Using Azolla and Cyanobacteria to Reduce Nitrate Accumulation in Lettuce Leaves and Optimize Nitrogen Fertilization in Hydroponics

Shahad. Mohamed. bander 1* , Ali Abbas Kadhum 2 , Mohamed Obaid Sallume [1] Ministry of Agriculture/ Anbar Directorate of Agriculture

[2] Department of Soil and Water Resources, College of Agriculture, University of Anbar, Anbar , Iraq

[3] Department of Soil and Water Resources, College of Agriculture, University of Anbar, Anbar , Iraq

Abstract

A study was conducted in Anbar Governorate, Fallujah District, located near the Euphrates River west of Baghdad, to evaluate the use of cyanobacteria and *Azolla* in reducing nitrate accumulation in lettuce (*Lactuca sativa L.*) leaves under hydroponic conditions, as well as optimizing nitrogen fertilization rates. Two hydroponic systems were employed: the pond system and the nutrient film technique(NFT) system.

A factorial experiment was designed with two factors. The first factor consisted of biotic treatments, including cyanobacteria, Azolla, and a combination of Azolla with cyanobacteria. The second factor included three nitrogen levels (72, 108, and 144 mg kg^{-1}), in comparison with the full recommended nitrogen fertilization rate.

The results demonstrated a significant superiority of the pond system in reducing nitrate concentration in lettuce leaves. Moreover, the combined biotic treatment (Azolla + cyanobacteria) significantly decreased nitrate levels and improved nitrogen use efficiency. This effect was particularly pronounced at the 108 mg kg^{-1} (N_2) level in the pond system, where both nitrate reduction and fertilizer optimization were achieved.

Keywords: Azolla , Cyanobacteria , Hydroponics , Lettuce , Nitrogen Fertilization

Introduction

Lettuce (*Lactuca sativa L.*) is one of the most important vegetable crops worldwide, with a global production of approximately 27.66 million metric tons cultivated over 1.23 million hectares (9). Major producers include China, India, and Spain. Production has increased by 50% over the past two decades (2000–2020), largely due to lettuce being a moderate source of antioxidants and health-promoting compounds such as phenolics, anthocyanins, and carotenoids (22). Regular consumption contributes to protection against cancer, cardiovascular diseases, and other chronic conditions (15).

Nitrogen (N) is a key essential nutrient for plant growth. In hydroponic systems, nitrate (e.g., calcium nitrate and potassium nitrate) is the primary nitrogen source. Nitrogen is often the most limiting nutrient for productivity and plays a critical role in leafy vegetable quality (26), as it is a fundamental component of proteins and chloroplast structure and function (1).

**This research is extracted from the first author's thesis*

ISSN 2072-3857

Lettuce requires relatively high nitrogen inputs for optimal growth (25).

However, excessive nitrogen fertilization can negatively affect crop quality, particularly by increasing nitrate accumulation in edible tissues (21). Lettuce, especially when grown hydroponically, tends to accumulate high nitrate levels in vacuoles even under low nitrate supply (10). Although nitrate itself has low toxicity, its reduction to nitrite in the human body poses health risks. Nitrite can react with amines to form nitrosamines, some of which are carcinogenic (6).

Therefore, there is a need to develop strategies to reduce nitrate accumulation by optimizing nutrient management. Biofertilizers such as Azolla and cyanobacteria represent promising alternatives due to their symbiotic relationship and ability to supply nitrogen biologically (14). These approaches contribute to sustainable agriculture by reducing reliance on chemical fertilizers while maintaining crop yield and quality (8).

This study aimed to evaluate the use of Azolla and cyanobacteria as biological nitrogen sources in hydroponic lettuce production to reduce nitrate accumulation and minimize mineral nitrogen inputs.

Materials and Methods

1. Preparation of Nutrient Solution

The nutrient solution was prepared according to (4) using distilled water and the following components:

- Calcium nitrate: 9 g per 10 L (for 50%, 75%, and 100% N levels)
- Potassium sulfate: 5.57 g per 10 L
- Magnesium sulfate: 4.95 g per L
- Chelated iron: 0.2 g per L
- Micronutrients: 0.2 g per L

Phosphorus was supplied using:

- DAP: 1.9 g L^{-1} (100% level)
- 1.43 g L^{-1} + 0.65 ml H_3PO_4 (75% level)
- 0.96 g L^{-1} + 1.30 ml H_3PO_4 (50% level)

Azolla was applied at 10 t ha^{-1} (16), while cyanobacteria were added at 0.5 ml L^{-1} (2)

.

2. Experimental Design

Two hydroponic systems were used:

A. Pond System (Experiment 1)

A factorial experiment (2 factors) was conducted:

- **Factor A: Biotic treatments**
  1. Cyanobacteria
  2. Azolla
  3. Azolla + Cyanobacteria
- **Factor B: Nitrogen levels**
  - 100% recommended N
  - 75% recommended N
  - 50% recommended N

Total treatments: 9 × 3 replicates = 27 experimental units + control treatments.

B. nutrient film technique(NFT) **System (Experiment 2)**

ISSN 2072-3857

The same treatments and experimental design were applied using a nutrient film technique(NFT)-based hydroponic system.

3. Cyanobacteria Cultivation

Cyanobacteria were cultured in polyethylene tanks (30 × 60 cm, 15 cm depth) at a 1:10 inoculation ratio. Aeration was provided using an air compressor every two hours until 6:00 PM. Manual mixing was performed to prevent stagnation. Water levels were maintained daily to compensate for evaporation.

4. Azolla Propagation

Azolla was cultivated in lined pits (20–25 cm depth) under shaded conditions. Soil (10–15 kg) and superphosphate were added, followed by water filling (10–30 cm depth). After stabilization, 2–3 kg Azolla inoculum was introduced. Biomass production reached 1–3 kg daily after two weeks.

5. Planting : Lettuce seeds (cv. *Summer Green*) were sown on 15/9/2025. Seedlings were treated with a fungicide (Carbendazim) before transplanting into hydroponic systems on 10/10/2025.

6. Nitrate Determination

Nitrate content was measured starting two weeks after transplanting, at 6-day intervals, using a Horiba LAQUAtwin NO_3 meter. Leaf sap was extracted, and average nitrate concentrations were calculated.

7. Statistical Analysis

Data were analyzed using ANOVA under a Randomized Complete Block Design (RCBD). Treatment mean were compared using LSD at 5% probability level via GenStat softwar

Results and Discussion

Nitrate Content in Lettuce Leaves

Pond System: The results presented in Table (1) indicate that the control treatment showed a significant effect compared to the other treatments in terms of mean nitrate content in lettuce leaves Furthermore, the results demonstrate that biotic treatments had a significant effect on the average nitrate concentration. Treatment B_2 (cyanobacteria) recorded the highest value (2614 mg kg^{-1}), followed by treatment B_1 (Azolla) with a value of 2552 mg kg^{-1}, whereas treatment B_3 (Azolla + cyanobacteria) showed the lowest nitrate content (2408 mg kg^{-1}). In contrast, nitrogen fertilization levels and their interaction with biotic treatments did not show any significant effect across all levels. This study is consistent with (28) who demonstrated that cyanobacteria in hydroponic systems improve nitrogen use efficiency and reduce excessive nitrate accumulation through nitrogen (N_2) fixation and regulation of its uptake.

ISSN 2072-3857

Table (1): Effect of biotic treatments and nitrogen fertilization on the mean nitrate content in lettuce leaves (mg kg^{-1}) under the pond hydroponic system

Biotic Treatments (B)	**Nitrogen Fertilization (N)**			**Mean of Biotic Treatments**
	N_1 (72 mg L^{-1})	N_2 (108 mg L^{-1})	N_3 (144 mg L^{-1})	
Azolla (B_1)	2580	2517	2558	2552
Cyanobacteria (B_2)	2650	2633	2558	2614
Azolla + Cyanobacteria (B_3)	2517	2308	2400	2408
Mean of Nitrogen Levels	2582	2486	2506	
Control	2667			
LSD (0.05):	• B = 154.1 • N = N.S (Not Significant) • B × N = N.S			

nutrient film technique(NFT) System: The results presented in Table (2) indicate that there were no significant differences between the control treatment and the other treatments in terms of mean nitrate content in lettuce leaves Furthermore, all studied factors, including the interaction levels between them, did not show any significant effect on the average nitrate concentration in the leaves.

However, the combined biotic treatment (B_3 : Azolla + cyanobacteria) recorded the lowest mean nitrate content in lettuce leaves, reaching 2478 mg kg^{-1}. In contrast, the values for treatments B_1 (Azolla) and B_2 (cyanobacteria) were relatively close, at 2508 and 2528 mg kg^{-1}, respectively.

Regarding nitrogen fertilization levels, the control treatment recorded the highest mean nitrate content (2692 mg kg^{-1}), whereas the N_1 level (72 mg L^{-1}) showed the lowest mean nitrate value (2447 mg kg^{-1}). This study is consistent with(27) and (12), who reported that the use of biofertilizers in hydroponic systems improved nutrient uptake, reduced nitrate accumulation in the leaves, and enhanced lettuce quality.

ISSN 2072-3857

Table (2): Effect of biotic treatments and nitrogen fertilization on the mean nitrate content in lettuce leaves (mg kg^{-1}) under nutrient film technique(NFT) (hydroponic system

Biotic Treatments (B)	Nitrogen Fertilization (N)			Mean of Biotic Treatments
	N_1 (72 mg L^{-1})	N_2 (108 mg L^{-1})	N_3 (144 mg L^{-1})	
Azolla (B_1)	2483	2600	2442	2508
Cyanobacteria (B_2)	2467	2533	2583	2528
Azolla + Cyanobacteria (B_3)	2392	2542	2500	2478
Mean of Nitrogen Levels	2447	2558	2508	
Control	2692			
LSD (0.05)	N = N.S (Not Significant) B = N.S B × N = N.S			

The results of the present study and the statistical analysis (Table 2) showed that nitrate concentration in lettuce leaves was lowest under the biotic treatment (B_3), which involved the combined application of cyanobacteria and *Azolla*. In addition, nitrate levels were further reduced at the nitrogen application rate of 108 mg L^{-1} in both hydroponic systems (ponds and nutrient film technique(NFT).

The reduction of nitrate content in lettuce leaves is considered beneficial from a human health perspective due to the potential harmful effects of excessive nitrate accumulation. These findings highlight the positive role of the interaction between *Azolla* and cyanobacteria in reducing nitrate levels in plants. This effect can be attributed to their ability to assimilate nitrogen from the growth medium and incorporate it into their biomass, thereby decreasing the availability of nitrate in the solution and ultimately reducing its accumulation in plant tissues compared to mineral fertilizers (20).

The symbiotic relationship between *Azolla* and cyanobacteria also contributes to improving lettuce quality indicators. Instead of relying entirely on chemical nitrogen sources such as calcium nitrate or potassium nitrate, *Azolla* provides a slow-release nitrogen source, which limits excessive nitrate accumulation in lettuce leaves an important quality parameter in hydroponic production (18).

ISSN 2072-3857

The significant reduction in nitrate concentration observed when *Azolla* and cyanobacteria were applied together is also associated with the high capacity of *Azolla* to absorb nitrogen from the aquatic medium. In hydroponic systems, both organisms act as biological filters, absorbing nitrate and converting it into organic compounds within their tissues. Moreover, the rapid growth rate and increased biomass production of *Azolla* enhance nitrogen uptake from the nutrient solution, thereby reducing nitrate concentration in the system (5).

Additionally, *Azolla* and cyanobacteria may enhance nitrate reductase activity within lettuce plants, promoting the conversion of absorbed nitrate into organic compounds such as amino acids and proteins, which reduces nitrate accumulation in leaves (17). This process also supports improved vegetative growth and increased biomass production, enabling the plant to utilize nitrate for structural synthesis rather than storing it in leaf tissues (Singh, 1977). Consequently, the reduction in nitrate accumulation improves the nutritional and health quality of lettuce, as high nitrate levels in leafy vegetables are a major food safety concern (23).

The lack of significant differences in nitrate content among treatments in the nutrient film technique(NFT) system, compared to the pond system, may be attributed to differences in hydroponic system design. Open pond systems provide more stable environmental conditions, including better light exposure, aeration, and oxygen exchange, which enhance the activity of beneficial microorganisms and algae. Furthermore, the larger volume and stability of the nutrient solution in ponds create a more favorable environment for the growth and activity of *Azolla* and cyanobacteria, allowing them to effectively fix nitrogen and gradually supply it to plants, thereby reducing nitrate accumulation in lettuce leaves.

In contrast, nutrient film technique(NFT) systems have smaller solution volumes and are more prone to rapid fluctuations in nutrient concentration, which may limit the effectiveness of these microorganisms (12). Additionally, ponds allow for greater biomass development of *Azolla* and cyanobacteria, increasing their capacity for nitrogen fixation and nitrate uptake, whereas nutrient film technique(NFT) systems provide limited space for their growth, reducing their biological impact (17).

Another contributing factor is the residence time of the nutrient solution. In pond systems, the solution remains relatively static for longer periods, allowing sufficient time for microbial processes such as nitrogen fixation and nitrate reduction to occur. In contrast, nutrient film technique(NFT) systems involve continuous nutrient flow, reducing the time available for these biological transformations (19).

Differences in light and aeration conditions may also play a role. *Azolla* and cyanobacteria require adequate light and oxygen for optimal growth. Pond systems generally provide more favorable and stable conditions, whereas nutrient film technique(NFT) systems may limit these factors, reducing microbial activity (13).

Furthermore, in nutrient film technique(NFT) systems, the continuous flow of nutrient solution around plant roots allows rapid nitrate uptake directly by the plant before *Azolla* or cyanobacteria can significantly influence nitrate concentration. This may explain the absence of significant differences among treatments (24)

These findings are consistent with those of (3), who reported that the biological interaction between *Azolla* and cyanobacteria effectively reduces nitrate accumulation in lettuce leaves by supplying nitrogen in a slow-release form, thereby lowering nitrate concentration in plant tissues compared to conventional mineral fertilization.

Similarly, (27) demonstrated that the presence of *Azolla* and cyanobacteria enhances microbial processes involved in the nitrogen cycle, such as denitrification, where nitrate is converted into gaseous nitrogen forms, resulting in reduced nitrate levels in the aquatic environment. The rate of these processes depends largely on environmental conditions within the hydroponic system.

Conclusions

1. The combined application of *Azolla* and cyanobacteria in hydroponic systems reduced nitrate concentration in lettuce leaves compared to the treatment based on the full recommended mineral fertilization. This is attributed to the ability of these microorganisms to fix atmospheric nitrogen and convert it into forms readily assimilated by plants without causing nitrate accumulation in plant tissues.
2. The use of *Azolla* and cyanobacteria improved nitrogen use efficiency in lettuce, as they act as a biological nitrogen source that releases nitrogen gradually, thereby reducing excessive nitrate uptake over short periods and decreasing

ISSN 2072-3857

dependence on mineral fertilizers.

3. The pond hydroponic system showed a significant superiority over the nutrient film technique(NFT) system in reducing nitrate accumulation in lettuce leaves.
4. The reduction in nitrate accumulation in lettuce leaves led to improved crop quality from both nutritional and health perspectives, as high nitrate levels in leafy vegetables are considered a major food safety concern.

References

1. .Barker, A.V., Bryson, G.M., 2007. Nitrogen. Handbook of plant nutrition. CRC Press, Boca Raton, Florida, USA, pp. 21–50. Barker, A.V., Pilbeam, D.J.
2. Bito, T., Ohishi, N., Hatanaka, Y., Takenaka, S., Nishihara, E., Yabuta, Y., & Watanabe, F. (2013). Production and characterization of cyanocobalamin-enriched lettuce (Lactuca sativa L.) grown using hydroponics. Journal of Agricultural and Food Chemistry, 61(16), 3852–3858. https://doi.org/10.1021/jf305033s.
3. Brouwer, P., Schluepmann, H., Nierop, K. G. J., Elderson, J., Bijl, P. K., van der Meer, I., de Visser, W., Reichart, G.‐J., Smeekens, S., & van der Werf, A. (2018.(Growing Azolla to produce sustainable protein feed: The effect of differing species and CO_2 concentrations on biomass productivity and chemical composition. Journal of the Science of Food and Agriculture, 98(12): 4759–4768 .
4. Carvalho, J. M., Ramos, S. J., Furtini Neto, A. E., Gastauer, M., Caldeira Jr, C. F., Siqueira, J. O., & Silva, M. L. (2018). Influence of nutrient management on growth and nutrient use efficiency of two plant species for mineland revegetation. Restoration Ecology, 26(2), 303-310.
5. Chakraborty, S., Ahmed, S., Ghosh, R., Banerjee, A., & Mukherjee, S. (2025). Eco-friendly nutrient management in fish ponds: the role of Azolla in nitrate and phosphate reduction. Discover Plants, 2, 128. https://doi.org/10.1007/s44372-025-00213-4
6. Ciriello, M., Formisano, L., Pannico, A., El-Nakhel, C., Fascella, G., Duri, L.G., Carillo, P., 2021. Nutrient solution deprivation as a tool to improve hydroponics sustainability: yield, physiological, and qualitative

response of lettuce. Agronomy 11 (8), 1469.

7. Divya, K., Suresh, P., Ramesh, T., & Prasad, K. (2020) Azolla as a sustainable biofertilizer for improving crop productivity – A review. International Journal of Creative Research Thoughts (IJCRT), 8(4).

8. Dudziak, A., & Kocira, A. (2022). Preference-Based Determinants of Consumer Choice on the Polish Organic Food Market.*International Journal of Environmental* **Research and Public Health**, **19**(17), 10895. https://doi.org/10.3390/ijerph191710895
9. FAOSTAT, 2024. Food and agriculture organization corporate statistical database. http://faostat3.fao.org/home/index.html, 18/03/2024.
10. Guffanti, D., Cocetta, G., Franchetti, B.M., Ferrante, A., 2022. The effect of flushing on the nitrate content and postharvest quality of lettuce (Lactuca sativa L. Var. Acephala) and rocket (Eruca sativa Mill.) grown in a vertical farm. Horticulturae 8, 604.

11. Horiba Scientific. (2022). *LAQUAtwin NO_3 $^-$ Ion Meter Instruction Manual*. Kyoto, Japan: Horiba Ltd.
12. Ikiz, B., Dasgan, H. Y., & Gruda, N. S. (2024). Utilizing plant growth-promoting rhizobacteria to reduce mineral fertilizer and improve yield and quality of hydroponic lettuce. Scientific Reports, 14, 1616. https://doi.org/10.1038/s41598-024-51818-w
13. Kannaiyan, S. (2002). Biofertilizers for Sustainable Crop Production. Scientific Publishers.
14. Kollah, B., Patra, A. K., & Mohanty, S. R. (2016) .Aquatic microphylla Azolla: A perspective paradigm for sustainable agriculture, environment and global climate change. Environmental Science and Pollution Research, 23(5), 4358–4369.
15. lSamarrai, Ismail Khalil & Faris, Mohammed Suhail. (2018). *Concepts and Applications of Soil Microbiology*. Central Press / University of Diyala.
16. Nicolle, C., Cardinault, N., Gueux, E., Jaffrelo, L., Rock, E., Mazur, A., Amouroux, P., R´ em´ esy, C., 2004. Health effect of vegetable-based diet: lettuce consumption improves cholesterol metabolism and antioxidant status in the rat. Clin. Nutr. 23, 605–614.
17. Rai, A. N., Söderbäck, E., & Bergman, B. (2000).

Cyanobacterium–Plant Symbioses. New Phytologist.

18. Resh.,H. M. (2013). Hydroponic Food Production. CRC Press.
19. Rouphael, Y. et al. Nutritional quality of hydroponically grown basil in response to salinity and growing season. Acta Hortic. 1227, 693–698 (2017).
20. Santamaria, P. (2006). Nitrate in Vegetables: Toxicity, Content, Intake and Regulations. Journal of the Science of Food and Agriculture.
21. Simko, I., 2019. Genetic variation and relationship among content of vitamins, pigments, and sugars in baby leaf lettuce. Food Sci. Nutr. 7, 3317–3326.
22. Singh, P. K. (1977). Azolla plants as fertilizer and feed. Economic Botany.
23. Taiz, L., Zeiger, E., Møller, I. M., & Murphy, A. (2015). Plant Physiology and Development. Sinauer Associates.
24. Thapa, U., Nandi, S., Rai, R., Upadhyay, A., 2022. Effect of nitrogen levels and harvest timing on growth, yield and quality of lettuce under floating hydroponic system. J. Plant Nut. 45 (17), 2563–2577.
25. Yoldas, F., Ceylan, S., Yagmur, B., Mordogan, N., 2008. Effects of nitrogen fertilizer on yield quality and nutrient content in broccoli. Plant Nut 31 (7), 1333–1343.
26. Zhao, Y., Liu, X., Zhang, Q., & Chen, J. (2023). Microbial regulation of nitrogen cycling in aquatic ecosystems. Environmental Research, 216, 114473.
27. Dasgan, H.Y., Yilmaz, D., Zikaria, K., Ikiz, B. and Gruda, N.S., 2023. Enhancing the yield, quality and antioxidant content of lettuce through innovative and eco-friendly biofertilizer practices in hydroponics. Horticulturae, 9(12), p.1274.
28. Renganathan, P., Puente, E.O.R., Sukhanova, N.V. and Gaysina, L.A., 2024. Hydroponics with microalgae and cyanobacteria: Emerging trends and opportunities in modern agriculture. *BioTech*, 13(3), p.27.

ISSN 2072-3857