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Ammonium enhances growth and stress defense of *Medicago sativa* under salinity

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Abstract
This study investigated the effects of nitrate (NO_3^-) and ammonium (NH_4^+) nutrition on the growth and physiology of *Medicago sativa* L. under salt stress. Plants were grown for 40 days in sand–peat (2:1) medium supplied with either 3 mM NO_3^- or 3 mM NH_4^+, with or without 100 mM NaCl. Growth, ion content, chlorophyll concentration, and total polyphenol content were assessed in shoots. In control conditions, NO_3^- nutrition promoted higher biomass production than NH_4^+. Salt stress reduced growth by 68% in NO_3^--fed plants but increased biomass by 24% in NH_4^+-fed plants. NH_4^+ nutrition under salinity lowered Na^+ accumulation in shoots and enhanced chlorophyll and polyphenol contents compared with NO_3^-. These findings indicate that *M. sativa* is more tolerant to salinity when supplied with NH_4^+, likely due to reduced Na^+ uptake and improved antioxidant capacity. NH_4^+ fertilization could thus mitigate salt stress effects in alfalfa cultivation.

Keywords: Ammonium, *Medicago sativa*, mineral nutrition, NaCl stress, nitrate, polyphenols

Introduction

Nitrogen (N) is an essential macronutrient for plant growth and development, serving as à structural component of proteins, chlorophyll, nucleic acids, phytohormones, and numerous secondary metabolites [1, 2]. It also plays a regulatory role in photosynthetic enzyme production, ATPase activity, photosystem function, and electron transport in chloroplasts [3]. Plants absorb N mainly in two inorganic forms—nitrate (NO_3^-) and ammonium (NH_4^+). While NO_3^- is generally the preferred source for most species, including many Fabaceae [4, 5], NH_4^+ can be advantageous under certain conditions [6], especially as excessive NO_3^- accumulation in leaves can be harmful to animal and human health. The physiological response to N form varies among species. Exclusive NH_4^+ nutrition can reduce root and shoot biomass in some plants [7, 8], mainly due to rhizosphere acidification from proton release during NH_4^+ assimilation [9], which disrupts nutrient uptake. This growth inhibition is often linked to reduced root K^+ content, suggesting NH_4^+/K^+ competition at low pH [10]. Additionally, NH_4^+ may directly cause cytoplasmic toxicity at high concentrations and compete with K^+, Mg^{2+}, and $Ça^{2+}$ for uptake sites [1, 11]. Salinity is a major and growing threat to agriculture, causing osmotic stress, ion toxicity, and nutrient imbalances [13]. The use of saline irrigation water contributes to soil salinization, and FAO projections indicate that up to 50% of arable land could be affected by 2050 [14]. Fertilization strategies can influence plant responses to salinity, as N form affects ion balance, osmotic adjustment, and nitrogen metabolism [15, 16]. While NaCl can inhibit nitrification and increase soil NH_4^+ levels [17], plant responses to N form under salinity are species-specific: for example, *Populus simonii* grows better under NO_3^--rich conditions in saline environments [18], whereas Carrizo citrange exhibits greater salt resistance with NH_4^+ nutrition [19]. *Medicago sativa* (alfalfa) is a globally important forage crop valued for its adaptability, high biomass production, and nutritional quality, with considerable genetic variability in salt tolerance [20, 21]. However, despite the recognized importance of N form in modulating salinity tolerance, the specific physiological and biochemical mechanisms underlying this interaction in *M. sativa* remain unclear.

This study provides the first detailed comparative analysis of NO_3^- and NH_4^+ nutrition in *M. sativa* under salt stress, focusing on growth, ion homeostasis, photosynthetic pigment content, and antioxidant activity. We aimed to (i) determine the preferred N form under non-saline conditions, and (ii) elucidate how each N form influences plant tolerance to 100 mM NaCl. By integrating physiological and biochemical parameters, we identify key mechanisms through which NH_4^+ mitigates salt-induced damage, offering new insights into fertilization strategies for improving alfalfa production in saline soils.

Material and Methods

Growth Condition and Nitrogen Treatments

Under accession number NGBTUN1469 from the National Gene Bank of Tunisia (NGBT), seeds of *Medicago sativa* L. var. *Gabès*—a local Tunisian cultivar—were obtained. The seeds, collected in 2023, were provided by Dr. Sabah M'rah from El Kef, Tunisia, and are stored at the NGBT for research and conservation purposes. The plant material was formally identified by Pr. Mouhiba Ben Nasri Ayachi, former Professor of Botany and Curator of the Herbarium at the Faculty of Sciences of Tunis (FST)**,** University of Tunis El Manar**.** A voucher specimen of *Medicago sativa* L. var. *Gabès* was deposited in the Herbarium of the Faculty of Sciences of Tunis (FST): its voucher number is currently being processed. The collection and use of the plant material comply with national regulations and were conducted with permission from the NGBT**.** Alfalfa seeds (*Medicago sativa* L.) were surface-sterilized with 20% (v/v) bleach and sown in black plastic pots containing a pre-sterilized sand-peat mixture (2:3 ratios). The substrate underwent dual sterilization—treatment with sulphuric acid followed by autoclaving at 120°C for 90–150 minutes. Plants were grown under controlled environmental conditions: a 16-h photoperiod with 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, day/night temperatures of 22°C/18°C, and relative humidity levels of 60% (day) and 80% (night).

Experimental Treatments

At the start of the experiment, plants were divided into two groups receiving different nitrogen sources: one group was irrigated with modified Hoagland solution (1950) [22] containing 3 mM NO_3^- (nitrate), while the other received the same solution with 3 mM NH_4^+ (ammonium). On day 20 post-germination, each group was further split into two subgroups - a control without NaCl addition and a treatment group receiving 100 mM NaCl. This resulted in four distinct growth media: M1 (Control, 3 mM NO_3^-), M2 (3 mM NO_3^- + 100 mM NaCl), M3 (3 mM NH_4^+), and M4 (3 mM NH_4^+ + 100 mM NaCl). The nutrient solutions were refreshed every three days to maintain stable nutrient concentrations and minimize potential depletion effects.

Harvesting Plants, Growth Measurements and Analysis

After 40 days of treatment exposure, plants were harvested for physiological and biochemical analyses. Due to sampling difficulties with root systems, only aerial parts were used in this study. The harvested shoots were immediately placed in pre-weighed kraft paper pouches. Fresh weight was measured using an analytical balance (OPTIKA, precision ±0.1 mg), while dry weight was determined after 72 hours of oven-drying at 60°C. Shoot length was measured using a standard ruler.

Water Content

Dry matter (DM) content was determined by drying both roots and shoots at 70°C for 48 hours, followed by weighing using an analytical balance with 0.1 mg precision (OPTIKA). Water content (WC) was calculated using the formula: WC = (FW - DW) /DW, where FW represents fresh weight and DW represents dry weight. The results were expressed as milligrams of water per gram of dry matter (mg H_2O/g DM). The water content expressed in ml. g^{-1} DW is estimated by the difference between fresh mass and dry mass, in relation to the unit of dry mass.

Mineral Element Assays

For cation analysis, potassium (K^+) and sodium (Na^+) were extracted from 20 mg aliquots of oven-dried shoot material using 25 mL of 0.5% nitric acid solution. Cation concentrations were determined by flame photometry and expressed as milliequivalents per gram dry weight (meq g^{-1} DW). Nitrate (NO_3^-) content was measured colorimetrically following the method of Miranda *et al*. (2001) [23], while ammonium (NH_4^+) concentration was quantified using the Berthelot reaction as described by Weatherburn (1967) [24].

Chlorophyll Determination

Chlorophylls were extracted from fresh leaf material in 80% acetone. Absorbance was measured with a Beckman DU 640 spectrophotometer and the chlorophyll concentration were calculated according to Torrecillas *et al*. (1984) [25].

Proteins Content

Leaves from separate plants were excised and immediately placed in liquid nitrogen. They were later ground in the presence of liquid nitrogen, in a 50mM pH 7.5 phosphate buffer containing 1mM EDTA, 1mM DTT, 5% glycerol and 5% polyvinylpyrrolidone, and centrifuged for 20min at 15000g. The concentration of proteins was determined according to the method of Bradford (1976) [26] using bovine serum albumin (BSA) as the standard.

Total Polyphenol Content

Colorimetric quantification of total polyphenol content (TPC) was conducted using the Foline Ciocalteu reagent, as previously described by Singleton and Rosi (1965) [27] and slightly modified by Dewanto *et al*. (2002) [28]. The absorbance was read at 760nm using an UV/VIS spectrophotometer (Perkin Elmer, lamda 25,). The TPC was expressed as milligram gallic acid equivalent per gram of dry weight (mg GAE.g^{-1} DW), through the calibration curve of gallic acid (0–500mg l^{-1}).

Statistical Analysis

The results are the averages of six independent biological replicates. The data (individual values) were subjected to a one-way ANOVA analysis using the CoStat software and the means were compared according to the Tukey test at a significance level of P=0.05.

Results

Plant Growth and Water Content

Clear differences in shoot growth of *Medicago sativa* were observed among the four treatments after 40 days (Figure1).

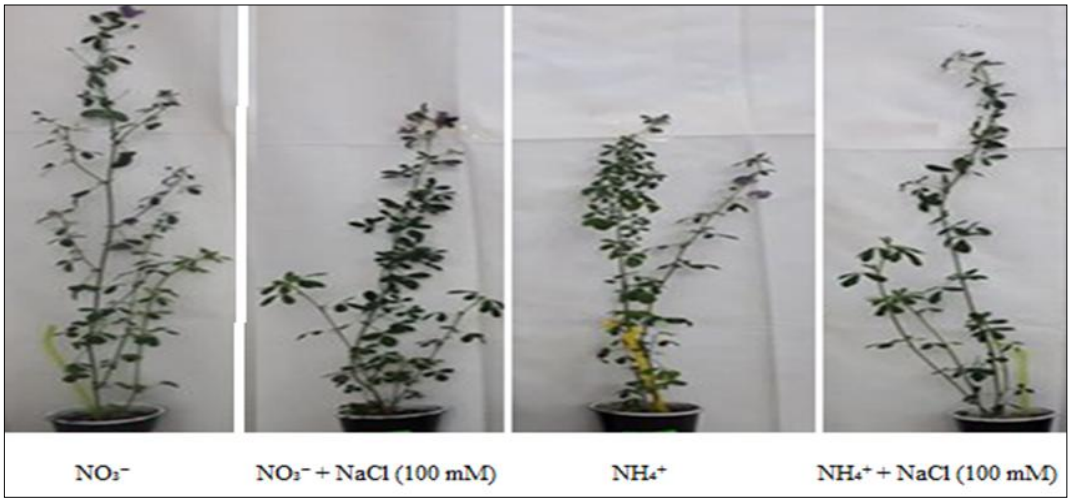

Fig 1: Shoot morphology of *Medicago sativa* after 40 days of growth under different nitrogen and salinity treatments: NO_3^-, NO_3^- + NaCl (100 mM), NH_4^+, and NH_4^+ + NaCl (100 mM)

Plants grown with NO_3^- alone developed tall shoots with moderate branching and flowering, while the addition of NaCl (NO_3^- + NaCl) strongly inhibited growth, leading to reduced leaf density, severe yellowing, and stunted development. By contrast, plants supplied with NH_4^+ showed vigorous growth, greener foliage, and more compact shoots compared to the NO_3^- treatment. Importantly, even under salinity (NH_4^+ + NaCl), ammonium-fed plants maintained relatively better shoot elongation, higher leaf vitality, and sustained flowering compared to NO_3^- + NaCl plants. These findings confirm that ammonium nutrition mitigates the detrimental effects of salinity on Medicago sativa by promoting shoot growth and preserving stress tolerance. Plants fed only with NO_3^- (3 mM) as a nitrogen source showed higher biomass production than crops in the presence of 3 mM NH_4^+ (Figure 2 A).

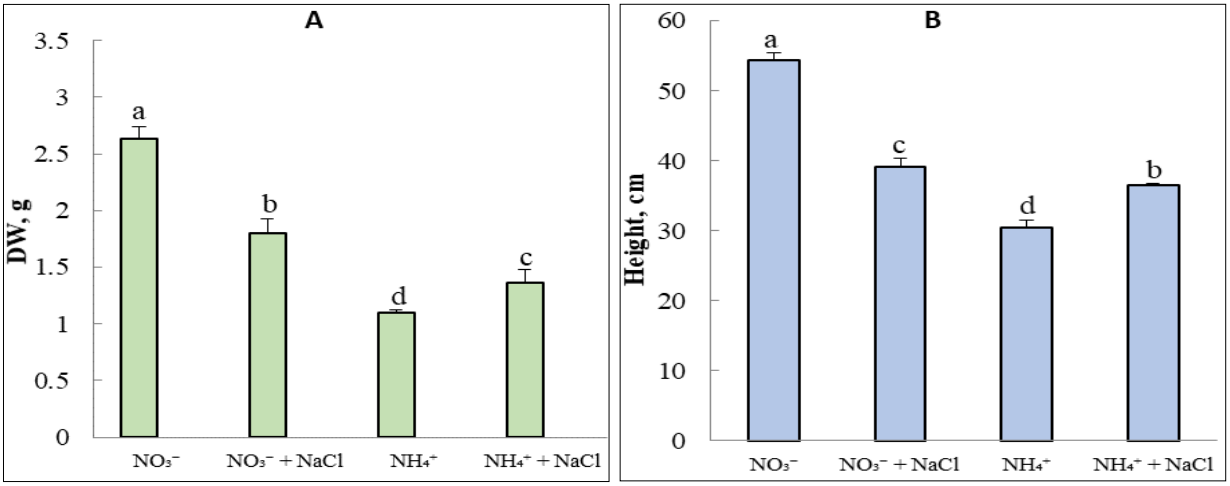

Fig 2: Variation of the dry weight (A) and the height (B) of the shoot of *Medicago sativa* grown on NO_3^- or NH_4^+, in the absence (0mM) or the presence (100mM) of NaCl during 40 days. Means of six replicates±standard error. For each organ, bars labeled with different letters are significantly different according to Tukey's multiple-range test at 5%

In fact, on a strictly ammoniacal medium, plant growth is reduced by 58.17% compared to that of plants grown on a nitrate medium. The addition of 100 mM of NaCl to the medium induced a significant restriction of the growth of the crop plants in the presence of NO_3^- (68%). Whereas the addition of 100 mM of NaCl in the presence of 3 mM of ammonium induced a 24% increase in the above-ground biomass of plants compared to that of plants receiving only ammonium and not subjected to salt. On a strictly nitric medium, the height of the plants decreased by 28% in the presence of salt. Plants grown in the presence of ammonium have a shoot height that has decreased by 44% compared to that of plants fed with nitrate (Figure 2 B). On the other hand, on an ammoniacal medium, salt increased this parameter by 33%. Shoot moisture content was reduced in NaCl-treated plants grown in the presence of NO_3^-, but was increased by salt if the plants were grown only in the presence of NH_4^+ (Figure 3).

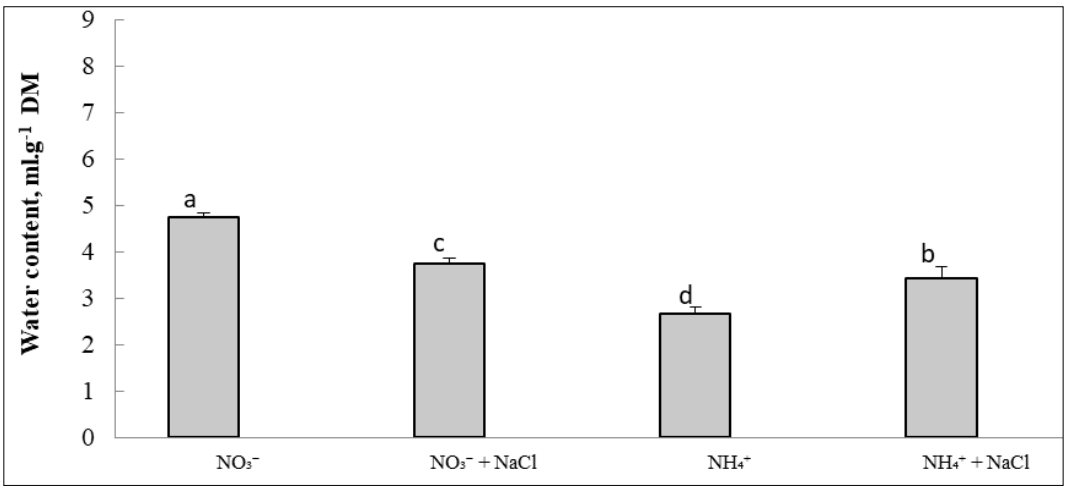

Fig 3: Water content in the shoot of *Medicago sativa* grown on NO_3^- or NH_4^+, in the absence (0 mM) or the presence (100mM) of NaCl during 40 days. Means of six replicates±standard error. For the same organ, bars labeled with different letters are significantly different according to Tukey's multiple-range test at 5%

Protein content in *Medicago sativa* shoots was significantly influenced by the nitrogen source and the presence of salt stress (Fig. 4). Plants supplied with nitrate (NO_3^-) exhibited the highest protein content, reaching 13.5 mol g^{-1} FW, while those grown with ammonium (NH_4^+) accumulated 9.0 mol g^{-1} FW, corresponding to a 33% decrease compared with nitrate-fed plants. The addition of NaCl markedly reduced protein accumulation under both nitrogen regimes. In nitrate-fed plants, protein content declined to 6.8 mol g^{-1} FW, representing a 50% reduction compared with nitrate

alone. In ammonium-fed plants, protein levels dropped to 4.2 mol g^{-1} FW, corresponding to a 53% reduction compared with Ammonium alone and a 69% decrease compared with nitrate-fed control plants. Overall, the lowest protein content was observed in the NH_4^+ + NaCl treatment.

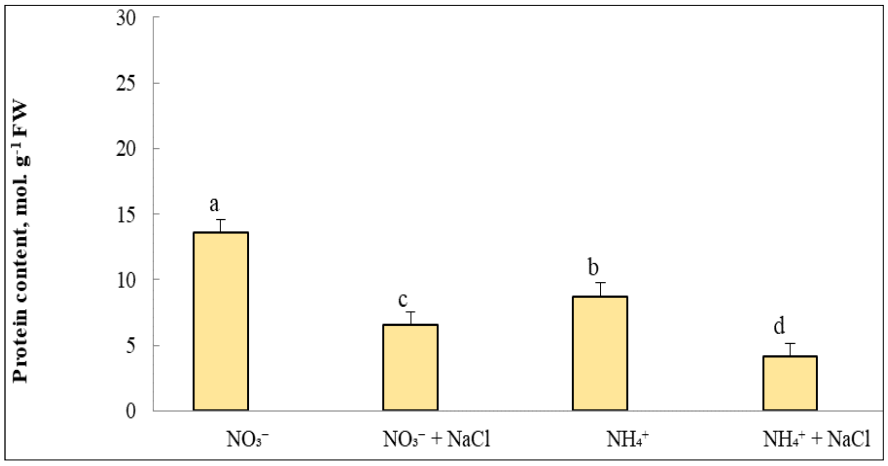

Fig 4: Protein content in the shoot of *Medicago sativa* grown on NO_3^- or NH_4^+, in the absence (0 mM) or the presence (100mM) of NaCl during 40 days. Means of six replicates±standard error. for the same organ, bars labeled with different letters are significantly different according to Tukey's multiple-range test at 5%

Ion Accumulation

In the absence of NaCl, the substitution of nitrate by ammonium in the culture medium restricted the accumulation of K^+ in shoots (Table 1). Indeed, the K^+ content increases from 2.09 meq g^{-1} DW in the shoots of plants receiving only NO3 to 0.70 in plants grown in the presence of only NH_4^+ (Table 1). The presence of 100mM NaCl further limited the accumulation of K^+. In all cases, potassium levels in the presence of ammonium without or with salt are always lower than those in the presence of NO_3^-. NaCl-treated plants showed a significant increase in the concentration of Na^+ in tissues compared to untreated plants. With respect to the effect of the N source on Na+ accumulation, plants accumulated more Na^+ in their shoots when grown in a medium containing NO_3^-. In fact, in the presence of nitrate, the sodium content in the shoots is 1.5 times higher than that in the presence of ammonium (Table 1). Regarding the variation of the accumulation of NO_3^- and NH_4^+ in the aerial tissues as a function of the nitrogen source and salinity, Table 1. shows that on control medium the addition of 3 mM of NO_3^- or 3 mM of NH_4^+ in the nutrient solution induced a respective and equitable increase of NO_3^- or NH_4^+ in the shoots. Our result also shows that NaCl exerted a contradictory effect on the accumulations of NO_3^- and NH_4^+ in the photosynthetic parts of the plant. Indeed, the addition of NaCl (100 mM) to the medium induced a significant decrease in the accumulation of NO_3^- in the organs of the treated plants. It should be noted, on a strictly ammoniacal medium, the increase in the NH_4^+ content in the tissues of plants treated with NaCl compared to those grown in the absence of salt. This increase is 24% (Table 1).

Table 1: K+, Na+, NO3- and NH4+ in shoots of *Medicago sativa* grown on NO3- or NH4+ in the absence (0mM) or presence (100mM) of NaCl during 40 days. Each value is the mean of six replicates corresponding to different plants. For each element, values marked with different letters are significantly different according to Tukey's multiple-range test at 5%

Concentration	NO_3^-(3mM)		NH_4^+(3mM)	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
K^+ (méq. g^{-1} DW)	2,09 ±0,29^a	1,06 ±0,09^b	0,70 ±0,09^c	0,41 ±0,10^d
Na^+ (méq. g^{-1} DW)	0,05 ±0,01^c	2,90 ±0,06^a	0,03 ±0,00^c	1,90 ±0,04^b
NO_3^- (µmol. g^{-1} DW)	30,00 ±3,01^a	20,50 ±1,09^b	3,15 ±0,19^c	2,87 ±0,12^d
NH_4^+ (µmol. g^{-1} DW)	3,11 ±1,00^c	2,99 ±0,89^c	30,49 ±1,11^b	37,87 ±1,04^a

Total Chlorophyll and Total Polyphenol Contents

NaCl 100 mM, induced a significant reduction in total chlorophyll content in nitrate-fed plants. On the other hand, on ammonia medium, the total chlorophyll content increased by 22% compared to that of plants grown in the absence of salt (Table 2).

Table 2: Total chlorophyll and total polyphenol in shoots of *Medicago sativa* grown on NO3- or NH4+ in the absence (0mM) or presence (100mM) of NaCl during 40 days. Each value is the mean of six replicates corresponding to different plants. For each element, values marked with different letters are significantly different according to Tukey's multiple-range test at 5%

Concentration	NO_3^-(3mM)		NH_4^+(3mM)	
	0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
Chlorophyll (µg.mg^{-1} FW)	2,86 ±0,24^a	1,99 ±0,14^b	1,29 ±0,03^d	1,57 ±0,18^c
Polyphenol (mg EAG.g^{-1} DW)	1,25 ±0,08^d	1,45 ±0,28^c	2,88 ±0,23^b	3,38 ±0,12^a

In this study, the determination of the total polyphenol content was carried out in plants growing on both nitrogen sources and in the presence or absence of NaCl (Table 2). Our results showed that the content of these antioxidant molecules increased with the addition of salt in both nitrate-fed and ammonium-fed plants. Plants grown in the presence of ammonium always have the highest levels in the presence and presence of salt. The addition of salt increased these levels by 8% and 17%, respectively, in plants fed nitrate and ammonium compared to their salt-free controls (Table 2).

Discussion

Fabaceae species are able to store and use nitrogen through two strategies that depend on soil nitrogen availability. In the absence of mineral nitrogen, they can fix atmospheric nitrogen through symbiosis with rhizospheric nitrogen-fixing bacteria [29]. However, this process is energetically costly for the plant. When nitrogen is abundant in soil, plants instead preferentially absorb it directly, saving energy. The two main mineral nitrogen forms available to plants are nitrate (NO_3^-) and ammonium (NH_4^+) [30].

Several studies have demonstrated that NO_3^- is the preferred nitrogen source for many species, as it promotes optimal growth compared to NH_4^+ [31, 32]. Our results confirm this, since *Medicago sativa* showed significantly higher biomass when supplied with NO_3^- rather than NH_4^+ at the same concentration. When NH_4^+ is the sole nitrogen source, it often restricts plant growth due to ammonium toxicity caused by excessive accumulation [33]. Nevertheless, NH_4^+ may also play regulatory roles by triggering oxidative stress signaling and activating metabolic pathways associated with reactive oxygen species [34]. In some species capable of storing NH_4^+ in vacuoles, ammonium nutrition can even be advantageous, as NH_4^+ uptake and assimilation require less energy than that of NO_3^- [35]. In our study, *M. sativa* grown with NH_4^+ alone maintained growth, although at a slower rate than plants fed with NO_3^-.

Nitrogen nutrition is highly sensitive to abiotic stressors such as salinity [36]. The effect of salinity on growth depends strongly on whether nitrogen is supplied as NH_4^+ or NO_3^-. Literature results are sometimes contradictory: Gholamnejad *et al*. (2023) [37] reported that replacing NO_3^- with NH_4^+ increased salt sensitivity in tomato, whereas Botella *et al*. (1997) [38] and Miranda *et al*. (2015) [35] found that wheat and sorghum grown with NH_4^+ tolerated salt better than those fed with NO_3^-. Our findings support the latter, as *M. sativa* grown under NaCl stress with NO_3^- suffered greater growth inhibition than those supplied with NH_4^+. This highlights that the combined effect of nitrogen source and salinity is species-specific and may even vary within the same species.

The higher protein content observed under nitrate nutrition compared to ammonium indicates that nitrate is more efficiently assimilated into amino acids and proteins in *M. sativa*. This finding is consistent with earlier studies reporting that nitrate promotes nitrogen metabolism and enhances protein biosynthesis in legumes [39, 40]. By contrast, ammonium-fed plants accumulated less protein, which may be attributed to the energy costs and metabolic imbalances associated with ammonium assimilation [11].

Salt stress significantly reduced protein content under both nitrogen regimes. This reduction may result from ionic toxicity and osmotic stress, which limit nitrogen assimilation and impair protein biosynthetic pathways [13, 41]. The most pronounced decline occurred in NH_4^+ + NaCl-treated plants, suggesting that ammonium-fed plants are more sensitive to salinity. This sensitivity is likely due to the combined effects of ammonium toxicity and Na^+/Cl^- stress, which together intensify metabolic disruptions [42]. Overall, these results demonstrate that nitrate nutrition is more favorable than ammonium for sustaining protein metabolism in *M. sativa*, and it may confer a relative advantage in mitigating the adverse effects of salt stress. We also found that this interaction is linked to ion homeostasis. Supplying 3 mM NH_4^+ together with 100 mM NaCl reduced Na^+ accumulation in the shoots of *M. sativa* (Table 1). This may result from competition between NH_4^+ and Na^+ for root uptake sites, thereby limiting sodium transport to shoots and partially restoring growth [43]. However, reduced Na^+ accumulation did not improve K^+ levels under NH_4^+ + NaCl treatment, in line with Assaha *et al*. (2017) [44], who showed that K^+ uptake is highly sensitive to NaCl competition, leading to potassium deficiency and associated toxicity symptoms.

Salinity also affected nitrogen assimilation. NaCl reduced NO_3^- content in shoots (Table 1), likely by inhibiting absorption, xylem loading, and translocation [45]. Conversely, NH_4^+ content increased under salt stress, as also observed by Nour *et al*. (2024) [46], suggesting that NO_3^- uptake is more negatively impacted by salt than NH_4^+. Interestingly, while NH_4^+ generally decreased photosynthesis in sorghum and tobacco (Miranda *et al*., 2015) [35], under salt stress it was found to increase stomatal conductance and regulate photosynthetic activity. This is consistent with our results, where total chlorophyll decreased under NH_4^+ nutrition alone but increased when NaCl was present, possibly due to restricted Na^+ accumulation in leaves, which preserved photosynthetic capacity.

Finally, our data suggest that NH_4^+ nutrition under salinity may help sustain growth through antioxidant defenses. The highest total polyphenol content was observed in *M. sativa* plants grown with NH_4^+ + NaCl, compared with those supplied with NO_3^- + NaCl. Since NaCl induces reactive oxygen species (ROS) generation [47], plants rely on antioxidant systems—enzymatic and non-enzymatic—to mitigate oxidative damage [48]. The elevated polyphenol accumulation under NH_4^+ + NaCl therefore likely contributed to enhanced ROS scavenging and protection of plant tissues, explaining the relatively better growth maintenance under these conditions.

Conclusion

This study demonstrates that while *Medicago sativa* grows best with nitrate (NO_3^-) nutrition under non-saline conditions, ammonium (NH_4^+) nutrition confers greater tolerance to salt stress. Under 100 mM NaCl, NH_4^+-fed plants exhibited increased biomass, reduced Na^+ accumulation in shoots, higher chlorophyll levels, and enhanced polyphenol content compared with NO_3^--fed plants. These findings suggest that NH_4^+ nutrition mitigates NaCl-induced damage through three main mechanisms: Limiting Na^+ uptake and translocation to shoots; Preserving photosynthetic pigments under stress; Stimulating antioxidant defenses to counteract reactive oxygen species.

From an agronomic perspective, partial or full substitution of NO_3^- with NH_4^+ in fertilization regimes could improve alfalfa productivity in saline environments. Future work

should investigate the optimal NO_3^-: NH_4^+ ratio for balancing maximum growth and salt tolerance, as well as the molecular pathways underlying NH_4^+-mediated Na^+ exclusion and antioxidant activation.

Declarations

Data Availability: All data are presented in article.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

Ethics Approval: Not applicable

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Author Contributions

Conceptualization, CCH, methodology, SM, Software, SM; validation, CCH, Investigation, GC; Writing—original draft preparation, MR, SMR; project administration, CCH; funding acquisition, CCH. All authors reviewed the results and approved the final version of the manuscript.

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