

Agronomic Performance and Molecular Insights into Nitrogen Management of Citrus Sinensis L. Using Bio-Fertilizers and Nitrification Inhibitors

EL-Sayed M. Qaoud^{1*}, Mohamed A. Abdein^{2*}, Hoda A.A. Desoky³, Safia M. Ezzat⁴, Fatma M.A. Elkady⁵, Ahmed Y. El-Saman⁶, Zeinab A. Zaki⁶, Huda M.H. Ismaiel⁶, Randa E.Y. Habasy⁶, Lames Abd El-Ati⁶, Dina S.M. Ibrahim⁶, Osama S. Mokhtar⁶, Faisl M.A. El Shaib⁶, Amgad M. Elazazy⁶, Wafaa A.M. Shehata⁶, Rania A.E. Abdelzاهر⁶, Antar M.A. Badran⁷, Amira S.A. Abd El-Rahman³

1. Horticultural Department, Faculty of Agriculture, Suez Canal University, Ismailia 41522, Egypt
2. Seeds Development Department, El-Nada Misr Scientific Research and Development Projects, Turrell, Mansoura, 35511, Egypt
3. Horticulture Department, Faculty of Agriculture, Benha University, Moshtohor, Toukh, Qalyoubia, 13736, Egypt
4. Statistics Department, Faculty of Commerce, Al-Azhar University, Girls' Branch, Cairo, Egypt
5. Botany Department, National Research Center, Dokki, Cairo, Egypt
6. Horticulture Research Institute, Agricultural Research Centre, Giza 12619, Egypt
7. Plant Production Department, Desert Research Center, Cairo, Egypt

*Correspondence: Mohamed A. Abdein: abdeingene@yahoo.com and EL-Sayed M. Qaoud: s_qaoud@agr.suez.edu.eg

Abstract

The experiment examined the effects of various nitrogen fertilization treatments on 20-year-old Baladi orange trees at two locations in Egypt over the 2023/2024 and 2024/2025 growing seasons. It employed a split-plot design with bio-fertilizer treatments as main plots and multiple mineral nitrogen sources as sub-plots. Key findings indicated significant effects of both mineral and bio-nitrogen sources, along with their interaction, on fruit weight, quality traits, and nutrient content across both locations. Analysis showed that the highest performance was associated with ammonium sulfate combined with nitrification inhibitors, while the application of bio-nitrogen consistently outperformed non-bio treatments across all measured traits. Most agricultural traits showed significant interaction effects across environments. Only the fruit juice (%) trait exhibited stable performance without any significant interactions. Mean comparisons revealed that the interaction between Bio-nitrogen and location was notably significant, with better performance in Ismailia (L1) compared to Qena (L2). Nitroben (bio-nitrogen) in a rate of 50 g/tree (twice in February and June) interacted with 2.864 kg/tree/year ammonium sulphate 20% N plus inhibitor exhibited significant positive effects for average fruit weight, fruit size, fruit juice as well as the superiority for positive effect of N, P, K of leaf content, indicating the possibility of combine both high yield and good quality characters under various environmental conditions. Principal component analysis (PCA) demonstrated marked differences between bio-nitrogen and non-bio treatments, highlighting bio-nitrogen's effectiveness, especially in Ismailia location. Overall, these findings suggest that the effectiveness of bio-fertilizers is influenced by environmental conditions and should be tailored accordingly.

Keyword: Bio-fertilizer, Nitroben, Orange fruit, Molecular docking, Nitrapyrin inhibitor, AMO enzyme, PCA.

Introduction

Baladi orange trees (*Citrus sinensis* L.) are a key juice variety in Egypt, well-suited to the Nile Delta's climate but challenged by soil and environmental factors. Their productivity and quality are largely affected by cultivation region, with new reclaimed sandy areas needing

specialized management techniques such as bio-nitrogen fertilizers and soil conditioners to compete with the fertile Delta's yields. The Delta remains the primary cultivation area, while arid regions require distinct irrigation strategies. In Upper Egypt, Heat stress has become an important determining factor for citrus production. As temperature fluctuation at the beginning of the growing seasons and that rising of day/night temperature above optimum range in the period from June to August (above 40 °C) impact on growth, flowering, setting and productivity of citrus which performs best at temperatures between 15.6 and 30 °C (Parsons and Beck, 2004). Optimal fertilizer uses and practices are crucial in maintaining fruit quality. Can be prevent the effect of abiotic stresses by amelioration the conditions of plant growth through improve the nutritional status of plants. (Kai and Koh, 2014; Yakhin, et al., 2017; Sharma and Manjeet, 2020, Albalwe et al., 2024, Mosallam et al., 2024).

Nitrogen (N) is one of the most important nutrients that have an important impact on plant growth and fruiting (Canovas et al., 2018). In addition, it is an essential component of acids, chlorophyll and many phytohormones may studies have confirmed that its deficiency has many symptoms and a negative impact on growth, yield and fruit quality (Marschner, 2012, Elazazi et al., 2024).

Nutrition management considered an important part of agricultural performs that reduce pollution soil and groundwater resources, help protect the environment and though, and keeping of different natural resources (Abobatta, 2018).

Using nitrification inhibitors represents an effective strategy for improving nitrogen use efficiency, as these inhibitors reduce the activity of Nitrosomonas bacteria in the soil, which are responsible for oxidizing ammonium ion to nitrite ion using an enzyme known as ammonia monooxygenase (AMO), this reaction is the first step in the nitrification process, and further. Subsequently, Nitrobacter bacteria oxidize nitrite ion to nitrate ion. Nitrification inhibitors suppress the process of converting ammonium ion to nitrite ion in the soil by inhibiting the activity of the ammonia monooxygenase enzyme, maintaining nitrogen in the stable ammonium form, and reducing loss of nitrogen fertilizers by leaching, thus improving nitrogen use efficiency. (Trenkel, 2010, Ruser, and Schulz, 2015 and Nevins et al., 2020).

Improving nitrogen fertilizer efficiency is a key factor to improve crop productivity and fruit quality under different environmental conditions. This can be achieved by evaluating different nitrogen management practices including the use of different mineral nitrogen sources, bio-fertilization and nitrification inhibitors which delay nitrogen transformation and enhance its availability for plants. Understanding the interaction of these approaches with environmental factors is crucial for the development of efficient and sustainable fertilization practices.

Proper nutrient management is essential to optimize yield and productivity of orange and reducing the environmental hazard. However, environmental variables according to Allard and Bradshaw (1964) are classified into predictable (under human control, such as soil type, planting date, row spacing, plant population and rates of nutrient application) and unpredictable (inconsistently fluctuating) include rainfall, temperature, and relative humidity.

To identify superior fertilization strategies with stable performance, it is essential to evaluate treatments over several locations and then use the components of variance to compare treatment x Environment interaction. Accordingly, this study aimed to investigate the variability in yield response of Baladi orange (*Citrus sinensis* L.) to different nitrogen fertilization strategies, assessing the role of nitrification inhibitors and bio-nitrogen in improving nitrogen use efficiency and to evaluate their interactions with specific locations. The goal is to optimize a planting system that maximizes return to planting management,

whether it is optimal agriculture or early due to weather. Generally, the study intended to evaluate the yield performance and fruit quality characteristics of Baladi orange (*Citrus sinensis*) grown under different environmental conditions across two locations.

Materials and Methods

Experimental sites and seasons

The experiment was conducted during two successive growing seasons of 2023/2024 and 2024/2025, at two different locations. Locations included the Wadi El-Mollak region, Ismailia governorate (30° 44' 47.6" N, 32° 0' 16.16" E) represents to locality 1 and Nag Hammadi region, Qena governorate of Upper Egypt on the west bank of the Nile River (26° 3' 0" N, 32° 15' 0" E) represents to locality 2, Egypt. The physical and chemical analyses of soils are shown in Table (1).

Table 1. Analysis of the orchard soils of both locations

Item	Locations	Locality 1	Locality 2
Physical properties	Organic matter (%)	1.2	1.8
	Textural class	Loamy Sand	Clay loam
Chemical properties			
pH		8.4	7.89
Electric conductivity E.C. (ds/m)		0.4	0.7
CaCO₃		0	2.48
CEC (meq/100g) soil		4.65	17.62
Soluble ions, meq / L	Soluble anions	HCO ₃ ⁻	1.77
		CL ⁻	1.43
		SO ₄ ⁻²	1.17
	Soluble cations	Ca ⁺²	1.77
		K ⁺	0.56
		Na ⁺	1.71

Each location–season combination was treated as an independent environment.

Tree were planted at a distance of 5 × 4 m, with a drip irrigation system, to study the impact of mineral Nitrogen sources including Nitrification inhibitors and bio-nitrogen (Nitrobenin) fertilization on 20-year-old Baladi orange (*Citrus sinensis* L.) budded on sour orange rootstock (*Citrus aurantium* L.).

The trees were selected as uniform as possible in growth, vigor, fruiting, and free from any disease. Standard cultural practices recommended for the orange including fertilizer (except nitrogen), pruning, irrigation, and pest control were uniformly applied throughout the experiment, except for the imposed fertilization treatments.

Experimental design: The experiment was laid out in a split-plot design with three replicates at each location in each season (Plate 1).

- **Main plots:** Bio-fertilizer treatments
- **Sub-plots:** Mineral nitrogen fertilization treatments

Each replicate consisted of all bio-fertilizer treatments assigned randomly to the main plots, and within each main plot, nitrogen fertilization treatments were randomly allocated to the sub-plots.

Replicate 1		Replicate 2		Replicate 3	
Main plot		Main plot		Main plot	
Bio1 (50 g/tree)	Bio2 (Untreated)	Bio1 (50 g/tree)	Bio2 (Untreated)	Bio1 (50 g/tree)	Bio2 (Untreated)
control	control	control	control	control	control
AS100	AS100	AS100	AS100	AS100	AS100
AS50	AS50	AS50	AS50	AS50	AS50
AN100	AN100	AN100	AN100	AN100	AN100
AN50	AN50	AN50	AN50	AN50	AN50
AS100+inh	AS100+inh	AS100+inh	AS100+inh	AS100+inh	AS100+inh
AS50+inh	AS50+inh	AS50+inh	AS50+inh	AS50+inh	AS50+inh
AN100+inh	AN100+inh	AN100+inh	AN100+inh	AN100+inh	AN100+inh
AN50+inh	AN50+inh	AN50+inh	AN50+inh	AN50+inh	AN50+inh
SCU100	SCU100	SCU100	SCU100	SCU100	SCU100
SCU50	SCU50	SCU50	SCU50	SCU50	SCU50

Plate 1: Experimental layout diagram (Split-plot with three replicates)

Key:

AS = Ammonium sulfate, AN = Ammonium nitrate, AS+inh = AS + inhibitor, AN+inh = AN + Inhibitor, SCU = Sulfur-coated urea, 50 and 100 represent to 50 unit and 100 unit

Bio-fertilizer treatments (Main plots): Two bio-fertilizer levels were tested:

1. **Without bio-fertilizer (Non-bio)**
2. **With bio-fertilizer (Bio)**

Bio-nitrogen (Nitrobein) was applied twice during the growing season, in February and June, at a rate of 50 g tree⁻¹ per application.

Mineral nitrogen fertilization treatments (Sub-plots):

Six nitrogen fertilizer sources were evaluated at two nitrogen levels (50 and 100 units):

1. **Ammonium sulfate (AS)**
 - 50 units: 179 g tree⁻¹ month⁻¹, equivalent to 1,432 g tree⁻¹ year⁻¹
 - 100 units: 357 g tree⁻¹ month⁻¹, equivalent to 2,864 g tree⁻¹ year⁻¹
2. **Ammonium nitrate (AN)**
 - 50 units: 109 g tree⁻¹ month⁻¹, equivalent to 872 g tree⁻¹ year⁻¹
 - 100 units: 218 g tree⁻¹ month⁻¹, equivalent to 1,744 g tree⁻¹ year⁻¹
3. **Ammonium sulfate + nitrification inhibitor (AS+Inh)**
 - Nitrogen rates identical to AS
 - Nitrification inhibitor applied at 50 mg or 100 mg tree⁻¹
4. **Ammonium nitrate + nitrification inhibitor (AN+Inh)**
 - Nitrogen rates identical to AN
 - Nitrification inhibitor applied at 50 mg or 100 mg tree⁻¹
5. **Sulfur-coated urea (SCU)**
 - 50 units: 793 g tree⁻¹ year⁻¹
 - 100 units: 1,586 g tree⁻¹ year⁻¹
 - Applied once annually without monthly splitting
6. **Bio-nitrogen (Nitrobein)**
 - Applied as described under bio-fertilizer treatments
 - Nitrobein (a bio-fertilizer containing non-symbiotic nitrogen-fixing bacteria

Azotobacter chroococcum) obtained from General Authority for Agricultural Equalization Fund, Ministry of Agriculture and Land Reclamation, Egypt)

Nitrogen applications for the treatments of both AS and AN were split monthly from March to October, while SCU was applied as a single annual dose.

Data recorded:

The following parameters were measured during the two growth seasons of each location:

Leaf content of N, P and K: forty mature leaves of Spring growth cycle in the non-fruiting shoots (Summer, 1985) were randomly collected in end September from each replicate, cleaned, washed and oven dried at 70°C to a constant weight, then ground and digested according to Evenhuis and DeWaard (1980) using H₂SO₄ and H₂O₂ to determine the elements N, P and K in leaves using the Micro-Kjeldahl method (Pregl, 1945), color-metrically (Murphy and Riley, 1962) and flame photometer (Brown and Lillelland, 1974), respectively

Fruit weight per tree: at harvesting time, in the end of December, the fruits were harvested from each tree and weighed as a whole and recorded the fruit weight/ tree (FW, kg)

Fruit quality properties: twenty fruits were taken randomly from each tree (5 fruit/ each direction) to determine the fruit properties, i.e., average fruit weight (AFW, g), size (FS, cm³), and peel thickness (PT, cm) as well as fruits chemical properties (average juice volume/fruit, JV cm³, fruit juice percentage (FJ%), T.S.S./acidity ratio and Vitamin C content (ascorbic acid content per 100 ml of juice) according to AOAC, (2006).

Statistical analyses:

The data were first subjected to analysis of variance for each location using the procedure illustrated by Gomez and Gomez (1984) for a complete randomized block design (CRBD) and the means of the different treatments were compared by Duncan's New Multiple Range Test (DMRT) using OPSTAT Software, as described by Sheoran et al. (1998).

A combined analysis of variance was computed using the same software as for the single site analysis (OPSTAT) in order to study the bio-nitrogen × environment (Bio× env.) and mineral nitrogen × environment (MF× env.) interactions, as well as to find out the implications of the confounding of bio-nitrogen × mineral nitrogen fertilization (Bio×MF) by different separately ways:

- Effect of bio-nitrogen and mineral nitrogen within each location and year, and the relative magnitude of Bio×MF.
- Locality effect, over the averages of the two seasons in each location, and the relative magnitude of Site × Bio, Site × MF and Site × MF × Bio interactions.

A principal component analysis (PCA) based on the bio-nitrogen main effect on all eleven studied traits on the two locations (over seasons) was conducted in a biplot-style dispersion plot. PCA yielded eigenvalues, which were then used to ascertain the axes' and their corresponding characteristics' relative discriminative power (Pradhan et al., 2015). A bi-plot figure was used to classify the treatments and compare them using cluster analysis.

Results

Analysis within individual environments

The analysis of variance for AFW, FW/tree and fruit quality traits (FS, PT, JV, TSS/A and VC) as well as nitrogen (N%), and potassium (K%) percentages conducted separately for each environment (L1S1, L1S2, L2S1 and L2S2) revealed highly significant effects ($P < 0.001$) of mineral nitrogen (MF), bio-nitrogen (Bio), and their interaction (MF $\times$ Bio) in all four environments (Tables 2 & 3).

Table (2): Mean square of bio-nitrogen (Bio) and mineral nitrogen (MF) combined analysis for Leaf contents, Fruit quality properties and Fruit weight per tree during 2023/2024 (S1) and 2024/2025 (S2) in Ismailia location (L1)

Source of Variation	D F	N	P	K	AFW	FW /tree	FS	JV	FJ	PT	TSS /A	VC
Mean Squares												
L1S1												
MF	10	1.02 **	0.003 **	0.436 **	3474 **	3355 **	10184 **	353.2 **	9.22 **	0.038 **	20.99 **	308.8 **
Bio	1	2.55 **	0.009 **	1.176 **	10259 **	9104 **	24049 **	906.8 **	29.06 **	0.104 **	58.43 **	1065.5 **
MF $\times$ Bio	10	0.04 **	0.0001 **	0.017 **	100.8 **	1425 **	541.74 **	15.16 **	0.13 **	0.002 **	1.75 **	22.99 **
Error	42	0.01	0.0001	0.002	30.1	5.5	33.27	1.33	0.29	0.0001	0.03	1.41
L1S2												
MF	10	1.09 **	0.003 **	0.451 **	4267.8 **	3891.7 **	7117.6 **	444.8 **	11.41 **	0.042 **	21.61* *	329.06* *
Bio	1	3 **	0.009 **	1.26 **	13163.5 **	10660 **	18541.37 **	1355.7 **	32.3 **	0.117 **	57.61* *	1147.5* *
MF $\times$ Bio	10	0.03 **	0.0001 **	0.016 **	158.3 **	172.3 **	174.06 **	11.93 **	0.3 **	0.001 **	1.36 **	26.06 **
Error	42	0.01	0.00007	0.002	23	4.8	25.01	2.17	0.73	0.00002	0.02	1.18

In environment 1 (Ismailia in first season, L₁S₁), mineral nitrogen (MF) explained a substantial proportion of the total variability in all traits ($P < 0.001$), while bio-nitrogen (Bio) exerted an even stronger effect ($P < 0.001$). The significant MF $\times$ Bio interaction ($P < 0.001$) indicates that the response of all traits to mineral nitrogen (MF) varied depending on the level of bio-nitrogen (Bio) (Table 2) except P% and fruit juice (%) in which MF $\times$ Bio interaction was not significant.

As for Bio- nitrogen, Bio-1 significantly outperformed non bio-nitrogen (Bio-2) in all environments for all studied traits. Also, across all environments, mineral nitrogen MF5 (AS₁₀₀+inh) produced the highest values for all traits, followed by MF7 (AN₁₀₀+inh), MF1, MF3 and MF9 in descending order with no significant differences between MF1 and MF3 for phosphorus percentage (P%) in 2nd season and between MF3 and MF9 for P% and FJ % of Qena region as well as between MF5 and MF7 for P% (1st season) and FJ% (both seasons) in both Ismailia and Qena regions. whereas control MF treatment consistently recorded the lowest values (Tables 4a&b-5a&b).

Table 3. Mean square of bio-nitrogen (Bio) and mineral nitrogen (MF) combined analysis for Leaf contents, Fruit quality properties and Fruit weight per tree during 2023/2024 (S1) and 2024/2025 (S2) in Qena location (L2)

Source of Variation	D F	N	P	K	AFW	FW/ tree	FS	JV	FJ	PT	TSS /A	VC
Mean Squares												
L2S1												
MF	10	0.73 **	0.002 **	0.313 **	2491 **	2406 **	7302 **	253.2 **	6.6 **	0.027 **	15.04 **	221.5 **
Bio	1	1.82 **	0.007 **	0.85 **	73575 **	6528.8 **	17245.5 **	650.63* *	20.9 **	0.073 **	41.81 **	764.2 **
MF × Bio	10	0.03 **	0.0001	0.013 **	72.3 **	102.22 **	388.42 **	10.85 **	0.09	0.001 **	1.25 **	16.49 **
Error	42	0.01	0.000071	0.001	21.6	3.95	23.85	0.96	0.21	0.00002	0.02	1.01
L2S2												
MF	10	0.83 **	0.002 **	0.339 **	3206.6 **	2923.9 **	5347.8 **	334.25 **	8.58 **	0.031 **	16.23 **	247.3 **
Bio	1	2.31 **	0.007 **	0.953 **	9891.7 **	8007.9 **	13932.85 **	1018.52 **	24.37 **	0.089 **	43.25 **	862.2 **
MF × Bio	10	0.03 **	0.0001	0.012 **	118.9 **	129.51 **	130.59 **	8.97 **	0.22	0.001 **	1.02 **	19.57 **
Error	42	0.01	0.00004	0.001	17.2	3.61	18.75	1.63	0.55	0.00002	0.01	0.88

A similar trend was observed in Environments 2 (Ismailia, L1S2), 3 (Qena, L2S1), and 4 (Qena, L2S2), where both main factors and their interaction were consistently highly significant ($P < 0.001$; Tables 2–3) in the same traits.

Table 4a. NPK properties as affected by mineral and bio-nitrogen, as well as their interaction in the two environments of Ismailia location (two seasons)

	N		P		K	
Treatments	Bio	Non-Bio	Bio	Non-Bio	Bio	Non-Bio
Environment 1 (L1S1), Ismailia 2023/2024						
Control	1.397h	1.357g	0.073f	0.073f	0.977g	0.950i
MF1 (AS ₁₀₀)	2.647c	2.403ab	0.150a	0.120ab	1.843ab	1.490c
MF2 (AS ₅₀)	2.300ef	1.777de	0.117cde	0.093de	1.403e	1.123fg
MF3 (AN ₁₀₀)	2.613c	2.330b	0.147a	0.120ab	1.787bc	1.447c
MF4 (AN ₅₀)	2.090g	1.673ef	0.103e	0.083ef	1.263f	1.090gh
MF5 (AS ₁₀₀ +inh)	3.033a	2.543a	0.153a	0.133a	1.890a	1.700a
MF6 (AS ₅₀ +inh)	2.473cd	1.953c	0.127bc	0.103cd	1.560d	1.217e
MF7 (AN ₁₀₀ +inh)	2.823b	2.507a	0.150a	0.130a	1.870a	1.617b
MF8 (AN ₅₀ +inh)	2.437de	1.883cd	0.123cd	0.093de	1.530d	1.190ef
MF9 (SCU ₁₀₀)	2.577cd	2.263b	0.140ab	0.113bc	1.743c	1.347d
MF10 (SCU ₅₀)	2.193fg	1.567f	0.110de	0.083ef	1.277f	1.037h
Environment 2 (L1S2), Ismailia 2024/2025						
Control	1.437a	1.397a	0.077a	0.077a	1.023a	0.993a
MF1 (AS ₁₀₀)	2.877a	2.430a	0.153a	0.123a	1.900a	1.537a
MF2 (AS ₅₀)	2.333a	1.813a	0.120a	0.093a	1.453a	1.190a
MF3 (AN ₁₀₀)	2.730a	2.363a	0.150a	0.120a	1.840a	1.477a
MF4 (AN ₅₀)	2.167a	1.757a	0.110a	0.090a	1.330a	1.137a
MF5 (AS ₁₀₀ +inh)	3.110a	2.597a	0.163a	0.140a	2.017a	1.760a
MF6 (AS ₅₀ +inh)	2.507a	2.000a	0.130a	0.107a	1.613a	1.270a
MF7 (AN ₁₀₀ +inh)	2.937a	2.540a	0.157a	0.133a	1.933a	1.687a
MF8 (AN ₅₀ +inh)	2.453a	1.923a	0.127a	0.100a	1.577a	1.250a
MF9 (SCU ₁₀₀)	2.647a	2.283a	0.150a	0.113a	1.790a	1.397a
MF10 (SCU ₅₀)	2.223a	1.623a	0.110a	0.087a	1.363a	1.103a

Table 4b. NPK properties as affected by mineral and bio-nitrogen, as well as their interaction in the two environments of Qena location (two seasons)

	N		P		K	
Treatments	Bio	Non-Bio	Bio	Non-Bio	Bio	Non-Bio
Environment 3 (L2S1), Qena 2023/2024						
Control	1.180a	1.150a	0.063a	0.060a	0.827a	0.803a
MF1 (AS ₁₀₀)	2.240a	2.037a	0.127a	0.100a	1.560a	1.260a
MF2 (AS ₅₀)	1.950a	1.503a	0.100a	0.077a	1.187a	0.950a
MF3 (AN ₁₀₀)	2.210a	1.980a	0.127a	0.100a	1.517a	1.223a
MF4 (AN ₅₀)	1.770a	1.417a	0.090a	0.070a	1.067a	0.923a
MF5 (AS ₁₀₀ +inh)	2.567a	2.153a	0.130a	0.113a	1.600a	1.440a
MF6 (AS ₅₀ +inh)	2.093a	1.650a	0.107a	0.087a	1.323a	1.030a
MF7 (AN ₁₀₀ +inh)	2.390a	2.123a	0.130a	0.110a	1.583a	1.367a
MF8 (AN ₅₀ +inh)	2.067a	1.590a	0.103a	0.083a	1.297a	1.007a
MF9 (SCU ₁₀₀)	2.180a	1.920a	0.120a	0.093a	1.477a	1.140a
MF10 (SCU ₅₀)	1.860a	1.330a	0.090a	0.067a	1.080a	0.877a
Environment 4 (L2S2), Qena 2024/2025						
Control	1.257a	1.227a	0.067a	0.067a	0.883a	0.863a
MF1 (AS ₁₀₀)	2.523a	2.130a	0.133a	0.103a	1.647a	1.330a
MF2 (AS ₅₀)	2.047a	1.590a	0.100a	0.083a	1.260a	1.030a
MF3 (AN ₁₀₀)	2.390a	2.073a	0.130a	0.103a	1.593a	1.280a
MF4 (AN ₅₀)	1.900a	1.540a	0.093a	0.080a	1.153a	0.983a
MF5 (AS ₁₀₀ +inh)	2.727a	2.273a	0.140a	0.120a	1.747a	1.523a
MF6 (AS ₅₀ +inh)	2.200a	1.753a	0.113a	0.090a	1.400a	1.100a
MF7 (AN ₁₀₀ +inh)	2.577a	2.230a	0.137a	0.113a	1.677a	1.463a
MF8 (AN ₅₀ +inh)	2.153a	1.687a	0.110a	0.083a	1.370a	1.083a
MF9 (SCU ₁₀₀)	2.320a	2.003a	0.130a	0.100a	1.553a	1.210a
MF10 (SCU ₅₀)	1.950a	1.423a	0.097a	0.073a	1.180a	0.953a

Table 5a. Average fruit weight (AFW, g), fruit size (FS, cm³), peel thickness (PT, cm) and fruits weight per tree (FW, kg) as affected by mineral and bio-nitrogen, as well as their interaction in Ismailia location at 2023/2024 and 2024/2025 seasons

	AFW		FW/tree		FS		PT	
Treatments	Bio	Non-Bio	Bio	Non-Bio	Bio	Non-Bio	Bio	Non-Bio
Environment 1 (L1S1), Ismailia 2023/2024								
Control	188.283g	183.333g	31.080j	30.260h	137.620g	134.000f	0.343i	0.333h
MF1 (AS ₁₀₀)	264.333ab	233.667b	104.387b	77.683b	258.000b	223.333b	0.590bc	0.513b
MF2 (AS ₅₀)	225.000e	205.000de	65.163g	43.253f	198.667e	155.000d	0.490f	0.387f
MF3 (AN ₁₀₀)	262.333bc	229.000bc	100.133c	72.127c	255.000bc	216.000b	0.580cd	0.507b
MF4 (AN ₅₀)	215.333f	197.000ef	52.613i	38.650g	171.000f	145.333e	0.437h	0.360g
MF5 (AS ₁₀₀ +inh)	273.000a	249.333a	114.833a	90.000a	269.333a	243.667a	0.607a	0.553a
MF6 (AS ₅₀ +inh)	239.667d	211.667d	83.723e	49.747e	230.333d	164.000d	0.537e	0.427d
MF7 (AN ₁₀₀ +inh)	269.000ab	243.000a	108.243b	87.447a	262.000ab	236.333a	0.600ab	0.547a
MF8 (AN ₅₀ +inh)	234.667d	208.333d	79.613f	46.933ef	226.667d	160.000d	0.527e	0.413e
MF9 (SCU ₁₀₀)	254.333c	222.000c	94.457d	60.817d	247.000c	192.000c	0.570d	0.470c
MF10 (SCU ₅₀)	219.333ef	188.667fg	56.747h	35.680g	176.333f	142.333ef	0.453g	0.350g
Environment 2 (L1S2), Ismailia 2024/2025								
Control	191.363a	186.333a	32.473a	31.617a	155.077a	151.000a	0.350a	0.340a
MF1 (AS ₁₀₀)	272.667a	237.000a	109.780a	80.443a	266.000a	231.667a	0.620a	0.523a
MF2 (AS ₅₀)	228.333a	207.667a	67.273a	46.103a	219.667a	185.667a	0.503a	0.403a
MF3 (AN ₁₀₀)	269.000a	231.333a	104.917a	74.713a	261.667a	225.000a	0.600a	0.513a
MF4 (AN ₅₀)	217.333a	200.333a	54.907a	41.017a	208.000a	177.000a	0.453a	0.380a
MF5 (AS ₁₀₀ +inh)	292.333a	255.000a	128.137a	94.347a	283.667a	250.667a	0.647a	0.570a
MF6 (AS ₅₀ +inh)	246.333a	213.667a	87.210a	52.067a	240.333a	199.667a	0.543a	0.443a
MF7 (AN ₁₀₀ +inh)	280.667a	251.000a	115.553a	91.780a	274.667a	245.000a	0.630a	0.560a
MF8 (AN ₅₀ +inh)	239.667a	210.000a	83.233a	49.127a	233.333a	192.333a	0.533a	0.423a
MF9 (SCU ₁₀₀)	261.667a	224.333a	99.177a	63.703a	257.000a	216.333a	0.587a	0.483a
MF10 (SCU ₅₀)	221.000a	193.000a	59.180a	37.327a	213.333a	169.667a	0.470a	0.370a

Table 5b. Average fruit weight (AFW, g), fruit size (FS, cm³), peel thickness (PT, cm) and fruits weight per tree (FW, kg) as affected by mineral and bio-nitrogen, as well as their interaction in Qena location at 2023/2024 and 2024/2025 seasons

	AFW		FW/tree		FS		PT	
Treatments	Bio	Non-Bio	Bio	Non-Bio		Bio	Non-Bio	Bio
Environment 3 (L2S1), Qena 2023/2024								
Control	159.440a	155.243a	26.317a	25.623a	116.533a	113.470a	0.293a	0.283a
MF1 (AS ₁₀₀)	223.837a	197.870a	88.400a	65.780a	218.477a	189.120a	0.500a	0.437a
MF2 (AS ₅₀)	190.530a	173.593a	55.180a	36.627a	168.233a	131.253a	0.410a	0.327a
MF3 (AN ₁₀₀)	222.143a	193.920a	84.793a	61.073a	215.937a	182.907a	0.493a	0.427a
MF4 (AN ₅₀)	182.347a	166.820a	44.550a	32.730a	144.803a	123.067a	0.373a	0.303a
MF5 (AS ₁₀₀ +inh)	231.180a	211.133a	97.237a	76.210a	228.070a	206.337a	0.510a	0.473a
MF6 (AS ₅₀ +inh)	202.953a	179.240a	70.893a	42.127a	195.043a	138.877a	0.453a	0.360a
MF7 (AN ₁₀₀ +inh)	227.790a	205.773a	91.660a	74.050a	221.863a	200.127a	0.503a	0.463a
MF8 (AN ₅₀ +inh)	198.717a	176.413a	67.417a	39.740a	191.940a	135.490a	0.447a	0.350a
MF9 (SCU ₁₀₀)	215.367a	187.990a	79.983a	51.500a	209.160a	162.587a	0.480a	0.397a
MF10 (SCU ₅₀)	185.730a	159.760a	48.053a	30.213a	149.320a	120.527a	0.383a	0.293a
Environment 4 (L2S2), Qena 2024/2025								
Control	165.877a	161.513a	28.147a	27.407a	134.420a	130.883a	0.303a	0.297a
MF1 (AS ₁₀₀)	236.347a	205.430a	95.157a	69.727a	230.567a	200.807a	0.537a	0.453a
MF2 (AS ₅₀)	197.917a	180.007a	58.313a	39.963a	190.407a	160.933a	0.437a	0.350a
MF3 (AN ₁₀₀)	233.170a	200.520a	90.940a	64.763a	226.813a	195.030a	0.520a	0.443a
MF4 (AN ₅₀)	188.387a	173.647a	47.590a	35.557a	180.293a	153.427a	0.393a	0.330a
MF5 (AS ₁₀₀ +inh)	253.397a	221.033a	111.067a	81.780a	245.880a	217.277a	0.560a	0.493a
MF6 (AS ₅₀ +inh)	213.523a	185.203a	75.593a	45.133a	208.320a	173.070a	0.473a	0.383a
MF7 (AN ₁₀₀ +inh)	243.280a	217.567a	100.160a	79.557a	238.080a	212.363a	0.547a	0.483a
MF8 (AN ₅₀ +inh)	207.743a	182.027a	72.143a	42.580a	202.253a	166.717a	0.463a	0.367a
MF9 (SCU ₁₀₀)	226.813a	194.450a	85.967a	55.220a	222.767a	187.517a	0.507a	0.420a
MF10 (SCU ₅₀)	191.560a	167.293a	51.297a	32.357a	184.920a	147.067a	0.410a	0.320a

The coefficients of variation (CV%) ranged from 3.5-4.4 (N), 6.7-8.2 (P), 2.7-3.03 (K), 2.05-2.4 (AFW), 3.01-3.4 (FW/tree), 2.3-2.9 (FS), 1.14-1.43 (JV), 1.26-1.99 (FJ), 1.09-1.42 (PT), 1.16-1.57 (TSS/A) and 1.81-2.03 (VC) %, reflecting high experimental reliability.

Homogeneity of error variances and pooled analysis

Bartlett's test (Fig.1) indicated homogeneity of error variances across environments ($\chi^2 =$ from 2 to 19.92, $P = 1.000$ for all traits), justifying a pooled analysis over environments. Pooled analysis of variance across four environments revealed that site effects were highly significant for all traits, demonstrating pronounced environmental influence on all traits.

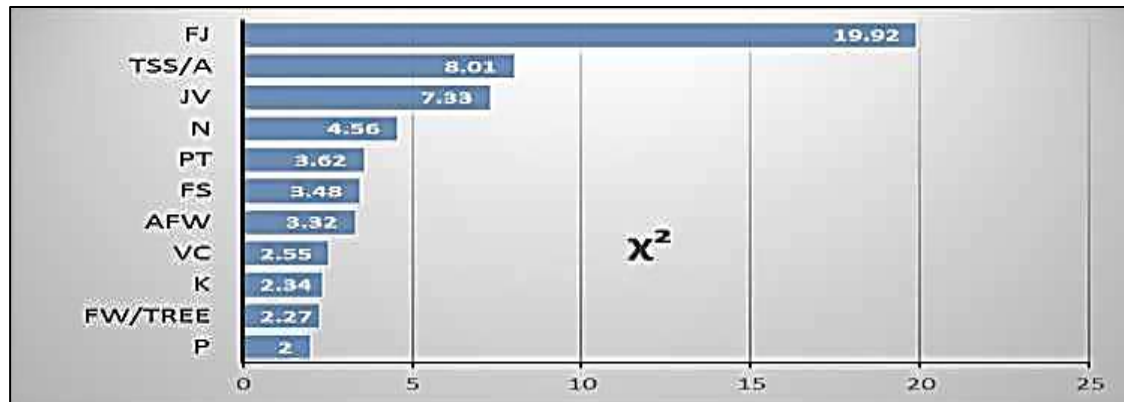


Fig. 1: Bartlett's test uses a chi-square (χ^2) distribution for studied traits across environments

Combined ANOVA across sites and seasons (Table 6) was conducted to quantify environmental and interaction effects. As shown in Table 6, mineral nitrogen (MF) and Bio-Nitrogen (Bio) significantly influenced all traits, confirming their importance. Interaction effects varied among traits showed that N, P, K%, AFW, FW/tree, TSS/A, and VC exhibited significant two-way interactions, whereas FS, JV and PT (Table) showed a significant Env. $\times$ MF $\times$ Bio-Nitrogen interaction, indicating differential treatment responses across environments. In contrast, FJ trait showed no significant interaction effects, suggesting stable performance of treatments across sites.

Significant Env. $\times$ MF and Env. $\times$ Bio-Nitrogen interactions indicated that the magnitude of response to individual factors varied across environments for all traits except N%, P% and fruit juice (FJ). The MF $\times$ Bio-Nitrogen interaction was also highly significant ($P < 0.001$) for all traits except FJ, confirming that the combined effect of the two factors (MF and Bio-Nitrogen) was non-additive. However, the three-way interaction (Env. $\times$ MF $\times$ Bio-Nitrogen) was non-significant for all traits except FS, JV and PT, suggesting that the MF $\times$ Bio interaction pattern was consistent across environments for most traits. On the other hand, FS, JV and PT are the most complex traits, where all main effects and all interactions are significant. Thus, treatment combinations respond differently in each environment.

Table 6. Mean square of environments, bio-nitrogen (Bio) and mineral nitrogen (MF) combined analysis for Leaf contents, Fruit quality properties and Fruit weight per tree'

Source of Variation	D F	N	P	K	AFW	FW/ tree	FS	JV	FJ	PT	TSS /A	VC
		Mean Squares										
Env.	3	2.31 **	0.007 **	1.063* *	25145 **	2647 **	28440 **	4908 **	845.3* *	0.118 **	55.74 **	1657 **
Rep within Env.	8	0.005	0.0002	0.004	28.36	13.95	67.01	2.12	0.986	0.001	0.152	2.305
MF	10	3.64 **	0.011 **	1.53 **	13301 **	12475 **	29353 **	1369 **	35.4 **	0.138 **	73.39 **	1099 **
Env. × MF	30	0.009	0.00003	0.003* *	46.15 **	33.95 **	199.4 **	5.51 **	0.14	0.0004 **	0.16 **	2.44 **
Bio	1	9.61 **	0.032 **	4.213* *	40249 **	34031 **	73061 **	3866 **	105.9* *	0.381 **	199.9 **	3814 **
Env. × Bio	3	0.03 **	0.00004	0.009* *	140.6 **	89.926 **	235.24* *	21.968* *	0.238	0.001 **	0.401 **	8.315 **
MF × Bio	10	0.13 **	0.0004 **	0.057* *	422.58* *	534.39 **	1026 **	39.29 **	0.516	0.004 **	5.303 **	84.34 **
Env.× MF× Bio	30	0.002	0.0001	0.001	9.251	4.088	69.382* *	2.533 *	0.075	0.00007* *	0.024	0.256
Error	168	0.007	0.0001	0.001	22.992	4.466	25.219	1.524	0.444	0.00003	0.02	1.12

Mean impacts of MF and Bio-Nitrogen (pooled):

Pooled mean comparisons showed wide and significant variation among levels of mineral (MF) nitrogen (Figs. 2&3). The highest pooled values were recorded for MF5 (AS₁₀₀+inh), followed by MF 7 (AN₁₀₀+inh) for all traits with no significant differences between them in phosphorus percentage, while MF control treatment exhibited the lowest values. Differences among treatments were statistically significant ($P < 0.05$).

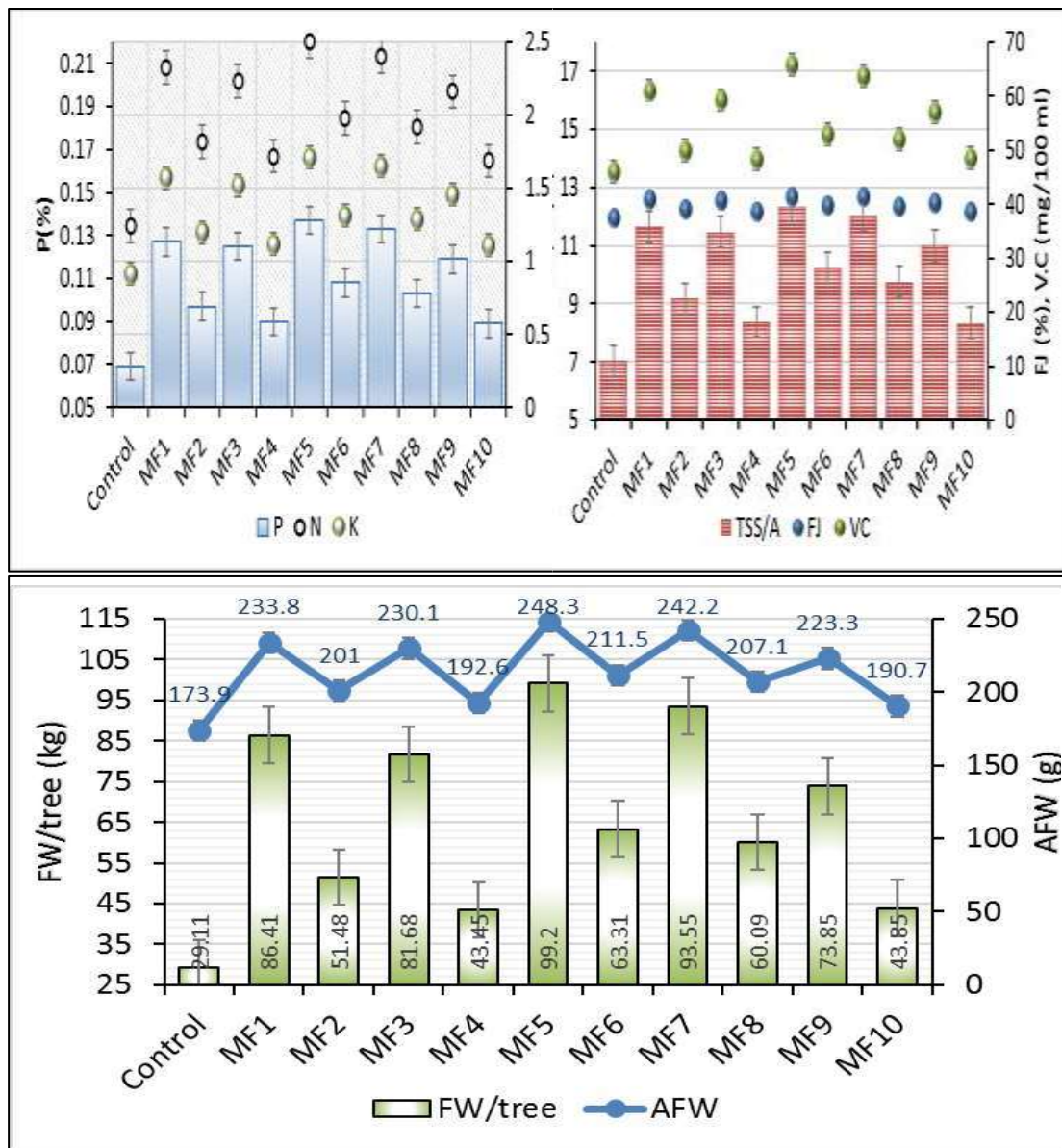


Fig. 2: N%, P%, K%, AFW, FW/tree, FS, JV, FJ, PT, TSS/A and VC as affected by Mineral Nitrogen (MF) over pooled environments

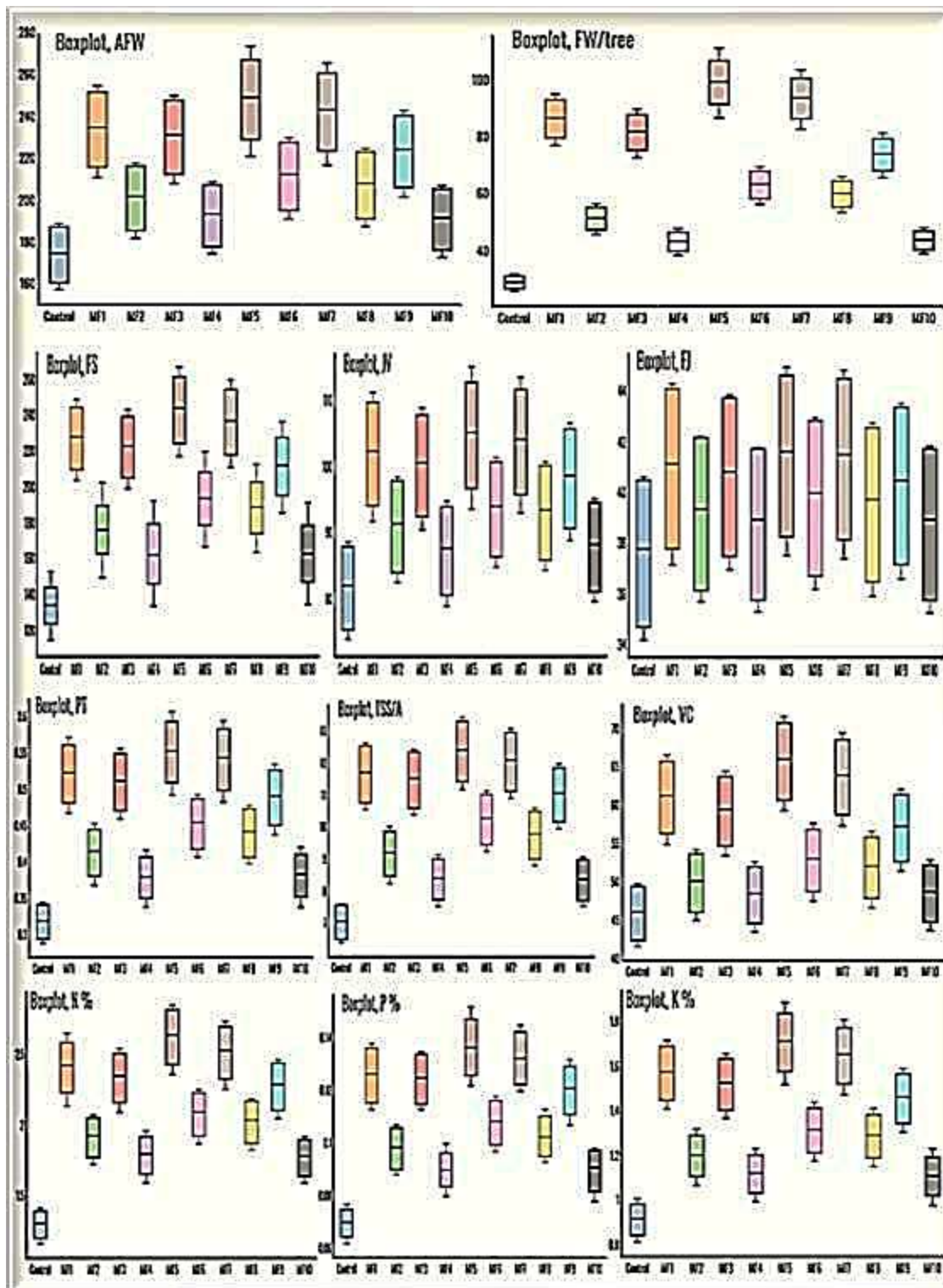


Fig. 3: Pox plot of all studied traits as affected by mineral-nitrogen (MF) treatments over pooled environments

For Bio-nitrogen, Bio 1 (50 g/tree/year added twice in the year) produced a significantly higher pooled values compared with non-application (Bio2) for all studied traits (Fig. 4).

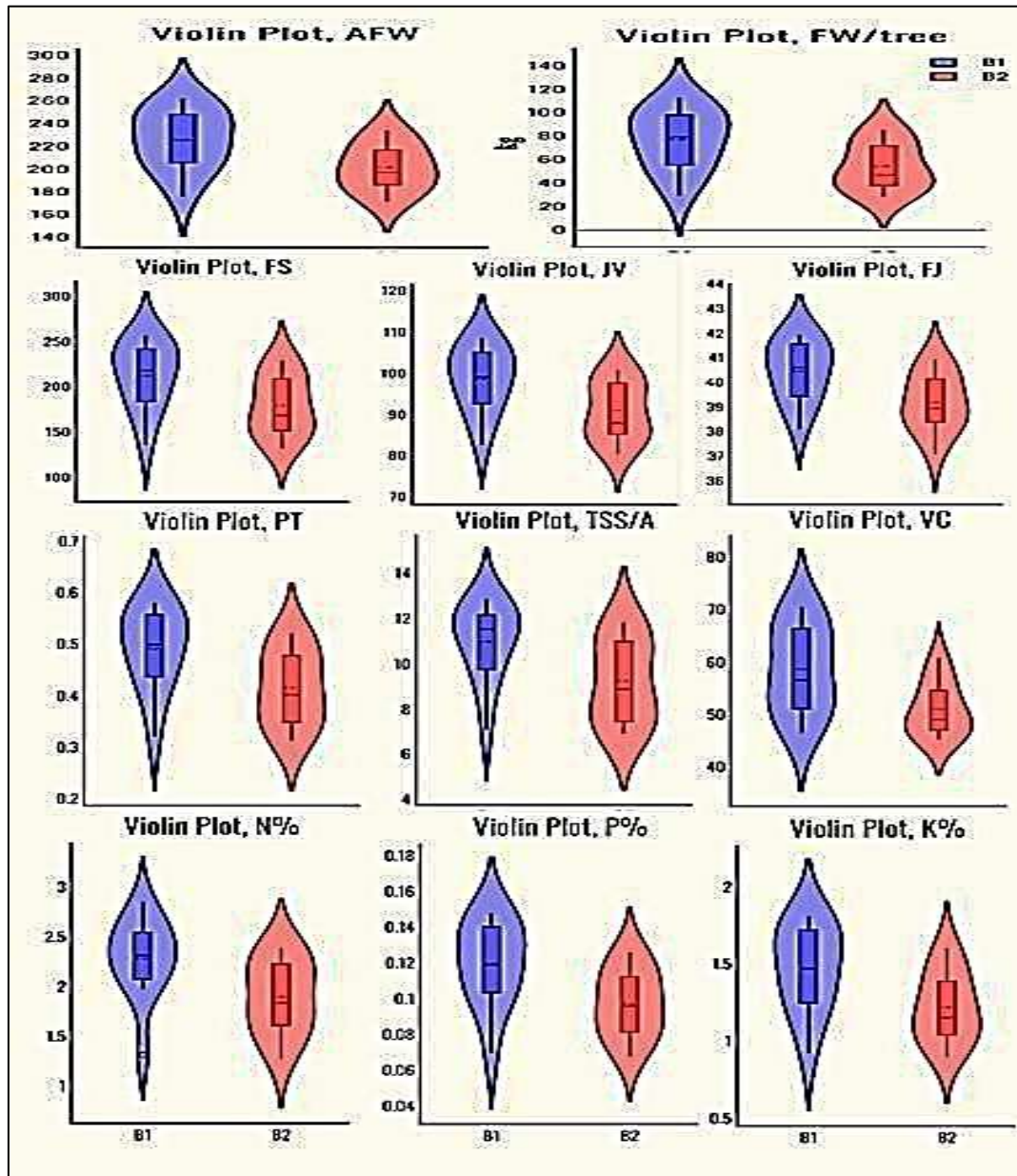


Fig. 4: Violin plot of all studied traits (N%, P%, K%, AFW, FW/tree, FS, JV, FJ, PT, TSS/A and VC) as affected by bio-nitrogen treatments over pooled environments

Table 7. NPK properties, average fruit weight (AFW, g), fruit size (FS, cm³), peel thickness (PT, cm) and fruits weight per tree (FW, kg) as affected by the interaction of mineral and bio-nitrogen over all environments

	N		P		K			
Treatments	Bio	Non- Bio	Bio	Non-Bio	Bio	Non- Bio		
Control	1.318i	1.283i	0.070d	0.069g	0.927h	0.903h		
MF1 (AS ₁₀₀)	2.572bc	2.250bc	0.141a	0.112bc	1.738bc	1.404c		
MF2 (AS ₅₀)	2.158g	1.671fg	0.109bc	0.087ef	1.326f	1.073f		
MF3 (AN ₁₀₀)	2.486cd	2.187cd	0.138a	0.111bc	1.684cd	1.357c		
MF4 (AN ₅₀)	1.982h	1.597gh	0.099c	0.081fg	1.203g	1.033fg		
MF5 (AS ₁₀₀ +inh)	2.859a	2.392a	0.147a	0.127a	1.813a	1.606a		
MF6 (AS ₅₀ +inh)	2.318ef	1.839e	0.119b	0.097de	1.474e	1.154e		
MF7 (AN ₁₀₀ +inh)	2.682b	2.350ab	0.143a	0.122ab	1.766ab	1.533b		
MF8 (AN ₅₀ +inh)	2.278f	1.771ef	0.116b	0.090ef	1.443e	1.133e		
MF9 (SCU ₁₀₀)	2.431de	2.118d	0.135a	0.105cd	1.641d	1.273d		
MF10 (SCU ₅₀)	2.057gh	1.486h	0.102c	0.078fg	1.225g	0.993g		
	AFW		FW/tree		FS	PT		
	Bio	Non-Bio	Bio	Non-Bio	Bio	Non-Bio	Bio	Non-Bio
Control	176.241h	171.606f	29.504k	28.727i	135.913h	132.338g	0.323k	0.313k
MF1 (AS ₁₀₀)	249.296bc	218.492b	99.431c	73.408b	243.261bc	211.232b	0.562c	0.482c
MF2 (AS ₅₀)	210.445f	191.567d	61.483h	41.487f	194.243f	158.213e	0.460h	0.367h
MF3 (AN ₁₀₀)	246.662c	213.693bc	95.196d	68.169c	239.854cd	204.734b	0.548d	0.473d
MF4 (AN ₅₀)	200.850g	184.450e	49.915j	36.988g	176.024g	149.707f	0.414j	0.343i
MF5 (AS ₁₀₀ +inh)	262.478a	234.125a	112.818a	85.584a	256.738a	229.487a	0.581a	0.523a
MF6 (AS ₅₀ +inh)	225.619e	197.444d	79.355f	47.268e	218.508e	168.903d	0.502f	0.403f
MF7 (AN ₁₀₀ +inh)	255.184b	229.335a	103.904b	83.208a	249.153b	223.456a	0.570b	0.513b
MF8 (AN ₅₀ +inh)	220.198e	194.193d	75.602g	44.595e	213.548e	163.635de	0.493g	0.388g
MF9 (SCU ₁₀₀)	239.545d	207.193c	89.896e	57.810d	233.982d	189.609c	0.536e	0.443e
MF10 (SCU ₅₀)	204.406fg	177.180f	53.819i	33.894h	180.977g	144.898f	0.429i	0.333j

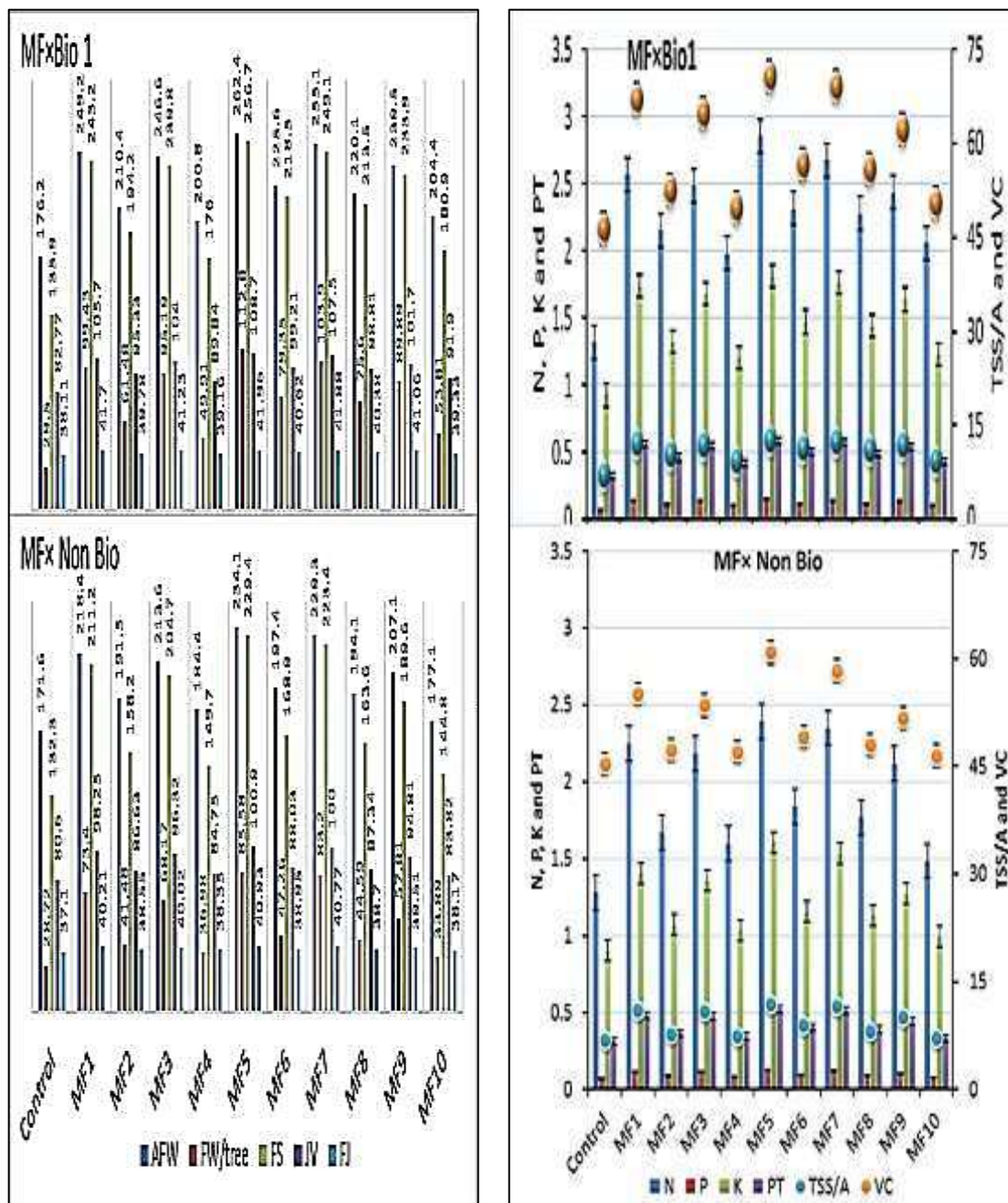


Fig.5: N%, P%, K%, AFW, FW/tree, FS, JV, FJ, PT, TSS/A and VC traits as affected by mineral $\times$ bio-nitrogen interaction over all environments

Are treatment rankings stable on average across locations?

Data were averaged over seasons for each location to assess overall treatment performance across sites. The results of locality effect (Table 8) were somewhat consistent with the above analysis (Table 6) of the four environments.

In the analysis based on season-averaged data within locations (ANOVA Table 8), the location $\times$ MF $\times$ Bio interaction for fruit size (FS, cm³), Juice volume (JV, cm³) and peel thickness (PT, cm) traits and the location $\times$ MF interaction for AFW were not significant, indicating relative stability of treatment performance when seasonal variation was removed. However, when locations and seasons were analyzed jointly (ANOVA Table 6), the corresponding environment $\times$ treatment interactions became highly significant, revealing strong season-dependent responses that were masked by averaging. The key takeaway is that seasonal averaging reduces environmental heterogeneity and interaction variance, potentially underestimating treatment sensitivity (Gomez and Gomez, 1984; Steel et al., 1997). Therefore, ANOVA of locality effect (over seasons, Table 8) is appropriate for assessing overall mean performance, whereas ANOVA of 4 environments (Table 6) is recommended for evaluating environmental responsiveness, stability, and treatment $\times$ environment interactions (Yan and Kang, 2003; Piepho et al., 1998).

Table 8. Mean square of locations (over seasons), bio-nitrogen (Bio) and mineral nitrogen (MF) combined analysis for Leaf contents, Fruit quality properties and Fruit weight per tree

Source of Variation	D F	N	P	K	A F W	FW /tree	FS	JV	FJ	PT	TSS	VC
Mean Squares												
Location (L)	1	3.171 **	0.009 **	1.426 **	35927 **	3406 **	30048 **	7056 **	1245 **	0.162 **	80.54 **	2367 **
Rep within site	4	0.005	0.00002	0.004	26.62	13.704	37.53	1.676	0.624	0.001	0.14	2.302
MF	10	1.818 **	0.005 **	0.766 **	6650 **	6237 **	14676 **	684.5 **	17.69 **	0.069 **	36.70 **	549.6 **
L $\times$ MF	10	0.01	0.00003	0.005 **	39.36	36.91 **	88.48 **	4.046 **	0.11 **	0.004 **	0.218 **	3.26 **
Bio	1	4.803 **	0.016 **	2.099 **	20123 **	17015 **	36529 **	1932 **	52.92 **	0.19 **	99.90 **	1907 **
L $\times$ Bio	1	0.027 **	0.000093	0.012 **	119.38 **	100.79 **	219.6 **	11.46 **	0.381	0.001 **	0.594 **	11.31 **
MF $\times$ Bio	10	0.064 **	0.000155 **	0.028 **	211.43 **	267.26 **	513.48 **	19.65 **	0.267	0.002 **	2.654 **	42.15 **
L $\times$ MF $\times$ Bio	10	0.0004	0.0000009	0.00017	1.116	1.559	3.176	0.107	0.005	0.000014	0.016	0.251
Error	84	0.006	0.00006	0.0013	21.825	4.224	21.412	1.361	0.305	0.00002	0.016	1.066

The results obtained from each location (over seasons) indicated that Bio-nitrogen, generally, superior in Ismailia location (L1) than Qena one (L2). The interaction between Bio-nitrogen and location (Bio $\times$ L) were highly significant for all traits (Table 8) revealing that Bio-nitrogen was superior in both locations, as an average of MF treatments. In the meantime, Bio $\times$ location effect was greater than the interaction of MF $\times$ Bio $\times$ L. These results showed that the differential response of Bio-treatment in the individual location was sufficiently similar in the different mineral levels to warrant the previously conclusion that, these differential responses may be permanent features for these locations.

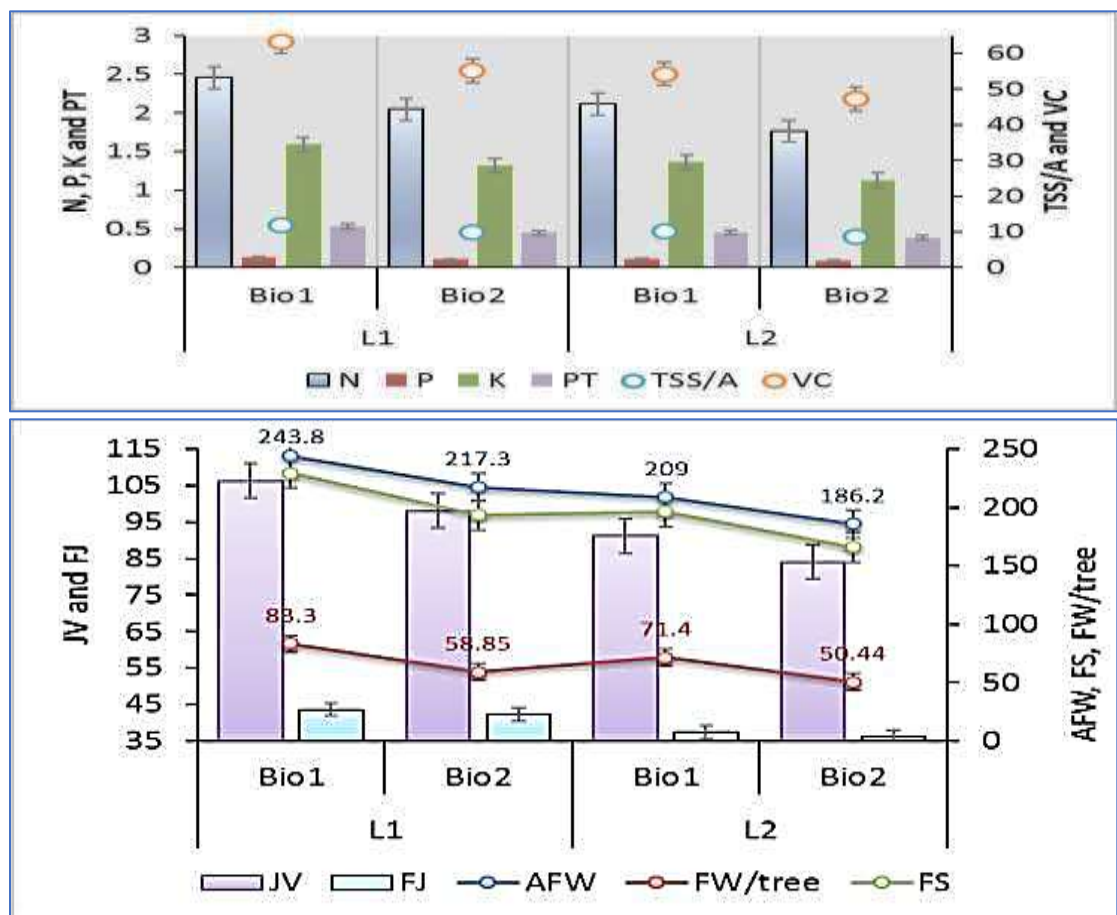


Fig.6: N%, P%, K%, AFW, FW/tree, FS, JV, FJ, PT, TSS/A and VC studied traits as affected by bio-nitrogen $\times$ location interaction

Brief comparison for results:

Field experiments were carried out to study the impact of various mineral nitrogen sources and levels as well as bio-fertilization on yield, and quality of summer oranges in two locations, i.e., Ismailia and Qena governorates at two seasons. Accordingly, comparing the performance of the treatments combinations on the basis of fruit yield (kg/tree) (Common NPK fertilization in Egypt as general control) and highest desirable response for yield (% over corresponding control) as well as the effects of the treatments on other traits was done. The best combination, which classified based on these parameters, are shown in Table (9). Twelve out of the twenty-two studied combinations were classified as a good affect source in desirable trend on yield. One out of these 12 treatments namely BIO-AS2/100 (100 unit ammonium sulphate 20% N + inhibitor combined with 50 g/tree/year Nitrobein (bio- nitrogen) added twice in February and

June) exhibited significant positive effects for average fruit weight, fruit size, fruit juice as well as the superiority for positive effect of N, P, K of leaf content, indicating the possibility of combine both high yield and good quality characters under various environmental conditions.

Table 9. The best treatment combinations chosen based on mean yield along with desirable significant effect for other traits comparing with corresponding control in Ismailia and Qena locations (on average of two seasons)

Fertilizer treatments		Increment over control						
		Value and percentage (%) of yield/tree (over corresponding control)						
		Ismailia		Qena		Range increment percentage of other traits responses		
		Kg/tree	%	Kg/tree	%			
	Nonbio	30.939	100%	27.515	100%			
	Control	30.939	100%	27.515	100%	Juice quality of fruit	Fruit size	NPK of leaf
BIO	MF1 (AS ₁₀₀)	121.484	292.66%	104.154	278.53%	13.1% -85.8%	94.00 %	101.2% -122.9%
	MF2 (AS ₅₀)	111.896	261.67%	95.909	248.57%	12.9% -79.5%	88.26 %	95.8% -109.2%
	MF3 (AN ₁₀₀)	107.084	246.12%	91.777	233.55%	12.4% -76.3%	83.81%	92.6% -106.0%
	MF4 (AN ₅₀)	102.524	231.38%	87.867	219.34%	11.1% -74.2%	81.24%	86.7% -102.1%
	MF5 (AS ₁₀₀ +inh)	96.816	212.93%	82.975	201.56%	10.7% -72.3%	76.80%	81.9% -94.7%
NON	MF6 (AS ₅₀ +inh)	92.170	197.91%	78.993	187.09%	10.3% -71.0%	73.40%	78.1% -86.5%
	MF7 (AN ₁₀₀ +inh)	89.613	189.64%	76.802	179.12%	9.9% -68.0%	68.85%	70.0% -83.2%
BIO	MF8 (AN ₅₀ +inh)	85.465	176.24%	73.244	166.20%	9.5% -66.7%	65.11%	63.3% -80.7%
	MF9 (SCU ₁₀₀)	81.424	163.18%	69.782	153.61%	8.8% -63.0%	61.36%	59.9% -77.5%
NON	MF10 (SCU ₅₀)	79.061	155.54%	67.753	146.24%	8.4% -59.8%	59.61%	55.7% -75.4%
	Control	73.420	137.31%	62.919	128.67%	7.9% -56.7%	54.70%	50.4% -70.5%
BIO	MF1 (AS ₁₀₀)	66.218	114.03%	56.746	106.23%	7.2% -53.2%	46.78%	47.0% -68.1%

PCA analysis-based location-dependent reaction to bio-nitrogen:

Treatments and locations were clearly separated using principal component analysis (PCA), with the top two principal components (PC₁ = 94.63%, PC₂ = 5.37%) accounting for 99.99% of the total variation (Fig.7). Consequently, PC₁ drove nearly all treatment differentiation, and PC₂ caught secondary, location-related variance. Across the two locations (L1 and L2),

the score plot demonstrated a significant difference between the bio-nitrogen and non-bio treatments. Trees showed a strong positive reaction to bio-nitrogen at Ismailia location (L1), as evidenced by its position far on the positive side of PC_1 (score = 4.09). Non-bio treatment, on the other hand, occupied the extreme negative side of PC_1 (-3.80), indicating the worst overall nutritional state and performance in the absence of bio-nitrogen, especially under Qena (L2) conditions. This pattern was further supported by the standardized data. While Non-bio treatment consistently displayed negative values for every attribute, Bio-nitrogen consistently recorded high positive standardized values for N, P, K, and Peel thickness (PT). This implies that, particularly in L1, bio-nitrogen significantly increased nutrient storage.

Despite Qena (L₂)'s good response to Non-bio treatment, its PCA position ($PC_1 = -0.20$; $PC_2 = -0.90$) showed a poorer response than Bio-L1. This discrepancy along PC_2 indicates a location impact, indicating that the effectiveness of bio-nitrogen was somewhat limited by soil or ambient conditions at Qena (L₂). Although not to the same degree as at Ismailia (L1), the proximity of Bio-L2 to the origin along PC_1 suggests a moderate benefit over untreated plants. All evaluated attributes contributed positively and nearly equally to PC_1 , according to eigenvector loadings, indicating that PC_1 is a general productivity and nutritional status axis. Therefore, rather than being an isolated effect on a particular characteristic, the improved bio-treated L1 location along PC_1 implies a broad enhancement across all investigated qualities. Overall, the PCA shows that bio-nitrogen application was more successful at location L1 than L2, suggesting a significant relationship between bio- nitrogen and the environment. These findings highlight the fact that the agronomic effectiveness of bio-fertilizers varies depending on the region and should be tailored to the local soil and climate.

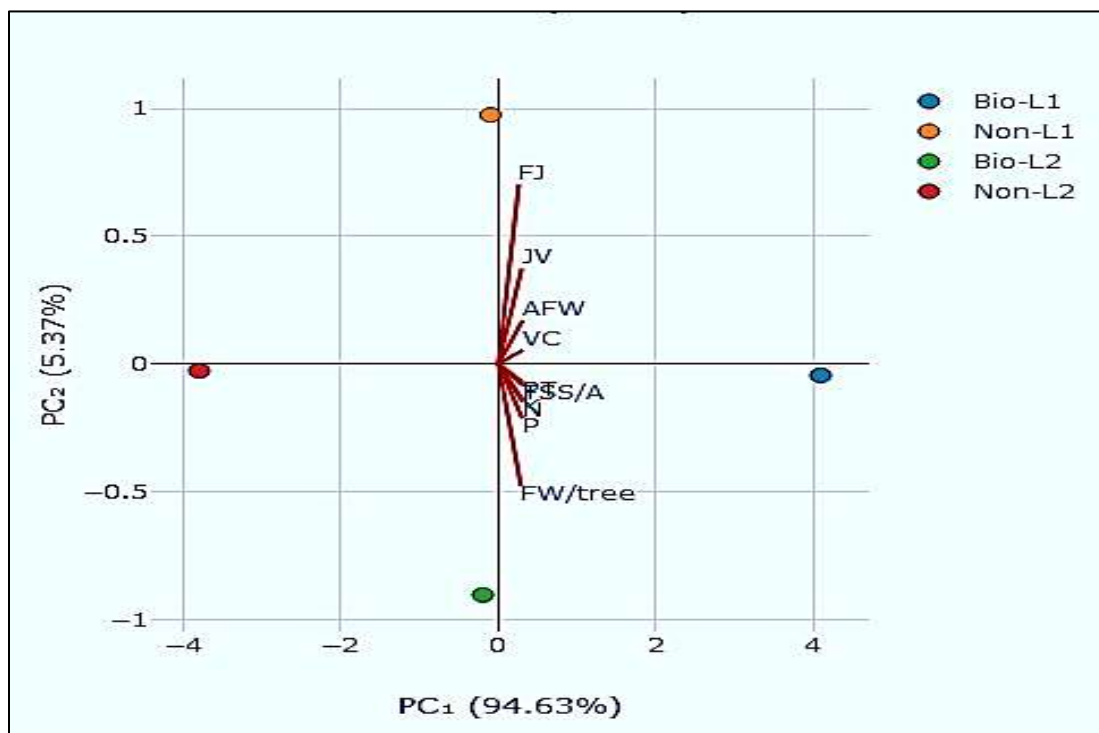


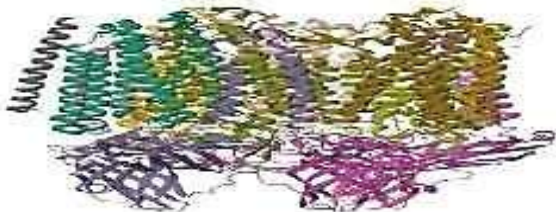
Fig.7: Biplot Principal component analysis for the two bio-nitrogen $\times$ 2 locations interacted treatments, based on the studied traits, using first and second components; a treatment in corresponding location is represented by each dot.

Molecular Docking Results

The interaction between the Nitrapyrin inhibitor and the AMO enzyme was examined using molecular docking analysis. The expected Gibbs free energy of binding (ΔG) was used to quantify binding affinity, and docking simulations produced a comprehensive picture of ligand binding inside the active site of the enzyme. In order to enhance binding site resolution and accuracy, docking was carried out on both the entire protein structure and its constituent subunits (α -subunit, β -subunit, subunit C, and the super-helix) due to the target protein's comparatively high size and structural complexity. Table 1 summarizes the docking scores (ΔG) for each structural unit.

The more negative ΔG values in the docking results show that Nitrapyrin has the strongest binding affinity toward both subunits C and the whole protein. On the other hand, ligand docking was not supported by the super-helix structure since it lacked a discernible binding cavity.

Table 1. Docking results showing the predicted Gibbs free energy of binding (ΔG) for Nitrapyrin with the AMO enzyme and its subunits

Structural unit	Docking score (kcal mol ⁻¹)	Protein structure ID
Whole protein PDB_00009C16	-5.8	
Alpha subunit	-4.9	
Beta subunit	-5.1	
Subunit C	-5.6	
Super helix	No detectable cavity or binding site	

Visualization of Docking Interactions

Molecular visualization tools were employed to analyze the binding modes and interaction patterns between Nitrapyrin and the AMO enzyme. Three-dimensional (3D) and two-dimensional (2D) interaction maps were generated to identify key intermolecular interactions, including hydrogen bonds and hydrophobic contacts, as well as to measure bond lengths and interaction distances. The visualization results for each protein component are presented as follows:

- ✚ **Figure 8:** 3D visualization of Nitrapyrin interaction with the whole protein structure
- ✚ **Figure 9:** 3D and 2D visualization of Nitrapyrin interaction with the α -subunit
- ✚ **Figure 10:** 3D and 2D visualization of Nitrapyrin interaction with the β -subunit
- ✚ **Figure 11:** 3D and 2D visualization of Nitrapyrin interaction with subunit C

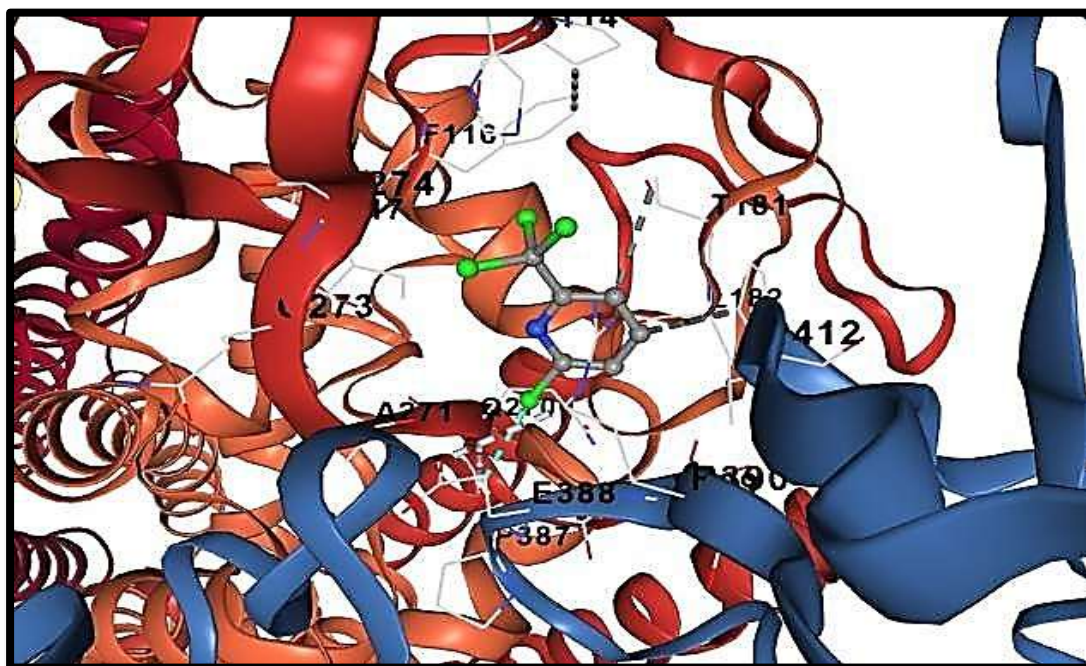


Fig. 8: 3D visualization of Nitrapyrin with whole protein

These visual analyses provide structural support for the docking scores by illustrating the spatial orientation of Nitrapyrin within the predicted binding pockets of the enzyme and its subunits. The observed interaction patterns are consistent with the calculated binding affinities and confirm the suitability of the identified binding regions.

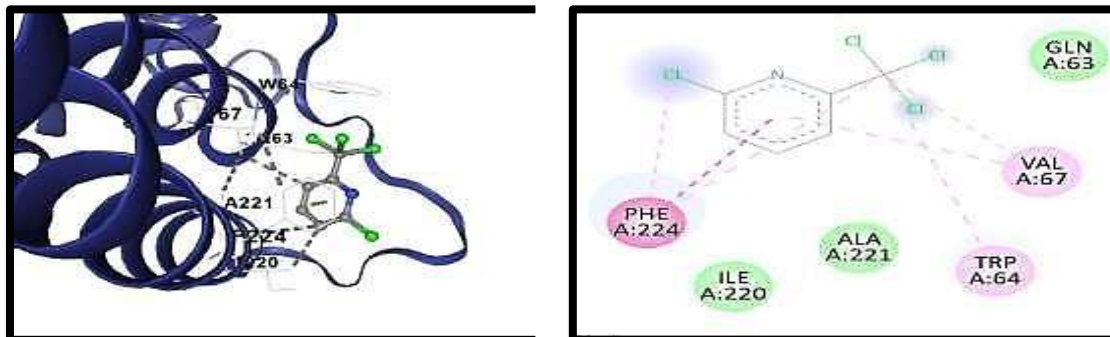


Fig. 9: 3D and 2D visualization of Nitrapyrin with alpha subunit

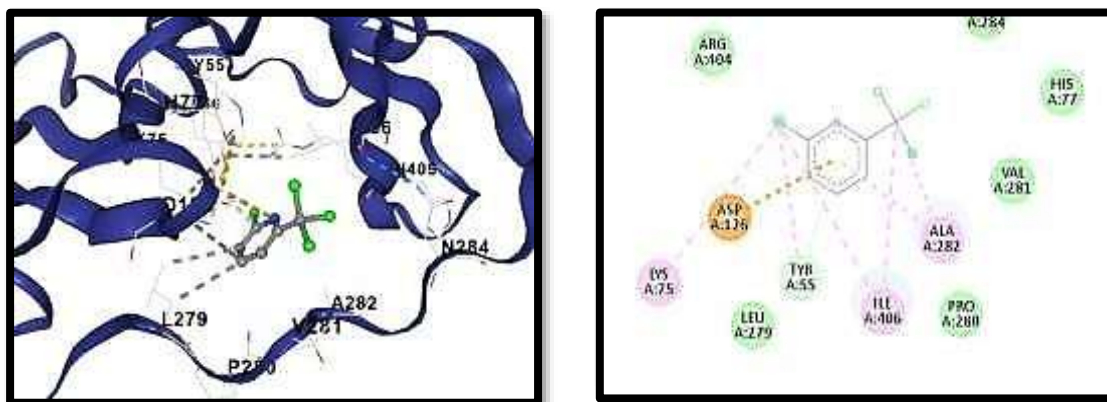


Fig. 10: 3D and 2D visualization of Nitrapyrin with beta subunit

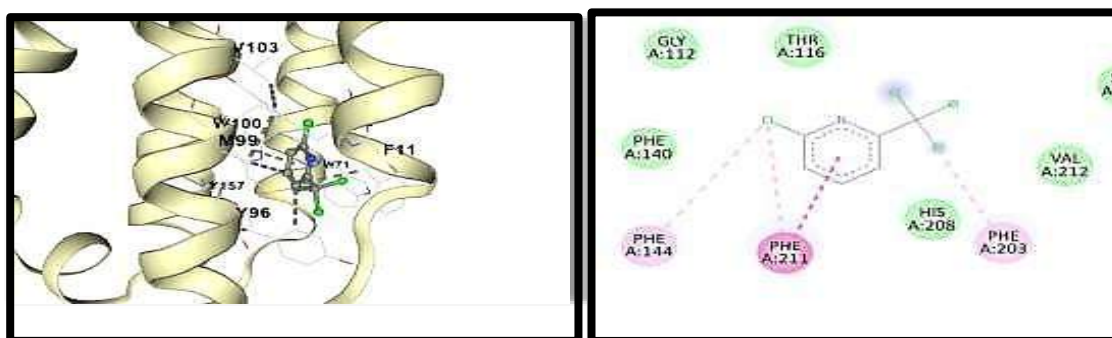


Fig. 11: 3D and 2D visualization of Nitrapyrin with Subunit C



Discussion

Nitrogen Fertilization's Relationship to the Site and Environment

The current study unequivocally shows that site-specific environmental factors have a significant impact on citrus trees' reaction to nitrogen fertilization. In the Ismailia location (L1) as opposed to Qena (L2), bio-nitrogen fertilization consistently yielded better responses across the seasons, suggesting that environmental conditions were crucial in regulating nutrient efficiency and plant performance. These variations most likely result from differences in the two locations' soil texture, organic matter content, climate, and microbial activity, all of which are known to have an impact on the availability of nutrients and the effectiveness of biological nitrogen fixation. Although the degree of response

differed between sites, the highly significant Bio $\times$ Location interaction for all variables under study (Table 8) suggests that bio-nitrogen fertilization was efficient in both environments. Ismailia's higher bio-nitrogen may be explained by the region's more favorable soil moisture regimes and temperate climate, which promote microbial survival, nitrogen fixation, and nutrient mineralization. Citrus and other fruit crops have shown similar site-dependent reactions to biofertilizers, where biological inputs were more effective in environments that supported microbial activity and root-microbe interactions (El-Badawy, 2017; Jugnake et al., 2017; Omari et al., 2020).

Interestingly, the Bio $\times$ Location interaction was stronger than the three-way interaction (MF $\times$ Bio $\times$ Location), suggesting that mineral nitrogen level had little bearing on how bio-nitrogen responded to environmental conditions. This implies that rather than being a temporary reaction to the supply of mineral nitrogen, the varied bio-fertilization efficiency between Ismailia and Qena is a reasonably constant and site-specific feature. This constancy lends credence to the idea that, independent of the intensity of mineral fertilization; environmental factors at each site have a dominant control over the effectiveness of bio-nitrogen application. Further evidence that bio-fertilization improves nutrient usage efficiency rather than simply increasing nitrogen availability comes from the consistency of bio-nitrogen superiority across mineral nitrogen levels. This result is consistent with other research showing that bio-nitrogen enhances fruit growth, quality, and nutrient uptake under a variety of fertilization regimes, especially when microbial activity is encouraged by the environment (Mohamed and Massoud, 2017; Pawar et al., 2020; Naidu, 2021). Overall, both sites showed success with the combination of mineral and bio-nitrogen fertilization; however, Ismailia's higher reactivity emphasizes the significance of site-specific nutrient management techniques. These findings highlight the need to customize bio-nitrogen fertilizer to local environmental circumstances in order to optimize its agronomic advantages. Therefore, when creating sustainable fertilizing recommendations for citrus orchards, the observed differences in responses between Ismailia and Qena may be regarded as intrinsic features of these production environments.

Fruit quality parameters as affected by nitrogen fertilization:

Nitrogen fertilization's effects on fruit quality parameters: Nitrogen fertilizer greatly increased fruit quality parameters, such as juice volume, peel thickness, TSS/acid ratio, and vitamin C content. Higher nitrogen levels have been shown to increase juice volume and peel thickness, which are frequently linked to increased cell expansion and water accumulation in fruit tissues (Quaggio et al., 2006; Barakat et al., 2012; Merwad et al., 2014; Brunetto et al., 2017). The results of Nguyen and Tai (2019) and Habasy (2017), who reported increased internal fruit quality under balanced nitrogen nutrition, are consistent with the observed rise in TSS/acid ratio and vitamin C content with nitrogen fertilizer. Juice volume, TSS/acid ratio, and vitamin C content were all significantly enhanced by bio-nitrogen fertilization at all nitrogen levels. According to reports by Jugnake et al. (2017), El-Badawy (2017), Omari et al. (2020), and Pawar et al. (2020), bio-fertilizers boost citrus fruits' antioxidant production and carbohydrate metabolism. When combined with mineral nitrogen, bio-nitrogen fertilizer consistently yielded better results than mineral fertilization alone. In line with earlier studies highlighting the agronomic and environmental advantages of integrated nutrient management systems, these results support the use of combined fertilization

strategies as an efficient and sustainable method for enhancing citrus nutritional status, yield, and fruit quality (El-Badawy, 2017; Yang et al., 2019; Naidu, 2021).

Molecular Docking:

A well-known nitrification inhibitor, Nitrapyrin reduces nitrate production, nitrogen leaching, and N_2O emissions from fertilized soils by inhibiting the activity of ammonia-oxidizing bacteria (Huang et al., 2002; Wang et al., 2017). Ammonia mono-oxygenase (AMO), the enzyme that catalyzes the initial stage of nitrification the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH) in ammonia-oxidizing bacteria and archaea is the main cause of its inhibitory effect. Fig.12 showed Graphical Abstract for Molecular and Agronomic Mechanism of Nitrapyrin as a Nitrification Inhibitor

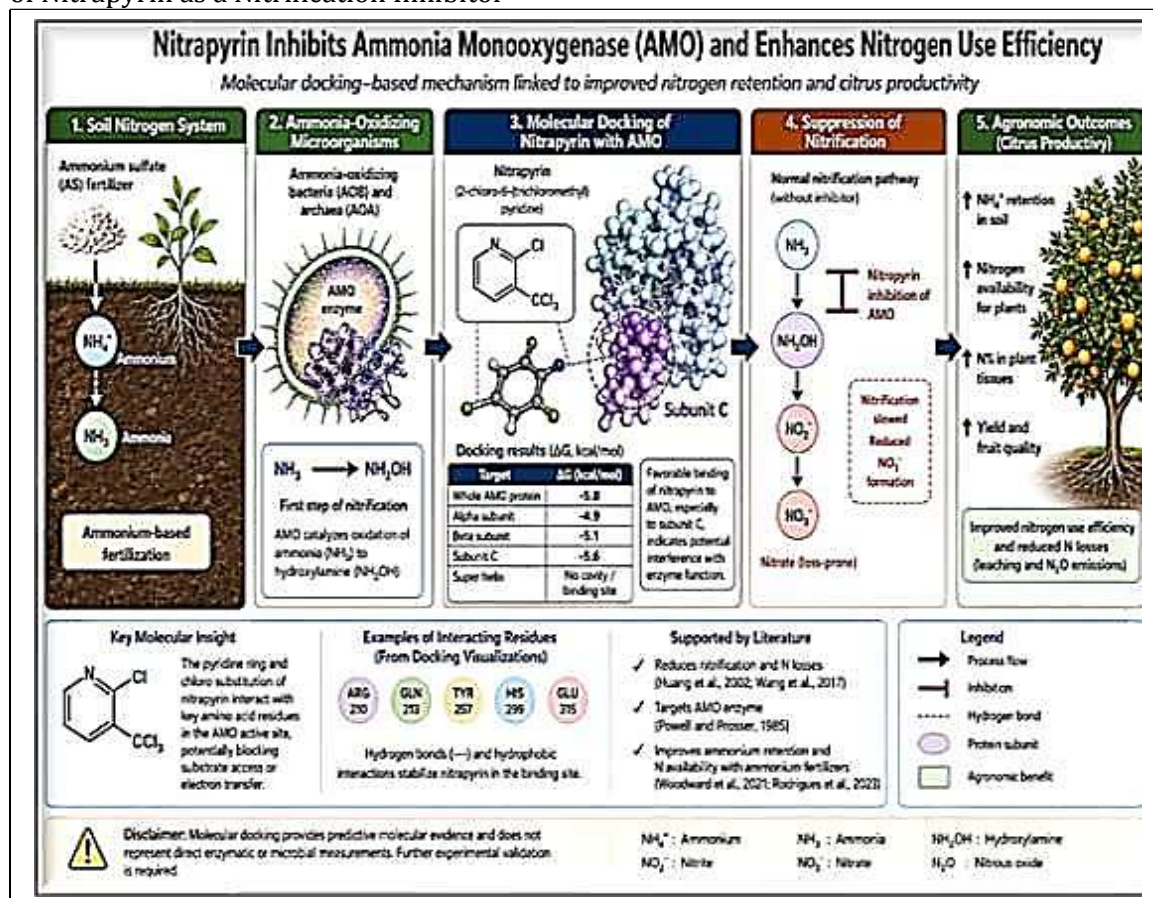


Fig.12: Molecular and Agronomic Mechanism of Nitrapyrin as a Nitrification Inhibitor

Schematic representation of the proposed mechanism underlying the effect of Nitrapyrin as a nitrification inhibitor. Molecular docking predicts favorable binding of Nitrapyrin to the ammonia mono-oxygenase (AMO) enzyme, particularly within the intact protein structure and subunit C, suggesting potential interference with the first step of nitrification. This interaction may reduce the oxidation of ammonia to hydroxylamine, thereby enhancing ammonium retention and nitrogen availability in soil. The proposed mechanism is consistent with observed improvements in nitrogen content and yield quality under ammonium-based fertilization combined with Nitrapyrin.

Docking results provide predictive molecular support and do not represent direct enzymatic or microbial measurements.

The current study's molecular docking results, which demonstrate Nitrapyrin's favorable binding affinity for the complete AMO protein and subunit C while no binding cavity was found in the super-helix region, offer structural evidence for this method of action. These results imply that Nitrapyrin does not bind nonspecifically to the protein surface, but rather interacts with particular functional areas of the enzyme.

Although the exact chemical mechanism is still unknown, earlier research has suggested that Nitrapyrin's inhibitory effect is connected to the pyridine ring structure and chloro substitution (Powell and Prosser, 1985). In this aspect, the anticipated docking interactions involving Nitrapyrin's pyridine ring are in line with previous theories about its capacity to disrupt AMO function. The predicted binding patterns are consistent with agronomic studies that show improved nitrogen availability and increased ammonium retention when Nitrapyrin is applied in combination with ammonium-based fertilizers like ammonium sulfate, despite the fact that molecular docking does not directly measure enzyme inhibition or nitrogen transformation dynamics (Woodward et al., 2021; , Ibrahim et al., 2022, Rodrigues et al., 2023). Therefore, rather than being direct evidence of enzymatic inhibition, the docking results should be viewed as supporting molecular evidence that enhances field observations. Based on the combined molecular docking and agronomic evidence, a conceptual mechanism can be proposed whereby Nitrapyrin binds to functionally relevant regions of the AMO enzyme, potentially restricting ammonia oxidation during the initial stage of nitrification. This inhibition may slow the conversion of ammonium to nitrate, reducing nitrogen losses through leaching and gaseous emissions and prolonging ammonium availability in the soil. Enhanced nitrogen retention can subsequently improve plant nitrogen uptake efficiency, contributing to higher nitrogen content and yield quality. While this mechanism is supported by docking predictions and field observations, further validation using enzyme kinetics, microbial assays, and molecular dynamics simulations is required to confirm the proposed inhibitory pathway.

Conclusion

This study demonstrates that integrating mineral and Bio-nitrogen fertilization significantly enhances the yield, fruit quality, and nutrient uptake of 20-year-old Baladi orange trees (*C. sinensis* L.). The combination of Nitroben (Bio-fertilizer) with ammonium sulfate and a nitrification inhibitor consistently achieved the highest agronomic performance across both studied environments. Furthermore, spatial variations heavily influenced fertilizer efficiency, with the Ismailia location showing a more pronounced positive response compared to Qena due to localized soil and climatic factors. Mechanistically, molecular docking confirmed that the nitrification inhibitor effectively binds to the ammonia mono-oxygenase (AMO) enzyme, particularly subunit C, slowing down nitrogen conversion and reducing leaching losses. Ultimately, adopting site-specific, combined bio-mineral fertilization regimes offers a sustainable approach to maximizing citrus productivity while optimizing nitrogen use efficiency.

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