

YIELD AND BIOCHEMICAL ATTRIBUTES OF POLYHOUSE GROWN SPINACH AS AFFECTED BY PROPORTIONS OF NO_3^- : NH_4^+ IN THE GROWING MEDIA

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ABSTRACT

Spinach (*Spinacia oleracea* L.) was cultivated under polyhouse conditions using a soilless growing medium, while a parallel crop was established in open field conditions following conventional agronomic practices. In both environments, sowing was carried out three times annually: October (main season), February (off-season), and July (off-season). In the polyhouse system, plants were supplied with nitrogen through nutrient solutions containing varying nitrate (NO_3^-) to ammonium (NH_4^+) ratios: 100:0 (T_1), 75:25 (T_2), 50:50 (T_3), 25:75 (T_4), and 0:100 (T_5). Foliage was harvested 30 days after sowing, with subsequent harvests conducted at 30-day intervals. At each harvest, various growth parameters, including plant height, leaf area, fresh and dry shoot biomass, and total yield per plant were recorded. Additionally, physiological attributes such as total chlorophyll content, carotenoids, soluble sugars, starch, soluble proteins, total free amino acids, and proline content were measured. The activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were also assessed. Across all sowing dates, spinach grown under polyhouse conditions and supplied with a 75:25 NO_3^- : NH_4^+ ratio (T_2) exhibited significantly higher yields compared to the open field-grown crop. The T_2 nutrient treatment also resulted in enhanced enzymatic activity and improved physiological quality parameters. These findings suggest that a 75:25 NO_3^- : NH_4^+ ratio is the most effective for optimizing spinach production under polyhouse conditions.

Keywords: Ammonium, Nitrate, Quality, Spinach, Yield

Spinach (*Spinacia oleracea* L.) is a popular leafy vegetable which is rich in vitamins and minerals. To meet the year round demand of this vegetable, it is sown three times in a year, i.e., in the months of October (main season), February (off season) and July (off season). However, because of poor yield of off season crop, its cultivation in soilless media under protective conditions is becoming popular. Soilless culture involves cultivation on growth media and hydroponics etc. It is free from the constraints of traditional agriculture and has advantages of saving water, fertilizer, and labour; producing high yield; and reducing pollution. Among the fertilizers, it is pertinent to optimize the use of nitrogen as its supra optimum content may not benefit the plant much but contributes to environmental pollution. Plants use N in two forms, i.e. cation (NH_4^+), and anion (NO_3^-). As compared to NO_3^- uptake, lower energy costs are associated with absorption and assimilation of NH_4^+ therefore roots of most plants prefer taking up NH_4^+ in micro-molar concentrations (Hachiya and Sakakibara, 2017). However, at milli-molar concentration, NH_4^+ is toxic for metabolism (Britto and Kronzucker, 2013). NO_3^- as sole source of N results in an efflux of OH^- ions in the rhizosphere solution leading to an increase in its

alkalinity (Zeng *et al.*, 2012). This can also lead to OH^- imbalance and adversely affect nutrient absorption by the plant and consequently has negative effects on the plant growth and metabolism. It is important to identify appropriate ratio of NO_3^- and NH_4^+ in the nutrient solution for optimum growth of each species. In previous studies on coriander (Kumari *et al.*, 2021) and lettuce (Kumari *et al.*, 2020), higher proportions of nitrate (NO_3^-) in the nutrient solution were found to significantly improve yield and quality parameters compared to ammonium (NH_4^+).

However, under the agro-climatic conditions of North India, there is a lack of research investigating the effects of varying NO_3^- : NH_4^+ ratios on spinach grown in soilless media across different seasons. Therefore, the present study was undertaken to evaluate the influence of nitrogen form, specifically, the relative proportions of NO_3^- and NH_4^+ on the growth, yield, and biochemical composition of spinach cultivated in soilless conditions, with particular attention to seasonal variation and sowing environment.

MATERIALS AND METHODS

The experiments were conducted during 2016-17 and 2017-18 at the polyhouse facility of the College of Agricultural Engineering and Technology

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Research Farm, Department of Vegetable Science, and in the laboratories of the Department of Botany, Punjab Agricultural University (PAU), Ludhiana. The experimental site is located at 30°56' N latitude, 75°52' E longitude, and an elevation of 243 meters above sea level. Seeds of spinach (*Spinacia oleracea* L., cv. Punjab Green) were procured from the Department of Vegetable Science, PAU. Under polyhouse conditions, spinach crops were sown three times per year to correspond with the main and off-season periods: October (main season), February (off-season), and July (off-season). At each sowing date, a corresponding control crop was established under open field conditions in 2 m² plots following standard agronomic practices (Anonymous, 2016). In the polyhouse, seeds were directly sown in plastic pots (30 cm diameter × 30 cm height) filled with a soilless potting mixture comprising coco peat, perlite, and vermiculite in a 3:1:1 ratio (Table 1), sourced from local suppliers. After germination, seedlings were thinned to four plants per pot. Each season consisted of three successive harvests, with the first occurring 30 days after sowing and subsequent harvests at one-month intervals. Foliage was harvested from the base using scissors. The polyhouse environment was managed to maintain optimal growing conditions. Temperature control was achieved using heaters in winter and coolers in summer. During summer months, green shade nets were employed to mitigate excessive solar radiation. Average daytime maximum temperatures were recorded as 25.2°C during the main season (October-January), 28.1°C (February-May), and 28.3°C (July-October), while average minimum temperatures were 11.4°C, 16.7°C, and 16.9°C, respectively. The temperature range was 18.2-25.2°C for the main season crop and 16.2-28.3°C for the off-season crops. Plants in the polyhouse were fertigated with a modified Hoagland's nutrient solution (Cao *et al.*, 2010), containing all essential macronutrients (Table 2), and supplemented with a commercially available micronutrient formulation. Five nitrogen treatments were established by altering the NO₃⁻:NH₄⁺ ratio while maintaining a constant total nitrogen level. Nitrate (NO₃⁻) was supplied as calcium nitrate [Ca(NO₃)₂], and ammonium (NH₄⁺) as ammonium sulfate [(NH₄)₂SO₄]. The pH of the nutrient solution was adjusted to 6.0 ± 0.2 using dilute sodium hydroxide (NaOH) or hydrochloric acid (HCl). Approximately 2 L of nutrient solution per pot was applied during the main season crop, and 3 L during off-season crops. To avoid microbial contamination and pH drift, fresh nutrient solution was prepared every 10 days. At each harvest, plant growth parameters were recorded. Fresh leaf biomass was measured immediately after harvest. Dry biomass was determined after drying the leaves in a hot air oven at 60°C for 48 hours. Plant height was measured from the base to the tip of the highest leaf using a ruler. Leaf

area was measured with a LI-COR leaf area meter (Model LI-3100). Yield was calculated as the cumulative fresh weight (g) of harvested leaves per plant at each monthly interval. Leaf water potential was measured using a PSYPRO system (WESCOR®, ELI Tech Group). For each sample, 6 mm diameter leaf discs were excised with a cork borer and immediately placed in the instrument's disc chamber. Stable readings were recorded after 30 seconds.

Biochemical analyses were conducted on both fresh and dried leaf samples. Total chlorophyll and carotenoid contents were quantified immediately after harvest from fresh leaf tissue. Dried leaves were used to estimate total soluble sugars, total starch, total soluble proteins, total free amino acids, and proline content. Fresh leaf samples were also used to determine the concentrations of ascorbic acid and α-tocopherol, as well as the enzymatic activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), following the protocol described by Malik and Singh (1980). Data collected over the two-year period were pooled and subjected to statistical analysis using SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to assess treatment effects, and means were compared using Tukey's post hoc test at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

The highest mean fresh and dry biomass of spinach was recorded in plants supplied with nutrient solution T₂ (NO₃⁻:NH₄⁺ = 75:25) (Fig. 1a; 1b), while the lowest values were observed in those treated with T₅ (NO₃⁻:NH₄⁺ = 0:100). The reduced biomass under T₅ may be attributed to excessive free NH₄⁺ accumulation in plant tissues, which necessitates greater carbohydrate assimilation to mitigate its toxic effects. According to Schortemeyer *et al.* (1997), increased carbohydrate allocation to roots under high NH₄⁺ conditions helps detoxify ammonium ions, thereby reducing the availability of assimilates for shoot growth. This partitioning away from shoot tissues ultimately results in decreased above-ground biomass (Britto and Kronzucker, 2013). Similar findings have been reported in other leafy vegetables. For instance, in Swiss chard, Barickman and Kopsell (2016) observed increased fresh biomass and enhanced chlorophyll and carotenoid concentrations when the NH₄⁺:NO₃⁻ ratio was 25:75 comparable to the T₂ treatment in the present study. Likewise, maximum fresh and dry weights were reported in coriander (Kumari *et al.*, 2021), lettuce (Kumari *et al.*, 2020), and kale (Assimakopoulou *et al.*, 2019) when grown with nutrient solutions containing NO₃⁻:NH₄⁺ ratios similar to 75:25.

Across all three growing seasons, the highest mean plant height and leaf area were observed in spinach plants treated with nutrient solutions T₁ (NO₃⁻:NH₄⁺ =

Table 1. Sowing and harvesting schedule of polyhouse and open field raised spinach crop

	Main Season	Off season	Off season
	October	February	July
Month of sowing	3 rd week of October (2016 and 2017)	2 nd week of February (2017 and 2018)	2 nd week of July (2017 and 2018)
Month of harvesting	3 rd week of January (2017 and 2018)	1 st week of May (2017 and 2018)	1 st week of October (2017 and 2018)

100:0) and T₂ (75:25) (Fig. 1c, 1d). A progressive decline in both parameters was recorded with decreasing NO₃⁻ and increasing NH₄⁺ concentrations in the nutrient solutions. The lowest plant height and leaf area were consistently noted in T₅-treated plants (NO₃⁻:NH₄⁺ = 0:100). These results suggest that elevated NH₄⁺ levels in the root zone negatively affect vegetative growth. The reduction in plant height and leaf expansion with increasing NH₄⁺ can be attributed to several physiological and biochemical disturbances, including ammonium toxicity, rhizosphere acidification, reduced cytokinin biosynthesis, carbon-to-nitrogen (C:N) imbalance, and enhanced oxidative stress. Collectively, these factors hinder cell division and elongation, impair nutrient uptake, and ultimately suppress shoot development and canopy expansion.

The yield per plant (Fig. 1e) of spinach grown in the polyhouse and supplied with nutrient solution T₂ (NO₃⁻:NH₄⁺ = 75:25) was significantly higher than that of soil-grown spinach across all three sowing seasons. Specifically, T₂-treated plants yielded 14.40%, 33.72%, and 60.87% more in the October (main season), February (off-season), and July (off-season) sowings, respectively, compared to their open-field counterparts. In contrast, polyhouse-grown spinach receiving nutrient solutions T₃, T₄, and T₅, containing higher proportions of NH₄⁺, produced lower yields than field-grown spinach in both seasonal and off-season crops. These findings align with previous reports suggesting that nitrate (NO₃⁻) is the preferred nitrogen form for promoting

optimal growth and productivity in leafy vegetables (Urlicé *et al.*, 2017). In the present study, all vegetative growth parameters responded positively to nutrient solutions containing a lower proportion of ammonium (0% or 25%), further supporting the view that NO₃⁻-dominant nutrition enhances plant development under soilless, controlled-environment conditions.

Similarly, previous studies have demonstrated that a nutrient solution containing NO₃⁻ and NH₄⁺ in a 75:25 ratio is optimal for vegetative growth in leafy vegetables. Assimakopoulou *et al.* (2019) in kale, Kumari *et al.* (2021) in coriander, and Kumari *et al.* (2020) in lettuce reported maximum plant height, leaf area, and biomass under this nutrient regime. Among the treatments in the present study, the highest mean proline content was recorded in spinach plants supplied with T₂ (NO₃⁻:NH₄⁺ = 75:25) across all three sowing seasons (Fig. 2c). Proline accumulation in T₂-treated plants was 156.72%, 152.78%, and 154.64% higher than in soil-grown spinach during the October, February, and July crops, respectively. In parallel, plants treated with T₄ and T₅ exhibited higher (less negative) leaf water potential values (Fig. 1f), indicating reduced water stress. The observed changes in leaf water potential can be associated with increased proline accumulation, a well-known osmoprotectant in plants. Proline stabilizes cellular membranes through interactions with phospholipids, scavenges hydroxyl radicals, and serves as a reserve of energy and nitrogen during stress. A similar pattern of proline

Table 2. Nutrient solution compositions (mmol L⁻¹) with varying NO₃⁻/NH₄⁺ ratios at a constant total N concentration (8 mmol L⁻¹)*

Treatment	NO ₃ ⁻ /NH ₄ ⁺ ratio	Source of nutrients (mmolL ⁻¹) (mgL ⁻¹)						
		KNO ₃	Ca(NO ₃) ₂ ·4H ₂ O	CaCl ₂ ·2H ₂ O	(NH ₄) ₂ SO ₄	KH ₂ PO ₄	MgSO ₄ ·7H ₂ O	KCl
T ₁	100:0	4.8	1.6	3.4	0.0	1.0	2.0	1.2
T ₂	75:25	2.8	1.6	3.4	1.0	1.0	2.0	3.2
T ₃	50:50	0.8	1.6	3.4	2.0	1.0	2.0	5.2
T ₄	25:75	2.0	0.0	5.0	3.0	1.0	2.0	4.0
T ₅	0:100	0.0	0.0	5.0	4.0	1.0	2.0	6.0

(*Cao *et al.*, 2010)

*KNO₃: Potassium nitrate, Ca(NO₃)₂·4H₂O: Calcium nitrate tetrahydrate, CaCl₂·2H₂O: Calcium chloride dehydrate, (NH₄)₂SO₄: Ammonium sulphate, KH₂PO₄: Potassium dihydrogen phosphate, MgSO₄·7H₂O: Magnesium sulfateheptahydrate, KCl: Potassium chloride.

*N concentration was kept constant at 8 mmol L⁻¹.

NO₃⁻ to NH₄⁺ ratios: 100:0 (T₁), 75:25 (T₂), 50:50 (T₃), 25:75 (T₄), 0:100 (T₅)

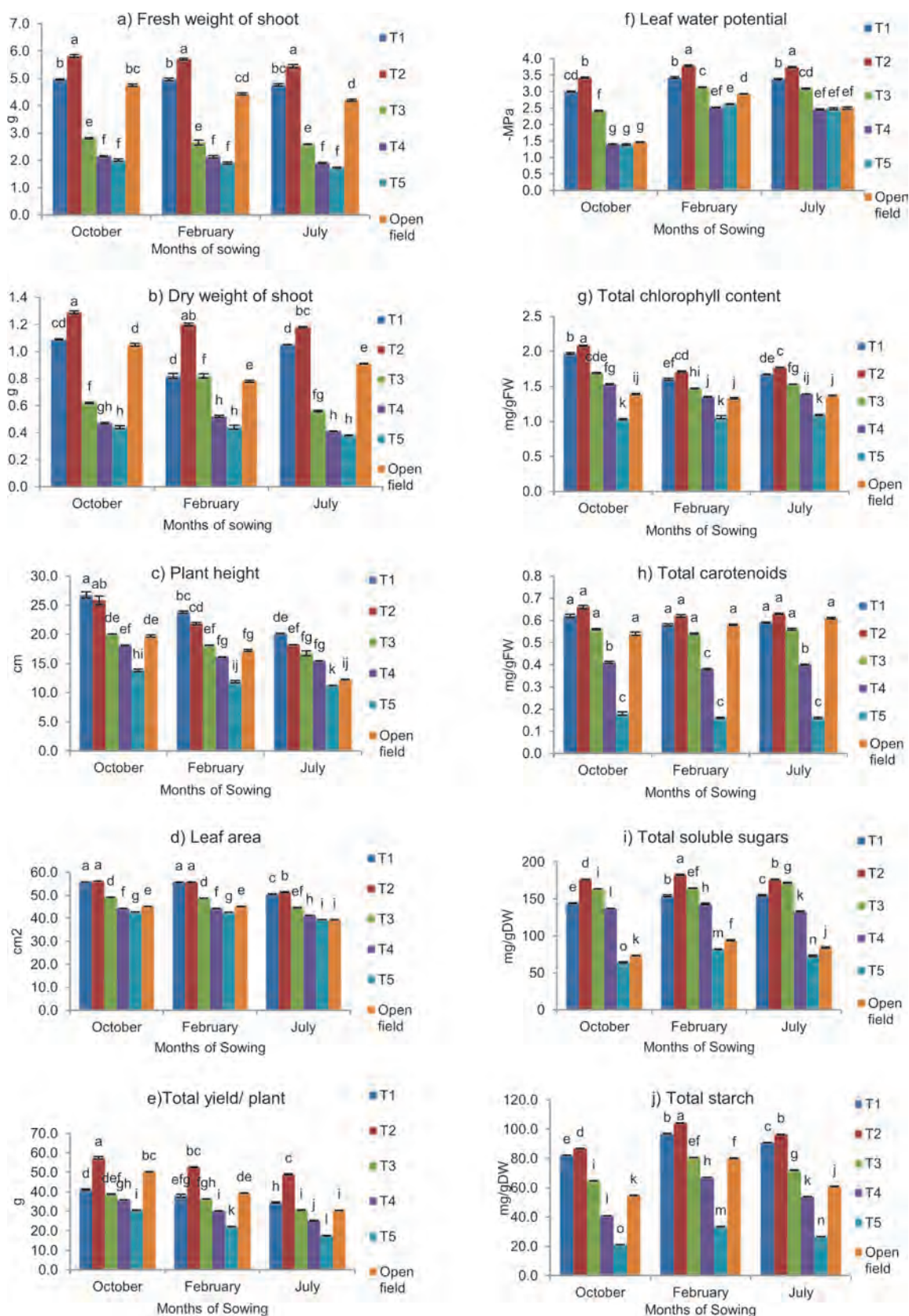


Fig. 1. Effects of different $\text{NO}_3^-:\text{NH}_4^+$ ratios in the nutrient solution on (a) Fresh weight of shoot (b) Dry weight of shoot (c) Plant height (d) Leaf area (e) Total yield/ plant (f) Leaf water potential (g) Total chlorophyll content (h) Total carotenoid content (i) Total soluble sugars (j) Total starch

enhancement at a 75:25 $\text{NO}_3^-:\text{NH}_4^+$ ratio was also reported in lettuce by Kumari *et al.* (2020). Interestingly, open-field crops exhibited minor seasonal fluctuations in proline content in response to variations in leaf water potential. These fluctuations suggest that stress acclimation mechanisms and osmotic adjustments were active in field-grown spinach. In contrast, the polyhouse-grown plants, subjected to more controlled environmental conditions, displayed reduced stress perception, thereby diminishing the expected proline response to variations in leaf water potential. These results highlight the distinct physiological and stress-response behaviours of spinach grown under protected versus open-field conditions, emphasizing the influence of cultivation environment on osmotic regulation and stress signalling pathways.

The highest mean total chlorophyll content (Fig. 1g) across all three sowing seasons was recorded in spinach plants supplied with nutrient solution T_2 ($\text{NO}_3^-:\text{NH}_4^+ = 75:25$), while the lowest values were observed in plants treated with T_5 ($\text{NO}_3^-:\text{NH}_4^+ = 0:100$). Carotenoid content (Fig. 1h) was highest in plants treated with T_1 , T_2 , and T_3 during the October (main season), February, and July (off-season) sowings, respectively. However, no statistically significant differences in carotenoid content were found between polyhouse-grown plants supplied with T_1 , T_2 , or T_3 and their open-field counterparts in any of the seasons. The lowest carotenoid concentrations were consistently observed in plants treated with T_5 . These findings suggest that increasing the proportion of NH_4^+ in the nutrient solution up to 50% can enhance chlorophyll and carotenoid accumulation. However, a further increase beyond this threshold, as in treatment T_5 , results in a marked decline in pigment content. This pattern may be attributed to the adverse effects of excessive ammonium, such as metabolic toxicity and oxidative stress, which can impair chloroplast development and pigment biosynthesis. Similar trends have been reported in other leafy vegetables. For example, Barickman and Kopsell (2016) observed increased chlorophyll and carotenoid content in Swiss chard grown with a $\text{NO}_3^-:\text{NH}_4^+$ ratio of 75:25, while Assimakopoulou *et al.* (2019) reported comparable findings in kale.

The highest total soluble sugar (TSS) content was recorded in spinach plants supplied with nutrient solution T_2 ($\text{NO}_3^-:\text{NH}_4^+ = 75:25$). Compared to field-grown spinach sown on the same dates, T_2 -treated plants exhibited 140.64%, 94.05%, and 109.28% higher TSS in the October, February, and July sowings, respectively. In addition to sugars, T_2 -treated plants also showed the highest accumulation of total starch (Fig. 1j). These results suggest that a balanced nitrate-dominant nitrogen supply facilitates optimal carbon

assimilation and storage in the form of soluble sugars and starch. Similar findings have been reported in celery, where nutrient solutions with $\text{NO}_3^-:\text{NH}_4^+$ ratios of 100:0 and 80:20 significantly increased sugar content (Saleh *et al.*, 2019).

The highest total soluble protein content across all three sowing seasons was observed in spinach plants supplied with nutrient solutions T_1 ($\text{NO}_3^-:\text{NH}_4^+ = 100:0$) and T_2 (75:25), while the lowest protein levels were consistently recorded in T_5 -treated plants ($\text{NO}_3^-:\text{NH}_4^+ = 0:100$) (Fig. 2a). Similarly, total free amino acid (FAA) content was significantly higher in off-season crops (February and July) treated with T_2 and T_1 , and lowest in those supplied with T_5 (Fig. 2b). During the main season (October sowing), maximum FAA accumulation was recorded in plants treated with T_2 . These results are consistent with previous findings in celery, where $\text{NO}_3^-:\text{NH}_4^+$ ratios of 100:0 and 80:20 were reported to enhance total soluble protein content (Saleh *et al.*, 2019). The favourable response to nitrate-rich treatments can be attributed to enhanced nitrogen assimilation and increased synthesis of nitrogenous compounds such as amino acids and proteins. FAA content varied between polyhouse and field-grown crops, as well as across seasons, reflecting differences in environmental stress and nitrogen metabolism. In field crops, FAAs accumulated to higher levels under favourable environmental conditions, possibly due to more efficient nitrogen assimilation and elevated protein turnover. In contrast, polyhouse-grown crops, subjected to relatively low-stress conditions, exhibited reduced FAA levels during the main season. However, during off-season periods, FAA levels in polyhouse crops remained relatively stable, while those in field crops declined possibly due to reduced nitrogen assimilation efficiency and lower metabolic activity under suboptimal conditions. The seasonal and cultivation-specific differences in FAA accumulation possibly result from the complex interaction of nitrogen form, environmental stress signals, and the plant's internal nitrogen transformation and protein metabolism dynamics.

The highest mean ascorbic acid content was recorded in spinach plants supplied with nutrient solution T_2 ($\text{NO}_3^-:\text{NH}_4^+ = 75:25$), while the lowest levels were observed in those treated with T_5 (0:100) across all three growing seasons (Fig. 2d). The reduction in ascorbic acid in T_5 -treated plants is possibly due to ammonium-induced toxicity, which can disrupt physiological processes and inhibit antioxidant biosynthesis. These results align with findings by Saleh *et al.* (2019), who reported maximum ascorbic acid content in celery under $\text{NO}_3^-:\text{NH}_4^+$ ratios of 100:0 and 80:20. A comparable pattern was observed for α -tocopherol content, which peaked in T_2 -treated plants and was lowest in those

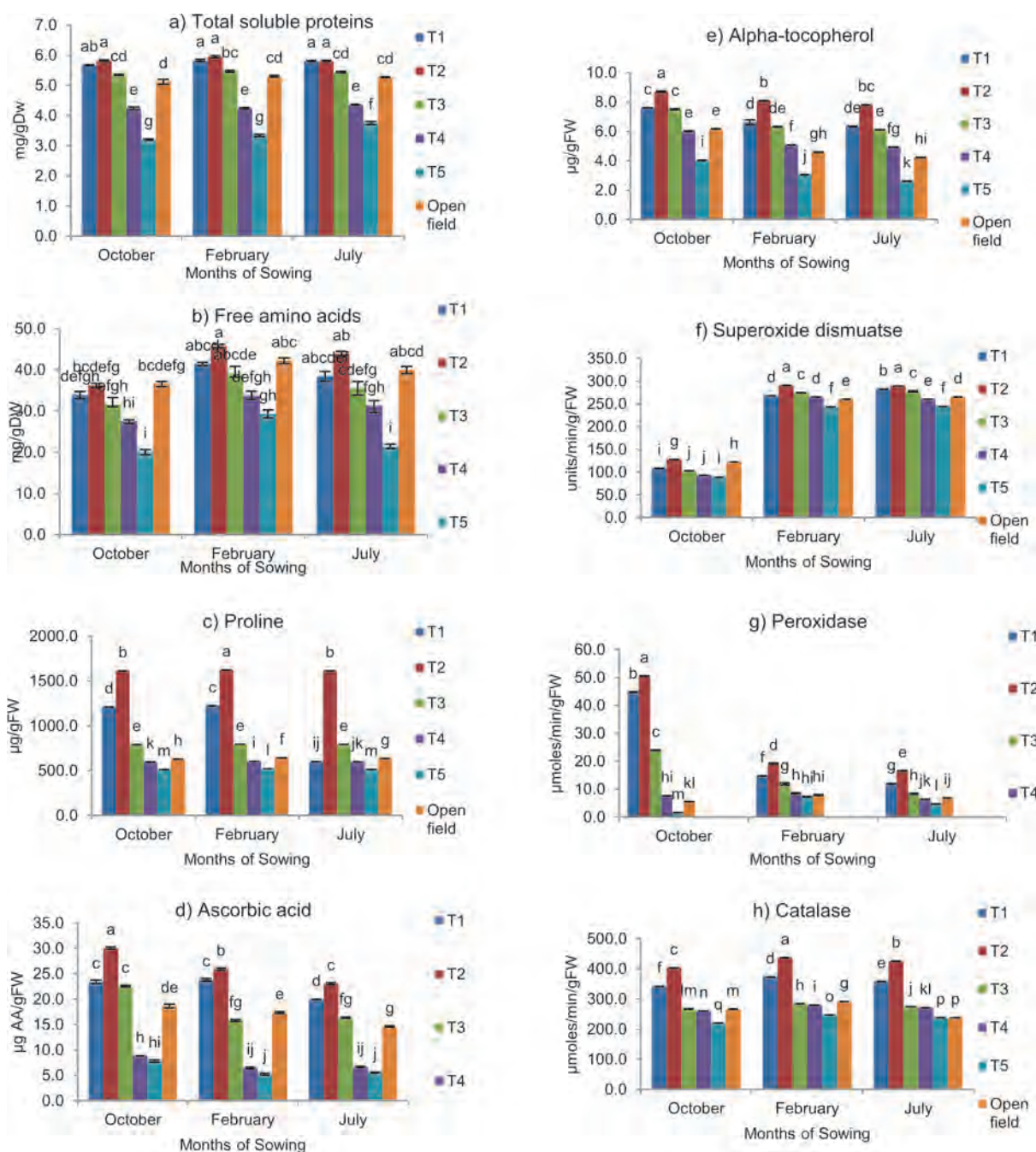


Fig. 2. Effects of different $\text{NO}_3^-:\text{NH}_4^+$ ratios in the nutrient solution on (a) Total soluble proteins (b) Free amino acids (c) Proline (d) Ascorbic acid (e) Alpha-tocopherol (f) Superoxide dismutase (g) Peroxidase (h) Catalase

receiving T_5 (Fig. 2e). In open-field crops, the highest α -tocopherol concentration was recorded in the October-sown crop ($6.17 \mu\text{g/g FW}$), while the February and July crops yielded 4.58 and $4.22 \mu\text{g/g FW}$, respectively. Under off-season conditions, hydroponically grown spinach in the polyhouse accumulated more ascorbic acid and α -tocopherol than field-grown plants. These findings corroborate earlier reports by Buchanan and Omaye (2013), who found elevated α -tocopherol levels in hydroponically grown lettuce compared to soil-grown

counterparts. Similarly, Kumari *et al.* (2020, 2021) reported enhanced ascorbic acid and α -tocopherol accumulation in coriander and lettuce when supplied with nitrate-dominant nutrient solutions. Importantly, the antioxidant content in hydroponically grown off-season spinach was comparable to that of main-season, soil-grown crops.

Superoxide dismutase (SOD) activity was significantly higher in off-season crops (February and

July sowings) compared to the main season crop (October) (Fig. 2f). Within the off-season crops, the highest SOD activity was recorded in plants treated with nutrient solutions T₂ (NO₃⁻:NH₄⁺ = 75:25) and T₃ (50:50), while during the main season, maximum SOD activity was observed in T₂-treated plants. Peroxidase (POD) activity followed a different trend, with higher levels observed in the main season crop (October) than in off-season crops. Nevertheless, in both main and off-season crops, plants supplied with T₂ consistently exhibited the highest POD activity (Fig. 2g).

Similarly, catalase (CAT) activity was significantly elevated in off-season crops, with the highest levels recorded in plants treated with T₂, followed by T₁ (Fig. 2h). These antioxidant enzymes i.e. SOD, POD, and CAT play critical roles in mitigating oxidative stress by scavenging reactive oxygen species (ROS), thereby protecting cellular components such as membranes from lipid peroxidation. The enhanced activity of these enzymes under the T₂ nutrient regime suggests that a NO₃⁻:NH₄⁺ ratio of 75:25 promotes better oxidative stress management in spinach under both seasonal and off-season conditions. These results are in agreement with previous studies. Sun *et al.* (2014) reported that NO₃⁻:NH₄⁺ ratios of 50:50 and 75:25 were optimal for protecting Chinese chive against oxidative stress by enhancing antioxidant enzyme activity. Similarly, Kumari *et al.* (2020, 2021) found that coriander and lettuce exhibited maximum SOD, POD, and CAT activity when supplied with a NO₃⁻-dominant nutrient solution at a 75:25 ratio.

CONCLUSION

The present study demonstrates that, across all sowing dates, spinach grown in a polyhouse using soilless media produced significantly higher yields compared to field-grown crops. Among the various nitrogen treatments, the nutrient solution with a NO₃⁻:NH₄⁺ ratio of 75:25 (T₂) consistently resulted in the highest yield, enhanced growth parameters, superior nutritional quality, and elevated antioxidant enzyme activities (SOD, POD, and CAT). While polyhouse-grown spinach supplied with higher proportions of ammonium (T₃, T₄, and T₅) showed reduced performance, field-grown crops occasionally outperformed these treatments in terms of yield. These findings highlight the importance of optimizing nitrogen form and ratio in nutrient solutions to maximize the benefits of soilless cultivation.

It is therefore recommended that a NO₃⁻:NH₄⁺ ratio of 75:25 be used for hydroponic spinach production under polyhouse conditions, as this combination promotes robust growth, maximizes yield, and enhances nutritional and biochemical quality throughout

the year including during off-season periods when field cultivation is suboptimal.

Authors' contribution

Conceptualization of research work and designing of experiments (SB, RK, ASD, VPS); Execution of field/lab experiments and data collection (RK); Analysis of data and interpretation (RK); Preparation of manuscript (RK, SB).

Conflicts of interest

Authors declare that they have no conflicts of interest.

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