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Synergistic Effects of Humic Acid and Seeding Rate on Berseem Clover Productivity under Arid Conditions

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Abstract

Optimizing forage productivity in arid regions requires integrated strategies that enhance plant growth and resource efficiency. This study assessed the interactive effects of seeding rate and humic acid on berseem clover (*Trifolium alexandrinum* L.). A factorial experiment based on a randomized complete block design with three replications was conducted in Kut, Iraq, involving three seeding rates (20, 25, and 30 kg ha^{-1}) and three humic acid levels (0, 5, and 10 L ha^{-1}). Results indicated that the seeding rate significantly influenced morphological traits and yield, with the highest fresh forage yield (40.95 t ha^{-1}) recorded at 30 kg ha^{-1}. Humic acid application significantly improved vegetative growth, notably stem number and leaf count. The interaction between factors was significant for biological yield and protein content. Under low density, humic acid enhanced individual plant growth, compensating for reduced stand count, whereas under high density, it mitigated competitive stress, supporting biomass accumulation. The combination of 30 kg ha^{-1} seed and 10 L ha^{-1} humic acid resulted in the highest biological yield (58.18 t ha^{-1}) and crude protein content. These findings suggest that integrating optimal plant density with humic acid biostimulants is a viable strategy for maximizing forage yield and quality in arid agro-ecosystems.

Keywords: Arid agriculture; Biostimulants; Crude protein; Plant density; *Trifolium alexandrinum*.

Introduction

Livestock production is inextricably linked to the availability of high-quality forage. In arid and semi-arid regions, such as the Middle East, forage crops serve a dual purpose: meeting the nutritional demands of livestock and conserving soil resources against erosion (Ayas & Gülser, 2005). Among forage species, annual legumes are indispensable due to their high protein content and ability to enhance soil fertility through biological nitrogen fixation (Heydarzadeh et al., 2022). Berseem clover (*Trifolium alexandrinum* L.) is a key annual forage legume characterized by rapid establishment, vigorous regrowth, and adaptability to irrigated systems (Delouriti et al., 1988).

Despite its potential, berseem clover production in regions like Iraq faces significant agronomic constraints. Soils often suffer from low organic matter, salinity, and nutrient imbalances. Furthermore, the long-term dependency on chemical fertilizers has led to soil degradation and environmental pollution (Wang et al., 1999). Consequently, there is a growing interest in sustainable management practices, such as the use of organic biostimulants.

Humic substances, which constitute 60–70% of soil organic matter, are critical for soil health and plant physiology. Humic acid improves soil structure, enhances water retention, and stimulates root development (Canellas et al., 2015). Physiologically, it acts as a biostimulant by influencing nutrient uptake, particularly phosphorus and micronutrients, and inducing hormone-like responses that enhance stress tolerance (Canellas & Olivares, 2014). These attributes are particularly valuable under arid conditions, where water and nutrient efficiency are paramount.

In addition to soil amendments, plant density management is a fundamental determinant of canopy architecture and resource use. Seeding rate dictates light interception and competition intensity; while high densities maximize biomass per unit area, they may induce stress due to competition for light and nutrients (Umesh et al., 2022). While the individual effects of seeding rate and humic acid have been studied, their interaction—specifically how biostimulants modulate density-dependent stress—remains less understood. This study hypothesized that humic acid would alleviate competitive stress at high densities and enhance compensatory growth at low densities. Therefore, the objective was to evaluate the interactive effects of seeding rate and humic acid on the yield and quality of berseem clover under the arid conditions of Kut, Iraq.

Materials and Methods

Experimental Site and Design

The study was conducted during the 2025 growing season in Kut, Wasit Province, Iraq (32.51° N, 45.82° E; 18 m a.s.l.). The region has a hot desert climate with mean annual precipitation of approximately 138 mm. The soil texture was silty loam (Table 1). The experiment was arranged as a factorial (3 × 3) in a randomized complete block design (RCBD) with three replications. The first factor was seeding rate (20, 25, and 30 kg ha^{-1}). The second factor was humic acid application (0, 5, and 10 L ha^{-1}), applied as a commercial granular formulation (Super Humate) at sowing.

Table 1-Physicochemical properties of the soil at the experimental site

Soil texture	Organic matter (%)	EC (dS m^{-1})	pH	Potassium (mg kg^{-1})	Phosphorus (mg kg^{-1})	Nitrogen (%)
Silty loam	0.73	0.72	7.44	395	9.1	0.09

Ethical and compliance statement

All field experiments and plant handling procedures were performed in accordance with the institutional and national guidelines of the Republic of Iraq and the Department of Agronomy and Plant Breeding, Ilam University. The plant material (Berseem clover, cultivar Giza-6) was obtained from commercial sources and cultivated under standard agronomic practices. No wild plant species were collected, and no specific permits were required for the described cultivated studies.

Crop Management

Basal fertilizers were applied uniformly based on soil analysis. Sowing was performed manually on October 20, 2025, using cultivar Giza-6. Plot dimensions were 1.5 × 2.5 m. Row spacing was adjusted according to seeding rate: 25 cm for 20 kg ha^{-1}, 20–25 cm for 25 kg ha^{-1}, and 15–20 cm for 30 kg ha^{-1}. Irrigation was managed via the furrow method.

Data Collection Four harvests were conducted at 30-day intervals starting January 17, 2026. In each harvest, ten random plants per plot were sampled. Measured traits included:

- **Morphological traits:** Plant height, stem length, number of stems per plant, and number of leaves per plant.
- **Yield components:** Fresh forage yield (FFY), dry forage yield (DFY), biological yield (BY), and harvest index (HI = [FFY/BY] × 100).
- **Quality trait:** Crude protein content (CP) determined via the Kjeldahl method. Data were analyzed as mean values across harvests.

Statistical Analysis Analysis of variance (ANOVA) was performed using SAS (v9.4). Mean comparisons were conducted using Fisher's Least Significant Difference (LSD) test at $p \leq 0.05$.

Results and Discussion

The analysis of variance (ANOVA) revealed that seeding rate exerted a highly significant effect ($p \leq 0.01$) on the majority of measured traits, including stem number, plant height, stem length, leaf number, fresh and dry forage yield, biological yield, and crude protein content (Table 2). This confirms that planting density is a primary determinant of canopy architecture and

biomass production in berseem clover. Humic acid application also significantly affected most vegetative traits and crude protein content, while its influence on fresh forage and biological yield was significant. However, the effect of humic acid on dry forage yield was not statistically significant. The interaction between seeding rate and humic acid was significant for several key traits (Table 2), indicating that the plant's response to the biostimulant is dependent on population density. In contrast, the harvest index remained unaffected by treatments, suggesting that the interventions primarily increased total biomass rather than altering the partitioning of assimilates to the economic yield.

Table 2 - Analysis of variance of data related to the traits of number of stems per plant, clover plant height, stem length, leaves per plant, fresh forage yield, dry forage yield, biological yield, harvest index and crude protein of Berseem clover are affected by seed rate and humic acid application.

S.O.V	D.F	Number of stems per plant	Plant height	Stem length	Leaves per plant	Fresh forage yield	Dry forage yield	Biological yield	HI	Cr/ude protein
Rep	2	1.4599	1.951	6.373	8.619	2.3509	0.1467	0.6908	27.1444	0.2341
Seed rate	2	20.9909**	353.247**	238.765**	322.972**	66.2217*	14.8213**	86.2646**	10.5252 ns	13.5069**
Error (a)	4	0.8390	15.454	11.798	13.482	7.7726	0.2819	2.4706	22.3731	0.0624
Humic acid	2	61.8456**	80.796**	74.701**	196.179**	55.2268*	0.8568 ns	94.3551*	7.3213 ns	7.7714**
Humic acid × Seed rate	4	2.7862*	141.360**	80.756**	196.774**	31.2188 ns	0.8203 ns	22.4254*	42.8938 ns	3.0767**
Error	12	0.5942	11.188	7.730	12.425	8.1282	0.4347	0.4849	21.6713	0.2558
C.V (%)	-	11	7.1	10	10.4	12.2	9.9	12.7	6.11	5.4

ns, *, **: Represent non-significance, and significance at the 5% and 1% probability levels, respectively.

Morphological Traits

Number of Stems per Plant Mean comparison showed that the number of stems per plant was significantly influenced by the interaction between seeding rate and humic acid application (Figure 1). The highest stem number (approximately 18 stems per plant) was observed at the seeding rate of 30 kg ha^{-1} combined with 5 or 10 L ha^{-1} humic acid. Conversely, the lowest stem number (≈10 stems per plant) occurred at 20 kg ha^{-1} without humic acid application. Across all seeding rates, the application of humic acid consistently increased stem number. This effect suggests that humic acid enhanced shoot proliferation. The increase in stem number can be attributed to the improved soil conditions and enhanced physiological activity induced by humic substances. Humic acids promote nutrient uptake—particularly nitrogen and phosphorus—and stimulate microbial activity in the rhizosphere. This leads to increased lateral root development and higher photosynthetic capacity, which collectively support the

development of auxiliary shoots and branching (Canellas & Olivares, 2014; Shah et al., 2018). Furthermore, at higher seeding rates, the competition for light stimulates the plant's architecture to adjust, increasing stem formation to optimize light capture. The combination of improved nutrition via humic acid and optimal plant density created a favorable environment for maximum stem production.

Plant Height of Berseem Clover

A significant interaction between seeding rate and humic acid was observed for plant height (Figure 2). The tallest plants (48.09 cm) were recorded at the highest seeding rate (30 kg ha^{-1}) combined with the highest level of humic acid (10 L ha^{-1}), whereas the shortest height (33.39 cm) occurred at the lowest seeding rate (20 kg ha^{-1}) in the control group. These results highlight the role of humic acid as a biostimulant capable of modulating plant growth under varying density conditions. Humic substances promote cell division and elongation through hormone-like (auxin-like) activity and improve the availability of micronutrients such as iron and zinc, which are essential for chlorophyll synthesis and metabolic activity (Zandonadi et al., 2010; Calvo et al., 2014). Under high plant density, competition for resources typically restricts growth; however, humic acid appeared to alleviate these limitations by enhancing root function and nutrient acquisition, thereby sustaining vertical growth even under competitive stress.

Stem Length

The mean comparison indicated that stem length was significantly affected by the interaction between seeding rate and humic acid (Figure 3). The maximum stem length (54.38 cm) was obtained at 30 kg ha^{-1} combined with 10 L ha^{-1} humic acid, while the minimum value (36.69 cm) was observed at 20 kg ha^{-1} without humic acid. The response pattern suggests a density-dependent effect. At the lower seeding rate (20 kg ha^{-1}), humic acid had a pronounced influence on stem elongation, likely due to reduced inter-plant competition allowing individual plants to fully exploit the growth-promoting effects of improved nutrient uptake and hormonal stimulation (Calvo et al., 2014). Interestingly, at the intermediate seeding density (25 kg ha^{-1}), the maximum stem length occurred in the untreated control. This suggests a "biological optimum" where plants achieved their intrinsic growth potential without requiring external stimulation. Introducing humic acid at this density may have disrupted endogenous hormonal balances or caused inefficient metabolic allocation (Rose et al., 2014). However, at the highest density (30 kg ha^{-1}), the positive response to humic acid resumed. Here, under intensified competitive stress, humic acid likely acted as a stress-alleviating agent, improving resource

acquisition efficiency and modifying root architecture to support continued elongation (Canellas et al., 2015).

Number of Leaves per Plant

Statistical analysis revealed a significant interaction between seeding rate and humic acid application on the number of leaves per plant. Mean comparisons indicated that the highest leaf production (approximately 78 leaves per plant) was achieved with the combined treatment of 30 kg seed ha^{-1} and 10 L humic acid ha^{-1}, which differed significantly from other treatments. Conversely, the lowest leaf number (around 40 leaves per plant) occurred at the seeding rate of 20 kg ha^{-1} without humic acid (Figure 4). These findings demonstrate that humic acid influences the expansion of the plant's photosynthetic apparatus. An increased leaf number directly elevates the Leaf Area Index (LAI), which is a primary determinant of light interception and biomass production potential (Tokatlidis & Koutroubas, 2004). Beyond yield, greater leaf abundance enhances forage quality, as leaves typically possess higher protein concentrations and digestibility than stems. The mechanism is attributed to the capacity of humic substances to improve nitrogen availability and uptake, which is critical for protein synthesis and chlorophyll formation in developing tissues (Calvo et al., 2014). Although high-density conditions generally constrain leaf initiation due to competition, humic acid alleviated these limitations by stimulating root system development, ensuring a supply of nutrients and water to support leaf expansion.

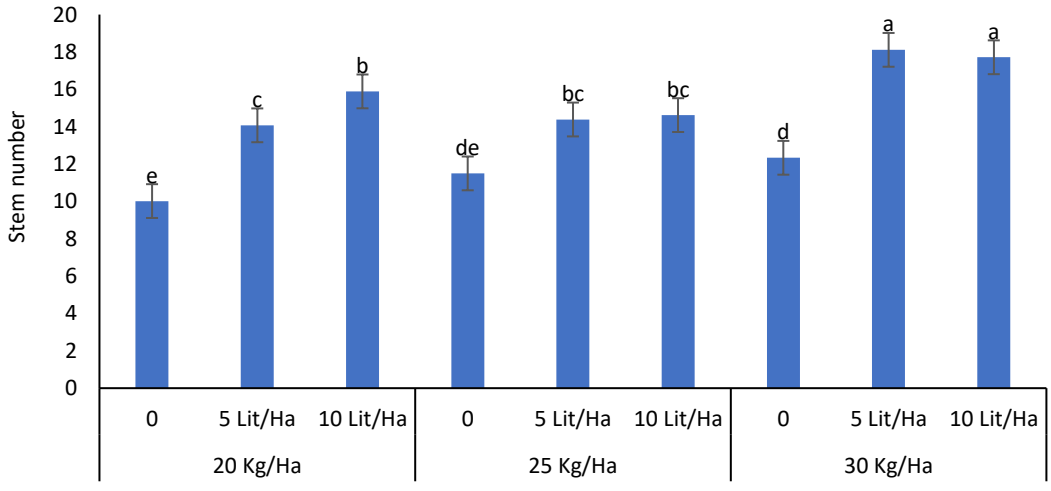
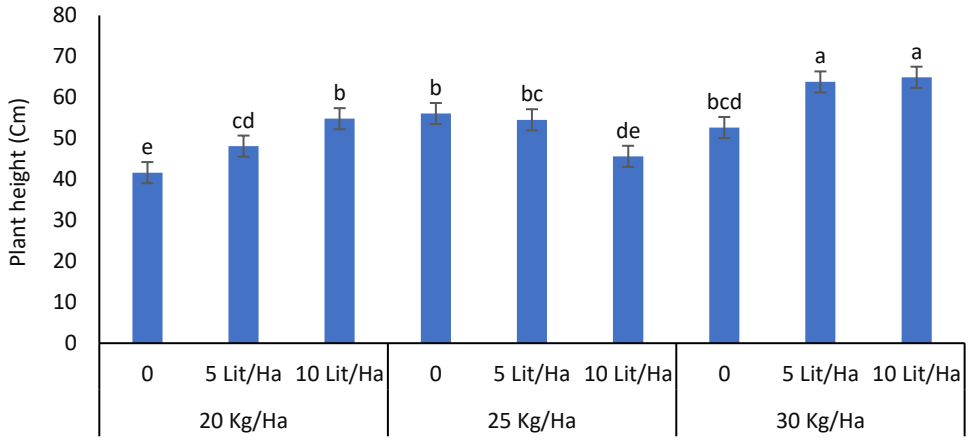
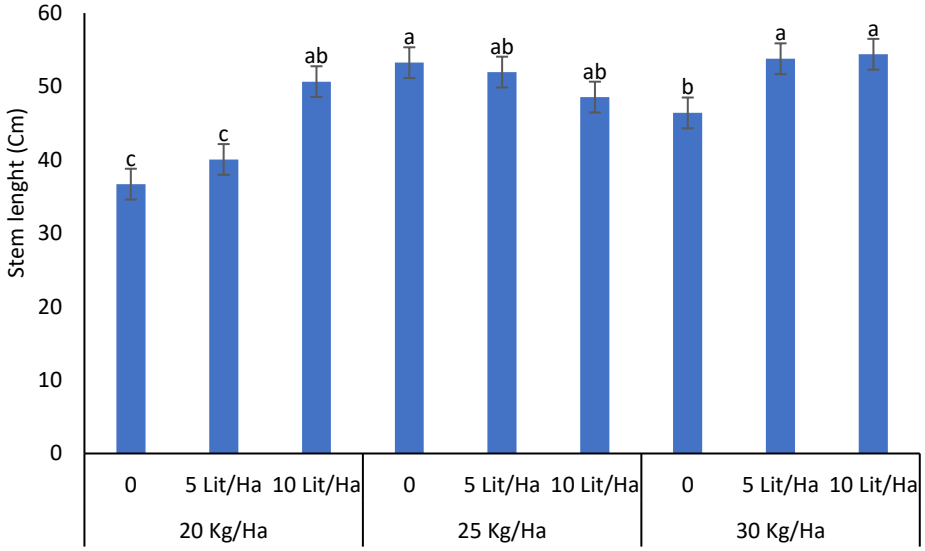


Figure 1 - Interaction between seed rate and humic acid application on the stem number in clover. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).



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172 Figure 2 - Interaction between seed rate and humic acid application on the plant height of Berseem clover.
173 Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).



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175 Figure 3 - Interaction between seed rate and humic acid application on the stem length of Berseem clover.
176 Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

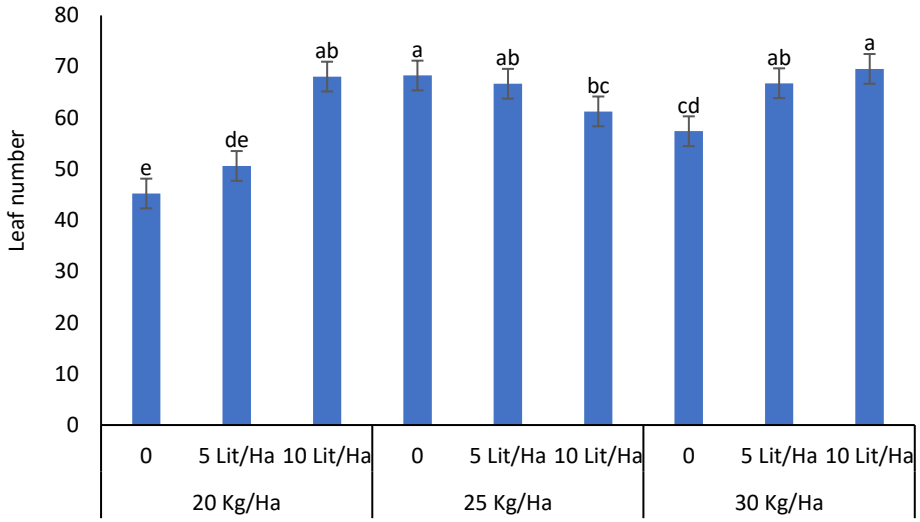


Figure 4 - Interaction between seed rate and humic acid application on leaf number in a clover plant. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

Yield Components

Fresh Forage Yield

The analysis of experimental data showed a significant effect of seeding rate on the fresh forage yield of berseem clover (Figure 5). The highest yield (40.95 t ha^{-1}) was obtained at the seeding rate of 30 kg ha^{-1}, whereas the lowest yield (35.55 t ha^{-1}) occurred at 20 kg ha^{-1}. The intermediate treatment (25 kg ha^{-1}) produced 37.77 t ha^{-1} and was not statistically different from the highest rate. The increase in fresh forage yield with higher seeding rates is explained by the improved ground coverage and efficient capture of environmental resources (light, water, nutrients) at higher populations (Tokatlidis & Koutroubas, 2004; Weiner, 2017). The lack of a significant difference between 25 and 30 kg ha^{-1} suggests a yield plateau, where increased inter-plant competition at very high densities offsets the gains from having more plants (Rose et al., 2014). From an economic perspective, the 25 kg ha^{-1} rate may offer a better cost-benefit ratio, although the absolute maximum was achieved at 30 kg ha^{-1}. Regarding humic acid, the application significantly affected fresh forage yield. The highest yield (40.27 t ha^{-1}) was recorded with 10 L ha^{-1} humic acid, whereas the lowest (35.39 t ha^{-1}) occurred in the control (Figure 6). Although the 10 L ha^{-1} treatment produced the maximum yield, it did not differ significantly from the 5 L ha^{-1} treatment, suggesting that 5 L ha^{-1} is a sufficient and cost-effective dose. The enhancement in yield is driven by the improvements in vegetative traits (height and leaf number) discussed earlier, which boost the photosynthetic capacity and biomass accumulation of the crop.

Dry Forage Yield

Statistical analysis revealed that seeding rate significantly affected dry forage yield. The highest yield (8.06 t ha^{-1}) was obtained at the highest seeding rate, while the lowest (5.67 t ha^{-1}) occurred in the untreated control (Note: Based on Figure 7 data interpretation). The yield at the highest seeding level differed significantly from both the control and the intermediate rate (Figure 7). These findings suggest that achieving a critical plant density is essential for maximizing dry biomass. At the intermediate rate, density was likely insufficient to fully activate the crop's production potential, resulting in incomplete resource utilization (Willey & Heath, 1969). Unlike fresh yield, dry matter accumulation seems more strictly dependent on the plant population per unit area in this study, as humic acid did not show a statistically significant main effect on dry forage yield (Table 2). This implies that while biostimulants improve the water content and fresh vigor of the plants, the ultimate structural dry matter accumulation is governed predominantly by the stand density.

Biological Yield

Mean comparison analysis demonstrated that the interaction between seeding rate and humic acid application significantly affected the biological yield of berseem clover (Figure 8). The highest yield (58.18 t ha^{-1}) was obtained with the combined treatment of 30 kg seed ha^{-1} and 10 L humic acid ha^{-1}, whereas the lowest yield (42.15 t ha^{-1}) occurred in the treatment receiving 20 kg seed ha^{-1} without humic acid. The significant improvement in biological yield under higher seeding rates combined with humic acid highlights the synergy between optimal plant density and enhanced physiological processes. At the lowest seeding rate (20 kg ha^{-1}), humic acid significantly increased biological yield, acting as a growth stimulant to compensate for the lack of plant population. This allowed for acceptable biomass production with fewer seeds. Conversely, at the intermediate seeding rate (25 kg ha^{-1}), the response to humic acid was less evident. However, at the highest seeding rate (30 kg ha^{-1}), humic acid exerted a positive effect once again, yielding the maximum biological yield. This suggests that under intense inter-plant competition, humic acid alleviates negative effects by improving the efficiency of limited resource utilization (Canellas et al., 2015).

Harvest Index

Harvest index (HI) was not significantly affected by either seeding rate or humic acid application. This indicates that while treatments substantially increased total biomass and quantitative yield, they did not alter the partitioning of assimilates within the plant. HI is

generally considered a genetically controlled trait with limited phenotypic plasticity (Hay, 1995). In this study, both seeding rate and humic acid functioned as growth-enhancing factors, simultaneously increasing both harvestable yield and total biomass proportionally. Therefore, the HI remained unchanged, suggesting that manipulating harvest index in berseem clover would likely require genetic interventions or specific plant growth regulators that directly modify source-sink relationships.

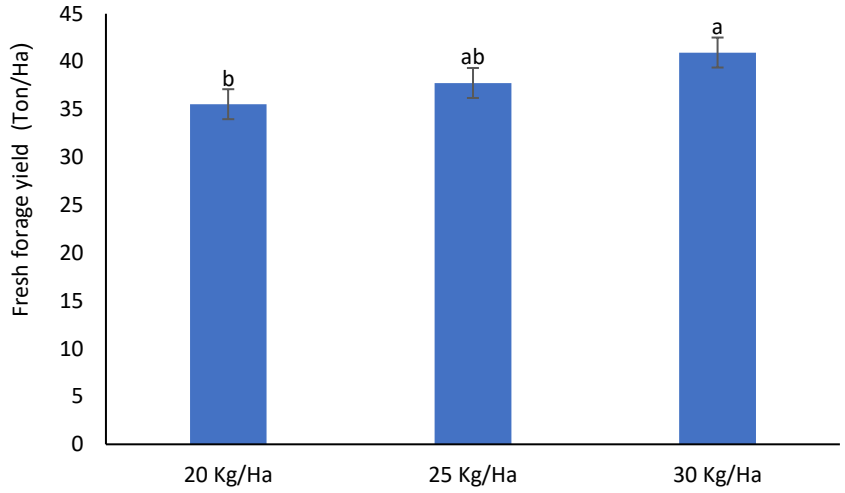


Figure 5 - Effect of seed consumption rate on fresh forage yield of clover. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

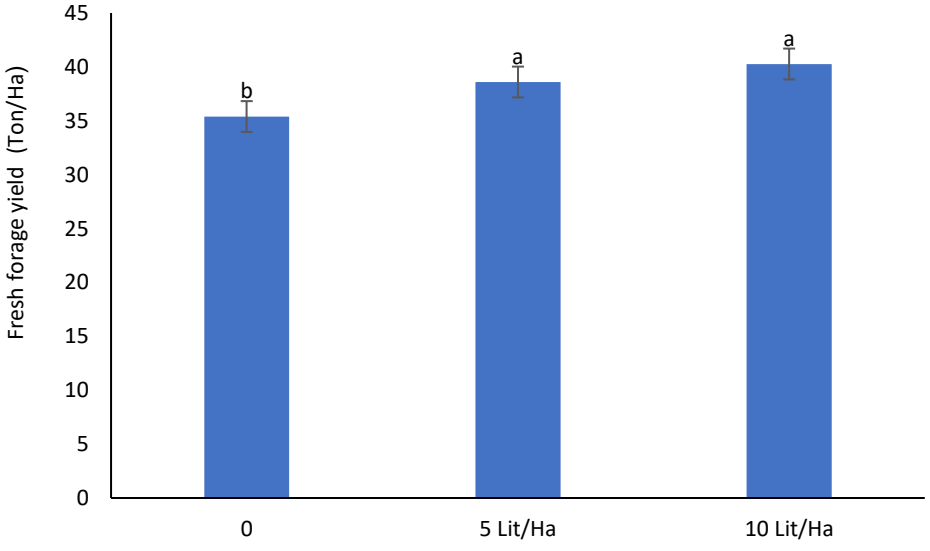


Figure 6 - Effect of humic acid application on fresh forage yield of Berseem clover. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

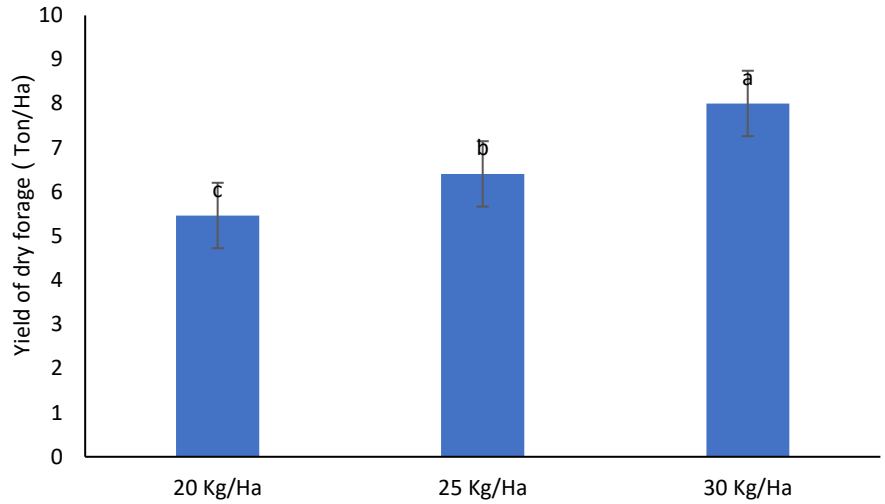


Figure 7 - Effect of seed consumption rate on yield of dry forage of clover. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

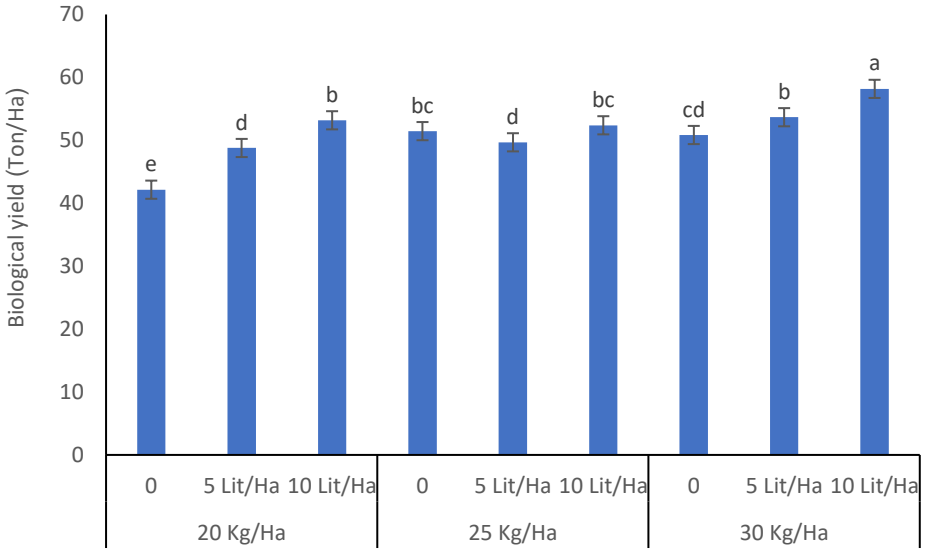


Figure 8 - Interaction between seed rate and humic acid application on the biological yield of berseem clover. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

Leaf Protein Content of Berseem Clover

Mean comparison analysis revealed that the combination of seeding rate and humic acid application significantly affected the leaf protein content (Figure 9). The highest protein content (21.94%) was recorded at 30 kg seed ha^{-1} without humic acid, while the lowest (17.60%) occurred at 20 kg seed ha^{-1} without humic acid. These results suggest that higher plant density can enhance nitrogen accumulation and protein synthesis, likely due to improved canopy structure and increased nitrogen fixation efficiency under optimal stand establishment. The interaction indicates a complex relationship between density and biostimulants. At the

lowest seeding rate (20 kg ha^{-1}), humic acid significantly increased leaf protein compared to the control. This response is attributed to the role of humic substances in enhancing nitrogen uptake; they increase root cell membrane permeability and facilitate nitrate and ammonium uptake, providing greater substrate availability for protein biosynthesis (Nardi et al., 2016). Notably, the highest protein content was observed at 30 kg seed ha^{-1} without humic acid. This phenomenon may be explained by "controlled metabolic stress." At high densities, intense competition for light may trigger stress-related signaling pathways, leading to the synthesis of defense and stress-tolerance proteins, or alter assimilate partitioning resulting in greater accumulation of nitrogenous compounds in leaf tissues (Jindo et al., 2020). The mechanisms by which humic acid influences protein content are multifaceted. Studies show that humic substances can stimulate the expression of genes involved in nitrogen metabolism, including those encoding nitrate reductase, glutamine synthetase, and glutamate synthase (Schiavon et al., 2010). This transcriptional activation enhances the activity of key enzymes involved in nitrogen assimilation. Furthermore, improved leaf protein content may be linked to enhanced photosynthetic efficiency driven by humic acids, which increase chlorophyll content and carbohydrate production. These carbohydrates provide the carbon skeletons required for amino acid and protein synthesis, resulting in a positive interaction between carbon and nitrogen metabolism.

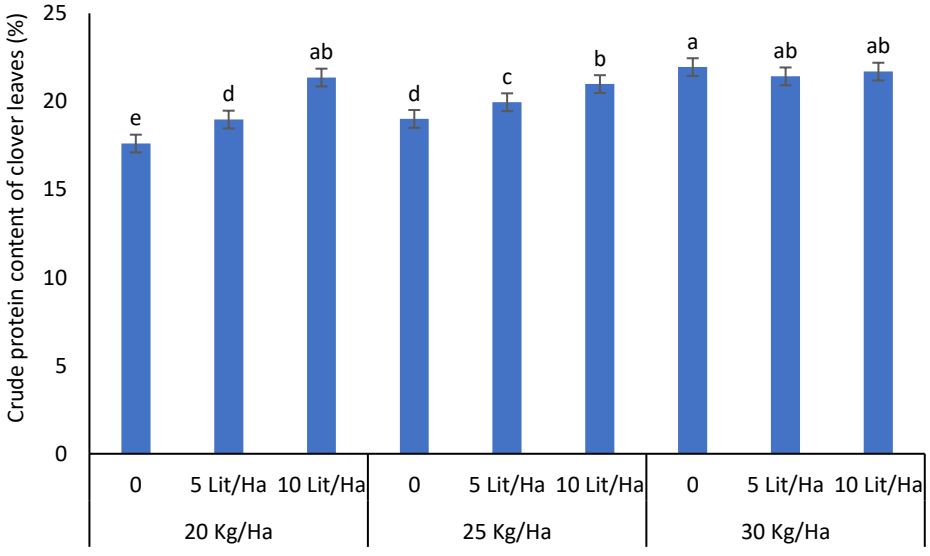


Figure 9 - Interaction between seed rate and humic acid application on the crude protein content of berseem clover leaves. Different letters indicate statistically significant differences according to Fisher's LSD test ($P \leq 0.05$).

Correlation Analysis

Strong, positive correlations were observed between most morphological traits and forage yield components. Plant height, stem number, and leaf number showed significant positive correlations with fresh forage yield, dry forage yield, and biological yield (Table 3), indicating that improvements in vegetative growth directly contributed to higher biomass accumulation. Dry forage yield exhibited a very strong correlation with biological yield ($r = 0.87$), confirming that total biomass production was largely driven by dry matter accumulation. In contrast, harvest index showed weak or non-significant correlations with most morphological traits, suggesting that management practices primarily enhanced total biomass rather than altering assimilate partitioning. Crude protein content showed strong positive correlations with vegetative traits, confirming that improved growth conditions enhanced not only forage quantity but also forage quality.

ble 3- Pearson correlation coefficients among morphological traits, yield components and quality traits of Berseem clover

Traits	Stems per plant	Plant height	Stem length	Leaves per plant	Fresh forage yield	Dry forage yield	Biological yield	HI	Crude protein
Stems per plant	1.0000								
	0.0000								
Plant height	0.7779	1.0000							
	0.0000	0.0000							
Stem length	0.5544	0.6843	1.0000						
	0.0019	0.0001	0.0000						
Leaves per plant	0.6361	0.8146	0.8814	1.0000					
	0.0003	0.0000	0.0000	0.0000					
Fresh forage yield	0.7464	0.7186	0.8819	0.7468	1.0000				
	0.0000	0.0000	0.0000	0.0000	0.0000				
Dry forage yield	0.8282	0.9196	0.6679	0.7383	0.7356	1.0000			
	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000			
Biological yield	0.8061	0.8216	0.7525	0.8290	0.7801	0.8693	1.0000		
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		
HI	0.1782	0.1160	0.4587	0.1534	0.6091	0.0834	−0.0203	1.0000	
	0.3551	0.5482	0.0112	0.4267	0.0004	0.6679	0.9161	0.0000	
Crude protein	0.8738	0.7402	0.6881	0.6619	0.7568	0.8273	0.7842	0.2256	1.0000
	0.0000	0.0000	0.0001	0.0002	0.0000	0.0000	0.0000	0.2413	0.0000

Conclusion

The findings of this study elucidate the critical and synergistic roles of seeding rate and humic acid in optimizing the productivity of berseem clover under arid conditions. While high plant density (30 kg ha^{-1}) proved essential for maximizing resource interception and biomass accumulation per unit area, humic acid application acted as a pivotal physiological modulator. A key revelation of this research is the context-dependent efficacy of humic acid: it functioned as a stress-alleviating agent under high-density conditions, mitigating competitive stress, and as a growth compensator under low-density conditions, enhancing individual plant vigor to offset reduced stand counts.

These results underscore that crop management inputs cannot be viewed in isolation; rather, optimal agronomic performance is achieved through an integrated strategy that balances population dynamics with biostimulant application. Consequently, the combined application of 30 kg seed ha^{-1} and 10 L humic acid ha^{-1} is recommended as a robust, environmentally sustainable strategy for farmers in the Kut region. This approach not only maximizes forage yield and crude protein content but also enhances resource-use efficiency, offering a viable pathway toward sustainable intensification in arid agro-ecosystems.

Declarations

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Plant guidelines

The experimental research and field studies on plants (*Trifolium alexandrinum* L.), including the collection of plant material, were conducted in compliance with relevant institutional, national, and international guidelines and legislation. The plant material used in this study (cultivar Giza-6) was commercially obtained and cultivated in a research field; no wild or protected species were collected.

Permissions

Not applicable (The study was conducted on a research field where permissions were secured through institutional agreements).

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Authors' contributions

H.S.N. and E.Z. performed the field experiments and data collection. Y.A. and E.Z. analyzed the data. H.S.N. and Y.A. wrote the main manuscript text. Y.A. supervised the project. All authors reviewed and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

During manuscript preparation, an AI-assisted tool was used exclusively for language editing. The authors take full responsibility for the scientific content and the final version of the manuscript.

References

1. Annicchiarico, P., Barrett, B., Brummer, E. C., Julier, B., & Marshall, A. H. (2015). Achievements and challenges in improving temperate perennial forage legumes. *Critical Reviews in Plant Sciences, 34*(1–3), 327–380. https://doi.org/10.1080/07352689.2014.898462
2. Ashoori, M., Barati, M., & Ghanbari, A. (2021). Forage yield and quality of clover species under different agronomic practices. *Grassland Science, 67*(3), 213–221. https://doi.org/10.1111/grs.12298

3. Ayas, H., & Gülser, C. (2005). The effect of different management practices on soil erosion in forage crop systems. *Soil & Tillage Research, 83*(1), 85–94. https://doi.org/10.1016/j.still.2004.08.005

4. Calvo, P., Nelson, L., & Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant and Soil, 383*, 3–41. https://doi.org/10.1007/s11104-014-2131-8

5. Canellas, L. P., & Olivares, F. L. (2014). Physiological responses to humic substances as plant growth promoter. *Chemical and Biological Technologies in Agriculture, 1*, 3. https://doi.org/10.1186/2196-5641-1-3

6. Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., & Piccolo, A. (2015). Humic and fulvic acids as biostimulants in horticulture. *Scientia Horticulturae, 196*, 15–27. https://doi.org/10.1016/j.scienta.2015.09.013

7. Capstaff, N. M., Miller, A. J., & Kronzucker, H. J. (2020). Nitrogen dynamics in legumes: Regulation of nodulation and biological nitrogen fixation. *Journal of Experimental Botany, 71*(17), 5044–5056. https://doi.org/10.1093/jxb/eraa273

8. Castillo, J. A., Pérez-Jiménez, M., & López-Bellido, L. (2022). Plant density effects on growth, yield and resource-use efficiency in forage legumes. *Field Crops Research, 276*, 108371. https://doi.org/10.1016/j.fcr.2021.108371

9. Cimrin, K. M., & Yilmaz, I. (2005). Humic acid applications to lettuce do not affect yield but improve phosphorus availability. *Acta Agriculturae Scandinavica, Section B, 55*(1), 58–63. https://doi.org/10.1080/09064710510008636

10. Delfine, S., Tognetti, R., Desiderio, E., & Alvino, A. (2005). Effect of foliar application of N and humic acids on growth and yield of durum wheat. *Agronomy for Sustainable Development, 25*(2), 183–191. https://doi.org/10.1051/agro:2005017

11. Delouriti, M. M., El-Shatnawi, M. K., & Turki, M. A. (1988). Growth and productivity of berseem clover under different cutting regimes. *Field Crops Research, 18*(1), 45–54. https://doi.org/10.1016/0378-4290(88)90043-7

12. du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae, 196*, 3–14. https://doi.org/10.1016/j.scienta.2015.09.021

13. Eyheraguibel, B., Silvestre, J., & Morard, P. (2008). Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize.

Bioresource Technology, 99(10), 4206–4212. https://doi.org/10.1016/j.biortech.2007.08.082

14. Ferraz-Almeida, R., Silva, S. C., & Sbrissia, A. F. (2022). Plant density effects on forage yield and quality in legume-based systems. *Crop Science, 62*(1), 312–324. https://doi.org/10.1002/csc2.20658

15. Gao, Y., & Chu, C. (2020). Hormonal regulation of plant responses to drought stress. *Journal of Experimental Botany, 71*(1), 15–28. https://doi.org/10.1093/jxb/erz365

16. García, A. C., De Souza, L. G. A., Pereira, M. G., Castro, R. N., García-Mina, J. M., Zonta, E., & Lisboa, F. J. G. (2016). Structure–property–function relationship of humic substances in modulating soil and plant physiology. *Scientia Horticulturae, 196*, 15–23. https://doi.org/10.1016/j.scienta.2016.01.006

17. Hay, R. K. M. (1995). Harvest index: A review of its use in plant breeding and crop physiology. *Annals of Applied Biology, 126*(1), 197–216. https://doi.org/10.1111/j.1744-7348.1995.tb05015.x

18. Heydarzadeh, S., Moradi, P., & Rezaei, M. (2022). Performance of annual forage legumes under semi-arid conditions. *Journal of Agricultural Science and Technology, 24*(4), 921–933. https://doi.org/10.52547/jast.24.4.921

19. Jindo, K., Olivares, F. L., Malcher, D. J. P., Sánchez-Monedero, M. A., Kempenaar, C., & Canellas, L. P. (2020). From molecular structure to plant biostimulant action: The role of humic substances in plant metabolism. *Science of the Total Environment, 703*, 134750. https://doi.org/10.1016/j.scitotenv.2019.134750

20. Jones, D. L., Edwards, A. C., & Murphy, D. V. (2004). Bioavailability of phosphorus in humic substances. *Soil Biology and Biochemistry, 36*(8), 1343–1351. https://doi.org/10.1016/j.soilbio.2004.03.017

21. Ku, Y. S., Sintaha, M., Cheung, M. Y., & Lam, H. M. (2018). Plant hormone signaling crosstalks between biotic and abiotic stress responses. *International Journal of Molecular Sciences, 19*(10), 3206. https://doi.org/10.3390/ijms19103206

22. Lithourgidis, A. S., Vasilakoglou, I. B., Dhima, K. V., Dordas, C. A., & Yiakoulaki, M. D. (2006). Forage yield and competition indices of common vetch intercropped with oat and triticale. *Field Crops Research, 97*(2–3), 237–247. https://doi.org/10.1016/j.fcr.2005.10.008

23. Malamura, C., Ercoli, L., Mariotti, M., & Masoni, A. (2024). Plant density regulation of biomass production and nitrogen fixation in annual forage legumes. *European Journal of Agronomy, 151*, 126683. https://doi.org/10.1016/j.eja.2023.126683

24. Mirzaei, A., Ardakani, M. R., & Madani, H. (2012). Effects of humic acid on soil physical properties and root development. *International Journal of Agriculture and Biology, 14*(3), 441–446. https://doi.org/10.17957/IJAB/14.3.441

25. Monteith, J. L. (1977). Climate and the efficiency of crop production in Britain. *Philosophical Transactions of the Royal Society B: Biological Sciences, 281*(980), 277–294. https://doi.org/10.1098/rstb.1977.0148

26. Nardi, S., Pizzeghello, D., Muscolo, A., & Vianello, A. (2002). Physiological effects of humic substances on higher plants. *Soil Biology and Biochemistry, 34*(11), 1527–1536. https://doi.org/10.1016/S0038-0717(02)00174-8

27. Nardi, S., Pizzeghello, D., Ertani, A., & Schiavon, M. (2021). Physiological effects of humic substances in plant growth: From soil to plant molecular responses. *Molecules, 26*(8), 2252. https://doi.org/10.3390/molecules26082252

28. Nardi, S., Schiavon, M., & Francioso, O. (2016). Chemical structure and biological activity of humic substances define their role as plant growth promoters. *Molecules, 21*(4), 523. https://doi.org/10.3390/molecules21040523

29. Ramos, A. C., Dobbss, L. B., Santos, L. A., Fernandes, M. S., Olivares, F. L., Aguiar, N. O., & Canellas, L. P. (2015). Humic matter elicits proton and calcium fluxes and signaling dependent on Ca^{2+}-dependent protein kinase at early stages of lateral root development. *Chemical and Biological Technologies in Agriculture, 2*, 3. https://doi.org/10.1186/s40538-014-0030-0

30. Reynolds, M., Foulkes, M. J., Slafer, G. A., Berry, P., Parry, M. A. J., Snape, J. W., & Angus, W. J. (2009). Raising yield potential in wheat. *Journal of Experimental Botany, 60*(7), 1899–1918. https://doi.org/10.1093/jxb/erp016

31. Rose, M. T., Patti, A. F., Little, K. R., Brown, A. L., Jackson, W. R., & Cavagnaro, T. R. (2014). A meta-analysis and review of plant-growth response to humic substances: Practical implications for agriculture. *Advances in Agronomy, 124*, 37–89. https://doi.org/10.1016/B978-0-12-800138-7.00002-4

444 32. Schiavon, M., Ertani, A., & Nardi, S. (2010). Effects of an alfalfa protein hydrolysate
445 on the gene expression and activity of enzymes involved in nitrogen assimilation in
446 maize. *Journal of Agricultural and Food Chemistry, 58*(23), 12355–12360.
447 https://doi.org/10.1021/jf102473g

448 33. Tan, K. H., & Tantiwiramanond, D. (1983). Effect of humic acids on plant growth. *Soil*
449 *Science Society of America Journal, 47*(4), 728–732.
450 https://doi.org/10.2136/sssaj1983.03615995004700040026x

451 34. Tokatlidis, I. S., & Koutroubas, S. D. (2004). A review of maize hybrids' dependence
452 on high plant populations and its implications for crop yield stability. *Field Crops*
453 *Research, 88*(2–3), 103–114. https://doi.org/10.1016/j.fcr.2003.11.013

454 35. Trevisan, S., Francioso, O., Quaggiotti, S., & Nardi, S. (2010). Humic substances
455 biological activity at the plant–soil interface. *Plant Signaling & Behavior, 5*(6), 635–
456 643. https://doi.org/10.4161/psb.5.6.11211

457 36. Umesh, M. R., Ravi, S., & Kumar, S. (2022). Seeding rate and plant density effects on
458 forage legumes. *Agronomy Journal, 114*(2), 1189–1200.
459 https://doi.org/10.1002/agj2.20995

460 37. Wang, Y., Huang, Q., & Zhu, H. (1999). Long-term fertilization effects on soil organic
461 matter and structure. *Soil Science, 164*(10), 760–768.
462 https://doi.org/10.1097/00010694-199910000-00005

463 38. Weiner, J. (2017). Applying plant ecological knowledge to increase agricultural
464 sustainability. *Journal of Ecology, 105*(4), 865–870. https://doi.org/10.1111/1365-
465 2745.12792

466 39. Willey, R. W., & Heath, S. B. (1969). The quantitative relationships between plant
467 population and crop yield. *The Journal of Agricultural Science, 72*(2), 301–318.
468 https://doi.org/10.1017/S0021859600024870

469 40. Xu, C., Zhang, Y., & Wang, Z. (2021). Plant density regulation and weed suppression
470 in forage cropping systems. *Agriculture, Ecosystems & Environment, 312*, 107356.
471 https://doi.org/10.1016/j.agee.2021.107356

472 41. Yu, Z., Duan, X., Luo, L., Dai, S., Ding, Z., Xia, G., & Sun, J. (2020). How plant
473 hormones mediate salt stress responses. *Trends in Plant Science, 25*(11), 1117–1130.
474 https://doi.org/10.1016/j.tplants.2020.06.008

42. Zandonadi, D. B., Canellas, L. P., & Façanha, A. R. (2007). Indolacetic and humic acids induce lateral root development through activation of H^+ pumps. *Planta, 225*(6), 1583–1595. https://doi.org/10.1007/s00425-006-0454-2