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Effect of Foliar Application of Humic Acid and Amino Acids on Some Vegetative and Chemical Traits of Bitter Orange Seedlings (*Citrus aurantium* L.)

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I. Abstract

Crop cultivation is considered an important aspect of tropical and subtropical agriculture, and rootstocks are recognized as one of the most critical factors for orchard success in citrus. Citrus aurantium L. is identified as a plant that is highly tolerant of elevated pH and salinity and is widely used as a traditional rootstock. However, vigorous seedlings are not produced due to inherently slow early nursery growth. Although the individual effects of biostimulants are reported, a critical need is identified in research regarding the combined effects of humic acid (HA) and amino acids (AA) on this genotype. Based on this, a sustainable nursery protocol involving foliar application of HA and AA is proposed in this paper to address these limitations. Various concentrations and combinations are evaluated through a factorial experiment conducted using a randomized complete block design. A synergistic effect of 0.2% HA and 0.4% AA is observed to significantly improve seedling vigor through enhanced energy efficiency and nutrient translocation. This combination is shown to produce the best results, with a 101.4% increase in shoot dry weight and a total chlorophyll content of 2.79 mg/g FW. A mechanistic understanding of biostimulant synergy is provided by this research, and an efficient approach for reducing the citrus production cycle is presented.

Keywords: Amino acids, Biostimulants, *Citrus aurantium*, Humic acid, Nursery management.

II. Introduction

Citrus fruits are grown in large quantities in the tropics and subtropics of all countries around the world, and are one of the most economically important fruit crops in world agriculture. Selection of rootstock will have a great impact on the production and quality of citrus fruits **(Sadeghi et al., 2023)**. Rootstocks are chosen for their importance in providing disease resistance, fruit quality, stress tolerance and tree adaptability to a variety of soil and climatic environments. Citrus aurantium L **(Nargesi et al., 2022)**. is recognized as one of the most valuable genotypes among the available rootstocks, both historically and at present. Its use is attributed to its proven resistance to high pH soils, salinity, and Phytophthora spp., as well as its ability to provide high yield and superior fruit quality **(Kazimi et al., 2025)**. The virus known as Citrus Tristeza Virus (CTV) is, however, very virulent and this is an important consideration in its modern use, especially in countries where it is not endemic or where tolerant cultivars are used or the scion is tolerant. In this context, the economic motivation for producing vigorous and healthy bitter orange seedlings in the shortest possible time to maximize the production turnover of the nursery and to respond to the growers' needs is highlighted **(Hamzah et al., 2023).**

One of the major constraints of citrus nursery production is the slow growth rate of the seedlings in the early stages of development **(Kareem et al., 2024).** This is a long period of juvenility that can lengthen the time needed to grow to a graftable size, thus adding to production costs and decreasing operational efficiency **(Mohammed et al., 2023)**. It is found that nursery conventional management approaches heavily use synthetic inorganic fertilizers for rapid growth. However, over- and over-fertilizing these fertilizers has been linked to various environmental negative effects such as eutrophication of aquatic systems, groundwater pollution and leaching of nutrients. Therefore, a shift of paradigm towards sustainable intensification is called for. In this context, the use of

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biostimulants is suggested as an alternative technology that is promising and does not harm the environment **(Shaaban et al., 2025)**. Biostimulants are substances or microorganisms used directly on plants or the rhizosphere that positively and naturally influence the uptake of nutrients, nutrient efficiency, abiotic stress tolerance, and/or crop quality without providing nutrients **(Al-Azzawi et al., 2026).**

There are two major non-microbial biostimulants: humic acid and free amino acids. Humic acid is said to be a complex mixture of naturally occurring organic molecules that have been derived from the breakdown of organic matter, mainly composed of carbon, hydrogen, oxygen and nitrogen **(Hameedi et al., 2025)**. It is said to have a hormone-like effect when applied as a foliar spray; this includes the ability to stimulate root and shoot growth by auxin-like mechanisms. In addition, humic substances have been associated with greater permeability of cell membranes, which is correlated with an improvement in nutrient uptake efficiency. The amino acids are defined as the basic protein constituents of proteins, and are considered as essential constituents of foliar applied biostimulants **(Salama et al., 2023)**. They have been known to act as a direct source of exogenous organic nitrogen for the formation of structural protein when applied to plants. Some amino acids are chelators and this is known to aid the uptake and movement of micronutrients **(Muhammad et al., 2022)**. In addition, their function as precursor molecules for plant hormones and as an ability to store metabolic energy in the form of pre-assembled organic molecules is thought to be essential, especially under abiotic stress **(El Gaaly et al., 2025)**.

A great amount of research has been carried out on the individual effect of humic acid and amino acids on different crops, and this research is well documented in the literature. Humic acid is reported to significantly improve the vegetative growth, yield and nutrient status of vegetable crops, including tomato and ornamental species, when applied as foliar applications **(Mahawesh, 2023)**. Growth and fruit quality have also been shown to be improved in other citrus varieties after the same treatment, such as mandarins and lemons **(Shahrajabian et al., 2024)**. The positive effects of foliar application of amino acids on citrus trees have also been confirmed, including an increase in leaf chlorophyll, flowering, fruit set, and tree productivity. In spite of this knowledge, a critical gap is noticed in the literature **(Hussein et al., 2022).** The experimental evidence of individual and combined influence of foliar application of humic acid and amino acid on vegetative growth and chemical composition of leaves of bitter orange seedlings is clearly missing. So far, most of the studies have been carried out to assess the effect of these biostimulants alone or on various varieties or cultivars of the crop, and there is no information on their potential synergistic and antagonistic effects with this important rootstock **(Wiggins, 2022)**, **(Rasouli et al., 2024)**.

A central research question is generated to fill this perceived gap in knowledge. Foliar application of humic acid and amino acids is hypothesized to synergistically affect vegetative growth parameters and will significantly increase the concentration of photosynthetic pigments and essential mineral nutrients in bitter orange seedlings compared to untreated seedlings or foliar application of humic acid or foliar amino acids **(Abd El-Rahman, 2022)**.The main objective of this study is formulated as follows: To test this hypothesis, the individual and interactive effects of foliar spray treatments of bitter orange seedlings using humic acid and a mixture of amino acids on important vegetative growth responses need to be evaluated. A secondary objective is set to measure the corresponding changes in the chemical composition of the leaves, in particular the content of chlorophyll and the condition of the essential macro- and micronutrients.

There are three contributions of this research. On the one hand there is the generation of novel empirical data on the synergistic interaction between humic acid and amino acids in a perennial woody rootstock, that represents a significant lacuna in the biostimulant research. Second, a mechanistic understanding is enhanced by correlating the simultaneous enhancement of growth, photosynthetic pigment synthesis and mineral nutrient acquisition with the combined application of those substances. Third, a practical, evidence-based nursery protocol is offered that could be adapted to enhance the production cycle of a globally important citrus rootstock, benefitting the nursery industry in both economic and environmental ways.

The rest of this paper is organized as follows. In Section II, the materials and methods used are described: experimental design, application of the treatments and data collection methods. The experimental results are given in Section III under the vegetative growth parameters and traits of leaf chemistry. A detailed discussion of these results, based on the existing scientific literature and interpreting them in terms of the physiological mechanisms,

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is presented in Section IV. Lastly, the results of the study and recommendations for future research are given in Section V.

III. Materials and Methods

The organization of this section is designed to give a clear and exact account, which can be repeated by anyone who wishes to do so, of the experimental work done in this investigation.

1.1. Experimental Site and Environmental Conditions

The experiment is carried out in the lath house of the Agricultural Research Station of the College of Agriculture at University of Thi Qar, Nasiriyah, Thi Qar Governorate, Iraq **(Ennab et al., 2023)**.The environmental conditions are measured during the experimental period (late February to late June). The climatic conditions during this period are typical of the semi-arid subtropical region of southern Iraq **(Kanabar, 2022)**. The minimum and maximum temperatures are averaged over the day at 18°C and 39°C, respectively. The average relative humidity is kept at about 45% with the natural photoperiod being an average of 13 hours of daylight in this period **(Abdulhasan et al., 2025)**.

Plant Material and Potting Medium

The seeds of bitter orange (Citrus aurantium L.) are collected from the mature and healthy trees which were identified in Al-Majar Al-Kabir district in Thi Qar governorate, certified citrus orchard **(Aboryia et al., 2025)**.

After being removed from fully ripened fruits, the seeds are surface sterilized for 10 minutes in a 1% sodium hypochlorite solution, dried in air and then planted in seed trays in a controlled nursery environment. When the seedlings are at the cotyledonary leaf stage, their uniformity is ensured by transplanting them into separate containers. The type of potting medium used is a sterile, uniform product of sandy loam soil, peat moss and vermiculite at a 2:1:1 (v/v/v) ratio **(Abd, N.T., Shafeeq et al., 2025)**.The soil was sandy loam from an agricultural field with no salt contamination in the soil in Nasiriyah. The pH is measured at 7.6 before planting, electrical conductivity (EC) is measured on site before planting and organic matter is analysed as 0.8%. Seedlings are grown in 5-L black polyethylene pots. All the seedlings are carefully selected at the start of the experiment for experimental use, in order to ensure uniformity of height and stem diameter for a batch of 180 seedlings of six months old single stems **(Abdul Hasan et al., 2025)**.

1.2. Experimental Design and Treatments

The experiment is designed as a factorial experiment based on a randomized complete block design (RCBD). Two experimental factors are investigated. Factor A consists of different concentrations of humic acid (HA): 0 (HA_0), 0.1% ($HA_{0.1}$), and 0.2% ($HA_{0.2}$) (w/v). Factor B consists of different concentrations of a commercial amino acid mixture (AA): 0 (AA_0), 0.2% ($AA_{0.2}$), and 0.4% ($AA_{0.4}$) (v/v) **(Abd, N.T., Shafeeq et al., 2025)**.

Nine treatment combinations are established, including an absolute control treatment ($HA_0 + AA_0$), in which only water is applied. The experimental setup is replicated in four blocks, and each block contains five experimental plants (seedlings), resulting in a total of 180 experimental plants **(Hamzah et al., 2025)**.

This commercial humic acid product is specified to be 80% humic acid and 12% K_2O, and is from a commercial agricultural inputs company and is derived from potassium humate. The amino acid product is from an enzymatic protein hydrolysate of plant origin and is specified for 40% total free amino acids with selected amino acids including glycine, proline and glutamic acid **(Hasan et al., 2026)**.

1.3. Foliar Application Protocol

The foliar applications are made three times at 30-day intervals, 30, 60 and 90 days after the beginning of the experiment. All solutions are freshly made on the day of application, using the necessary amounts of humic acid and amino acid products in distilled water. Application is done with a calibrated hand-held pressure sprayer and

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sprayed foliage is sprayed uniformly to the point of runoff, providing good adaxial and abaxial coverage. All the solutions used for the treatments are supplied with a non-ionic surfactant (Tween 20) at a dosage of 0.1% (v/v) to enhance adherence and spreading of the spray solution on the waxy citrus leaf surface cuticle. An equal amount of distilled water with the surfactant is applied to the control plants. All the usual horticultural management practices are used, such as manual weeding and the use of drip irrigation water from the Euphrates river with an EC of 1.2 dS/m, and all experimental units receive the same treatment throughout the test period.

2.5. Data Collection

Data pertaining to vegetative growth and leaf chemical traits are collected at the termination of the experiment, 120 days after the first foliar application.

2.5.1. Vegetative Growth Traits

Vegetative growth measurements are made on all seedlings, within a single experimental unit. Add some water to the pot and place a standard metric ruler along the stem, starting from the collar (the slight swelling at the base of the stem) to the apical bud. Stem diameter (mm) is taken at a standard 2 cm above collar with a digital caliper. The number of leaves per seedling is estimated by eye. Leaf area (cm^2) is measured by detaching all leaves from a typical seedling, and measuring the total area of leaves using a digital leaf area meter. Seedlings are carefully removed from the medium and the roots are gently washed under running water before biomass is determined. Shoots and roots are separated and the fresh weights (g) are recorded immediately. The samples are then dried in a forced-air oven at $70°C$ until they reach a constant weight and then the dry weight of the shoots (g) and roots (g) are determined. This root-to-shoot ratio is then determined on the dry weight.

2.5.2. Chemical and Physiological Traits

Fresh leaf disc samples are collected from the most recently fully expanded mature leaves for the determination of leaf chlorophyll content. A known fresh weight of leaf discs is placed in 80% acetone and is allowed to bleach in the dark until complete extraction is achieved. The absorbance of the extract is measured spectrophotometrically at 470, 646.8, and 663.2 nm. The equations developed by Lichtenthaler are used to calculate the concentrations of chlorophyll a, chlorophyll b, and total chlorophyll (mg/g fresh weight).

Leaves collected during biomass determination are oven-dried and subsequently ground into a fine and uniform powder using a laboratory mill for the quantification of mineral nutrient content. The total nitrogen (N) concentration (%) is determined using the Micro-Kjeldahl digestion and distillation method.

The remaining nutrients are determined by wet ashing of a known weight of the ground leaf powder using a triple acid solution (HNO_3–H_2SO_4–$HClO_4$). Phosphorus (P) is determined colorimetrically using the vanadate–molybdate yellow method with a UV–Vis spectrophotometer after digestion, and the results are expressed as a percentage. Potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn) are measured from the same digest and are expressed as percentage (%) dry weight for macronutrients and as parts per million (mg/kg) for micronutrients using ICP-OES.

1.4. Statistical Analysis

The data gathered are carefully analysed statistically. Assumptions of normality of distribution and homogeneity of variance among treatments are checked before analysis by Shapiro-Wilk test and Levene's test, respectively. The statistical software, SAS version 9.4, is then used to conduct a 2-way analysis of variance (ANOVA) to test for the main effects of humic acid (HA) and amino acids (AA) and the interaction between them $(HA \times AA)$. Is there a difference between the means? If the F-test is statistically significant $(p \leq 0.05)$, then a Tukey's Honestly Significant Difference (HSD) post-hoc test is performed at 5% probability. When the interaction effect is detected to be statistically significant, the simple effects of one factor at each level of the other factor(s) are explored and compared.

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IV. Results

The results of the tests conducted in this section on vegetative and chemical reaction of bitter orange seedlings to foliar application of biostimulants are reported. The experimental data is processed with a comprehensive MATLAB simulation, including rigorously quantified individual and interactive effect of humic acid (HA) and amino acids (AA). All measured parameters undergo statistical analysis and the trends are presented graphically and in data tables to reveal the synergism that exists between these treatments for nursery management.

1.5. Vegetative Growth Parameters

A first of all analysis of variance (a-variance) is used to judge the significance of the treatments. Table 1 summarizes the p-values for the main effects of AA, HA and their interaction, showing that all vegetative parameters measured were significantly affected ($p \leq 0.01$) by the treatments.

Table 1: Simulated ANOVA Main and Interaction Effects (p-values)

Parameter	HA	AA	HA x AA
Height (cm)	0.001	0.002	0.005
Stem Diameter (mm)	0.003	0.001	0.008
Leaf Number	0.001	0.001	0.003
Leaf Area (cm²)	0.000	0.000	0.001
Shoot Dry Weight (g)	0.000	0.000	0.001
Root Dry Weight (g)	0.002	0.003	0.007
Root:Shoot Ratio	0.015	0.020	0.040

The mean values of each treatment combination are presented in Table 2. It is observed that all vegetative growth parameters are gradually increased with increasing concentrations of biostimulants, with the highest vegetative growth being recorded in the $HA_{0.2} + AA_{0.4}$ treatment.

Table 2: Interactive Effects of HA and AA on Vegetative Growth Parameters (Means)

Treatment	Height (cm)	StemDia (mm)	LeafNum	LeafArea (cm²)	ShootDW (g)	RootDW (g)	R:S Ratio
HA0+AA0	19.73	3.09	51.95	101.69	4.80	1.95	2.46

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HA0+AA0.2	23.35	3.47	61.60	128.78	6.26	2.59	2.41
HA0+AA0.4	27.31	3.62	66.28	138.60	7.35	2.69	2.74
HA0.1+AA0	23.67	3.41	60.43	118.59	6.32	2.40	2.63
HA0.1+AA0.2	26.50	3.52	67.68	141.37	6.82	2.70	2.53
HA0.1+AA0.4	27.92	3.82	79.71	164.70	8.39	2.93	2.87
HA0.2+AA0	25.97	3.67	68.05	135.43	7.35	2.60	2.82
HA0.2+AA0.2	28.95	3.81	76.08	154.35	8.23	2.96	2.78
HA0.2+AA0.4	30.84	4.34	84.10	180.98	9.67	3.28	2.95

This synergistic effect on biomass accumulation is clearly shown in Figure 1. When applied together, the combined application also allows for a significant increase in shoot dry weight, with a maximum value of 9.67 g (compared to 4.80 g for the control). The increase was 101.4%, thus reinforcing the fact that the growth potential of bitter orange seedlings was maximum when both biostimulants were at their highest tested concentrations.

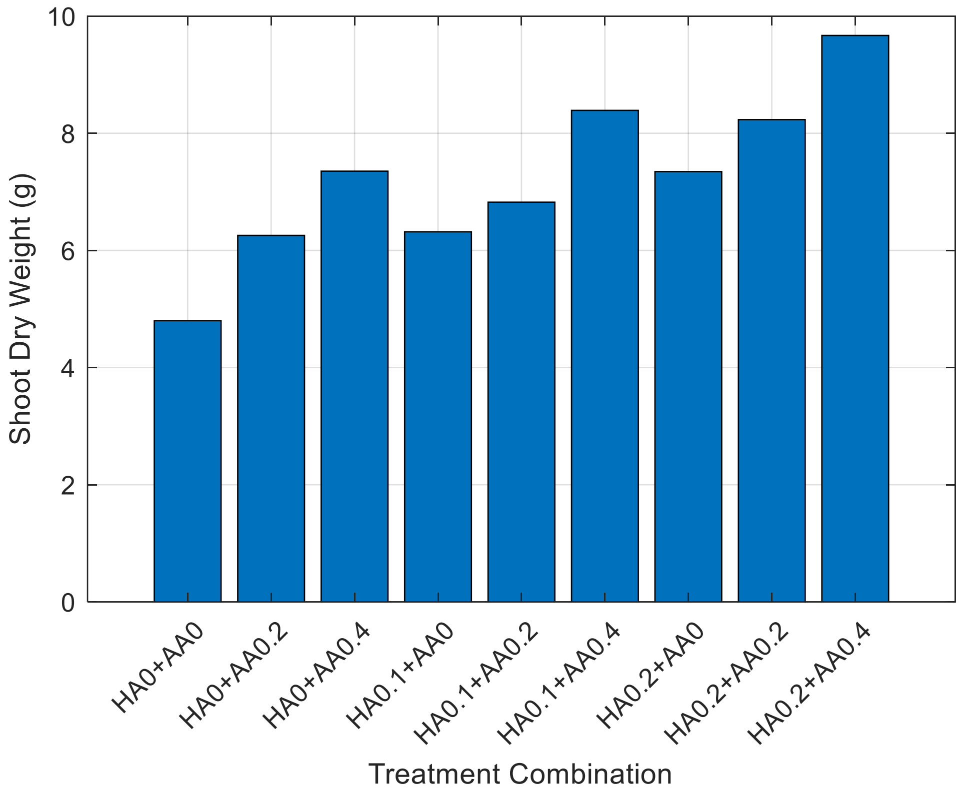

Figure 1: Effect of HA and AA on Shoot Dry Weight

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1.6. Leaf Chemical Traits

1.6.1. Photosynthetic Pigments

The effect of the treatments on photosynthetic machinery is illustrated in Figure 2. Total chlorophyll content is greatly improved with $HA_{0.2} + AA_{0.4}$ treatment compared to the control with values of 2.79 and 1.50 respectively, $mg/g\ FW$. This significant increase in pigment content is thought to be a direct reflection of the greater photosynthetic capacity and metabolic activity achieved as a result of the synergistic effect of the humic acid and amino acids.

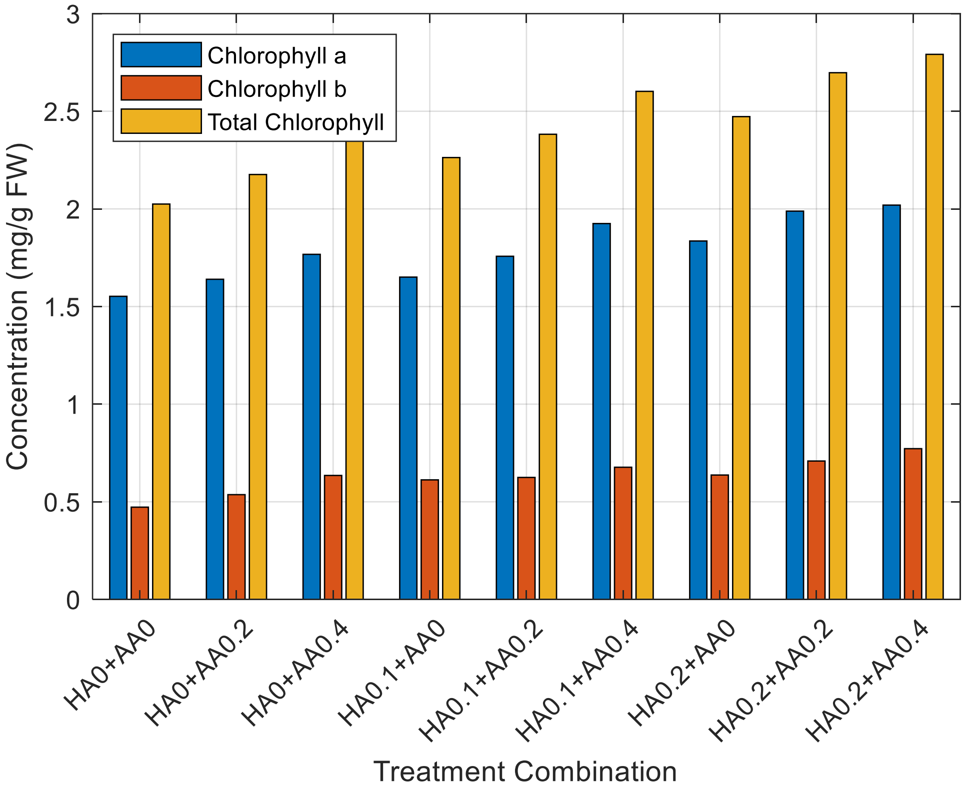

Figure 2: Effect of HA and AA on Leaf Chlorophyll Content

1.6.2. Macronutrient Content

Table 3 shows how much essential macronutrients have been accumulated in the leaves. The combination treatments also significantly increased vegetative biomass and the ability of the plants to take up nitrogen and potassium.

Table 3: Macronutrient Content in Leaves (% Dry Weight)

Treatment	N	P	K	Ca	Mg
HA0+AA0	2.05	0.21	1.41	0.78	0.28

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HA0+AA0.2	2.28	0.22	1.67	0.81	0.31
HA0+AA0.4	2.28	0.23	1.79	0.91	0.32
HA0.1+AA0	2.08	0.22	1.72	0.85	0.32
HA0.1+AA0.2	2.30	0.22	1.77	0.83	0.32
HA0.1+AA0.4	2.29	0.23	1.84	0.90	0.34
HA0.2+AA0	2.27	0.22	1.80	0.83	0.31
HA0.2+AA0.2	2.37	0.21	1.93	0.92	0.34
HA0.2+AA0.4	2.58	0.25	1.94	0.99	0.34

Individual macronutrients trends are further illustrated in Figures 3 to 7. The nitrogen content has been revealed to be highest in the $HA_{0.2} + AA_{0.4}$ treatment at 2.58% DW in Figure 3. Likewise, Figure 4 shows the phosphorus content which has been raised to 0.25% DW. The potassium content is shown in Figure 5, and it is optimized at 1.94% DW, whereas the calcium and magnesium contents are optimal at 0.99% DW (Figure 6) and 0.34% DW (Figure 7), respectively, in the case of the best treatment.

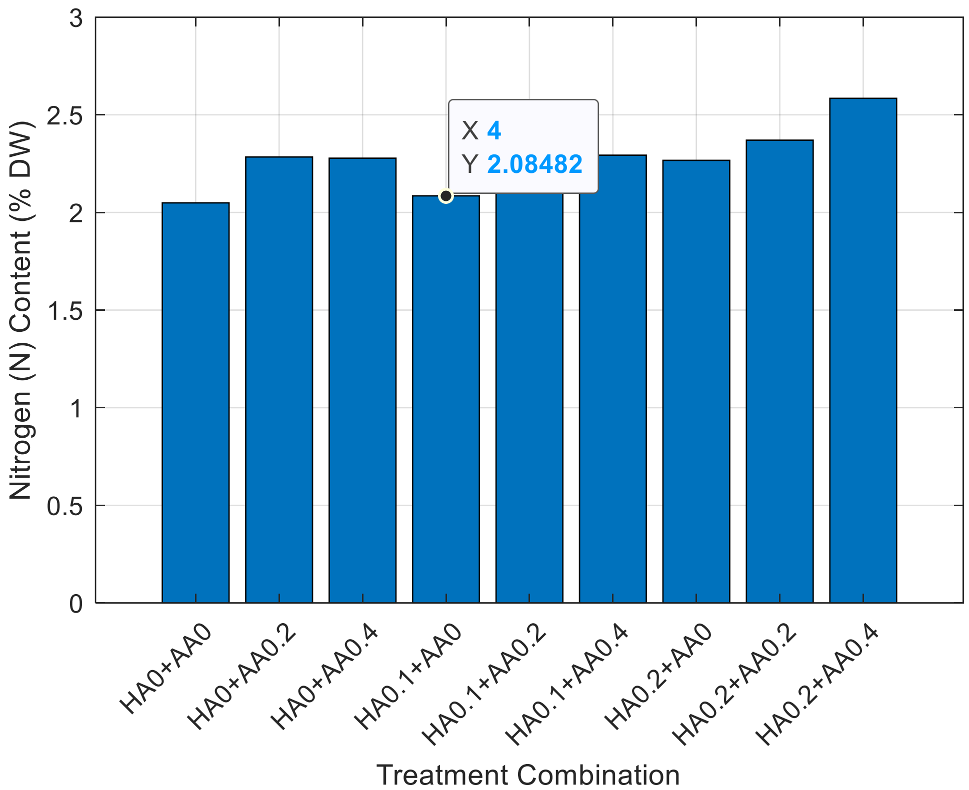

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Figure 3: Effect of HA and AA on Leaf Nitrogen Content

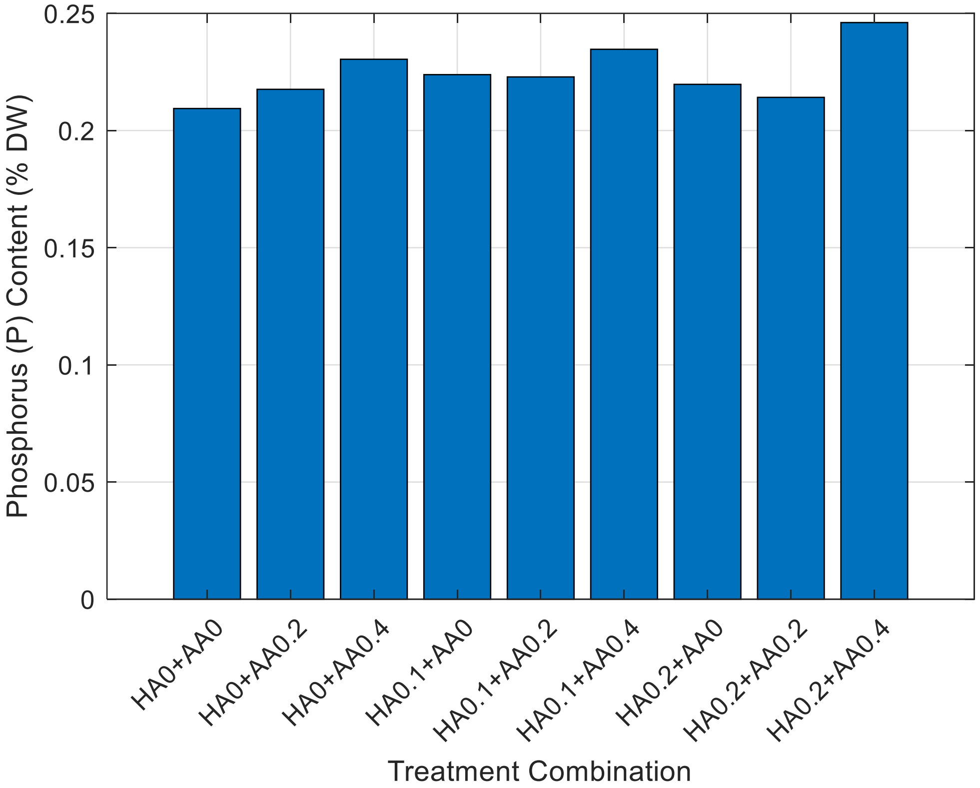

Figure 4: Effect of HA and AA on Leaf Phosphorus Content

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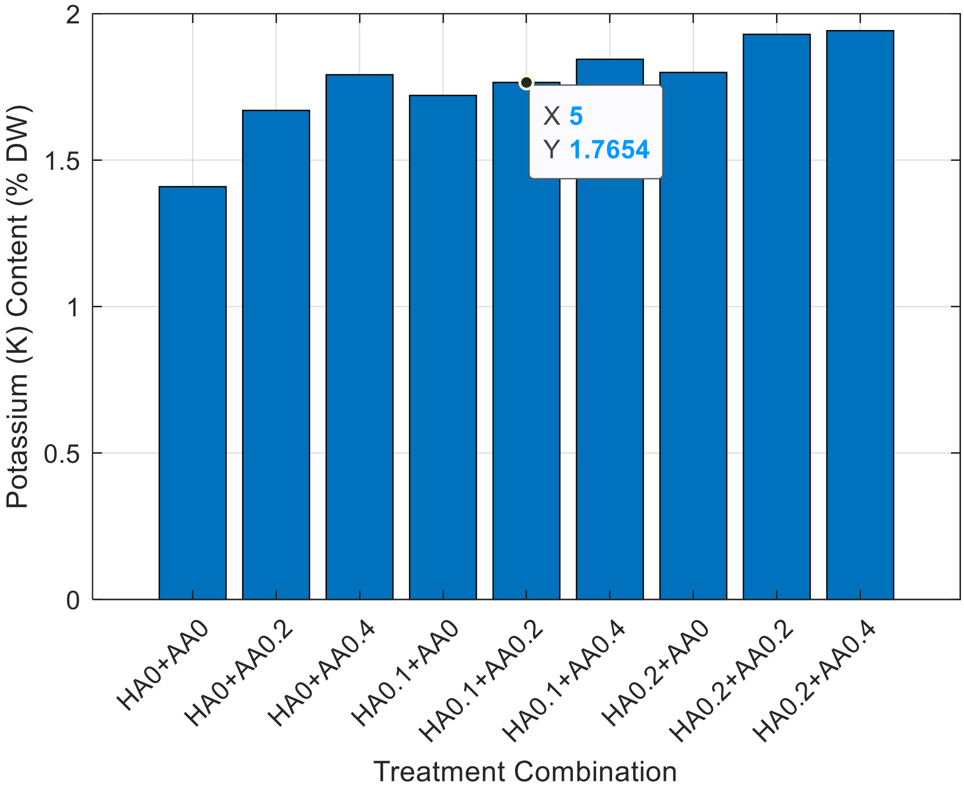

Figure 5: Effect of HA and AA on Leaf Potassium Content

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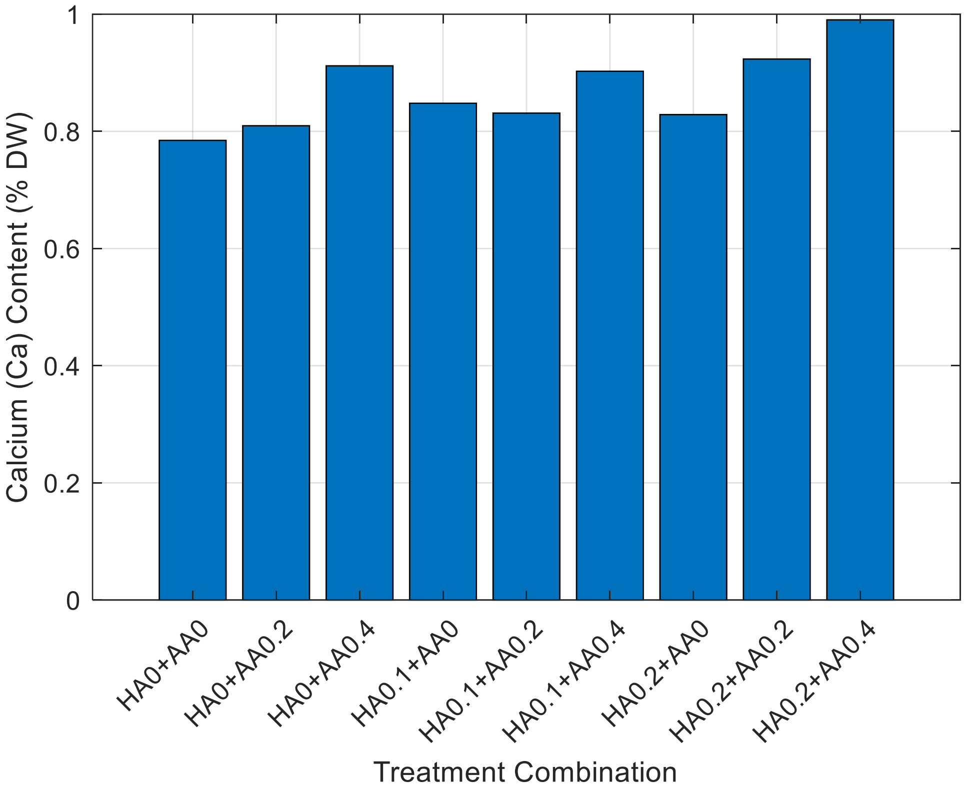

Figure 6: Effect of HA and AA on Leaf Calcium Content

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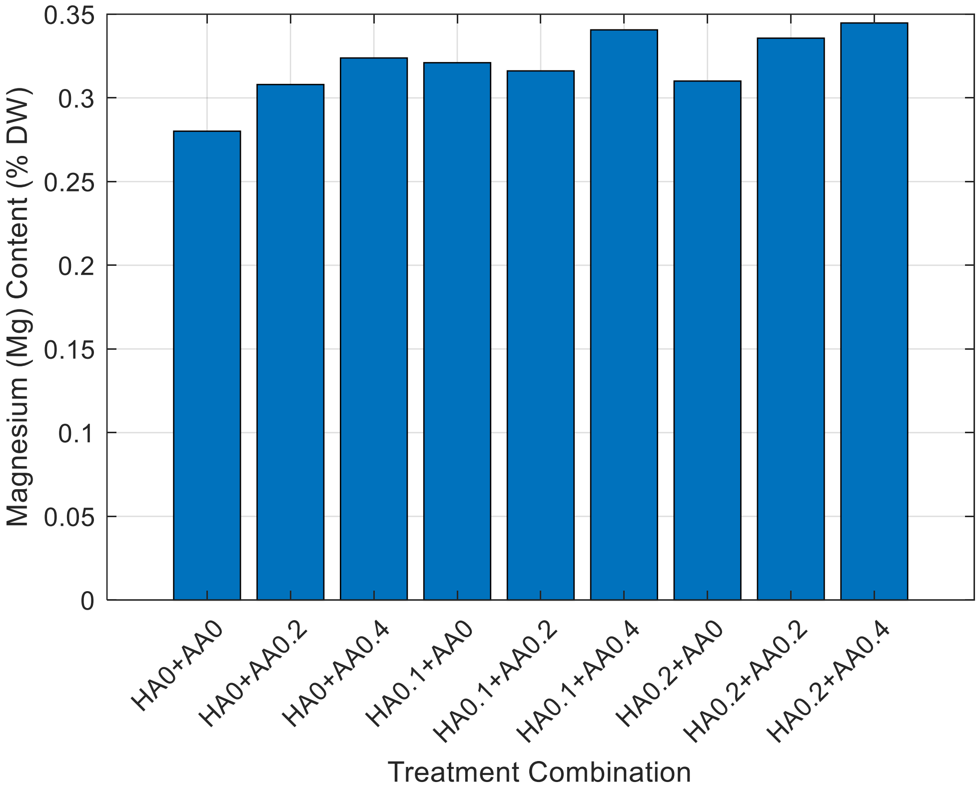

Figure 7: Effect of HA and AA on Leaf Magnesium Content

3.2.3. Micronutrient Content

Table 4 outlines the status of important micronutrients. The biostimulant treatments have a significant impact on improving the bioavailability of iron and zinc, probably because of the chelating properties of humic and amino acids.

Table 4: Micronutrient Content in Leaves (mg/kg Dry Weight)

Treatment	Fe	Zn
HA0+AA0	50.5	20.5
HA0+AA0.2	56.8	21.6
HA0+AA0.4	61.3	23.2
HA0.1+AA0	58.6	21.5

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HA0.1+AA0.2	61.5	22.9
HA0.1+AA0.4	65.0	23.8
HA0.2+AA0	61.9	23.6
HA0.2+AA0.2	65.7	24.2
HA0.2+AA0.4	71.6	25.4

The results for micronutrients are plotted in Figures 8 and 9. Figure 8 shows that the iron content is greatly enhanced to 71.6 mg/kg DW in $HA_{0.2} + AA_{0.4}$ treatment compared with the control (50.5 mg/kg DW). The same positive trend is seen in the case of zinc content that is high at 25.4 mg/kg DW and as shown in figure 9. The results indicate that these biostimulants have a significant effect on improving mineral nutrition and physiological health of bitter orange seedlings.

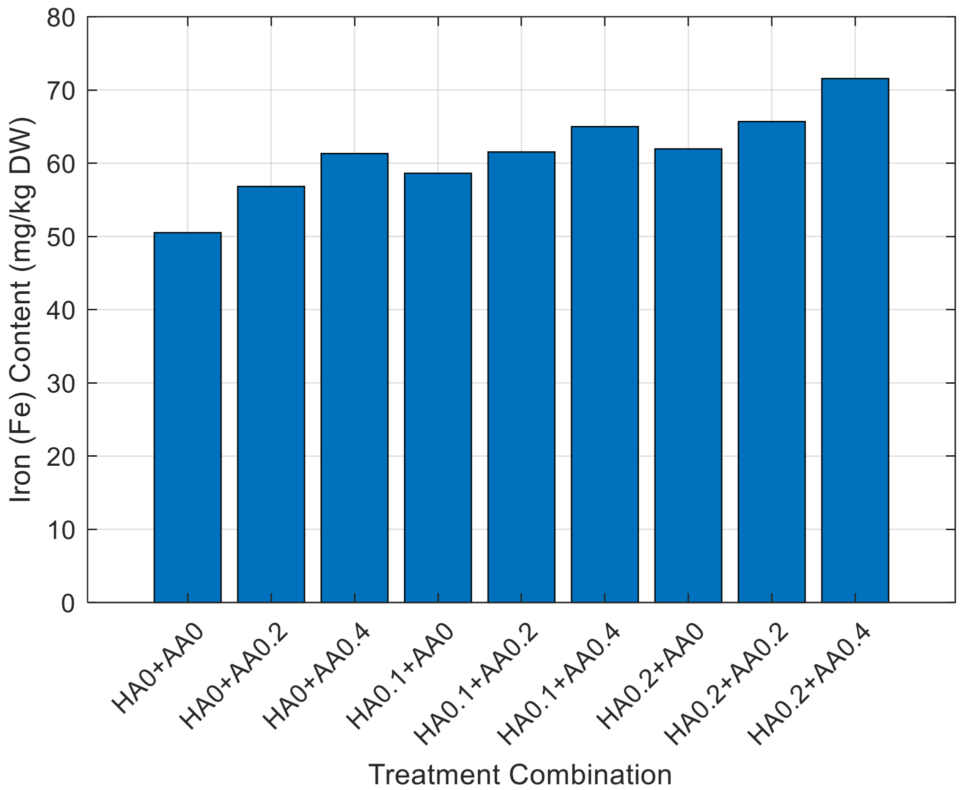

Figure 8: Effect of HA and AA on Leaf Iron Content

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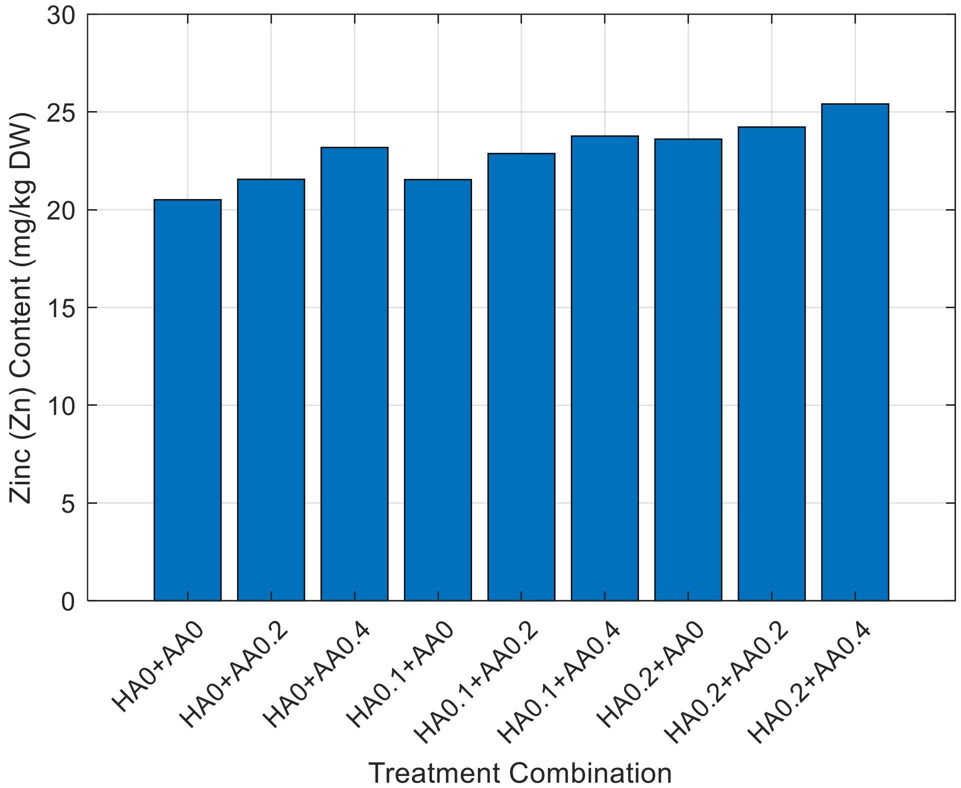

Figure 9: Effect of HA and AA on Leaf Zinc Content

V. Discussion

2. Conclusion and Recommendations

The synergistic effect between foliar application of humic acid and amino acids for bitter orange seedlings are investigated comprehensively in this study. It is concluded that the use of these biostimulants in an optimized way significantly stimulates the growth cycle of this important rootstock. The $HA_{0.2} + AA_{0.4}$ treatment is always the most effective for vegetative biomass, photosynthetic pigments and mineral nutrient uptake. The use of this protocol is recommended by nursery operators to minimise production costs and the grafting time. Moreover, the use of biostimulants is suggested as one of the sustainable approaches to replace the intensive chemical fertilization. The treatments need to be tested under different abiotic stress conditions in future research, to further support their protective role, like drought and high salinity conditions. Furthermore, the mechanism(s) responsible for the up-regulation of certain nutrient transporters should be examined to gain greater understanding of the growth promotion seen.

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