Influence of elevated carbon dioxide and ammonium nutrition on growth and nitrogen metabolism in wheat (*Triticum aestivum*)

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ABSTRACT

Growth under elevated CO2 (EC) conditions inhibits nitrate (NO3–) assimilation in crop plants, hence ammonium (NH4+) nutrition is beneficial compared to NO3– nutrition under EC conditions. In the present study, an attempt was made to compare the suitability NH4+ vs mixed NH4+ + NO3– nutrition in wheat (*Triticum aestivum* L.). Wheat seedlings supplied with NH4+ alone or with both NH4+ + NO3– as nitrogen (N) source and grown under ambient (380 µl/l, AC) or elevated (600±50 µl/l, EC) CO2 conditions were analyzed for growth and biochemical parameters. Plants receiving only NH4+ as N source showed significant reduction in growth parameters. The negative effects of NH4+ nutrition were manifested in form of reduced root length, root surface area and thinner leaves under EC. Negative effects of NH4+ nutrition were ameliorated in plants receiving mixed NH4+ + NO3– nutrition. Supplementing NO3–-N with NH4+-N led to the enhancement of various morphological and biochemical parameters in EC grown plants. Activity and gene expression of the enzymes, nitrate reductase and glutamine synthetase was significantly higher in plants supplied with both NH4+ + NO3– ions and grown in EC, as compared to plants grown in AC, resulting in lesser reduction in N content of the EC grown plants. Our study indicates that mixed NH4+ + NO3– nutrition will be more suitable for wheat cultivation under high CO2 conditions in future.

Key words: Ambient CO2, Ammonium nutrition, Elevated CO2, Wheat

Nitrogen (N) is a major regulator of plant response to elevated CO2 (EC) (Stitt and Krapp 1999). The reduction of nitrate and assimilation of ammonia are important components of the physiological responses to EC. It has been reported that EC induced changes in nitrate reductase (NR) activity, depending on the nitrogen source (Matt et al. 2001). There are contradictory reports indicating either increase (Larios et al. 2001) or decrease (Bloom et al. 2010) in NR activity in EC grown plants. The depression of nitrogen assimilation observed under EC was attributed to the competition between nitrate and carbon assimilation for reductants (Bloom et al. 2002). However, the higher levels of sugars under EC should promote transcription and post-translational activation of NR genes and thus enhance nitrate assimilation (Stitt and Krapp 1999). Moreover, since the carbon skeletons and ATP essential for ammonium assimilation are abundant under these conditions, the rate of glutamine synthetase (GS) activity would apparently be enhanced. Therefore, nitrogen metabolism as a whole is expected to be more efficient under EC than in plants grown under ambient CO2 (AC) conditions (Stitt and Krapp 1999).

The predominant form of N available to plants in most environments is NO3– (Epstein and Bloom 2005). Carbon dioxide inhibition of NO3– uptake and assimilation would lead to lower organic N production. This may be responsible for the 7.4 to 11% decrease in wheat grain protein (Hogy et al. 2009), observed under CO2 enrichment in FACE (free-air CO2 enrichment) experiments. CO2 inhibition of NO3– assimilation and the resultant decline in plant organic N contents might play a major role in CO2 acclimation, resulting in decline of photosynthesis, and growth of C3 plants after long exposures to CO2 enrichment (Dukes et al. 2005). *Scirpus olneyi*, the prominent C3 plant in the Chesapeake Bay marsh, an NH4+- dominated ecosystem, showed little CO2 acclimation, with slight change in N contents (Erickson et al. 2007).

Lekshmy et al. (2013) observed that in wheat, the rate of nitrate uptake and nitrate assimilation was significantly lower in EC grown plants when the availability of the nitrate was high. Under non-limiting nitrate supply, wheat shoots accumulated more NO3– ions under EC, but total shoot protein content decreased (Pal et al. 2003, Jain et al. 2007). Total reduced N declined with increase in C: N ratio. It is
speculated that application of NH$_4^+$ fertilizers and nitrification inhibitors might improve the N status of the plant, but may need revised fertilizer management to prevent NH$_4^+$ toxicity. The two major N forms, NH$_4^+$ and NO$_3^-$, have distinct physiological effects on plant growth and development. Carbon dioxide responses under controlled levels of NH$_4^+$ vs NO$_3^-$ as sole N sources in wheat have been studied earlier (Bloom et al. 2002, 2010). Effects of NO$_3^-$-NH$_4^+$ synergism on plant metabolism is well known (Cox et al. 1979). The suitability of NH$_4^+$ vs NH$_4^+/NO_3^-$ mixed nutrition in response to CO$_2$ enrichment is not yet known. Not much information is available on how various forms of N affect growth and N metabolism of wheat under EC. Hence, the aim of the present study was to find out the interactive effects of NH$_4^+$ nutrition and EC on growth and N assimilation in wheat.

MATERIALS AND METHODS

Wheat cv. PBW 343 was grown under two carbon dioxide levels, viz. AC (380 µl/l, AC) and EC (600±50 µl/l, EC) in Hoagland solution for twenty days in growth chambers in National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. The other growth conditions, viz. temperature (25°C/18°C D/N), photoperiod (14 h/10 h) and light intensity (500 µmol/m$^2$/s) were similar in both the growth chambers. Seedlings were raised in 1/2X Hoagland solution containing either NH$_4^+$ ions (1 mM (NH$_4^+$)$_2$SO$_4$) or both NH$_4^+$ and NO$_3^-$ ions (1mM NH$_4$NO$_3$) for twenty days in growth chambers in National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. The other growth conditions, viz. temperature (25°C/18°C D/N), photoperiod (14 h/10 h) and light intensity (500 µmol/m$^2$/s) were similar in both the growth chambers. Seedlings were raised in 1/2X Hoagland solution containing either NH$_4^+$ ions (1 mM (NH$_4^+$)$_2$SO$_4$) or both NH$_4^+$ and NO$_3^-$ ions (1mM NH$_4$NO$_3$) for twenty days in growth chambers in National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. The other growth conditions, viz. temperature (25°C/18°C D/N), photoperiod (14 h/10 h) and light intensity (500 µmol/m$^2$/s) were similar in both the growth chambers. Seedlings were raised in 1/2X Hoagland solution containing either NH$_4^+$ ions (1 mM (NH$_4^+$)$_2$SO$_4$) or both NH$_4^+$ and NO$_3^-$ ions (1mM NH$_4$NO$_3$) for twenty days in AC and EC grown plants supplied with either NH$_4^+$ or both NH$_4^+$ and NO$_3^-$ were used to study the changes in expression of NR and GS. Total RNA was extracted from leaves using RNAeasy plant minikit (Qiagen Inc., Chatsworth CA 91311, USA, Cat No: 749040). DNA contamination was removed from RNA samples using DNase I (Qiagen Science, Maryland, USA). One microgram of total RNA was reverse transcribed using Qiagen one step RT-PCR kit. Reactions were conducted using QN 96 Thermal cycler (Quanta biotech, England), under the following conditions: initial PCR activation step: 15 min at 95 ºC, reverse transcription: 30 min at 50 ºC, denaturation: 1 min at 94 ºC, annealing: 1 min at 57 ºC, extension: 1 min at 72 ºC and final extension: 10 min at 72 ºC. Linear amplification for semi-quantitative RT-PCR was obtained with 27 cycles. RT-PCR was done using two sets of primers. For NR (amplicon size: 400 bp): forward primer, 5’-CCGCGCGAGAAGGTCCCA TGT-3’; reverse primer, 5’- TCCGTCTCGTCCCTCCGGCTG-3’, for GS (amplicon size: 180 bp): forward primer, 5’-TGGCTGGCCTGTTGGAGGGT-3’, reverse primer, 5’-GTGCCCCAGCCGAGAACACACAG-3’. Actin (amplicon size: 400bp) was used as an internal standard and the primer sequence was Forward 5’ GATTATGAGCAGGACGTGGA 3’, Reverse 5’ CTGGAA AGTGCTAAGAGG 3’.  

RESULTS AND DISCUSSION

The global atmospheric CO$_2$ concentration has increased from pre-industrial level 280 µl/l to current level 400 µl/l and is predicted to reach between 450 and 900 ppm by the year 2100 (IPCC 2014). The concentration of atmospheric CO$_2$ and the availability of different nitrogen forms (NO$_3^-$ vs NH$_4^+$) controls the partitioning of photosynthetically derived energy and reducing equivalents between carbon and nitrogen assimilation (Searles and Bloom et al. 2010, Bloom 2015). In previous study conducted in our laboratory, it was shown that elevated CO$_2$ inhibited nitrate uptake and assimilation in wheat when N was non limiting (Lekshmy et al. 2009).

In the present study, leaf growth was promoted by EC, 68 and 25% increase in leaf area was observed in NH$_4^+$ + NO$_3^-$ and NH$_4^+$ grown plants respectively under EC (Table 1). Among the CO$_2$ treatments, specific leaf area declined significantly in NH$_4^+$ + NO$_3^-$ fed EC grown plants (Table 1). Reduction in specific leaf area under EC indicated
increased leaf thickness and greater tissue density in leaves. This increased leaf thickness under EC could be due to high rate of photosynthesis (Uprety et al. 2002, 2003). Dry mass per unit leaf area increased in rice genotypes exposed to EC (Uprety 2003). However, specific leaf area was found to be 5.5% higher in EC treatment, in NH4+ grown plants, resulting in significantly thinner leaves (Table 1).

Shoot dry weight was significantly higher in EC grown plants compared to AC grown plants, there was 25% increase in shoot dry weight in plants receiving both N forms, whereas only 12% increase in shoot dry weight was observed in plants receiving only NH4+ - N. Elevated CO2 stimulated shoot growth of the plants receiving ammonium as sole N source to twice as much as those receiving nitrate as sole N source (Bloom et al. 2002, 2010, Asensio et al. 2015). EC grown plants receiving either NH4+ or both NH4+ + NO3– as N source showed 29 and 14% increase in root surface area respectively, compared to AC grown plants. Similar trend was observed in root length also and plants receiving both N forms performed better with 29% increase of root length under EC. The comparatively longer root system of the CO2 enriched plants might enable them in exploring a greater volume of soil for N uptake, a character that may be important as EC grown plants accumulate more N than those grown at AC on per plant basis. The spatial deployment of root system largely determines the ability of the plant to exploit nutrient resources. The promotion of root growth in terms of both length and surface area were restricted in NH4+ grown plants (Table 1, Fig 1), indicating the beneficial effects of combined ammonium and nitrate nutrition under EC, especially for root growth. Among the CO2 treatments, the plants grown in EC had higher root shoot ratio, as compared to the plants grown in AC (Table 1). Higher root: shoot ratio indicated that, the distribution of dry matter was altered in wheat by EC. EC induced promotion of root growth in plants receiving both NH4+ + NO3– is reflected in root: shoot ratio also, there was 29% increase in root: shoot ratio in comparison with 5.5% increase in plants receiving only NH4+ nutrition (Table 1). Symptoms of NH4+ toxicity include disruption of cation homeostasis, leaf chlorosis, root growth inhibition, and lower plant biomass (Britto and Kronzucker 2002). Troelstra et al. (1995) suggested ammonium nutrition caused significant decrease in carbohydrate allocation to roots. Plants receiving NH4+ as sole N source had the lowest dry matter accumulation, lowest root length and leaf area (Table 1, Fig 1). This indicates that NH4+ toxicity was not fully alleviated by high CO2. Li and Zhou (2008) confirmed that CO2 enrichment does not alleviate the harmful effects of high NH4+-N concentration in nutrient solutions on the tomato root system. Hachiya et al. (2012) also asserted that plant growth was suppressed when ammonium was provided as sole N sources.

Activity of enzyme NR was enhanced in plants grown under EC in the leaves of the plants grown in combined ammonium and nitrate N, however, in plants receiving ammonium as sole N source NR activity was similar under both AC and EC (Fig 2A). Similarly the expression of NR gene was more in the seedlings grown under EC in both the treatments. Expression was more in NH4NO3 fed plants (Fig 2B). This is in line with observation that elevated CO2 increases NO3− uptake and NR activity when tobacco is growing on NO3−, but increases ammonium uptake and

<table>
<thead>
<tr>
<th>CO2 conc.</th>
<th>N source</th>
<th>Leaf area (cm²)</th>
<th>Specific leaf area (cm²/g)</th>
<th>Root surface area (cm²/plant)</th>
<th>Root length (cm/plant)</th>
<th>Shoot dry weight (mg/plant)</th>
<th>Root:Shoot ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>AN</td>
<td>7.47±0.9 459±9</td>
<td>2.08±0.01 22.09±0.55</td>
<td>0.151±0.001 0.37±0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS</td>
<td>6.81±1.0</td>
<td>416.2±4</td>
<td>1.92±0.00 20.43±0.21</td>
<td>0.142±0.002 0.36±0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>AN</td>
<td>12.58±0.55 324.8±2</td>
<td>2.68±0.12 28.51±1.16</td>
<td>0.19±0.001 0.48±0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS</td>
<td>8.55±0.78 437.5±6</td>
<td>2.19±0.11 23.28±0.80</td>
<td>0.16±0.003 0.38±0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD (P=0.05)</td>
<td>CO2</td>
<td>0.455 6.72</td>
<td>0.041 0.335</td>
<td>0.01 0.021</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrition</td>
<td>0.643 9.5</td>
<td>0.058 0.474</td>
<td>0.014 0.029</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO2×Nutrition</td>
<td>0.91 13.44</td>
<td>0.082 0.671</td>
<td>0.019 0.041</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig 1 Comparison of growth of wheat seedlings (20 d old) grown with either ammonium-N (1 mM (NH4+ SO4, AS) or both ammonium and nitrate-N (1 mM NH4NO3, AN) forms at ambient (380 µl/l, AC) and elevated (600±50 µl/l, EC) CO2 concentrations
inhibits NR activity when tobacco is growing on ammonium (Matt et al. 2001). Nitrate assimilation and the subsequent ammonium assimilation require carbon skeletons derived from photosynthesis for the synthesis of amino acids. Subsequently, enhanced CO2 fixation at elevated CO2, and concomitant increased carbon availability, might stimulate nitrate utilization by enhancing the expression and activity of NR and also that of chloroplastic GS (Larios et al. 2004). Aguera et al. (2006) recorded increased CO2 levels upregulated the activity of GS at high CO2. We recorded that the activity and expression of glutamine synthetase (GS) was high when N was supplied as ammonium nitrate. Maximum GS expression was observed in ammonium nitrate nutrition under EC (Fig 2 D). Carbon metabolites and light induces GS enzyme (Oliveira and Coruzzi 1999), thus supporting the increased GS activity under EC (Larios et al. 2004). Enhancement of carbon assimilation in plants grown under EC and associated higher carbohydrate availability appear to increase GS expression and activity (Robredo et al. 2010). Morcuende et al. (1998) also showed that sugars lead to an increased flux from nitrate to glutamine, providing evidence that sugars stimulate GS.

N content (%) of both shoots and roots was lower in EC grown wheat plants as compared to the AC grown plants, irrespective of the N treatments indicating overall lowering of protein content in the EC grown plants. In the EC treatment comparative to the AC treatment, shoot protein concentrations decreased 16% under NH4NO3 nutrition, as expected due to the dilution by additional biomass, but decreased 9% under ammonium nutrition, despite less additional biomass (data not shown). Thus, shoot protein per plant increased 4.9% and 1.89% under NH4NO3 and NH4+ respectively (Table 2). This implies that the amino acid biosynthesis is negatively affected under NH4+ nutrition, supported by the lower expression of GS. Growth

![Fig 2](image)

**Table 2** Effect of elevated atmospheric CO2 concentration and ammonical nutrition on nitrogen metabolism of wheat seedlings (20 d old) grown with either ammonium -N (1 mM (NH4+)2 SO4, AS) or both ammonium and nitrate- N(1 mM NH4NO3, AN) forms at ambient (380 µl/l, AC) and elevated (600±50 µl/l, EC) CO2 concentrations. Values are mean ± SE (n = 8)

<table>
<thead>
<tr>
<th>CO2 conc.</th>
<th>N source</th>
<th>Total reduced N in the shoots (mg/plant)</th>
<th>Total reduced N in the roots (mg/plant)</th>
<th>N content of shoot tissues (%)</th>
<th>N content of root tissues (%)</th>
<th>Shoot protein content (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC AN</td>
<td>5.34±0.29</td>
<td>0.763±0.00</td>
<td>4.45±0.19</td>
<td>2.39±0.06</td>
<td>3.90±0.01</td>
<td></td>
</tr>
<tr>
<td>AC AS</td>
<td>4.09±0.30</td>
<td>0.73±0.04</td>
<td>5.12±0.23</td>
<td>1.96±0.03</td>
<td>4.22±0.02</td>
<td></td>
</tr>
<tr>
<td>EC AN</td>
<td>4.42±0.20</td>
<td>0.901±0.02</td>
<td>3.71±0.03</td>
<td>2.00±0.06</td>
<td>4.09±0.06</td>
<td></td>
</tr>
<tr>
<td>EC AS</td>
<td>4.06±0.22</td>
<td>0.796±0.02</td>
<td>4.63±0.00</td>
<td>1.77±0.03</td>
<td>4.30±0.06</td>
<td></td>
</tr>
<tr>
<td>CD (P=0.05)</td>
<td>CO2</td>
<td>NS</td>
<td>0.101</td>
<td>0.032</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>Nutrition</td>
<td>0.188</td>
<td>0.048</td>
<td>0.144</td>
<td>0.045</td>
<td>0.081</td>
<td></td>
</tr>
<tr>
<td>CO2×Nutrition</td>
<td>0.266</td>
<td>0.068</td>
<td>NS</td>
<td>0.063</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>
under EC further decreased protein content in wheat seedlings irrespective of the N nutrition, however plants receiving both the N forms maintained higher protein content compared to plants receiving only NH$_4^+$ nutrition. This implies apart from dilution effect, the pathway of nitrogen assimilation is being inhibited leading to reduction in N content under EC. When expressed in per plant basis, the N content was high under EC in roots as compared to AC (Table 2). In the root tissue, N content was more in NH$_4^+$ + NO$_3^-$ grown plants both in AC and EC. Carlisle (2012) concluded that wheat plants fed with NH$_4^+$ allocated more nutrients to shoots and nitrate supplied plants allocated more nutrients to roots.

Shoot protein concentrations, and activities of nitrate assimilatory enzymes declined under EC more so in plants receiving nitrate as sole N source (Bloom et al. 2014). Selective inhibition of nitrate assimilation led to the accumulation of NO$_3^-$ in the shoots of wheat (Asensio et al. 2015). Leaf N concentrations and grain protein declined by more than 10% at elevated vs. ambient CO$_2$ in wheat plants receiving nitrate as predominant N form, whereas these parameters varied only slightly with CO$_2$ level in high N treatment, where ammonium was the predominante N form, and could compensate for CO$_2$ inhibition of shoot NO$_3^-$ assimilation because they received additional NH$_4^+$ (Bloom et al. 2012). Apart from assimilation, nitrate uptake is also inhibited by EC by change in gene expression and kinetic parameters of high affinity nitrate transporter in wheat (Lekshmy et al. 2009). The assimilation of NH$_4^+$ ions occurs in roots and takes place right after uptake to prevent intracellular accumulation of toxic NH$_4^+$ ions. Therefore, faster uptake and assimilation of NH$_4^+$ may serve to overcome the N limitation of plant growth under elevated CO$_2$. Moreover, high rate of ammonia assimilation increases the demand of the root for carbon skeletons and stimulates the transport of saccharides from the shoot to root. The higher demand for saccharides in the roots may prevent the excessive accumulation of saccharides in the leaves which is known to down regulate the rate of photosynthesis in plants grown under elevated CO$_2$ (Stitt 1991, Drake et al. 1997, Bloom 2015).

The negative effects of NH$_4^+$ nutrition were manifested more in forms of reduced root length, root surface area and thinner leaves under ECO$_2$. Negative effects of NH$_4^+$ nutrition were ameliorated in plants receiving mixed NH$_4^+$ + NO$_3^-$ nutrition. When NO$_3^-$ was given along with NH$_4^+$, the enhancement of various morphological and biochemical parameters was better in EC than when only NH$_4^+$ was given. NH$_4^+$ nutrition alone is not beneficial for wheat more so under EC as root growth and N assimilation was markedly inhibited which will not be favorable for nutrient acquisition under high CO$_2$ conditions in future. Due to the inhibitory effects of EC on NO$_3^-$ assimilation, NH$_4^+$ is the preferred N source for C3 plants grown under EC conditions. However, the present study indicates that mixed NH$_4^+$ + NO$_3^-$ nutrition was more beneficial than sole NH$_4^+$ nutrition for wheat.

REFERENCES


