Assessment of Exogenous Ammonium Nitrate and Urea as a Source of Nitrogen on Nitrate Assimilation, Photosynthetic Pigments and Biochemical Characteristics in Maize (*Zea mays* L.)

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Abstract

Nitrogen is one of the most important inorganic nutrients for plant growth. In this study, we examined the effects of ammonium nitrate (NH_4NO_3) and urea (CH_4N_2O) supplies on photosynthetic pigment content, nitrate accumulation and production of nitrite, total protein, free amino acids, proline, nitrate reductase activity and carbohydrate biosynthesis in ~~the~~ leaf of maize (*Zea mays* L.). Germinated seeds were planted in pots containing perlite, and plants were grown under standard conditions for 3 weeks. Three NH_4NO_3 and three CH_4N_2O concentrations were applied as treatments. Results showed that, with low concentrations of CH_4N_2O and NH_4NO_3, the photosynthetic pigment content increased. Nitrate starvation caused reduction in (i) nitrate accumulation and nitrite production; (ii) total free amino acid, proline and total protein contents; (iii) carbohydrate concentration; and (iv) nitrate reductase activity. Some amounts of NH_4NO_3 and CH_4N_2O increased all the above factors. In low concentrations, the nitrate induced its own assimilatory pathway, but in high levels, this effect was impaired. CH_4N_2O was more effective than NH_4NO_3 in accumulation of nitrate, increasing production of nitrite, amino acids, proteins, carbohydrates, and nitrate reductase activity. In addition, CH_4N_2O, as an inducer, had significant effects on the assimilatory and nitrate metabolism, in low concentrations. In high nitrate levels, nitrate assimilation was prevented by a negative regulatory mechanism and nitrate toxicity.

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Keywords

Maize
Ammonium nitrate (NH_4NO_3)
Urea (CH_4N_2O)
Nitrate reductase
Nitrate assimilation

1. Introduction

Naturally, nitrogen (N) exists in the earth's atmosphere. However, nitrogen

available in the atmosphere is in gas form, and plants do not directly use this form of nitrogen. Moreover, this form of nitrogen is considered as molecular nitrogen and is inefficient for plants (Rad et al. 2013). Nitrogen is a component of very important compounds, viz. amino acids, proteins, chlorophylls, nucleic acids, vitamins, some plant hormones and other relevant plant metabolites. It is the most important inorganic nutrient for plant growth. The origin of nitrogen in soil is not only from plant and animal residues, but also from fixation by Fabaceae plants. Therefore, farmers are forced to consume large amounts of chemical fertilizers containing nitrogen or animal fertilizers for growing the majority of cereals and non-legume grasses. Under appropriate conditions, the organic nitrogen is converted into inorganic nitrogen, available for ammonium nitrate (NH_4NO_3) and NO_3^- production. Nevertheless, mineral nitrogen is only a small fraction of total nitrogen in the soil. Today, urea (CH_4N_2O) is considered the most popular form of nitrogen fertilizer in agricultural activities (Vavrina and Obreza 1993). Plants receiving CH_4N_2O as nitrogen source exhibit a higher performance than plants receiving NH_4NO_3. This may be due to the higher content of nitrogen in CH_4N_2O than in NH_4NO_3. CH_4N_2O contains 46 % nitrogen, while NH_4NO_3 has 35 % nitrogen; this causes further increase in the growth of plants receiving CH_4N_2O compared with NH_4NO_3 (Bybordi and Gheibi 2009). CH_4N_2O cannot be directly used in plant metabolism; it has to be hydrolyzed to ammonia and carbon dioxide by urease (Ciurli et al. 1999). CH_4N_2O is an active substance that increases photosynthesis by activating proteolysis enzymes in the leaf and, subsequently, increases the flow of nitrogenous materials from leaf to seeds. CH_4N_2O, in high levels, is toxic to plant metabolism causing leaf burn (Krogmeier et al. 1989). Range of nitrogen concentration in the soil solution can be very diverse. In many natural and agricultural ecosystems, NH_4^+ is the predominant N source (Bijlsma et al. 2000), and it is usually present to some extent in the majority of ecosystems. Frequency of NH_4NO_3 in the soil solution is characterized by a number of different factors, such as the accumulation of organic matter, pH, temperature, soil oxygen status and the presence of allelopathic chemicals, which is considered as the most important factor (Rice and Pancholy 1972; Dijk and Eck 1995). NH_4^+ is a paradoxical nutrient ion, since it is a major nitrogen source whose oxidation state eliminates the need for its reduction in the plant cell. It is involved in many metabolic activities. On the other hand,

employing ammonium as the only nitrogen source in many plants will lead to toxicity symptoms (Vines and Wedding 1960). The reported symptoms of ammonium toxicity range widely. Generally, concentrations higher than 0.1–0.5 mmol/l ammonium led to the occurrence of observed symptoms (Schenk and Wehrmann 1979). Many studies have shown that the relative amounts of nitrate reduction and nitrate concentration, in root and shoot, largely depend on the external environment. When external concentration of nitrates is low, most of their regeneration to amino acids occurs in root tissues and then is transferred to the plant branches and leaf. In conditions of high external nitrate concentration, reduction is not enough into the root and a significant fraction of the nitrogen entering the vascular system moves to the branches and leaf. Thus, in such status, most of the nitrate is reduced to nitrite in the leaf (Rachmilevitch et al. 2004). Nitrate is not directly converted into proteins, but it has to be reduced to ammonium ion before being converted into organic compounds. NH_4NO_3 is converted in a two-stage process. In the first stage, cytoplasmic enzyme nitrate reductase (EC 1.6.6.1) reduces nitrate to nitrite (Der et al. 1998). Then, nitrite moves to plastids in the root or leaf chloroplast and is then reduced to ammonium by the enzyme nitrite reductase (EC 1.7.7.1) (Der et al. 1998). Nitrite is toxic and rarely accumulates in plants. Lack of accumulation of nitrite is due to higher nitrite reductase activity compared with the nitrate reductase one. Ammonium obtained from nitrate enters quickly into organic compounds by enzymatic system glutamine synthetase—glutamate synthase.

Maize (*Zea mays* L.), one of the most widely cultivated crops, is a major component in the diet of many developing countries and is one of the crops with the most biotechnological potential for energy production and other industrial applications (McLaren 2005). The production of high-yielding maize is associated with the application of large quantities of fertilizers at high cost, and, therefore, nitrogen pollution is becoming a threat to universal ecosystems because the majority of nitrogen fertilizers is lost to the atmosphere or leached into groundwater, lakes and rivers.

In this study, to gain more insight into these effects, we examined the effects of NH_4NO_3 and CH_4N_2O supplies on photosynthetic pigment content, nitrate accumulation and production of nitrite, total protein, total free amino acid, proline, nitrate reductase activity and carbohydrate biosynthesis in the leaf of

maize. Various concentrations of NH_4NO_3 and CH_4N_2O were investigated as two different sources of nitrogen.

2. Materials and Methods

2.1. Plant Materials and Treatments

The seeds of maize (var KSC.704) were obtained from the Iranian Agricultural Organization of Shiraz City. Primarily, the seeds were disinfected with sodium hypochlorite 1 % for 5 min. Seeds were immersed in sterile water and placed inside a growth chamber for germination. After a week, ten vigorous seedlings of the same size were selected and replanted in the pots containing 1 kg perlite. The seedlings were grown in Hoagland's solution (Hoagland and Arnon 1950) for 3 weeks. Plants were then divided into two groups: (1) grown in standard nutrient solutions (positive control), and (2) grown in media without nitrate (starved plants) for 2 weeks. Starved plants were subdivided into three groups: (1) grown in media without nitrate (negative control) for an additional period of 2 weeks, (2) grown in various concentrations of CH_4N_2O (Merck Company, Germany) (0.01, 0.05 and 0.09 g/kg soil) for an additional period of 2 weeks, and (3) grown in various concentrations of NH_4NO_3 (Merck Company, Germany) (0.01, 0.06 and 0.1 g/kg soil) for an additional period of 2 weeks. After nitrate supplementation, the leaves were harvested and frozen in liquid nitrogen.

2.2. Measurement of Photosynthetic Pigments

Measurements of the photosynthetic pigment contents of maize seedlings were carried out in control and treated plants. Leaf fragments were randomly selected; then, 200 mg of leaf tissue was weighed and pulverized with a mortar and pestle in liquid nitrogen. Large pieces were completely pulverized with 80 % acetone, and the final volume was brought to 25 ml. The resulting solution was centrifuged at 4800 rpm for 20 min and the supernatant was used for the measurements of chlorophyll a, b and carotenoids. Light absorption of the plant extract was determined by a spectrophotometer-UV (Shimadzu A160) set at wavelengths 470, 645, 646.8, 663 and 663.2 (Lichtenthaler 1987). Each treatment was replicated three times.

2.3. Nitrate/Nitrite Assay

The frozen leaves were powdered with a mortar and pestle, solubilized in phosphate-buffered saline (pH 7.4), and the extract was centrifuged at 9000 rpm for 15 min. The supernatant was stored at −70 °C. Nitrate was determined by Griess reagent using sodium nitrate as standard (Miranda et al. 2001). Briefly, to 100 µl of the culture medium, 100 µl of vanadium chloride (III) (8 mg/ml) was added. After 40 min, 50 µl of Griess reagents [1:1 (v/v) of 0.1 % naphthylethylenediaminedihydrochloride (NED) in H_2O+2 % sulphanilamide in 5 % H_3PO_4] was added and incubated at 37 °C for 10 min, and the absorbance was read at 540 nm. For nitrite assay, vanadium chloride (III) was omitted from the test.

2.4. Total Amino Acid Assay

Total amino acids were determined by reaction with ninhydrin using glycine as standard according to Sun et al. (2006). Briefly, to 500 solutions of amino acids 1 ml acetic acid 80 % and 1 ml Ninhydrin solution (2 mg ninhydrin in 50 % ethanol) were added, and then mixed for 15 min. The above mixture was heated at 100 °C for 15 min and then at 70 °C for 10 min. Finally, we added 5 ml of 2-propanol (50 %) and read the absorbance at 570 nm. A different concentration of the amino acid glycine was used for the standard curve.

2.5. Measurement of Total Protein

Total protein content was determined by the Bradford method using bovine serum albumin (BSA) as standard (Bradford 1976). To extract the protein, 1 g of leaf plant ground with liquid nitrogen and then homogenized in 4 ml of extraction buffer (Table 1) was mixed with 1 ml of Phenylmethanesulfonyl fluoride (PMSF). After homogenization, the solution was centrifuged for 3 min at speed of 10,000 rpm at 4 °C. The supernatant samples were transferred to a new tube and stored at −20 °C. To prepare the Bradford solution, 100 mg Comassi Blue was dissolved in 50 ml of ethanol 95 % and mixed with 100 ml of 85 % phosphoric acid. The final volume with distilled water reached 1 l and was brought to pass the filter paper. To determine the protein concentration of the extract dilutions of 0, 5, 10, 15, 20, 25,

30,35 µl/mlH_2O of ~~bovine serum albumin (BSA) with concentration of 1 mg/ml was prepared~~ and absorbance was measured at 595 nm by a spectrophotometer (Jenway 6405 UV models). To determine the amount of protein samples, to 20 mL of solution of protein extract 180 mL distilled water and 1 ml of Bradford reagent were added and absorbance was read at 595 nm and protein content of the sample was calculated using a standard curve.

Table 1

Protein extraction buffer

Chemical compounds	Amounts
Tris-hydrochloride (pH = 8)	0.1 M
Polyvinyl pyrrolidone (PVP)	0.01 M
Triton-x-100	1 %
Phenylmethanesulfonyl fluoride (PMSF)	10 mM
Ethylene diamine tetraacetic acid (EDTA)	5 mM
Dithiothreitol (DTT)	0.5 mM

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2.6. Measurement of Nitrate Reductase Activity

Nitrate reductase assays were performed by the reduction of nitrate to nitrite (Brunetti and Hageman 1979). One unit of enzyme activity is defined as the production of 1 µM nitrite per min.

2.7. Free Proline Amino Acid Assay

In this study, the extraction and measurement of proline were performed according to the method of Bates et al. (1973). Leaf samples (0.8 g) were extracted with 3 % sulphosalicylic acid. Extracts (1 ml) were held for 1 h in boiling water by adding 2 ml ninhydrin and 2 ml glacial acetic acid, after which cold toluene (4 ml) was added. Proline content was measured by a spectrophotometer (Shimadzu UV 1601, Japan) at 520 nm and calculated as µmol g^{-1} DW against standard proline.

2.8. Total Carbohydrate Determination

The carbohydrate content was determined by the phenol–sulfuric acid method (Rao and Pattabiraman 1989). Briefly, 50 µl 80 % phenol and then 3 ml 98 % sulfuric acid were added to 1 ml of sugar solution. The mixture was vortexed, kept at room temperature for 30 min and the absorbance read at 490 nm.

2.9. Statistical Analysis

The experimental design was a randomized complete block with three replicates. Analysis of variance (ANOVA) was calculated using SPSS v. 11.5 (IBM SPSS, New York, USA) and differences between treatment means were compared using Duncan's multiple range tests at $P < 0.05$.

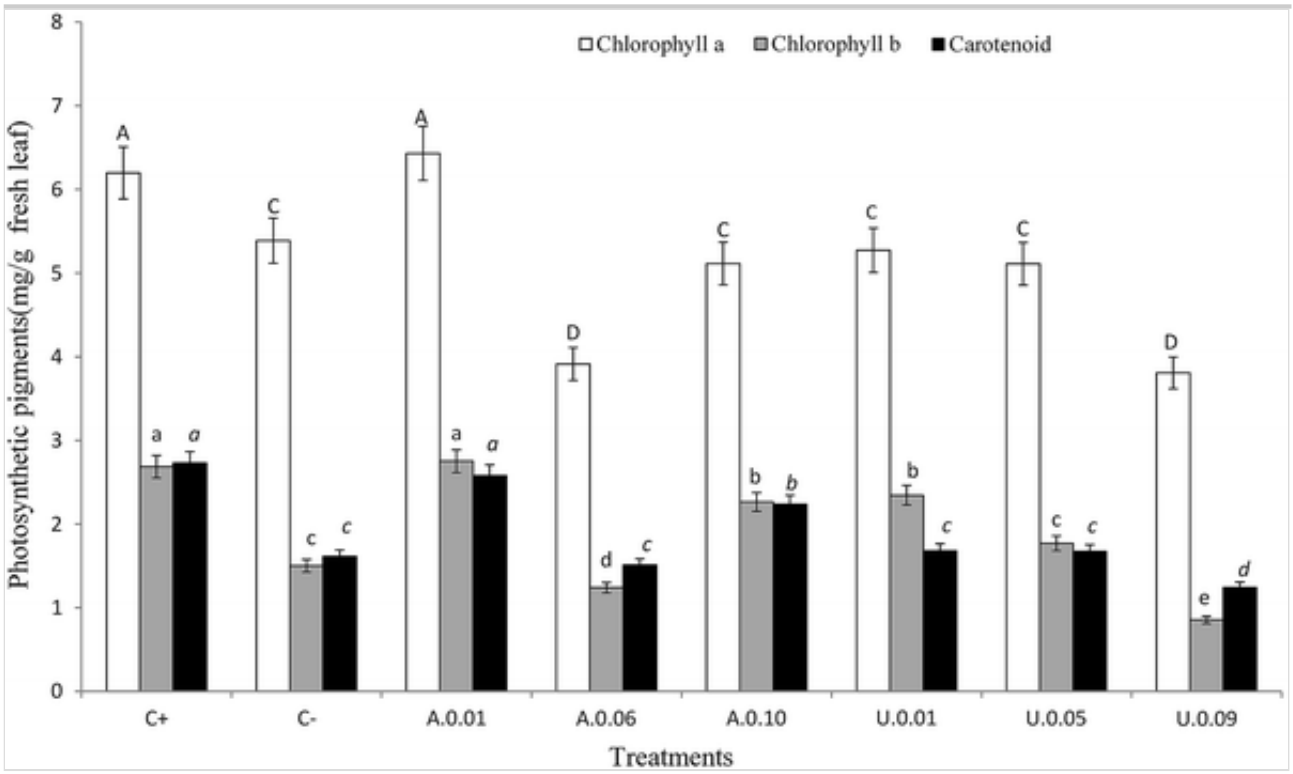
3. Results

Results of nitrogen supply on Chlorophyll (Chl) a, b and carotenoids are shown in Fig. 1. Concentrations of Chl a in C+ (positive control), C− (negative control), A.0.01(0.01 g/kg soil of NH_4NO_3), A.0.06 (0.06 g/kg soil of NH_4NO_3), A.0.10(0.10 g/kg soil of NH_4NO_3), U.0.01(0.01 g/kg soil of CH_4N_2O), U.0.05(0.05 g/kg soil of CH_4N_2O) and U.0.09(0.09 g/kg soil of CH_4N_2O) were 6.19, 5.38, 6.43, 3.91, 5.11, 5.27, 5.11 and 3.80 mg/g fresh leaf, respectively. These results showed no significant difference between A.0.01 and C+, but these two concentrations showed significant difference with all treatments ($P < 0.05$). Also, the concentrations of C−, A.0.1, U.0.01 and U.0.05 showed no significant difference at $P < 0.05$. The concentration of A.0.06 and U.0.09 showed the minimum concentration of Chl a, but these two concentrations were not significantly different from each other at $P < 0.05$. Among NH_4NO_3 treatments, significant differences in each concentration were observed (Fig. 1). Among CH_4N_2O treatments, U.0.01 and U.0.05 showed no significant differences but these two concentrations had significant difference with U.09 treatment ($P < 0.05$).

Fig. 1

Effects of various concentrations of NH_4NO_3 and CH_4N_2O (in g kg^{-1} soil) on the photosynthetic pigment contents in maize leaves. Means with the same

letter within an assay are not significantly different; *letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. (C+: positive control; C−: negative control; A.0.01: 0.01 g/kg soil of NH_4NO_3; A.0.06: 0.06 g/kg soil of NH_4NO_3; A.0.10: 0.10 g/kg soil of NH_4NO_3; U.0.01: 0.01 g/kg soil of CH_4N_2O; U.0.05: 0.05 g/kg soil of CH_4N_2O; U.0.09: 0.09 g/kg soil of CH_4N_2O)



Concentrations of Chl b in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 were 2.68, 1.49, 2.75, 1.23, 2.26, 2.34, 1.76 and 0.85 mg/g fresh leaf, respectively. The concentrations of A.0.01 and C+ showed no significant difference at $P < 0.05$. A.0.1 and U.0.01 showed no significant difference in Chl b concentrations at $P < 0.05$. The C- comparison with other treatments showed no significant difference with U.0.05 for Chl b concentrations. Among NH_4NO_3, treatments for Chl b concentrations observed that all treatments had significant difference at $P < 0.05$. Among CH_4N_2O, all treatments showed significant difference for Chl b concentration at $P < 0.05$.

The carotenoids contents in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 were 2.73, 1.61, 2.58, 1.50, 2.23, 1.67, 1.67 and 1.24 mg/g fresh leaf, respectively. The carotenoids contents showed no significant difference

in A.0.01 and C+ ($P < 0.05$) in comparison with other treatments. Among other treatments, the C−, A.0.06, U.0.01 and U.0.05 showed no significant difference ($P < 0.05$). Among all treatments, the minimum content of carotenoids was shown in U.09 treatment. Significant difference was observed among the NH_4NO_3 concentrations. In CH_4N_2O treatments, U.0.01 and U.0.05 showed no significant difference but U.0.09 treatment had significant difference with U.0.01 and U.0.09 treatments ($P < 0.05$).

A result of nitrogen supply on nitrate accumulation is shown in Table 2. The nitrate concentration in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 was 1000, 423, 1235, 1009, 802, 1017, 1241 and 808 μg/g fresh leaf, respectively. The results showed significant differences among NH_4NO_3 and CH_4N_2O treatments. We observed no significant difference between A.0.01 and U.0.05 treatments. Treatments A.0.10 and U.0.09 were not different. Treatments C+, A.0.06 and U.0.01 also showed no significant difference at $P < 0.05$. The minimum concentration of nitrate was observed in C− (423 μg/g fresh leaf). The U.0.05 treatment had the maximum concentration of nitrate with 1241 μg/g fresh leaf.

Table 2

Effects of different concentrations of NH_4NO_3 and CH_4N_2O (in g kg^{-1} soil) on the nitrate activity of maize leaves

	Treatments				
	Positive control (C+)	**Negative control (C −)**	**0.01 g/kg soil NH_4NO_3**	**0.06 g/kg soil NH_4NO_3**	**0.10 g/kg soil NH_4NO_3**
Nitrate concentration (μg/g fresh leaf)	1000 ± 0.01^b	423 ± 0.00^d	1235 ± 0.01^a	1009 ± 0.00^b	802 ± 0.0
Nitrite concentration (μM/g fresh leaf)	250 ± 0.00^b	98 ± 0.00^d	384 ± 0.1^a	232 ± 0.7^b	185 ± 1.0
Nitrate reductase activity (μmol/min)	3.99 ± 0.4^b	0.7 ± 0.0^d	5.2 ± 0.0^a	3.84 ± 0.1^b	2.43 ± 0.

Different letters indicate significant differences ($P < 0.05$) according to Duncan's mu

Values are expressed as mean ± SEM ($n = 3$)

Results of nitrogen supply on nitrite production are shown in Table 2. Nitrite production in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 was 250, 98, 384, 232, 185, 244, 371 and192 µM/g fresh leaf, respectively. We observed a similar trend with nitrate accumulation among all treatments. In NH_4NO_3 and CH_4N_2O treatments, we observed a significant difference among all concentrations at $P < 0.05$. The maximum and minimum concentration of nitrite was observed in A.0.01 (384 µM/g fresh leaf) and C− (98 µM/g fresh leaf), respectively. The result in comparison of C+ with other treatments showed that C+ was not different from A.0.06 and U.0.05 treatment. The results showed that with increasing the concentration of NH_4NO_3, nitrite production decreased. In CH_4N_2O treatments, the highest production was observed in U.0.05 treatment.

The result of nitrogen supply on nitrate reductase activity is shown in Table 2. The nitrate reductase activity in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 was 3.99, 0.7, 5.2, 3.84, 2.43, 3.69, 4.99 and 2.36 µmol/min, respectively. The results showed the same trend with nitrate accumulation and nitrite production in maize. The range of nitrate reductase activity in this study was between 0.7 and 3.99 µmol/min. The result showed that within two ~~grout~~ under treatment with different concentration of NH_4NO_3 and CH_4N_2O had a significant difference ($P < 0.05$). The C− treatment had the minimum nitrate reductase activity with 0.7 µmol/min. There was no significant difference among the treatments. Treatment U.0.05 with nitrate reductase activity of 4.99 µmol/min had maximum nitrate reductase activity among all treatments. The C+, A.0.06 and U.0.01 groups showed no significant different in nitrate reductase activity.

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The result of nitrogen supply on amino acid production is shown in Table 3. The amino acid production in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 was 24, 2.5, 9.2, 23, 13, 12, 22 and 10 µg/ml, respectively. Result showed that the amino acid production ranged between 2.5 and 24 µg/ml in

the maize cultivars studied. Treatments C+, A.0.06 and U.0.05 were not different in amino acid production. Also, in comparison with other treatment we observed no significant difference among A.0.01, A.0.10 and U.0.01 ($P < 0.05$). The C− was significantly different from all other treatments.

Table 3

Effect of different concentrations of NH_4NO_3 and CH_4N_2O (in g kg^{-1} soil) on the amir concentrations and proline content of maize leaves

	Treatments					
	Positive control (C+)	**Negative control (C −)**	**0.01 g/kg soil NH_4NO_3**	**0.06 g/kg soil NH_4NO_3**	**0.10 g/kg soil NH_4NO_3**	**0. so C**
Amino acid concentration (µg/ml)	24 ± 0.00^a	2.5 ± 0.00^c	9.2 ± 0.03^b	23 ± 0.01^a	13 ± 0.1^b	12
Total protein concentration (µg/g leaf)	352 ± 0.9^b	20 ± 0.06^e	154 ± 0.0^d	272 ± 1.0^c	455 ± 0.0^a	23
Carbohydrate concentration (µg/g leaf)	2.78 ± 0.3^a	1.4 ± 0.9^c	2.76 ± 0.0^a	2.1 ± 0.6^b	1.43 ± 0.1^c	2.
Proline content (mg/ml)	6.2 ± 0.0^d	14.4 ± 0.0^a	8.4 ± 0.8^c	8.0 ± 0.0^c	8.2 ± 0.0^c	12
Values are expressed as mean ± SEM ($n = 3$)						

The result of nitrogen supply on total protein production is shown in Table 3. The total protein production in C+, C−, A.0.01, A.0.06, A.0.10, U.0.01, U.0.05 and U.0.09 was 352, 20, 154, 272, 455, 239, 366 and 469 µg/g leaf, respectively.

Both NH_4NO_3 and CH_4N_2O supplementation decreased the total carbohydrate content in a dose-dependent manner. Differences among positive control, 0.01 g/kg soil of NH_4NO_3 and 0.01 g/kg soil of CH_4N_2O were not statistically significant (Table 3).

Only the negative control showed a significant increase in the proline content. All treatments significantly increased the proline content compared with positive control (Table 3).

4. Discussion

Morphological and physiological characteristics of plants are often influenced by the availability of resources, especially nitrogen fertilizer. The present result showed that an increase of nitrogen in treatment groups caused an increase in photosynthetic pigments of maize leaf. Plant growth and yield depend on the process of photosynthesis and nitrogen assimilation. Nitrogen can have a direct effect on the rate of photosynthesis per unit leaf area, besides influencing photosynthetic enzymes and increasing yield. Schnier et al. (1990) reported that an increase in leaf nitrogen concentration increased leaf area and photosynthesis in rice plants. Cassman et al. (1994) stated that, due to the presence of nitrogen in the structure of chlorophyll, there exists a strong positive association between leaf nitrogen and chlorophyll. In a study on CH_4N_2O as nitrogen source for maize, leaf nitrogen and chlorophyll increased significantly in the treated group compared with the control (Jeffrey and Gyles 2003). Nitrogen status has significant effects on the amount of carbohydrates. Results showed a negative correlation between NH_4NO_3 and nitrate/nitrite concentration in maize leaf. It may be due to the role of nitrogen in amino acid fixation that requires some Krebs cycle metabolites. Krebs cycle replaces these compounds and carbohydrates and their derivatives are required. In addition, reduced nitrate and nitrite require reducing power supplied through respiration and photosynthesis. If carbohydrates provided to respiration decline, as well as those arising from photosynthesis, less carbon dioxide is reduced to carbohydrate conversion (Minotti et al. 1969). Nitrate absorbed from soil by root cells is mediated by nitrate transporter. Nitrate is reduced in the roots or stored in cytoplasm of root cells and the excess is stored in vacuoles of leaf parenchyma cells. Therefore, nitrate accumulates in vacuoles, and the excess is transported to the leaf where it is reduced. The efficiency of net nitrate uptake is under negative feedback control by nitrate accumulation (Skrdleta et al. 1979; Chen et al. 2004). Consequently, when nitrate supply is higher than the plant needs, the decrease in nitrate accumulation may be due to the decrease of

nitrate uptake due to the negative feedback regulation by accumulated nitrate (Sivasankar et al. 1997; Stohr 1999). The rate of nitrate uptake relies on the activity of nitrate transport systems in the plasma membrane of root cells. External factors, such as nitrate concentration, as well as internal factors like nitrogen metabolites (ammonium and glutamine) all regulate the rate of nitrate uptake (Orsel et al. 2002). Exposure of roots to nitrate causes the induction of nitrate transport 2 transcripts, which leads to nitrate uptake by positive feedback, whereas metabolites resulting from nitrate reduction, most likely ammonia and glutamine, downregulate NRT2 (Remans et al. 2006; Walch-Liu and Forde 2008).

In conclusion, our results showed that nitrogen supply stimulates nitrate uptake, nitrate reduction, nitrite reduction, amino acid production, protein production and nitrate reductase activity at low concentrations of NH_4NO_3 and CH_4N_2O, by positive effects of nitrate on the nitrate assimilation pathway. Nevertheless, at high nitrogen supplies, nitrate metabolites, possibly ammonium and glutamine, suppress nitrate assimilation pathway and carbohydrate production by inhibiting the related rate-limiting enzymes in the biosynthetic pathway, as well as by reducing photosynthesis and ATP production. Proline amino acid content, in this study, did not show significant changes.

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