1999). Different N forms induce various effects on GS and GOGAT activity in crop roots and leaves (Yamaya, et al., 1995; Hirose et al., 1997; Li et al., 1999; Dong et al., 2006; Li et al., 2006) and, consequently, have been shown to affect and change the assimilating properties of NH_4^+ -N (Kronzucker et al., 1999; Li et al., 2003; Duan et al., 2007). Photosynthetic parameters such as RuBP carboxylase activity, optical electron transfer rate, and stomatal conductance may also be affected by different forms of N application (Cramer and Lewis, 1993; Xiao et al., 2000; Cao et al., 2003; Chen

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et al., 2005; Tabatabaei et al., 2006; Guo et al., 2007). A moderate increase in $\text{NH}_4^+\text{-N}$ levels can be enough to encourage higher overall net photosynthesis of some crops like rice or lettuce (Duan et al., 2007), while using much higher concentrations can have the opposite effect (Guo et al., 2006; Li et al., 2008; Bybordi, 2012). Some studies stated that $\text{NH}_4^+\text{-N}$ may be superior to $\text{NO}_3^-\text{-N}$ (Claussen and Lenz, 1999; Britto and Kronzucker, 2002), while others reported the opposite (Cramer and Lewis, 1993). These differences may be accounted for by certain species-specific criteria: rice and conifer species usually prefer $\text{NH}_4^+\text{-N}$ as a source of nitrogen, even in the presence of relatively high $\text{NO}_3^-\text{-N}$ concentrations (Gerendás et al., 1997; Britto and Kronzucker, 2002), while wheat prefers $\text{NO}_3^-\text{-N}$ to $\text{NH}_4^+\text{-N}$ (Cramer and Lewis, 1993). However, there are few studies that have investigated the effects of different nitrogen forms on woody plants or tea plants.

The tea plant *Camellia sinensis* L. has been widely cultivated and harvested in China, Sri Lanka, Turkey, and other countries for many centuries. Tea has been consumed socially and habitually since 3000 BC as a nonalcoholic beverage (Mondal et al., 2004) that is of benefit to human health (Cabrera et al., 2006). Today China has an annual yield of 1.62×10^6 t of tea (Chen, 2011) and cultivates more than 200 different tea cultivars (Chen, 2011), which makes it the largest consumer of N fertilizer in the world. In some tea plantations the application rate of N fertilizer can be up to 620–2600 kg ha⁻¹ (Watanabe, 1995; Han et al., 2007). However, nitrogen-use efficiency levels are often lower than 30% (Zhang et al., 2005; Han et al., 2007). The excessive use of fertilizer not only increases economic costs but also leads to environmental hazards such as soil acidification, degradation, and eutrophication (Guo et al., 2010). Worse yet is the widespread use of inorganic nitrogen in the form of ammonium for plant cultivation, which can cause more serious soil acidification or even reduce tea quality (Chen, 2011; Liu et al., 2013a). In addition, there are several types of biological growth properties that impact tea cultivar diversification. Various tea cultivars can be used to produce many different types of tea, e.g., green tea, black tea, or oolong tea (Chen, 2011). Therefore, we need to outline a sustainable management program for *C. sinensis* L. cultivation that makes the most efficient use of fertilizer and/or water while reducing the risks of using fertilizers.

In the past few decades, some studies have focused on N-use efficiency and genotypes of *C. sinensis* L., the relationship between N application rates and tea yield, or the effect of N forms on tea growth and any impact on the ability of roots to absorb N (Wang et al., 2005; Du et al., 2008; Du and Peng, 2010). In tea cultivation, ammonium nitrogen fertilizers are still widely used and different cultivars are often planted to produce different types of

tea (Han et al., 2007; Chen, 2011). It is therefore urgent and imperative that we understand the physiological mechanisms of inorganic N fertilization and provide the theoretical basis for tea cultivation. We hypothesized that a moderate mix of ammonium and nitrate nitrogen would encourage better tea growth than simply the addition of N alone. In this study, we attempted to answer the following questions: 1) Do nitrogen applications promote nitrogen assimilation and photosynthesis, and, if so, is the effect on the various cultivars different and what treatment is best? 2) Are the changes in photosynthesis regulated or affected by foliar N content and ammonium assimilation? 3) Do these influence water-use efficiency (WUE) and photosynthetic N-use efficiency (PNUE) in *C. sinensis* L.?

2. Materials and methods

2.1. Plant materials and experimental design

Four tea (*C. sinensis* L.) cultivars (2-year-old seedlings), Wuniuzao (WNZ), Yingshuang (YS), Zhenong139 (ZN139), and Longjing43 (LJ43), were grown in plastic pots (50 cm in height and 35 cm in diameter) for 1 year with 5 kg of soil per pot in a greenhouse at Nanjing Forestry University (32°04'34.53"N, 118°48'42.06"E). These 4 tea varieties are mainly cultivated in the area around the southern part of the Yangzi River and typically have different biological properties. For example, their initial sprouting times and/or their leaf sizes are different, and their cultivars are used for different types of tea production. LJ43 and YS have smaller leaves and also a smaller crown area. In the southern Yangzi River region, WNZ starts to germinate in the middle of February, while the germination time of LJ43 begins in the middle of March. The other cultivars germinate in the beginning of March. WNZ and LJ43 can be used to produce green tea with a relatively flat shape, and ZN139 and YS can be used to produce both green and black tea. The soil was collected from a subtropical evergreen broadleaf forest near Nanjing. The total N, total P, and total K values of the soil were 0.13%, 0.04%, and 0.96%, respectively. The concentrations of $\text{NO}_3^-\text{-N}$ and $\text{NH}_4^+\text{-N}$ were 10 mg kg⁻¹ and 18 mg kg⁻¹, respectively. The fertilization experiment trial was composed of 4 sample treatments: 1) a control treatment, 2) a treatment fed only with $\text{NO}_3^-\text{-N}$, 3) a treatment fed only with $\text{NH}_4^+\text{-N}$, and 4) a treatment fed with a mixture of $\text{NO}_3^-\text{-N}$ and $\text{NH}_4^+\text{-N}$ in a 1:1 ratio. Each treatment contained 15 pots, including 3 replications with 5 pots in every replication. All fertilized treatments received 1 g of N per pot. No fertilizer was used in the control sample. Dicyandiamide ($\text{C}_2\text{H}_4\text{N}_4$, CAS461-58-5, 0.5 g per plot) was added to help prevent soil nitrification. Sodium nitrate (NaNO_3 , CAS7631-99-4) and ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$, CAS7783-20-2) were used as fertilizers for the $\text{NO}_3^-\text{-N}$ and $\text{NH}_4^+\text{-N}$ -fed samples respectively. The

pots were watered as necessary during the course of the experiment.

2.2. GS and GOGAT activities in the leaf

Two months after the beginning of the trial it was possible to collect the third and fourth healthy young leaf samples for studies of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.1.14). The relevant activity measurements were performed. GS and GOGAT were extracted with 5 mL of Tris-HCl buffer (100 mM, pH 7.0) containing 1 mM $MgCl_2$ (CAS7791-18-6), 1 mM EDTA ($C_{10}H_{16}N_2O_8$, CAS60-00-4), and 10 mM 2-mercaptoethanol (C_2H_6OS , CAS60-24-2) and then placed into a centrifuge (13,000 rpm), which was operated at temperatures between 0 and 4 °C. The obtained liquid supernatant was then used for further analysis.

GS and GOGAT activities were determined with a synthetase reaction (Mifflin and Habash, 2002; Du et al., 2008). The reaction medium used to determine GS activity consisted of 0.6 mL of imidazole-HCl buffer (0.25 mol/L, pH 7.0), 0.4 mL of sodium glutamate ($C_5H_8NO_4Na$, CAS142-47-2, 0.30 mol/L, pH 7.0), 0.4 mL of ATP-Na ($C_{10}H_{14}N_5O_{13}P_3Na_2$, CAS987-65-5, 30 mM, pH 7.0), 0.2 mL of $MgSO_4$ (CAS7487-88-9, 0.5 M), and 1.2 mL of enzyme extract solution. The reaction was initiated by adding 0.2 mL of hydroxylamine (NH_2OH , CAS7803-49-8, 0.6 mM) and was stopped by adding 0.8 mL of $FeCl_3$ solution (TCA, 2%, w/v; $FeCl_3 \cdot 6H_2O$, 3.5%, w/v; HCl, 2%, v/v). The reaction was carried out over a 15-min period at a temperature of 30 °C. The reacting mixture was centrifuged (4000 rpm) for 15 min and the absorbance level of the supernatant solution was measured at 540 nm using a UV-Vis spectrophotometer (U-2800, Hitachi, Tokyo, Japan).

GOGAT activity was measured using a spectrophotometer at a temperature of 30 °C by monitoring the oxidation of NADH at 340 nm. The reaction medium used to measure GOGAT activity was a solution that consisted of 0.4 mL of L-glutamine ($C_{10}H_{20}N_4O_6$, CAS56-85-9, 20 mM), 0.5 mL of α -ketoglutaric acid ($C_5H_6O_5$, CAS328-50-7, 20 mM), 0.1 mL of KCl (CAS7447-40-7, 10 mM), 0.2 mL of NADH (CAS606-68-8, 3 mM), 0.3 mL of enzyme extract solution, and 1.5 mL of Tris-HCl buffer (25 mM, pH 7.6). GOGAT activity was measured at 340 nm with a UV-Vis spectrophotometer (Hitachi, U-2800) and recorded every 30 s during the reaction period.

2.3. Gas exchange

The net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO_2 content (Ci) were measured in a closed system with an ambient CO_2 level ($400 \mu mol mol^{-1}$), photosynthetic photon flux of $1000 \mu mol m^{-2} s^{-1}$ (maintained by a 6400-02 LED red and blue light source), 60% relative humidity, and a constant temperature of 28 °C using a LI-COR 6400XT

portable photosynthesis device (LI-COR Biosciences, Inc., Lincoln, NE, USA). All parameters were measured 5 times per treatment on sunny days from 0930 to 1100 hours. Young and healthy leaves, growing in the third and fourth positions in the plants, were selected for our samples. CO_2 was introduced into and maintained in the closed system by a dedicated CO_2 steel cylinder fitting for the LI-COR 6400XT device.

2.4. N content per unit leaf area (N_f), PNUE, and WUE

The leaf samples were collected after measuring the gas exchange, and their fresh weights, dry weights, and N contents were determined. The total N of the leaf samples was treated with the Kjeldahl digesting method and determined using a continuous-flow automatic analysis device (AutoAnalyzer III, Bran Luebbe, Inc., Norderstedt, Germany). PNUE and WUE were calculated as the ratios of Pn to N_f and Pn to Tr, respectively.

2.5. Statistical analysis

Statistical tests were performed on all the parameters listed above. We conducted 2-way ANOVA for each parameter, with treatments and cultivars incorporated as fixed-effect factors. Post hoc comparisons among groups were performed using Fisher's least significant difference (LSD) tests. All values shown in the tables are mean \pm standard deviation values. The dataset obtained was analyzed using Statistic Analysis System software (SAS 9.0, Cary, NC, USA).

3. Results

3.1. GS and GOGAT activity

We found that the effects of treatments and cultivars and their interaction effects on GS and most GOGAT activity were significant (Table 1). Different nitrogen applications all exhibited significantly higher GS and GOGAT activity than that of control plants for all cultivars, with no significant difference in GOGAT activity between NO_3^- -N-fed and control plants (Table 1). Compared with the control plants, the mixture-fed plants had increased GS activity by 110.2%, 65.18%, and 72.75% for WNZ, YS, and ZN139, respectively (Table 1).

The response of GS and GOGAT activity to different nitrogen applications was dependent on individual cultivars. For GS activity, an observable difference existed between mixture-fed and NH_4^+ -fed plants in most cultivars (excluding YS). For GOGAT activity, NO_3^- -N-fed plants achieved significantly lower results than mixed N-fed and NH_4^+ -N-fed plants in ZN139 and LJ43, while no major difference existed among different N applications in WNZ and YS. For YS, no obvious measurable differences existed in GS or GOGAT activity for N applications (excluding control).

Table 1. GS and GOGAT activity response to different inorganic N applications. Samples were harvested from 4 tea cultivars (2 years old), grown in a greenhouse and fed with different N applications. Samples were subsequently analyzed for GS activity and GOGAT activity. Data were calculated from 3 replicates (5 separate plants that underwent the same experimental treatments) and are stated as mean \pm SD. Letters indicate statistical differences ($P < 0.05$) according to LSD test ($n = 3$). F_p , N treatment effect; F_c , cultivar effect; $F_t \times F_c$, the interactive effect of N treatments and cultivars. NS, not significant; * $P < 0.05$; ** $0.001 < P < 0.01$; and *** $P < 0.001$.

Tea cultivars	Nitrogen treatments	GS activity ($\mu\text{mol g}^{-1} \text{h}^{-1}$)	GOGAT activity ($\mu\text{mol g}^{-1} \text{h}^{-1}$)
WNZ	Control	84.50 \pm 8.50fg	38.50 \pm 5.20g
WNZ	Nitrate-fed	127.80 \pm 13.80bcd	74.89 \pm 2.85abc
WNZ	Mixture-fed	175.66 \pm 10.81a	74.00 \pm 9.96abc
WNZ	Ammonium-fed	128.17 \pm 9.42bcd	74.91 \pm 7.56abc
YS	Control	78.60 \pm 10.20fg	44.50 \pm 7.30fg
YS	Nitrate-fed	121.88 \pm 16.87cd	63.66 \pm 8.38cde
YS	Mixture-fed	129.83 \pm 9.25bcd	72.28 \pm 4.23abc
YS	Ammonium-fed	114.57 \pm 3.61de	68.75 \pm 5.02bcd
ZN139	Control	86.54 \pm 10.54fg	39.80 \pm 6.20g
ZN139	Nitrate-fed	129.49 \pm 9.56bcd	58.54 \pm 7.05de
ZN139	Mixture-fed	149.50 \pm 14.75b	76.07 \pm 3.64abc
ZN139	Ammonium-fed	112.70 \pm 16.17de	73.18 \pm 5.73abc
LJ43	Control	72.41 \pm 12.41g	43.60 \pm 7.80fg
LJ43	Nitrate-fed	141.92 \pm 13.63bc	57.61 \pm 11.38de
LJ43	Mixture-fed	115.32 \pm 16.74de	83.29 \pm 7.61a
LJ43	Ammonium-fed	96.70 \pm 8.91f	77.25 \pm 5.02ab
	$P:F_c$	***	NS
	$P:F_t$	***	***
	$P:F_c \times F_t$	**	*

A significant difference in GS activity among the cultivars was found in mixture-fed and NH_4^+ -N-fed plants, while no significant difference in GOGAT activity was found in most treated plants (Table 1). In mixture-fed plants, WNZ showed significantly higher GS activity than the other cultivars. Both under mixture-fed and NH_4^+ -N-fed conditions, LJ43 had the lowest levels of GS activity. In NO_3^- -N-fed plants, WNZ had much higher levels of GOGAT activity than ZN139 and LJ43 due to the significant interaction effects of the cultivars and N treatments.

3.2. Net photosynthetic rate (Pn) and transpiration rate (Tr)

There were significant differences in Pn and Tr among treatments and cultivars, and the reciprocal effects were also clearly noticeable (Table 2). Mixed N-fed and NO_3^- -

N-fed plants showed much higher Pn and Tr levels than control plants in all cultivars (Table 2). These 2 applications increased Pn by more than 70% and 50%, respectively, compared to the control plants for most tea cultivars (excluding LJ43). No significant difference was found in Pn between NH_4^+ -N-fed and control plants in most cultivars, and the Pn of NH_4^+ -N-fed plants was significantly lower than that of the control plants for YS. As for Tr, NH_4^+ -N-fed plants achieved significantly higher levels than the control plants in LJ43 and ZN139, highlighting that the difference was not as great as it was for WNZ and YS samples (Table 2).

Clearly detectable differences in Pn and Tr were observed between mixed N-fed and NH_4^+ -N-fed samples in most cultivars. For LJ43, however, no significant difference in Pn was found for the 3 different N applications.

Table 2. Pn, Tr, Gs, and Ci responses to different inorganic N applications. The measurements were carried out on healthy leaves cut from similar positions on the plants. Samples were taken on sunny days. Data were calculated from 3 replicates. The results shown are mean \pm SD. Letters indicate statistical differences ($P < 0.05$) according to LSD test ($n = 3$). F, N treatments effect; F_c, cultivars effect; F_t \times F_c, the interactive effect of N treatments and cultivars. NS, not significant; * $P < 0.05$; ** $0.001 < P < 0.01$; and *** $P < 0.001$.

Tea cultivars	Nitrogen treatments	Pn ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Tr ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$)	Gs ($\text{mmol m}^{-2} \text{s}^{-1}$)	Ci ($\mu\text{mol mol}^{-1}$)
WENZ	Control	4.86 \pm 0.80gh	3.68 \pm 0.40ef	79.77 \pm 8.96ij	298.45 \pm 21.48fg
WENZ	Nitrate-fed	8.48 \pm 1.59bcde	4.68 \pm 0.45cd	123.67 \pm 10.69def	365.43 \pm 35.66abcde
WENZ	Mixture-fed	8.72 \pm 0.98bcd	5.18 \pm 0.54bc	128.4 \pm 9.80de	338.00 \pm 9.54cdefg
WENZ	Ammonium-fed	6.23 \pm 1.42fgh	4.14 \pm 0.25de	101.4 \pm 12.72fghi	395.00 \pm 25.01a
YS	Control	7.50 \pm 0.64cdef	4.20 \pm 0.38de	86.97 \pm 8.73hij	289.33 \pm 23.73g
YS	Nitrate-fed	12.00 \pm 1.30a	5.58 \pm 0.28ab	160.0 \pm 11.13ab	332.67 \pm 33.74defg
YS	Mixture-fed	12.87 \pm 1.84a	5.56 \pm 0.70ab	133.50 \pm 20.04cd	327.40 \pm 30.91defg
YS	Ammonium-fed	4.44 \pm 0.70h	4.14 \pm 0.48de	92.86 \pm 10.92ghij	381.44 \pm 17.83abc
ZN139	Control	6.45 \pm 0.98defg	3.70 \pm 0.28ef	114.28 \pm 9.56defg	289.70 \pm 24.82g
ZN139	Nitrate-fed	9.65 \pm 0.87b	4.54 \pm 0.46cd	155.00 \pm 16.26bc	321.22 \pm 24.50efg
ZN139	Mixture-fed	12.78 \pm 1.52a	6.13 \pm 0.76a	178.14 \pm 16.87a	343.33 \pm 25.72bcdef
ZN139	Ammonium-fed	5.65 \pm 0.64fgh	4.63 \pm 0.40cd	123.60 \pm 15.90def	403.89 \pm 25.24a
LJ43	Control	6.89 \pm 0.74defg	2.94 \pm 0.34f	69.89 \pm 7.99j	301.34 \pm 18.26fg
LJ43	Nitrate-fed	8.67 \pm 0.52bc	4.12 \pm 0.56de	105.17 \pm 10.01efg	389.23 \pm 32.26ab
LJ43	Mixture-fed	9.33 \pm 1.54bc	5.70 \pm 0.23ab	151.40 \pm 6.12bc	331.56 \pm 23.72defg
LJ43	Ammonium-fed	7.33 \pm 1.04cdef	4.48 \pm 0.53cde	85.84 \pm 13.18hij	372.67 \pm 20.64abcd
	P:F _c	***	*	***	NS
	P:F _t	***	***	***	***
	P:F _c \times F _t	***	*	**	NS

The major differences seen for Pn and Tr among cultivars might be due to N application. In control plants, a significant difference in Pn and Tr only existed between YS and WENZ and between YS and LJ43, respectively. Under NO₃⁻-fed and mixture-fed conditions, YS achieved a much higher Pn than any other cultivar. For NH₄⁺-fed plants, no detectable difference was found in Pn and Tr among cultivars (Table 2).

3.3. Stomatal conductance (Gs) and intercellular CO₂ content (Ci)

There were significant observable differences in Gs for treatments and cultivars, but a distinguishable difference in Ci occurred only for treatments (Table 2). Mixed-fed and NO₃⁻-N-fed plants showed significantly higher Gs than control plants and NH₄⁺-N-fed plants, while no perceivable difference existed between control plants

and NH₄⁺-N-fed plants. Mixture-fed applications led to increases in Gs at a rate of more than 50% compared with the control; e.g., Gs in LJ43 increased by 116.63% for mixture-fed plants compared with the controls (Table 2). The differences in Gs for cultivars occurred as a result of the N application. Under controlled mixture-fed and NH₄⁺-N-fed conditions, ZN139 had significantly higher Gs levels than any of the other cultivars. Under NO₃⁻-fed conditions, ZN139 and YS showed much higher Gs levels than WENZ and LJ43. Thus, the relatively high Gs difference in cultivars was mainly caused by ZN139.

As for Ci, N application encouraged Ci, and NH₄⁺-N-fed plants showed the highest Ci. Major differences in Ci were measured between NH₄⁺-fed plants and control plants for all cultivars. Clear differences in Ci levels between NO₃⁻-fed and control plants were found in WENZ

and LJ43, while similar variations between mixture-fed and control plants were only found in ZN139. Compared with the control samples, NH_4^+ -fed plants had increased Ci by more than 23%, while other applications only had increased Ci levels by less than 10%. However, our results revealed that NH_4^+ -fed plants had lower Pn, Gs, and Tr than mixture-fed and NO_3^- -fed plants (Table 2). These results indicate that there are other factors that influence the Pn levels in NH_4^+ -fed plants.

3.4. Foliar N content per unit area (N_f)

N applications, especially mixture-fed applications, encouraged higher N_f levels. Mixed N-fed plants had much higher N_f levels than control plants for all cultivars (Table 3). Compared with the control samples, most mixture-fed samples increased N_f by more than 63%, though only by 26.17% for WNZ plant types. For ZN139 plant types, N_f even increased to 6.6 g m^{-2} under mixed-feeding conditions. NO_3^- -N-fed applications presented similar results to those achieved by the mixture-fed samples. NH_4^+ -N feeding encouraged greatly enhanced N_f levels only for ZN139.

Important and clearly observable differences in N_f existed among the tea cultivars we investigated (Table 3). Under most conditions (without mixture-fed condition), WNZ had much greater N_f levels than any of the other cultivars, and LJ43 had the lowest N_f levels (Table 3).

3.5. Photosynthetic N-use efficiency and water-use efficiency

NO_3^- -N-fed applications significantly increased PNUE in most cultivars (excluding LJ43). Generally, mixed-N-fed applications only had a slight effect on PNUE, with a noticeable difference between mixture-fed plants and the control sample only achieved in LJ43 (Table 4). However, feeding with NH_4^+ -N resulted in a significant decrease in PNUE levels in YS and ZN139 (Table 4). Compared with the control, feeding with NO_3^- -N caused an increase in PNUE of 34.85%, 21.70%, and 8.13% for YS, ZN139, and LJ43, respectively. In contrast, following feeding with NH_4^+ -N, PNUE fell by 40.25%, 26.46%, and 6.91% for the same cultivars, respectively.

There were important differences in PNUE levels among the cultivars (Table 4). For WNZ plants, under

Table 3. N_f response to different N treatments in plants. The plants were fed with different inorganic N applications. This was carried out for several different tea varieties. Samples were collected at the same time as photosynthesis measurements were made. N content was determined using a continuous-flow automatic analyzer device. Data were calculated from 3 replicates. Letters indicate statistical differences ($P < 0.05$) according to LSD test ($n = 3$). Values given in g m^{-2} . P: $F_1 < 0.0001$, P: $F_2 < 0.0001$, P: $F_1 \times F_2 = 0.0018$.

Treatments	Tea cultivars			
	WNZ	YS	ZN139	LJ43
Control	4.70 ± 0.38de	3.13 ± 0.23hi	3.40 ± 0.29hi	2.79 ± 0.28i
Nitrate-fed	5.52 ± 0.40bc	3.69 ± 0.31gh	4.30 ± 0.28efg	3.25 ± 0.24hi
Mixture-fed	5.93 ± 0.51b	5.38 ± 0.45bc	6.56 ± 0.58a	4.57 ± 0.38def
Ammonium-fed	5.04 ± 0.42cd	3.14 ± 0.21hi	4.03 ± 0.23fg	3.30 ± 0.31hi

Table 4. PNUE response to different inorganic N applications. PNUE was related to Pn (Table 2) and N_f (Table 3) and was calculated as a ratio of Pn to N_f to illustrate the rate of N per unit area used for photosynthesis. Letters indicate statistical differences ($P < 0.05$) according to LSD test ($n = 3$). Values given in $\mu\text{mol s}^{-1} \text{ g}^{-1}$. P: $F_1 < 0.0001$, P: $F_2 < 0.0001$, P: $F_1 \times F_2 < 0.0001$.

Treatments	Tea cultivars			
	WNZ	YS	ZN139	LJ43
Control	1.03 ± 0.08h	2.41 ± 0.18bc	1.89 ± 0.12e	2.46 ± 0.22bc
Nitrate-fed	1.55 ± 0.10f	3.25 ± 0.24a	2.30 ± 0.19cd	2.66 ± 0.14b
Mixture-fed	1.32 ± 0.09fg	2.40 ± 0.20bc	1.90 ± 0.09e	2.10 ± 0.16de
Ammonium-fed	1.24 ± 0.11gh	1.44 ± 0.08fg	1.39 ± 0.11fg	2.29 ± 0.21cd

most conditions (except NH_4^+ -N feeding), PNUE was much lower than for any other cultivar. The PNUE of WNZ in combination with any of the N applications was lower than $1.6 \mu\text{mol s}^{-1} \text{g}^{-1}$. Under these conditions, YS and LJ43 showed much higher PNUE levels than ZN139. When fed with NH_4^+ , LJ43 achieved much higher PNUE values than any of the other cultivars, while no significant difference existed among the other 3 cultivars (Table 4).

WUE slightly improved under most conditions and decreased under NH_4^+ -fed conditions. Substantial differences in WUE only existed between NH_4^+ -fed and control plants of the YS cultivar and between the NH_4^+ -fed and mixed-N application plants of the ZN139 cultivar (Table 5). Differences among cultivars under different conditions were not pronounced (Table 5). Thus, our results indicate that NH_4^+ applications have a negative effect on both PNUE and WUE.

4. Discussion

4.1. Effects of inorganic N on gas exchange and ammonium assimilation

Generally, N application promoted higher gas exchange levels than were observable in the control, as nutrient levels may have been deficient in the control samples. Some studies have suggested that plants have higher Pn, Gs, and Tr values under mixture-fed conditions, which indicates that plants undergo more active carbon and water metabolization (Xiao et al., 2000; Chen et al., 2005). In our study, we found that the application of N can lead to a noticeable increase in Pn, Tr, Gs, and GS/GOGAT activities, which indicates that carbon and nitrogen metabolism were both activated by the addition of N (Bybordi, 2012), especially for mixture-fed samples. This is because different genes were present, which are related by different types of nitrogen (Gaur et al., 2012) and regulate GS and GOGAT activities; e.g., plastid type GS and NADH-GOGAT activities increase with the

supply of NO_3^- (Hayakawa et al., 1990), while GS family genes are regulated by ammonia (Cren and Hirel, 1999). Similar changes in Pn, Tr, and Gs occur under different applications, because Pn and Tr are closely related to Gs (Flexas and Medrano, 2002), with correlation coefficients equal to 0.66 ($P < 0.01$) and 0.65 ($P < 0.01$), respectively.

As compared with the control plants, the NH_4^+ -N-fed plants showed no significant effect on Pn, Tr, or Gs and only a slight effect on key enzyme activities for ammonium assimilation in most cultivars. These changes were noticeably different from the mixture-fed or NO_3^- -fed plants. These results are in accordance with some of the previous studies undertaken, which stated that too much NH_4^+ -N results in negative effects on plant Pn (Cramer and Lewis, 1993; Britto and Kronzucker, 2002; Li et al., 2008). Nonstomatal factors, e.g., lower mesophyll conductance and RuBP activity (Flexas et al., 2002, 2008), inhibit Pn. The accumulation of NH_4^+ caused a reduction in Pn, but higher Ci levels were still observable in the leaves (Flexas and Medrano, 2002; Jalloh et al., 2009). In our study, the Ci in NH_4^+ -fed plants was the highest for most cultivars. These results indicate that application of NH_4^+ -N alone is not the best way to feed the cultivars.

4.2. Numerous effects of inorganic N on various cultivars

Previous studies showed that the effect of N fertilizer on plant photosynthesis is species-specific. For example, Pn and Gs decreased when NH_4^+ -N was present in wheat (Lopes and Araus, 2006). This is, however, in contrast to French beans, rice, lettuce, etc. (Guo et al., 2002; Duan et al., 2007). In our study, no significant response to different N applications was found in GS and GOGAT activity in YS. A similar result was achieved for Pn in LJ43. Both YS and LJ43 are small-leaved cultivars (Chen, 2011). This might indicate that small-leaved tea cultivars exhibit more stability in terms of ammonium assimilation and Pn when they are subjected to different nitrogen treatments. Thus, during planting, the effects of different

Table 5. WUE response to different inorganic N applications in different tea varieties. WUE was related to Pn and Tr (Table 2) and was calculated as a ratio of Pn to Tr, which immediately helps illustrate water-use efficiency for photosynthesis. Letters indicate statistical differences ($P < 0.05$) according to LSD test ($n = 3$). Values given as $\mu\text{mol mmol}^{-1} \text{H}_2\text{O}$. P: $F_1 = 0.0003$, P: $F_c = 0.13$, P: $F_1 \times F_c = 0.05$.

Treatments	Tea cultivars			
	WNZ	YS	ZN139	LJ43
Control	$1.32 \pm 0.08\text{cd}$	$1.79 \pm 0.10\text{abc}$	$1.74 \pm 0.11\text{abcd}$	$2.34 \pm 0.14\text{a}$
Nitrate-fed	$1.81 \pm 0.14\text{abc}$	$2.15 \pm 0.19\text{ab}$	$2.12 \pm 0.13\text{ab}$	$2.10 \pm 0.17\text{ab}$
Mixture-fed	$1.68 \pm 0.09\text{abcd}$	$2.31 \pm 0.16\text{a}$	$2.08 \pm 0.18\text{ab}$	$1.64 \pm 0.17\text{abcd}$
Ammonium-fed	$1.50 \pm 0.12\text{bcd}$	$1.07 \pm 0.09\text{d}$	$1.22 \pm 0.11\text{cd}$	$1.64 \pm 0.15\text{abcd}$

types of nitrogen application on LJ43 or YS should be evaluated comprehensively along with other physiological parameters, such as PNUE, Tr, and Gs.

Based on the data obtained from all treatments, LJ43 showed higher PNUE and lower N_p , while WNZ had the opposite results. The Pn levels of most cultivars were similar (except YS). This indicates that the application of nitrogen should be tailored for different types of cultivars, e.g., more nitrogen for LJ43 might be used to encourage photosynthesis, while more nitrogen for WNZ might be used to encourage other physiological activities.

4.3. The relationship between gas exchange and foliar nitrogen

Gas exchange is a critical process of carbon metabolism in plants, which is also closely related to nitrogen and water metabolism (Mitchell and Hinckley, 1993; Evans, 1989; Champigny and Foyer, 1992; Ripullone et al., 2003). We found that gas exchange processes can be approximate indicators for nitrogen absorption and utilization. In our study, N_f increased with the application of N, especially under mixed-N-fed conditions. This indicates that N's absorption ability increased, as did its affinity to NH_4^+ -N and NO_3^- -N, which was affected by N fertilizer (Du and Peng, 2010). Any change in N_f can affect gas exchange in the cultivars (Niinemets, 1997; Ripullone et al., 2003). Han et al. (2009) stated that leaf N content increased after thinning and was positively related to photosynthesis; the correlation index ranged from 0.40 to 0.80 ($P < 0.01$). High N foliar presence was shown to cause significantly greater rates of transpiration (Mitchell and Hinckley, 1993). Our results also confirmed that N_f was positively related to Pn ($R = 0.43$, $P < 0.01$) and Tr ($R = 0.65$, $P < 0.01$). This is due to foliar N, represented by the proteins of the Calvin cycle and thylakoids, which are key ingredients that regulate plant growth and photosynthetic metabolism (Evans et al., 1989; Takashima et al., 2004).

The utilization of N in leaves also helped confirm certain patterns that are closely related to gas exchange, where understanding of the GS/GOGAT cycle is critical for successful application of inorganic N. This cycle provides organic N for transport so as to maintain N levels in plants (Suzuki and Knaff, 2005). We found that GS activity significantly correlated with Pn and Ci, with measured correlation coefficients of 0.40 ($P < 0.01$) and -0.39 ($P < 0.01$), respectively. Conversely, the GOGAT activity did not correlate well with Pn and Ci. As for Tr, it correlated significantly with both GS ($R = 0.65$, $P < 0.01$) and GOGAT ($R = 0.66$, $P < 0.01$) activity. As such, it can be said that there is a strong link between leaf N metabolism and photosynthesis (Novitskaya et al., 2002), due to the positive correlation of glutamine synthesis in the malate shuttle between the cytosol and stroma (Stitt et al., 2002). Gas exchange is very closely related to the GS/GOGAT

cycle and oxoglutarate is required for both GOGAT operations and carbon metabolism (Noctor and Foyer, 1998; Britto and Kronzucker, 2002; Bernard et al., 2009). Hence, in situations where the GS/GOGAT cycle changes (e.g., in our study, when our samples were affected by addition of different nitrogen forms), carbon metabolism (photosynthesis) may also be affected.

However, we found that Pn in NH_4^+ -fed plants was lower than in NO_3^- -fed and mixture-fed applications. This is related to the lower GS activity in NH_4^+ -N-fed plants, as GS can reassimilate the release of endogenous NH_4^+ -N by ammonium-evolving processes such as photorespiration (Oliveira et al., 2002; Gonzalez-Moro et al., 2003).

4.4. Variations in gas exchange levels led to different PNUE and WUE values

WUE and PNUE, which represent the rate of nitrogen and water uptake in photosynthesis, are very closely related to gas exchange. WUE was enhanced by N supply under nonlimiting water conditions (Liu et al., 2013b), with NO_3^- -fed plants having a higher water uptake rate than NH_4^+ -N-fed plants (Guo et al., 2002). We found that WUE increased in NO_3^- -N-fed and mixture-fed plants (except LJ43), but these effects were not significant. Some NH_4^+ -fed plants even experienced significantly decreased WUE levels. These different results are mainly due to the different genotypes causing contrasting responses for WUE when N is added (Gorny and Garczynski, 2002; Brueck, 2008).

Changes of Pn and N_p , which were both caused by the addition of N, led to changes in PNUE. PNUE is a key parameter that helps illustrate the proportion of N distributed during plant photosynthesis (Poorter et al., 1998; Habash et al., 2007). In our study, mixed-N-fed plants had the highest Pn and N_p , but NO_3^- -N-fed plants exhibited the highest PNUE levels. This indicates that when leaf N_f increases, the allocation of nitrogen to enable photosynthesis increases (Evans, 1989; Niinemets, 1997). However, if it increases too much and exceeds growth requirements, then Pn sometimes tends to cause increased curvature as leaf N content increases (Evans and Terashima, 1988). This can even increase the proportion of RuBisCo contents as nitrogen storage proteins (Stitt and Shulze, 1994; Hikosaka and Terashima, 1994). In our study, mixture-fed plants showed significantly higher N_f levels than other types of cultivars (Table 3).

Our study presents an integrative research framework, clearly showing how gas exchange, ammonium assimilation, nitrogen uptake, and water utilization in photosynthesis respond to different N fertilizers. We found that the application of N, especially for mixture-fed applications, improved tea photosynthesis physiology, increased the ammonium assimilation rate, and boosted N content in leaves. PNUE responded significantly to N applications, but NH_4^+ -N might lead to decreased WUE

in some cultivars. Furthermore, differences in values of PNUE, N_p and GS among the various cultivars are mainly caused by WNZ, and differences in gas exchange are mainly caused by YS or ZN139. In YS and LJ43, different applications (except the control) exerted no significant effect on key enzyme activity and Pn. The results indicate that N might regulate nitrogen content and its assimilation, thereby encouraging improved gas exchange. The changes observed in both gas exchange and N_i led to PNUE being affected. Therefore, mixture-fed treatments generally had the most positive effect on ammonium assimilation and

photosynthetic physiology for *C. sinensis* L. cultivation. These results help us to understand tea physiology and represent an important step forward in terms of studying the relationships that influence biological stoichiometry, nutrient managing, and abiotic stress in tea cultivation.

Acknowledgment

The study was funded in part by the Zhejiang Provincial Natural Science Foundation (Grant No. Y3110441) and the National Natural Science Foundation of China (Grant No. 31070545, 30872055).

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