



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

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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 $\text{NH}_4^+/\text{NO}_3^-$ 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 $\mu\text{l/l}$, AC) and EC (600 \pm 50 $\mu\text{l/l}$, 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 $\mu\text{mol/m}^2/\text{s}$) were similar in both the growth chambers. Seedlings were raised in 1/2X Hoagland solution containing either NH_4^+ ions (1 mM $(\text{NH}_4^+)_2\text{SO}_4$) or both NH_4^+ and NO_3^- ions (1mM NH_4NO_3) as N source. Solution was changed every alternate day without disturbing the root system.

Twenty day old plants were harvested from the growing media, excess water was removed from the roots and the root and shoot tissues were kept in an oven at 60°C and dried till constant tissue dry weight was achieved. Root: shoot ratio was calculated from the dry weight of roots and shoots. Leaf area was recorded using leaf area meter (LiCOR 3100, Lincoln Nebraska, USA). Leaves were then kept in an oven at 60°C till constant dry weight was recorded. Specific leaf area was calculated using leaf area and leaf dry weight and was expressed as cm^2/g . Root surface area was estimated following the method of Ansari *et al.* (1995), based on adsorption of NO_2^- ions on negatively charged root surface. The method was calibrated with wheat root sections of known surface area. The factor for calculating surface area was worked out to be 40.36 mmol NO_2^- for 100 cm^2 root surface area (Kalita 1999). The diameter of the same roots were measured at several points by vernier calipers. Root length was calculated using the following formula. Root surface area = $\pi d(l/2+1)$, l = length in cm, d = diameter in cm, A = area in cm^2 .

Total N in roots and shoots was measured separately using N_2 analyzer (Gerhardt, Turbotherm digestion unit and Gerhardt Vapodest distillation unit). Shoot protein content was calculated by multiplying with conversion factor 5.81 (Fujihara *et al.* 2008). Estimation of *in vivo* nitrate reductase activity was done by estimating nitrite formed by the enzyme present in cells and nitrite formed was then

diazotized using sulphanilamide in acidic medium and NEDD using the method of Nair and Abrol (1973). Nitrite was estimated by the method of Evans and Nason (1953). Glutamine synthetase activity was assayed following the method of Mohanty and Fletcher (1980). The data was analysed statistically using 2 factorial CRD. F-test was carried out to test the significance of the treatment differences and the least significant difference (LSD) was computed to test the significance of different treatments at 5 % level of probability by SPSS 10.0.

To confirm the data obtained from biochemical analysis, gene expression studies were conducted. Ten days old 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 RNeasy 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 °C, reverse transcription: 30 min at 50 °C, denaturation: 1 min at 94 °C, annealing: 1 min at 57 °C, extension: 1 min at 72 °C and final extension: 10 min at 72 °C. 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'-CGCGCGAGAAGGTCCCATGT-3'; reverse primer, 5'-TCCGTCTCGTCTCCGCGCTG-3', for GS (amplicon size: 180 bp): forward primer, 5'-TGGCTGGCCTGTTGGAGGGT-3', reverse primer, 5'-GTGCCCCGACGGAACCACAG-3'. Actin (amplicon size: 400bp) was used as an internal standard and the primer sequence was Forward 5' GATTATGAGCAGGAGCTGGA 3', Reverse 5' CTGGAA AGTGCTAAGAGAG 3'.

RESULTS AND DISCUSSION

The global atmospheric CO_2 concentration has increased from pre- industrial level 280 $\mu\text{l/l}$ to current level 400 $\mu\text{l/l}$ 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 $\text{NH}_4^+ + \text{NO}_3^-$ and NH_4^+ grown plants respectively under EC (Table 1). Among the CO_2 treatments, specific leaf area declined significantly in $\text{NH}_4^+ + \text{NO}_3^-$ fed EC grown plants (Table 1). Reduction in specific leaf area under EC indicated

Table 1 Effect of elevated atmospheric CO₂ concentration and ammonium nutrition on growth parameters of wheat seedlings (20 d old) grown with either ammonium -N (1 mM (NH₄⁺)₂SO₄, 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)

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

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



Fig 1 Comparison of growth of wheat seedlings (20 d old) grown with either ammonium-N (1 mM (NH₄⁺)₂SO₄, 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

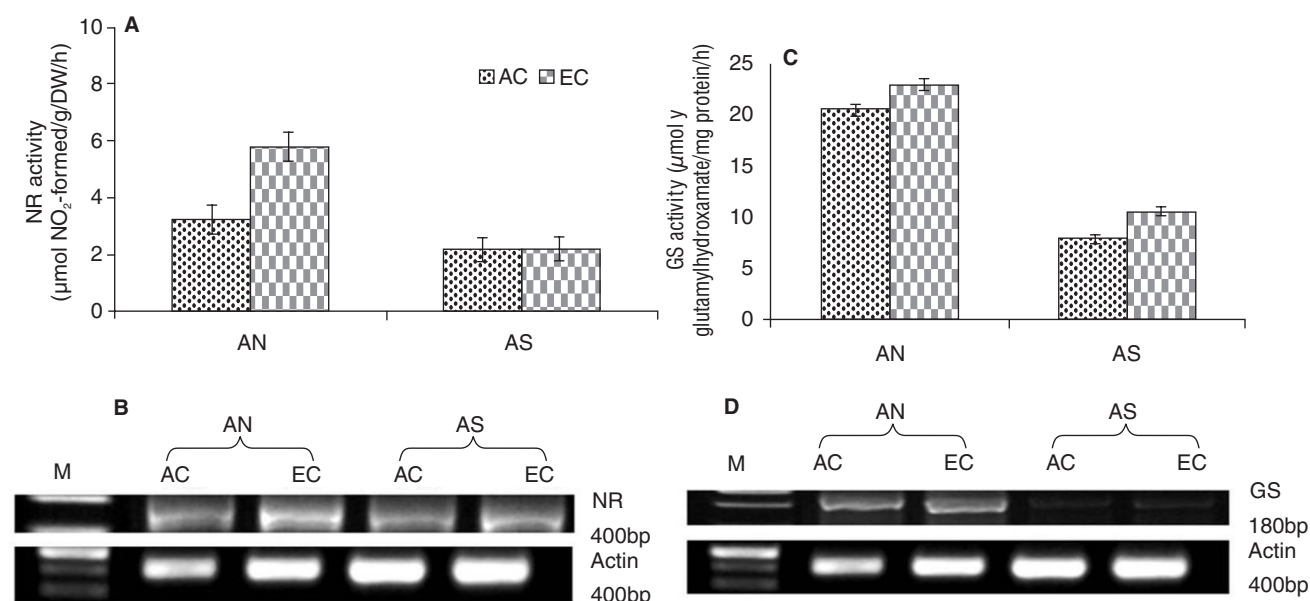


Fig 2 Nitrate reductase (NR) activity (A), *NR* gene expression (B), Glutamine synthetase (GS) activity (C) and *GS* gene expression (D) in wheat (PBW-343) seedlings grown with either ammonium - N (1 mM $(\text{NH}_4^+)_2\text{SO}_4$, AS) or both ammonium and nitrate -N (1 mM NH_4NO_3 , AN) forms at ambient (380 $\mu\text{l/l}$, AC) and elevated (600 \pm 50 $\mu\text{l/l}$, EC) CO_2 concentrations. M: Molecular weight ladder (1 Kb). Values are mean \pm SE (n = 12)

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 CO_2 fixation at elevated CO_2 , 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 CO_2 levels upregulated the activity of GS at high CO_2 . We recorded that the activity and expression of glutamine synthetase (GS) was high when N was supplied as ammonium nitrate. (Fig 2 C, D). 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 NH_4NO_3 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 NH_4NO_3 and NH_4^+ respectively (Table 2). This implies that the amino acid biosynthesis is negatively affected under NH_4^+ nutrition, supported by the lower expression of GS. Growth

Table 2 Effect of elevated atmospheric CO_2 concentration and ammonical nutrition on nitrogen metabolism of wheat seedlings (20 d old) grown with either ammonium -N (1 mM $(\text{NH}_4^+)_2\text{SO}_4$, AS) or both ammonium and nitrate- N(1 mM NH_4NO_3 , AN) forms at ambient (380 $\mu\text{l/l}$, AC) and elevated (600 \pm 50 $\mu\text{l/l}$, EC) CO_2 concentrations. Values are mean \pm SE (n = 8)

CO_2 conc.	N source	Total reduced N in the shoots (mg/plant)	Total reduced N in the roots (mg/plant)	N content of shoot tissues (%)	N content of root tissues (%)	Shoot protein content (mg/plant)
AC	AN	5.34 \pm 0.29	0.763 \pm 0.00	4.45 \pm 0.19	2.39 \pm 0.06	3.90 \pm 0.01
	AS	4.09 \pm 0.30	0.73 \pm 0.04	5.12 \pm 0.23	1.96 \pm 0.03	4.22 \pm 0.02
EC	AN	4.42 \pm 0.20	0.901 \pm 0.02	3.71 \pm 0.03	2.00 \pm 0.06	4.09 \pm 0.06
	AS	4.06 \pm 0.22	0.796 \pm 0.02	4.63 \pm 0.00	1.77 \pm 0.03	4.30 \pm 0.06
CD (P=0.05)	CO_2	NS	0.034	0.101	0.032	0.077
	Nutrition	0.188	0.048	0.144	0.045	0.081
	$\text{CO}_2 \times \text{Nutrition}$	0.266	0.068	NS	0.063	N.S.

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 $\text{NH}_4^+ + \text{NO}_3^-$ 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 predominate 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^+ . 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 $\text{NH}_4^+ + \text{NO}_3^-$ 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 $\text{NH}_4^+ + \text{NO}_3^-$ nutrition was more beneficial than sole NH_4^+ nutrition for wheat.

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