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Metabolic Profile Evolution of *Citrus sinensis* 'Navelina' Under Different Cultivation Techniques and Water-Saving Strategies

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Abstract: Citrus trees, particularly oranges, are a highly significant plant genus due to their consumption as fresh produce and the multiple compounds derived from them, which are extensively used in the food, cosmetic, and pharmaceutical industries. Despite recent advancements, the understanding of metabolic processes in the *Citrus* genus remains limited, especially in the context of variable agricultural practices. This study aimed to investigate the metabolomic evolution in leaves of sweet orange (*Citrus sinensis*) cultivated under different conditions over two key developmental periods: pre-winter (t_1) and spring sprouting and flowering (t_2). Using proton nuclear magnetic resonance (H-NMR) spectroscopy, this research identified 27 key metabolites across five distinct cultivation treatments (T0, T1, T2, T3, T4), including amino acids, organic acids, and sugars, and their variation over time. T0 represents the traditional crop of the control plot, while T1, T2, T3, and T4 incorporate different strategies aimed at water-saving, such as the use of weed control mesh and subsurface drainage systems, all designed to improve profitability and crop efficiency under the same soil and climatic conditions. The treatments were evaluated for their impact on plant growth parameters such as height, trunk diameter, and flower production, with a focus on reducing water usage without compromising crop performance. The results indicate that the use of weed control mesh significantly improves plant growth, increases flower production, and stabilizes key metabolite levels, contributing to a concept termed "plant metabolomic homeostasis." These findings are particularly relevant in regions like southeastern Spain, where water scarcity is a major concern. The study provides compelling evidence that the implementation of weed control mesh in orange cultivation can enhance water efficiency, promote healthier plant development, and maintain metabolic stability under variable growing conditions. These results suggest that such agricultural practices could be recommended for broader commercial application in citrus cultivation to improve sustainability and crop profitability.

Keywords: leaf *Citrus sinensis*; metabolomic profile; agricultural plastic mulch; metabolite mobilization; plant metabolomic homeostasis

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1. Introduction

Modern agriculture faces numerous global challenges, which necessitates the continuous implementation of new cultivation techniques to address issues such as resource scarcity, environmental impact, and sustainability in agricultural production. One of the most critical problems in agriculture is water scarcity, which directly affects plant vegetative growth, flower production, and ultimately crop survival [1,2]. This issue is particularly severe in southeastern Spain, where recurrent droughts have been a constant limitation for agricultural production. According to Melgarejo et al. [3], in this semi-arid region, it is essential to develop techniques that improve water use efficiency without compromising crop profitability.

Citrus cultivation, especially *Citrus sinensis*, is a key industry in Spain, one of the main citrus-producing regions in Europe. To enhance production sustainability, several studies have explored different agricultural techniques, such as irrigation management, soil covers, and rootstock selection, aimed at improving crop yield and plant health [3,4]. However, there remains a significant knowledge gap regarding the effects of these practices on the metabolomic profile of citrus trees. Metabolomics, defined as the identification and quantification of all metabolites present in an organism under specific conditions, has proven to be a powerful tool for investigating how plants respond to various environmental and management factors [5]. As noted by Son et al., specific metabolomic profiles can be used to identify "metabolic fingerprints" that provide valuable insights into plant responses to both internal and external factors, thus optimizing their management [2].

For the experimental study, a plot was divided into five subplots, each subjected to specific management conditions, including the use of weed control fabric and the presence or absence of an underground drainage system. Previous research has suggested that soil covers, such as weed control fabric, can offer significant benefits, including improved soil moisture retention, reduced herbicide use, and greater thermal stability in the soil [3–6]. Additionally, Zhu et al. [6] showed that soil covers can also influence the balance of essential metabolites in plants under stress conditions. In our study, we evaluate whether these techniques help maintain the trees' metabolic homeostasis, minimizing the metabolic imbalances observed in traditional cultivation without fabric or drainage [7–11].

In addition to the direct benefits of moisture retention and reduced pesticide use, recent studies suggest that the implementation of innovative agricultural techniques can improve overall crop efficiency by reducing abiotic stress, such as water stress [3,7]. According to Asai et al. [7], combining soil management techniques with metabolomic analysis can provide a holistic view of how agricultural practices impact plant metabolism, allowing for more precise recommendations for farmers.

In this context, this study not only offers valuable information on the metabolic state of citrus trees under different cultivation conditions but also provides a solid foundation for future practical recommendations. As the metabolomic analysis of the fruits progresses, we expect to complement these findings with studies that evaluate the impact of cultivation techniques on fruit quality and size, enabling more comprehensive recommendations for commercial citrus plantations.

Given the water scarcity in the southeastern region of Spain, it is crucial to implement techniques that reduce water consumption without compromising crop profitability. This study aims to evaluate the impact of five different cultivation conditions on the metabolic profile and physical development of *Citrus sinensis*, with a particular focus on water-use efficiency and plant health. By analyzing changes in metabolite levels across key developmental stages, this research seeks to identify practices that optimize resource use, promote sustainable growth, and support the long-term viability of citrus production in semi-arid regions. It appears that certain treatments, particularly those employing weed control nets and drainage, will demonstrate improved water retention, metabolic stability, and enhanced growth metrics, contributing valuable insights for future citrus management practices.

2. Materials and Methods

For this experiment, sweet orange trees, *Citrus sinensis* (L.) Osbeck, variety 'Navelina', grafted onto *Citrus macrophylla* Wester rootstocks, were used due to their commercial importance and representativeness in the study area. The trees, which were 1 year old, are located in an experimental plot of the Orihuela Polytechnic School at the Miguel Hernández University of Elche, in southeastern Spain (latitude 38°4′7.70″ N, longitude 0°59′1.11″ W).

In Orihuela, province of Alicante, the climate is dry, semi-arid, and subtropical Mediterranean, with more than 3000 h of sunshine per year and an average annual temperature that barely exceeds 20 °C degrees. The temperature throughout the year ranges between 5 °C and 32 °C degrees, with a summer period of more than 3 months where, recently,

temperatures have been exceeding 35 °C. The moderately cold season lasts approximately 4 months, with an average temperature of 20 °C and a minimum of about 5 °C. Rainfall is scarce throughout the year, the rainiest month being October, with an average of 34 L/m^2.

2.1. Plant Material, Experimental Design, and Growing Conditions

In the experimental plot, five different cultivation conditions or treatments were tested. These treatments differ from one another in the use or non-use of weed control mesh and/or an underground drainage system. The characteristics of each treatment are detailed in Table 1. Each treatment consisted of 21 trees, arranged in 3 rows of 7 trees each. The trees were planted in a 6 × 4 m pattern, following the traditional and commercial practices.

Table 1. Main characteristics of the theses/cultivation conditions tested in the study, irrigated by drip.

Thesis	Anti-Weed Mesh	Underground Drainage System	Others	Observations
T0	No	No	No	Traditional cultivation system. Control-test thesis
T1	Yes	Yes, located between tree rows	No	Simulation of established plantations with mature trees
T2	Yes	Yes, located beneath tree rows	No	Simulation of new plantations
T3	Yes	Yes, located beneath tree rows	Gravel trench	Simulates the traditional drainage system
T4	Yes	Yes, located beneath tree rows	Zeolite	Adds natural soil enhancers

All experimental treatments were equipped with a drip irrigation system consisting of two low-density polyethylene pipes with an external diameter of 16 mm (Azud tub PE model, manufacturer by Sistema Azud S. A. in Alcantarilla, Spain). Four self-compensating drippers, each with a flow rate of 2 L/h (PC dripper flat outlet model, Netafim Spain S.L, Riba-roja del Túria, Valenicia, Spain), were installed per tree, with two drippers per pipe and tree, spaced 0.8 m apart. The amount of water applied, as well as the timing and frequency of irrigation, was determined based on data from moisture and salinity probes (Sentek Drill and Drop Triscan probe, encapsulated with fixed sensors every 10 cm, Sentek Sensor Technologies, Stepney, Australia) installed in all evaluated treatments. These probes monitor real-time conditions, allowing precise irrigation adjustments according to the specific needs of the crop.

After planting the orange trees in March 2023, two leaf sampling sessions were conducted to track the metabolomic evolution of the trees before and after winter dormancy. The first sampling took place in October 2023, before winter dormancy (t_1), while the second sampling was conducted in May 2024, right after the first spring sprouting (t_2). These sampling times were selected to capture metabolomic changes during critical phases of the orange tree's growth cycle. The pre-winter sampling focused on analyzing the plant's reserves and metabolic adaptations accumulated before dormancy, whereas the post-winter sampling provided insights into metabolic reactivation and adaptations occurring during the spring growth resumption [8].

2.2. Metabolomic Profile

To understand the metabolomic profile, or metabolome, at a given moment, proton nuclear magnetic resonance spectroscopy (H-NMR) was employed. H-NMR is a highly sensitive analytical technique for determining functional groups, and with the use of specialized software (Chenomx NMR mixture analysis, version 11, Edmonton, AB, Canada) to interpret complex H-NMR spectra, it allows for both qualitative and quantitative identification of most metabolic compounds present in orange leaves. Additionally, measurements of plant height, trunk diameter, flower production, macroelement and microelement content in leaves, and other variables were conducted to further explore the relationship between the metabolome and the physical state of the plants.

In this study, the metabolomic evolution of the trees in all five experimental theses (T0, T1, T2, T3, T4), corresponding to different cultivation treatments, was analyzed. In

both periods (t_1 and t_2), the same procedure was followed to select the leaves for analysis. From the 21 trees in each thesis, approximately 5 leaves per tree were collected. All selected leaves were mature, well formed, practically undamaged, and taken from various orientations. The total number of leaves collected was more than 100 per treatment but finally that selection was reduced to 80. The number of leaves collected per treatment was determined based on the minimum sample size required to obtain representative data for each cultivation condition.

Immediately after collection, the leaves were washed with water to remove impurities and dust, then cut into small pieces to create a homogeneous sample. The chopped samples were immediately frozen using liquid nitrogen and stored at −80 °C until lyophilization (Christ Alpha 2-4 LSCplus, Martin Christ, Osterode am Harz, Germany). Once lyophilized, the samples were ground (TSM6A013, Taurus, Oliana, Spain) until a homogeneous mixture was obtained, and then stored again at −80 °C until analysis.

Once homogenized, three replicate samples were taken from each treatment (thesis), resulting in a total of 15 samples (5 treatments × 3 replicates) for metabolomic analysis. The data presented in the manuscript represent the averages of these three replicates per treatment, ensuring that the metabolomic profiles accurately reflect the overall condition of each treatment. By pooling the leaves and averaging across replicates, the influence of outlier trees or microenvironmental variability was minimized, providing a robust and reliable overview of the metabolomic state of each treatment.

To analyze the metabolomic profile of the leaf samples, polar and semi-polar compounds were first extracted following the protocol described by Sonia van der Sar et al. [9]. Briefly, 50 mg of lyophilized sample were weighed under sterile conditions and placed in a 2 mL Eppendorf-type polypropylene tube. A 1200 µL mixture of methanol and water (1:1, $MeOH_2O$) was added. The tubes were vortexed for at least 1 min, followed by ultrasonic agitation for 3 min in 1 min intervals, repeated three times. The tubes were then refrigerated at 4 °C for 30 min and centrifuged at 11,000× *g* for 20 min at 4 °C. The hydromethanolic phase was carefully pipetted to avoid any solid residue, transferred to a 1.5 mL Eppendorf tube, and evaporated using SpeedVac equipment at a maximum temperature of 30 °C, continuing overnight if necessary.

Once the hydromethanolic phase had completely evaporated, the resulting solid was resuspended in 800 µL of 100 mM potassium phosphate buffer (KH_2PO_4) at pH 6.0 (dissolved in 100% D_2O) with 0.58 mM TSP (internal standard). Finally, the samples were filtered using 0.45 µm nylon filters, and 600 µL of the filtrate was transferred to standard 5 mm NMR tubes for the quantification and identification of metabolites via H-NMR spectroscopy (Ascend NMR Magnet 500 MHz, Bruker, Billerica, MA, USA). All analyses for the five treatments were performed in triplicate (n = 3).

Although the H-NMR analysis detected a greater number of metabolites, only those that met the quantification criteria established for this study were included. The selection was based on the detection limits (LOD) and quantification limits (LOQ) defined by the method, with LOQ values between 5 and 10 µM and LOD greater than 3 µM. Metabolites detected at concentrations below these thresholds were not quantified to avoid reporting data that could be considered unreliable. These decisions were made to ensure the precision and robustness of the metabolomic data presented.

2.3. Evaluation of the Plant Development of Trees

To evaluate the growth rate and physical development of the trees in the different experimental treatments, measurements were taken periodically for all trees (n = 21) in each treatment (Medid flexometer with brake 5 m–19.0 mm). Parameters such as the height of the highest branch, crown diameter in four different directions, and trunk diameter of the scion (Mitutoyo ABS Digimatic digital caliper CD-15APX-150 mm. Mitutoyo Europe GmbH, Neuss, Germany) were measured at both t_1 and t_2. The results represent the average of the data collected from all trees studied (n = 21). The crown diameter of each tree was calculated as the arithmetic mean of the four measurements taken from different orientations.

2.4. Analysis of the Results

The sample results were analyzed both qualitatively and quantitatively using the Chenomx NMR mixture analysis software, version 11 (Edmonton, AB, Canada). For the comparative statistical study, means, standard deviations, one-way analysis of variance (ANOVA), and *t*-tests for related data were calculated using the free statistical software R version 4.2.2 (Copyright © 2022 The R Foundation for Statistical Computing, Vienna, Autria).

To perform a more comprehensive and functional metabolomic statistical analysis, the open-source JavaServer Faces web interface MetaboAnalyst 6.0 was used. Through MetaboAnalyst, several analyses were conducted, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), variable importance in projection (VIP) analysis, significance analysis of microarrays (SAM), hierarchical clustering heatmaps, and debiased sparse partial correlation (DSPC) calculations and visualizations.

3. Results and Discussion

This section presents the metabolomic profiles of the five experimental treatments studied at two different time intervals, t_1 and t_2. To facilitate data interpretation, a comparative analysis of the metabolomic profiles between t_1 and t_2 is included, highlighting the observed changes. Additionally, these profiles are correlated with phenotypic parameters such as height, trunk diameter, and canopy diameter, measured at both intervals. The observed increase between these measurements and the quantification of flowers during the spring of 2024 are also detailed.

3.1. Pre-Winter Metabolomic Profile (t_1)

In this initial analysis, corresponding to the pre-winter period, a total of 27 primary metabolites were detected across all samples. Of these, 12 belong to the amino acid group, 7 are organic acids, 4 are carbohydrates, and 4 belong to other functional groups. The results are presented in Table 2. A visual assessment revealed that amino acids were the most diverse group of compounds present in the leaves. From a quantitative perspective, organic acids dominated in terms of concentration, followed by sugars, while amino acids showed the lowest concentrations. These results are consistent with previous studies [10].

Table 2. Concentrations of metabolites (mM) identified in the pre-winter leaf samples (t_1) across the studied treatments. Values are presented as mean (±standard deviation), where the first value represents the average of three replicate measurements, and the value in parentheses represents the standard deviation.

Metabolite	Study Thesis				
	T0	T1	T2	T3	T4
Amino acids					
GABA	2.02 (0.28) a	1.96 (0.22) a	1.98 (0.09) a	1.69 (0.23) a	1.86 (0.39) a
Alanine	0.55 (0.07) a	0.66 (0.086) a	0.69 (0.08) a	0.54 (0.09) a	0.53 (0.09) a
Arginine	0.97 (0.12) a	1.30 (0.42) a	1.26 (0.36) a	0.84 (0.20) a	1.22 (0.24) a
Asparagine	0.99 (0.12) b	0.86 (0.08) ab	0.83 (0.06) ab	0.61 (0.16) a	0.77 (0.18) ab
Aspartate	0.68 (0.08) b	0.50 (0.02) ab	0.54 (0.05) ab	0.46 (0.09) a	0.56 (0.05) ab
Glutamate	0.80 (0.27) a	0.60 (0.16) a	0.61 (0.17) a	0.57 (0.36) a	0.68 (0.25) a
Glutamine	0.90 (0.28) a	0.92 (0.20) a	0.86 (0.11) a	0.96 (0.22) a	1.12 (0.19) a
Phenylalanine	0.20 (0.05) a	0.21 (0.05) a	0.21 (0.05) a	0.21 (0.08) a	0.24 (0.05) a
Proline	11.30 (1.71) a	11.04 (1.80) a	11.57 (0.79) a	10.12 (2.45) a	10.96 (1.84) a
Tryptophan	0.56 (0.16) a	0.52 (0.12) a	0.46 (0.07) a	0.46 (0.14) a	0.51 (0.05) a
Tyrosine	0.29 (0.008) a	0.27 (0.04) a	0.29 (0.05) a	0.27 (0.07) a	0.26 (0.03) a
Valine	0.09 (0.02) a	0.11 (0.01) a	0.10 (0.02) a	0.09 (0.02) a	0.12 (0.02) a
Organic acids					
Ascorbate	0.86 (0.13) a	0.86 (0.13) a	0.86 (0.18) a	0.87 (0.22) a	0.90 (0.18) a
Citrate	2.77 (0.36) ab	2.85 (0.45) ab	3.00 (0.48) b	1.70 (0.35) a	2.29 (0.51) ab

Table 2. *Cont.*

Metabolite	Study Thesis				
	T0	**T1**	**T2**	**T3**	**T4**
Formate	0.02 (0.008) a	0.02 (0.006) a	0.02 (0.01) a	0.02 (0.007) a	0.01 (0.005) a
Lactate	0.31 (0.05) a	0.33 (0.04) a	0.32 (0.05) a	0.24 (0.03) a	0.29 (0.06) a
Malate	10.11 (1.62) a	9.69 (1.80) a	8.95 (1.20) a	8.66 (2.22) a	8.88 (1.65) a
Quinic Acid	44.51 (7.63) a	39.55 (2.39) a	38.41 (5.09) a	43.73 (12.59) a	44.88 (8.40) a
Succinate	0.12 (0.02) a	0.10 (0.02) a	0.08 (0.02) a	0.09 (0.02) a	0.11 (0.02) a
Sugars					
Fructose	5.06 (0.98) a	6.99 (1.49) a	6.36 (1.76) a	3.81 (1.91) a	5.91 (1.78) a
Glucose	5.24 (0.87) a	7.46 (1.31) a	8.44 (1.62) a	10.02 (3.24) a	7.92 (1.68) a
myo-Inositol	1.78 (0.30) a	2.06 (0.40) a	2.13 (0.25) a	1.94 (0.58) a	1.95 (0.50) a
Sucrose	10.10 (1.62) a	9.24 (1.42) a	9.85 (1.21) a	10.16 (2.78) a	10.69 (1.88) a
Other metabolites					
Chlorogenate	0.24 (0.02) a	0.28 (0.02) a	0.26 (0.04) a	0.25 (0.061) a	0.33 (0.03) a
Choline	1.23 (0.19) a	1.43 (0.20) a	1.56 (0.19) a	1.44 (0.38) a	1.62 (0.35) a
Ethanol	1.35 (0.56) a	1.53 (0.65) a	1.71 (0.48) a	1.59 (0.51) a	1.56 (0.50) a
Trigonelline	0.04 (0.01) a	0.03 (0.007) a	0.03 (0.0005) a	0.03 (0.008) a	0.03 (0.01) a

T0, T1, T2, T3, and T4 represent the different theses or cultivation conditions studied. Different letters within the same row indicate statistically significant differences according to post hoc tests.

3.1.1. Amino Acids in the Pre-Winter Period (t_1)

The amino acid with the highest concentration across all samples was proline, with a range of 10.12 mM–11.57 mM. When comparing proline with all detected amino acids, differences of up to 98.96% were observed in comparison to the amino acid with the lowest concentration, valine. However, there were no significant differences among the study treatments. Previous studies report similar findings [11]. Proline plays a critical role in protein synthesis, osmotic adjustment, and protein protection under stress conditions. Additionally, it has been shown to participate in cell wall elongation, stem growth, and root and shoot development. Given its essential functions, it is unsurprising that proline had the highest concentration in the leaves of young orange trees. During the pre-winter period (t_1), citrus trees prepare for the winter by accumulating proline, as suggested by Yelenosky's study [12].

The amino acids GABA (gamma-aminobutyric acid), alanine, arginine, glutamate, glutamine, phenylalanine, proline, tryptophan, tyrosine, and valine were detected in all five treatments, without statistically significant differences. Among this group, GABA had the highest concentration. GABA is a key metabolite in plants, serving several primary and secondary metabolic functions [13,14], including providing carbon chains and energy, regulating cytosolic pH, and participating in nitrogen metabolism [15]. Proline and GABA are critical amino acids in the active growth of orange trees, explaining why their synthesis is prioritized in all trees, despite the differences in cultivation conditions.

On the other hand, significant differences were observed among the samples for asparagine and aspartate. In this regard, T0 had higher amounts of asparagine (0.99 mM) and aspartate (0.68 mM) than the other samples. These values were consistent with those reported by other studies [16]. However, for these same metabolites, T3 had the lowest amounts, with values of asparagine and aspartate at 0.61 mM and 0.46 mM, respectively. These lower values have also been quantified in different citrus species grown in Japan [7]. Asparagine is a nitrogen transport and storage molecule found in many plants. It is synthesized in the roots during nitrate assimilation, although evidence suggests it is also synthesized in the leaves [17]. Some of its transport functions include mobilizing nitrogen from sources to sinks [18]. The amino acid aspartate serves as an important metabolic hub for the biosynthesis of many metabolites, including asparagine, which is essential for plant growth and development [19]. The high concentration of these amino acids in T0, which

showed less development as reflected in Table 3, suggests that cultivation conditions may have influenced these differences.

Table 3. Tree height, crown diameter, and trunk diameter (cm) for the five studied treatments during the pre-winter period. Results represent the mean (n = 21/treatment) with the standard deviation in parentheses.

Parameter	Thesis/Cultivation Conditions Studied				
	T0	**T1**	**T2**	**T3**	**T4**
Tree height (cm)	91.00 (14.34) a	93.00 (11.22) a	105.67 (11,14) b	105.24 (12.18) b	100.76 (12.96) ab
Crown diameter (cm)	64.62 (12.71) a	86.86 (9.80) b	91.57 (8.37) b	93.52 (11.19) b	90.08 (14.69) b
Trunk diameter (cm)	13.29 (2.43) a	16.25 (2.20) b	17.67 (2.14) bc	18.36 (2.39) c	18.32 (3.00) bc

Different letters within rows indicate statistically significant differences according to Tukey's multiple comparison test.

T0 exhibited the least growth, with an average height of 91.00 cm. Based on the results, it can be inferred that T0 had higher amino acid levels in its leaves because it did not allocate them as intensively to vegetative growth, unlike the other treatments. This suggests that the cultivation conditions of T0 may not be the most favorable for the growth and development of orange trees, as T0 lacks both weed control mesh and a drainage system. On the other hand, T3 showed the greatest growth and development at this initial stage, with an average height of 105.24 cm. This might explain why T3 had the lowest total amino acid levels, as the amino acids were more extensively used during the vegetative period compared to the other treatments.

At t_1, amino acids appeared as the most diverse chemical group in all treatments. The orange trees were very young (less than a year old), with fully functional vascular systems and grown under favorable conditions, prioritizing metabolite formation for growth. The youth of the trees and the short exposure time to each treatment's specific conditions may explain the limited differences observed in amino acid profiles across treatments.

3.1.2. Organics Acids in the Pre-Winter Period (t_1)

Of the seven organic acids detected, quinic acid had the highest concentration across all treatments, ranging from 38.41 mM to 44.88 mM, without significant differences between treatments. Without classifying metabolites into chemical families, quinic acid was the most abundant compound overall. In contrast, formate had the lowest concentration, ranging from 0.01 mM to 0.02 mM, with no significant differences between treatments [20].

The high presence of quinic acid can be explained by the trees being in a vegetative and juvenile stage, a period of intense growth activity. During this phase, plants biosynthesize and store quinic acid in preparation for periods of accelerated growth [21]. Formate, on the other hand, typically accumulates under stress conditions, which was not the case in this experiment since all plants received continuous water and nutrients. The low formate concentration suggests that the trees were not experiencing stress and were growing uniformly [22]. With the results obtained, the high and consistent levels of quinic acid across the experimental treatments (T1, T2, T3, and T4) suggest that, while not all plants exhibited the same growth rate or used this compound for forming woody tissues at the same intensity, they were in a juvenile phase where the active storage of this metabolite occurred. This storage is essential, as it allows plants to quickly mobilize quinic acid when favorable agroclimatic conditions arise, facilitating an optimal growth response. The exact role of quinic acid and its derivatives in plants is still partially unknown, but studies suggest it may be incorporated into lignin in some plants, potentially relating it to the formation of supportive tissues [21].

Formate plays a multifaceted role in biosynthetic pathways related to photosynthesis. It can be formed as a byproduct of photorespiration and serves as a crucial substrate for oxidation in the mitochondria. Formate is especially relevant in stress situations such as darkness, hypoxia, wounds, cold, and drought, where an increase in formate oxidation

is observed [22]. Formate plays a multifaceted role in biosynthetic pathways related to photosynthesis. It can be formed as a byproduct of photorespiration and serves as a crucial substrate for oxidation in the mitochondria. Formate is especially relevant in stress situations such as darkness, hypoxia, wounds, cold, and drought, where an increase in formate oxidation is observed [23].

In the group of organic acids—ascorbate, formate, lactate, malate, quinic acid, and succinate—most concentrations were not statistically significant, except for citrate. Organic acids are considered relatively stable and homogeneous across the different treatments, likely due to the uniformity applied to all treatments in terms of irrigation and fertilization. However, T2 recorded the highest citrate concentration, while T3 had the lowest, with a 43.33% reduction compared to T2, which was significant [24]. Citrate plays a crucial role as a key metabolite, providing carbon skeletons for nitrogen assimilation and facilitating essential processes in energy metabolism [25]. The lower citrate concentration observed in T3 correlates with the greater growth observed in this treatment, as reflected by the larger canopy diameter of 93.52 cm documented in Table 2.

3.1.3. Sugars in the Pre-Winter Period (t_1)

The sugar with the highest concentration across all treatments was sucrose, ranging from 9.24 mM to 10.69 mM, while myo-inositol had the lowest concentration, ranging from 1.78 mM to 2.13 mM, with no significant differences between treatments [26]. These results are promising indicators, as sucrose is synthesized in the cytosol of plant cells and can be transported to storage tissues, where it is either used or stored. Sucrose also functions as a key metabolite and indicator molecule. Any modification in its synthesis, transport, or degradation directly affects plant growth, development, and physiology. Generally, high levels of sucrose translate into enhanced growth and differentiation, whereas sucrose deficiencies are likely to result in chlorosis, reduced photosynthetic rates, and, consequently, slower growth [27], while myo-inositol and its derivatives are crucial compounds for development and signaling in plants. They serve as metabolic intermediates or participate in various signaling pathways in response to stress, hormones, and nutrients [28]. Moreover, the metabolic pathways associated with myo-inositol are vast and highly effective in inducing stress-tolerance responses in plants [28]. As previously mentioned, myo-inositol was the sugar detected in the lowest concentration during the t_1 period, suggesting that the plants were not exposed to significant stress conditions.

Thus, during t_1, sugars, especially sucrose, serve as indicators of healthy plant performance. Since no significant differences in sugar concentrations were observed between the treatments, and the detected sucrose levels were relatively high, it can be concluded that the crop was performing well.

3.1.4. Other Metabolites in the Pre-Winter Period (t_1)

Regarding the secondary metabolites identified (chlorogenic acid, choline, ethanol, and trigonelline), the analyses did not reveal statistically significant differences in their concentrations across the treatments. For example, chlorogenic acid concentrations ranged from 0.24 mM in T0 to 0.33 mM in T4, while choline concentrations varied from 1.23 mM in T0 to 1.62 mM in T4. Similarly, ethanol showed minor variations, with concentrations ranging from 1.35 mM in T0 to 1.71 mM in T2, while trigonelline levels remained consistently low across all treatments, reflecting a stable pattern of production under the different experimental conditions.

This consistency in the concentrations of chlorogenic acid, choline, ethanol, and trigonelline suggests that the cultivation conditions did not induce significant changes in the biosynthesis of these compounds. The observed uniformity supports the hypothesis that agronomic management was homogeneous [29], and that the experimental conditions were effectively controlled throughout the study [30].

3.2. Post-Winter Metabolomic Profile (t_2)

For the second time point (t_2), samples were collected during the post-winter period, six months after the first analysis. A total of 25 primary metabolites were detected, of which 9 were amino acids, 8 were organic acids, 4 were sugars or glucids, and 4 were other secondary metabolites. Similarly, to t_1, amino acids were the most diverse group in terms of metabolite variety. However, organic acids were present in the highest concentrations, followed by sugars, with amino acids in the lowest concentrations.

Overall, in this second sampling, there was a reduction in the total number of detected metabolites, decreasing from 27 to 25, along with a significant reduction in the overall quantitative levels. This reduction was particularly notable in the amino acid concentrations and can be explained by well-known biological processes. The timing of the second sampling coincided with spring budding and the end of the orange blossom period, which are highly demanding vegetative phases in terms of metabolic requirements. During these phases, plants mobilize large quantities of metabolites and nutrients to support the rapid development and expansion of new tissues, which accounts for the observed decrease in amino acid levels.

3.2.1. Amino Acids in the Post-Winter Period (t_2)

Proline was the amino acid with the highest concentration across all study treatments, with levels ranging from 3.51 mM to 5.56 mM. A significant decrease in proline concentrations was observed at t_2 compared to t_1, with reductions of up to 65.31%. This reduction in proline, the amino acid with the largest difference in concentration (97.66%) compared to the one with the lowest concentration (valine, as at t_1), reflects the intense nutritional mobilization that plants undergo during the seasonal transition from autumn to spring, characterized by rising temperatures and longer daylight hours [31,32].

Phenylalanine, tryptophan, and valine were detected in all treatments, but their concentrations did not show statistically significant differences across the samples. However, GABA (gamma-aminobutyric acid), alanine, asparagine, aspartate, proline, and tyrosine did show statistically significant results. As shown in Table 4, T0 had the lowest concentration of five of these amino acids. Additionally, although phenylalanine, tryptophan, and valine did not show significant differences among treatments, T0 consistently had lower values than the other treatments. This is an important observation, as it suggests that T0 had the lowest total amino acid content, having utilized them largely for spring vegetative growth [33], as reflected in the tree development data shown in Table 5.

Tyrosine plays a key role in a common regulatory process known as tyrosine phosphorylation, which is directly involved in plant growth and development, and various stress response pathways under different environmental conditions [34]. Given its importance in plant growth mechanisms, it is logical that tyrosine levels were significantly higher at t_2 compared to t_1. Notably, T4 had the highest concentration of tyrosine, coinciding with this treatment's superior growth in terms of canopy and trunk diameter (Table 5).

The concentration of alanine observed at t_2 has decreased compared to the measurements at t_1, which indicates a positive adjustment. During t_1, the colder autumn conditions may induce episodes of root hypoxia, where alanine acts as a stress response molecule. This phenomenon is particularly reflected in T0, which has the lowest alanine concentration among all the theses, as shown in Table 4. This thesis is notable because it is the only one of the five that is cultivated without weed mesh or a drainage system, which could influence root dynamics and stress responses [35], in this case, it is worth mentioning that previous studies have shown that alanine and some of its derivatives accumulate as part of a general stress response in plants, providing protection against low temperatures, root hypoxia, and water scarcity [36]. The decrease in alanine levels at t_2 suggests a reduction in these stress conditions, especially in T0, where the reduction likely occurred more rapidly than in the other treatments. Without the installation of weed control mesh, T0 may have avoided potential root anoxia during the mild winter and quickly activated its metabolism to prepare for spring growth.

Table 4. Concentrations of metabolites (mM) identified in the post-winter leaf samples (t_2) across the studied treatments. Values are presented as mean (±standard deviation), where the first value represents the average of three replicate measurements, and the value in parentheses represents the standard deviation.

Metabolite	T0	T1	T2	T3	T4
Amino acids					
GABA	0.84 (0.20) a	1.15 (0.09) b	1.10 (0.04) ab	1.19 (0.07) b	1.21 (0.09) b
Alanine	0.22 (0.04) a	0.32 (0.02) b	0.27 (0.002) ab	0.34 (0.02) b	0.32 (0.02) b
Asparagine	0.34 (0.14) a	0.55 (0.008) b	0.58 (0.02) b	0.59 (0.08) b	0.64 (0.03) b
Aspartate	0.43 (0.09) a	0.69 (0.02) b	0.63 (0.005) b	0.66 (0.02) b	0.69 (0.01) b
Phenylalanine	0.14 (0.03) a	0.14 (0.01) a	0.13 (0.007) a	0.15 (0.01) a	0.16 (0.01) a
Proline	3.51 (0.76) a	5.16 (0.37) b	5.56 (0.21) b	5.29 (0.28) b	5.01 (0.32) b
Tryptophan	0.48 (0.04) a	0.53 (0.01) a	0.49 (0.02) a	0.50 (0.02) a	0.49 (0.04) a
Tyrosine	0.57 (0.07) ab	0.46 (0.03) a	0.48 (0.01) a	0.55 (0.003) ab	0.61 (0.03) b
Valine	0.10 (0.01) a	0.12 (0.009) a	0.13 (0.003) a	0.12 (0.006) a	0.10 (0.008) a
Organic acids					
Ascorbate	0.32 (0.05) a	0.31 (0.01) a	0.31 (0.01) a	0.31 (0.01) a	0.35 (0.03) a
Citrate	2.29 (0.44) a	2.52 (0.17) ab	2.17 (0.04) a	3.10 (0.16) b	2.17 (0.19) a
Formate	0.01 (0.004) a	0.01 (0.003) a	0.01 (0.001) a	0.02 (0.002) a	0.01 (0.002) a
Fumarate	0.12 (0.01) a	0.15 (0.03) a	0.16 (0.03) a	0.13 (0.01) a	0.12 (0.003) a
Lactate	0.14 (0.01) a	0.18 (0.01) a	0.17 (0.03) a	0.16 (0.006) a	0.18 (0.01) a
Malate	4.71 (0.67) a	9.14 (0.62) c	8.47 (0.16) bc	8.71 (0.43) bc	7.62 (0.35) b
Quinic	26.62 (7.34) ab	22.57 (1.46) a	19.05 (0.79) a	26.73 (1.91) ab	34.54 (2.49) b
Succinate	0.09 (0.04) a	0.12 (0.003) a	0.12 (0.002) a	0.12 (0.01) a	0.11 (0.008) a
Sugars					
Fructose	4.40 (0.64) a	3.55 (0.66) a	3.59 (0.14) a	4.89 (0.24) a	4.67 (0.57) a
Glucose	6.16 (1.27) bc	3.28 (0.20) a	2.78 (0.11) a	4.40 (0.20) ab	6.62 (0.74) c
myo-inositol	2.00 (0.55) a	2.04 (0.13) a	2.07 (0.20) a	2.20 (0.20) a	2.14 (0.04) a
Sucrose	6.58 (1.34) a	6.28 (0.62) a	6.46 (0.41) a	6.05 (0.31) a	6.73 (0.56) a
Other metabolites					
Chlorogenate	0.16 (0.06) a	0.21 (0.006) a	0.22 (0.002) a	0.22 (0.006) a	0.21 (0.01) a
Choline	0.75 (0.17) a	0.94 (0.08) a	1.00 (0.04) ab	1.30 (0.08) c	1.22 (0.09) bc
Ethanol	0.79 (0.06) ab	0.70 (0.02) a	0.65 (0.01) a	0.78 (0.10) ab	0.89 (0.02) b
Trigonelline	0.04 (0.008) a	0.04 (0.008) a	0.04 (0.002) a	0.05 (0.004) a	0.04 (0.002) a

Different letters in each row indicate significant differences according to the multiple comparison test of means (Tukey contrasts).

Table 5. Tree height, canopy diameter, and trunk diameter (cm) for the five studied treatments in the post-winter period. Results represent the mean (n = 21 per treatment) with the standard deviation in parentheses.

Parameter	Thesis/Cultivation Conditions Studied				
	T0	T1	T2	T3	T4
Tree height (cm)	112.52 (15.76) a	108.00 (20.57) a	116.14 (13.74) a	121.57 (14.83) a	118.67 (15.64) a
Crown diameter (cm)	92.31 (12.07) a	106.86 (10.90) b	110.38 (12.42) b	107.88 (12.88) b	112.40 (14.01) b
Trunk diámeter (cm)	22.27 (2.57) a	24.93 (4.74) ab	25.23 (3.87) ac	26.43 (4.02) bc	28.57 (4.08) c

Different letters in each row indicate significant differences according to the multiple comparison test of means (Tukey contrasts).

3.2.2. Organics Acids in the Post-Winter Period (t_2)

In the post-winter analysis, quinic acid remained the most prevalent organic acid across all samples, with concentrations ranging from 19.05 mM to 34.54 mM. In contrast, formate had the lowest concentration, with values ranging from 0.01 mM to 0.02 mM; furthermore, upon closer examination, it was observed that the concentrations of ascorbate,

formate, fumarate, lactate, and succinate showed no statistically significant differences across the samples from all treatments, indicating a consistency in their levels, despite seasonal and management variations. A notable finding during the t_2 sampling period was the detection of fumarate, which had not been identified in the first t_1 sampling. The appearance of fumarate at t_2 could indicate the activation of specific metabolic pathways, potentially in response to improved environmental conditions or a reconfiguration of the plant's energy metabolism during the seasonal transition [37].

However, citrate, malate, and quinic acid exhibited statistically significant variations. Specifically, quinic acid showed a marked reduction in its concentration, with a maximum decrease of 23.03% observed in the highest values. This decrease could be related to seasonal metabolic changes or adaptive responses to environmental conditions, which will be discussed further in the analysis.

Malate plays a crucial role in plant metabolism, especially in mineral nutrition, symbiotic nitrogen fixation, phosphorus acquisition through roots, and tolerance in aluminum-rich soils. Malate facilitates nitrogen assimilation into amino acids by providing carbon skeletons and is also secreted by roots under phosphorus deficiency conditions to mobilize this essential nutrient, making it more accessible to the plant [38]. Additionally, fumarate, a component of the tricarboxylic acid cycle (TCA cycle), can be metabolized to produce ATP and carbon chains for the synthesis of other compounds. Fumaric acid concentrations increase with plant age and light intensity in some species. Moreover, phloem exudates in certain plants contain significant amounts of fumarate, suggesting that fumarate may play a role in carbon transport [39]. The presence and variation of malate and fumarate in the post-winter samples (t_2) were expected and are statistically significant, reflecting their active involvement in the plant's metabolism, which is at full capacity during this phase of plant development.

All these changes in metabolic levels occurred at t_2, a period marked by three major events in the agricultural year: the transition from winter to spring, significant budding, and flower formation.

3.2.3. Sugars in the Post-Winter Period (t_2)

In the post-winter measurements, sucrose was the most abundant sugar across all treatments, with concentrations ranging from 6.05 mM to 6.73 mM. In contrast, myo-inositol had the lowest concentrations, ranging from 2.00 mM to 2.20 mM. While the variations in fructose, myo-inositol, and sucrose concentrations did not show statistically significant differences between the treatments, glucose exhibited significant variations.

A general decrease in glucose levels was observed from t_1 to t_2 across all treatments (Table 4). This reduction may be linked to climatic conditions; during t_1, when temperatures were cooler, plants likely increased glucose production as a protective mechanism against cold. As temperatures rose towards t_2, the need for glucose for thermal protection decreased, reflecting an adaptation to more favorable environmental conditions for citrus growth. Glucose is a sugar found throughout the plant kingdom. In addition to being a universal carbon source, glucose acts as an indicator compound that regulates various metabolic processes in plants. The effects of glucose are concentration-dependent, influencing photosynthesis, respiration, and nitrogen metabolism. Glucose levels in plants increase after exposure to various stresses, where its accumulation helps mitigate stress by enhancing the production of antioxidants and photosynthesis-related compounds. These compounds function as osmoregulatory agents within the cell, regulate pH balance, and reduce plasma membrane permeability under stress conditions [40].

3.2.4. Other Metabolites in the Post-Winter Period (t_2)

For chlorogenic acid and trigonelline, no significant differences were detected between the five treatments, whereas choline and ethanol exhibited significant variations. Some studies suggest that endogenous ethanol production helps maintain cellular energy status under specific stress conditions. Other studies associate ethanol production in response to

low temperatures with the maintenance of membrane function. Under drought and salinity stress, ethanol production triggers the formation of reactive oxygen species (such as hydrogen peroxide and peroxides), which are essential for protecting cellular functions. Choline, on the other hand, is a precursor to glycine betaine and phospholipids, playing a crucial role in plant tolerance to salinity and other abiotic stresses by facilitating osmotic adjustment, stabilizing membrane proteins, and protecting them from oxidative damage [41].

Both choline and ethanol are metabolites primarily associated with adverse conditions for cultivated plants. During the first sampling (t_1), slightly higher levels of choline and ethanol were recorded compared to the second sampling (t_2). This reduction may be attributed to improved climatic conditions at t_2, which alleviated some of the environmental stresses experienced during t_1, leading to a lower demand for these defensive metabolites at t_2.

3.3. Comparative t_1/t_2 Analysis and Metabolomic Evolution

To conduct the metabolomic evolution study between t_1 and t_2 and determine which of the proposed cultivation conditions were most favorable, it was necessary to compare the metabolomic profiles between the same samples and across all treatments. Additionally, it was essential to investigate the direct relationship between the metabolome at t_1 and t_2 and the observed physical plant development measured in the field.

Initially, a general analysis was conducted to identify differences in metabolite concentrations between the two sampling periods, t_1 and t_2, by grouping the data by metabolite type. This broad approach provided an overview of the metabolic changes occurring over time in the studied plants. A more detailed analysis by treatment was subsequently performed, facilitating a specific assessment of the variations within each experimental group. This sequential methodology ensured a thorough understanding of the metabolic dynamics at both the general and thesis-specific levels.

Below are the results (Table 6), which present the average concentrations from three replicates of the same sample for t_1 and t_2, along with the standard deviation and the p-value from the paired *t*-test.

Table 6. Concentrations of metabolites with statistically significant differences between the two time periods studied, where t_1 corresponds to pre-winter sampling and t_2 to the post-winter period. Values are presented as mean (±standard deviation), where the first value represents the average of three replicate measurements, and the value in parentheses represents the standard deviation.

Metabolite	Thesis	Time		*p*-Value
		t_1 (Pre-Winter)	t_2 (Post-Winter)	
Amino acids				
GABA	T0	2.02 (0.28)	0.84 (0.20)	0.03248
	T1	1.96 (0.22)	1.15 (0.09)	0.01077
	T2	1.98 (0.09)	1.10 (0.04)	0.007257
Alanine	T0	0.55 (0.07)	0.22 (0.04)	0.02398
	T1	0.66 (0.086)	0.32 (0.02)	0.02394
	T2	0.69 (0.08)	0.27 (0.002)	0.01248
	T4	0.53 (0.09)	0.32 (0.02)	0.0441
Asparagine	T0	0.99 (0.12)	0.34 (0.14)	0.005011
	T1	0.86 (0.08)	0.55 (0.008)	0.02253
	T2	0.83 (0.06)	0.58 (0.02)	0.04016
Aspartate	T0	0.68 (0.08)	0.43 (0.09)	0.03993
	T1	0.50 (0.02)	0.69 (0.02)	0.02663
Proline	T0	11.30 (1.71)	3.51 (0.76)	0.009521
	T1	11.04 (1.80)	5.16 (0.37)	0.02401
	T2	11.57 (0.79)	5.56 (0.21)	0.008875
	T4	10.96 (1.84)	5.01 (0.32)	0.02426

Table 6. *Cont.*

Metabolite	Thesis	Time		*p*-Value
		t_1 (Pre-Winter)	t_2 (Post-Winter)	
Tyrosine	T0	0.29 (0.008)	0.57 (0.07)	0.02366
	T1	0.27 (0.04)	0.46 (0.03)	0.009024
	T2	0.29 (0.05)	0.48 (0.01)	0.03383
	T3	0.27 (0.07)	0.55 (0.003)	0.02691
	T4	0.26 (0.03)	0.61 (0.03)	0.0001822
Organics acids				
Ascorbate	T0	0.86 (0.13)	0.32 (0.05)	0.0144
	T1	0.86 (0.13)	0.31 (0.01)	0.01796
	T2	0.86 (0.18)	0.31 (0.01)	0.03172
	T3	0.87 (0.22)	0.31 (0.01)	0.04319
	T4	0.90 (0.18)	0.35 (0.03)	0.02795
Citrate	T3	1.70 (0.35)	3.10 (0.16)	0.04183
Formate	T1	0.02 (0.006)	0.01 (0.003)	0.04237
Lactate	T0	0.31 (0.05)	0.14 (0.01)	0.03012
	T1	0.33 (0.04)	0.18 (0.01)	0.03366
Malate	T0	10.11 (1.62)	4.71 (0.67)	0.04053
Quinic Acid	T1	39.55 (2.39)	22.57 (1.46)	0.001447
	T2	38.41 (5.09)	19.05 (0.79)	0.02803
Sugars				
Fructose	T1	6.99 (1.49)	3.55 (0.66)	0.01868
Glucose	T1	7.46 (1.31)	3.28 (0.20)	0.0265
	T2	8.44 (1.62)	2.78 (0.11)	0.0298
Sucrose	T2	9.24 (1.42)	6.28 (0.62)	0.03486
	T4	10.69 (1.88)	6.73 (0.56)	0.03599
Others				
Choline	T1	1.43 (0.20)	0.94 (0.08)	0.0314
Trigonelline	T1	0.03 (0.007)	0.04 (0.008)	0.01506
	T2	0.03 (0.0005)	0.04 (0.002)	0.01282

The first significant difference detected when comparing samples from the same trees at different time points was the reduction in the number of detected metabolites. A widespread quantitative reduction was also observed. In the t_1 samples, a total of 27 metabolites were detected, whereas at t_2, this number dropped to 25. This reduction was due to the absence of arginine, glutamate, and glutamine at t_2, along with the appearance of fumarate, resulting in a slight reduction in amino acids and an increase in organic acids compared to t_1. Thus, the amino acid-to-organic acid ratio changed from 12:7 at t_1 to 9:8 at t_2.

Notably, tyrosine and ascorbate were the only metabolites that showed significant differences across all treatments between t_1 and t_2. Tyrosine, in particular, increased from t_1 to t_2, indicating a positive sign for crop development. Both tyrosine and ascorbate are involved in plant growth processes, which are the primary activities in plants in a juvenile state. This common growth process occurred across all treatments from t_1 to t_2.

This significant reduction in metabolites, coupled with the appearance of fumarate, aligns with the spring budding experienced by citrus trees in southeastern Spain, one of the most crucial phases following the winter vegetative rest.

3.3.1. Amino Acids in Comparative t_1/t_2 Analysis

The results from t_1 and t_2 were evaluated together, revealing a significant qualitative and quantitative reduction in amino acids. Several amino acids, such as GABA, alanine, asparagine, phenylalanine, and proline, experienced a notable quantitative reduction. In contrast, other amino acids like arginine, glutamate, and glutamine were not detected at all at t_2. Aspartate, tryptophan, and valine remained relatively stable, while only tyrosine significantly increased. Glutamate is a primary metabolite essential to plants. Besides its role as an amino acid, it acts as a key connection between carbon and nitrogen metabolism. One of the main functions of glutamate in plants is to serve as a nitrogen donor for the biosynthesis of amino acids and other nitrogenous compounds [42]. Similarly, glutamine is produced through the action of glutamine synthetase, which converts glutamate into glutamine in the presence of ammonium [43]. Glutamine, like glutamate, is crucial for protein synthesis and serves as a nitrogen donor for the synthesis of nitrogenous biomolecules [44], and tryptophan, an aromatic amino acid, is important because it functions as a precursor for secondary metabolism in plants. Among these secondary compounds are phytohormones such as auxin (indole-3-acetic acid, IAA) as well as serotonin and melatonin, which play roles in seed germination, root growth, senescence, flowering, fruit ripening, and responses to biotic and abiotic stresses [45].

As noted earlier, plants respond to abiotic stresses by accumulating branched-chain amino acids like valine. These amino acids accumulate in response to drought, salinity, nitrogen deficiency, and other environmental challenges [46].

3.3.2. Organic Acids in Comparative t_1/t_2 Analysis

Comparing the results from t_1 and t_2, it was observed that the concentrations of organic acids such as ascorbate, quinic acid, and lactate decreased. The reduction in quinic acid was particularly striking, with a decrease of 50.40% compared to t_1 samples. Ascorbate serves multiple functions in plants, including modulation of plant growth through phytohormones, protection against oxidation, acting as an enzyme cofactor, and being involved in cell division, growth, and senescence [47]. Under low-oxygen conditions, the metabolic profile of plant cells changes, resulting in the accumulation of glycolysis intermediates as well as increases in lactate, alcohols, and sugars [48].

Citrate, formate, malate, and succinate maintained relatively moderate levels between t_1 and t_2, while fumarate was detected only at t_2. Succinate production in plants is generally associated with anoxic conditions, although other potential functions of its production are being studied [49].

3.3.3. Sugars in Comparative t_1/t_2 Analysis

When evaluating the results from t_1 and t_2 together, a general reduction in sugars such as fructose, glucose, and sucrose was observed, while myo-inositol remained relatively stable. Fructans, which are long chains of fructose synthesized from sucrose, are stored in the vacuole. Their presence in the extracellular apoplast strongly indicates their involvement in plant stress tolerance [28].

3.3.4. Other Metabolites in Comparative t_1/t_2 Analysis

Among the remaining metabolites detected between t_1 and t_2, chlorogenate, choline, and ethanol decreased compared to t_1, while trigonelline remained relatively constant. Chlorogenic acids are important products of plant metabolism, involved in various developmental processes and environmental responses. Their main biological functions in plants include chemical defense against herbivores and protection against ultraviolet radiation [21]. Trigonelline is involved in various processes, such as nyctinasty (leaf closure) in response to drought and salt stress, as well as oxidative stress responses in many plants [50].

3.4. Metabolomic Evolution by Thesis

3.4.1. Metabolomic Evolution in Thesis T0

In Thesis T0, statistically significant differences were identified in a total of nine metabolites between periods t_1 and t_2. Of these, six were amino acids: GABA, alanine, asparagine, aspartate, proline, and tyrosine, while three were organic acids: ascorbate, lactate, and malate. It was observed that the most significant changes occurred in amino acids and organic acids, with no significant variations detected in sugars or other metabolites.

These metabolomic results, combined with the height, canopy diameter, and trunk diameter measurements recorded during the study, indicate that Thesis T0 experienced notable vegetative growth during the spring of 2024 compared to the other theses. This suggests that the mobilization of amino acids, which are directly involved in plant growth, was effective. Since the plants were in a fully juvenile state with vegetative tissues operating at "full capacity", it is reasonable that amino acids were the most prominent metabolites at both time points.

However, flower production was limited, with an average of 29.68 ± 29.78 flowers per tree, significantly lower than the numbers observed in the other theses (see Table 7). These findings suggest that Thesis T0 prioritized the allocation of nutritional and metabolic resources to vegetative growth rather than flowering, indicating a clear competition between the two processes.

Table 7. Flower count performed in spring 2024. Values are presented as mean (±standard deviation), where the first value represents the average of replicate measurements, and the value in parentheses represents the standard deviation.

Parameter	Thesis/Cultivation Conditions Studied				
	T0	**T1**	**T2**	**T3**	**T4**
Number of flowers	29.68 (29.78) a	347.93 (187.66) b	394.76 (222.17) b	428.63 (158.74) b	400.86 (316.01) b

Different letters between rows indicate significant differences according to the multiple comparison test of means (Tukey contrasts).

3.4.2. Metabolomic Evolution in Thesis T1

In Thesis T1, statistically significant differences were found in a total of 15 metabolites between t_1 and t_2. Thesis T1 showed the most notable changes among all the theses evaluated, with significant variations across all chemical groups: six amino acids (GABA, alanine, asparagine, aspartate, proline, and tyrosine), four organic acids (ascorbate, formate, lactate, and quinic acid), three sugars (fructose, glucose, and sucrose), and two other metabolites (choline and trigonelline). These results suggest that Thesis T1 underwent significant changes in both budding and flowering during the spring of 2024; that is why it is evident that this thesis effectively mobilized metabolites to support both budding and flowering. It recorded an average flower production of 347.92 ± 187.66 per tree, which, while lower than that of Theses T2, T3, and T4 (the highest flowering counts), was considerably higher than that of Thesis T0. Additionally, the phenotypic data, including height, canopy diameter, and trunk diameter (Tables 3 and 5), showed that Thesis T1 outperformed Thesis T0 in all measured dimensions. However, among the theses with weed control mesh, Thesis T1 showed the least development, possibly because the drainage tubes were positioned farther from the root zone, reducing the efficiency of salt leaching and root aeration.

3.4.3. Metabolomic Evolution in Thesis T2

In Thesis T2, a total of nine metabolites showed statistically significant differences between t_1 and t_2. Of these nine metabolites, five were amino acids: GABA, alanine, asparagine, proline, and tyrosine; two were organic acids: ascorbate and quinic acid; one was a sugar: glucose; and one, trigonelline, belonged to other metabolites. Thesis T2 ranked third in flower production, with 394.76 ± 222.16 flowers per tree. It can be said that this

thesis holds an intermediate position among the theses with anti-weed mesh in terms of flower production and overall vegetative development.

3.4.4. Metabolomic Evolution in Thesis T3

In Thesis T3, only three metabolites exhibited statistically significant differences between t_1 and t_2. Of these three metabolites, one was an amino acid: tyrosine, and two were organic acids: ascorbate and citrate. Based on these results, one might have suspected that Thesis T3 would show the least sprouting and flowering due to the few significant metabolite changes detected. However, Thesis T3 recorded the highest flower production of all the theses, with 428.62 ± 158.73 flowers per tree, and also demonstrated good sprouting, although it did not lead in terms of canopy or trunk diameter. These findings suggest that Thesis T3 may have been the most nutritionally balanced or that its specific cultivation conditions were the most favorable for sprouting and flowering. While it exhibited the fewest significant changes in metabolites, this could indicate that it maintained "plant metabolic homeostasis" more effectively than the other theses, resulting in successful sprouting and flowering. Although it did not lead in canopy or trunk diameter growth, Thesis T3 ranked highest in tree height, showing medium-high overall growth and the best flower production among all the theses.

3.4.5. Metabolomic Evolution in Thesis T4

In Thesis T4, five metabolites showed statistically significant differences between t_1 and t_2. Of these five metabolites, three were amino acids: alanine, proline, and tyrosine; one was an organic acid: ascorbate; and one was a carbohydrate: sucrose. Thesis T4 produced the second-highest number of flowers, following Thesis T3, with 400.85 ± 316.00 flowers per tree (Table 7).

To further distinguish Thesis T4 from Thesis T3, it is important to note that, while all the trees in the Thesis T3 experiment produced flowers, five of the 21 trees in Thesis T4 did not produce any flowers. This detail suggests that Thesis T4 may have directed its metabolites more toward vegetative growth than flower production. This hypothesis is supported by the data in Tables 3 and 5, where Thesis T4 recorded the highest growth values among the theses with anti-weed mesh installed. In this thesis, where zeolite was applied as a soil enhancer, certain salts may have been retained, slightly reducing their concentration in the wet bulb. This effect may have caused Thesis T4 to allocate some of its metabolites toward vegetative growth at the expense of flower production.

3.4.6. General Results and Recommendations

A detailed analysis of the data, comparing results among the different study theses, revealed differences between t_1 and t_2. Ordered from highest to lowest based on the total number of statistically significant metabolites, the theses are ranked as follows: T1 (15) > T0 (9) > T2 (9) > T4 (5) > T3 (3), with T0 and T2 tied at nine metabolites each. A common trait among T0, T1, and T2 is that amino acids showed the most substantial quantitative changes between the two sampling periods.

At the level of the experimental plots, there is a clear relationship between the concentration of metabolites detected at t_1, the general growth experienced, and the number of flowers produced at t_2. At t_2, plots with poor flowering generally show good vegetative growth, illustrating the competitive processes between budding and flowering. Amino acids, which were the most utilized, and, consequently, the most reduced metabolites, are associated with smaller plant size at t_1. Conversely, plots that showed greater vegetative size at t_1 produced more flowers but exhibited less vegetative growth at t_2, indicating that they were better able to maintain a "quantitative balance" of metabolites between t_1 and t_2. Given this, we suggest that the plots which best maintained their "metabolomic homeostasis" between t_1 and t_2 were T3 and T4. These plots demonstrated the most vegetative growth, produced the most flowers, and experienced the fewest significant metabolic changes between t_1 and t_2. They were followed by T2 and T0, with T1 ranking last.

Based on these observations and the notable results obtained, we strongly recommend that citrus growers consider the installation of anti-weed mesh before planting new crops. This practice could offer both short- and long-term advantages, as demonstrated in this study. The experimental plots with anti-weed mesh and a drainage system (T1, T2, T3, and T4) outperformed the plot without mesh (T0) in terms of growth, development, and production. While it is still too early to determine definitively which cultivation practice is the most effective, the anti-weed mesh appears to provide better conditions for crop development and production compared to traditional methods. With the anti-weed mesh installed, faster growth was observed in the three measured parameters (height, crown diameter, and trunk diameter), as well as higher flower production, which translates into better tree development and greater production. Additionally, the anti-weed mesh reduces water consumption, herbicide use, and the labor costs associated with herbicide application, while also mitigating the effects of temperature fluctuations between day and night. In the long term, this improvement could lead to earlier entry into full production compared to traditional cultivation. We hypothesize that this positive effect of the anti-weed mesh on navel orange cultivation may also extend to other citrus species. In commercial citrus plantations, the benefits would be noticeable from the outset, reducing water consumption, labor, herbicides, and tillage. By entering full production earlier, the profitability of the farm would be directly improved, and by better maintaining the metabolic balance, a more uniform harvest in terms of size and quality might be achieved.

Finally, it is worth noting that metabolites related to abiotic stress were detected in very low quantities, indicating that the crop is developing correctly and that the plants are receiving all the necessary inputs for proper biological function. This is expected to result in good experimental development and, ultimately, positive outcomes.

3.5. Statistical Metabolomic Analysis

The graphical results presented below were generated using the MetaboAnalyst 6.0 platform, an open-source tool specifically designed for comprehensive metabolomic data analysis. Extensive studies were carried out across all theses (T0, T1, T2, T3, and T4), employing one-way statistical analyses to compare independent samples at both t_1 and t_2, as well as the same analysis for paired samples. This approach enabled a detailed assessment of metabolomic variations both between and within the theses over time, providing a deeper understanding of the metabolic dynamics that influence the observed phenotypic traits.

Principal Component Analysis (PCA) is an unsupervised multivariate chemometric analysis that allows data to be visualized in a graphical representation [51]. It enables us to observe how samples naturally cluster. PCA is commonly used in omics analyses as an initial overview of the data to detect potential outliers. The data is reduced to fewer variables, known as scores, which represent the weighted averages of the original variables [52]. Figure 1 shows the PCA of our study. Component 1 accounts for 79.8% of the total variability of the metabolites across all theses, while component 2 accounts for 10.8% of the total variability [3]. Additionally, no sample falls outside the confidence thresholds (shaded areas).

Partial Least Squares Discriminant Analysis (PLS-DA) is a supervised statistical method that enhances class separation by maximizing the variance between groups. Figure 2 presents the graphical representation of the PLS-DA using a variable importance projection (VIP) plot, highlighting the most significant metabolites detected across all theses. The colored boxes on the right indicate the relative concentrations of each corresponding metabolite in the study theses, with red representing the highest concentration of the respective metabolite.

To visually demonstrate which metabolites were most significant across all theses at t_1 and t_2, we used Partial Least Squares Discriminant Analysis (PLS-DA) and also estimated the Variable Importance in Projection (VIP), with the results shown in Figure 2. Unlike PCA, PLS-DA is a supervised method, meaning the algorithm knows to which thesis each sample

belongs [53]. By using PLS-DA, we can identify which metabolites are most significant across all theses, and with VIP, we can determine which metabolites best distinguish each thesis. The results show that the metabolites with the highest concentrations are glucose, sucrose, proline, and quinic acid; all of these metabolites have a VIP score exceeding 1, making them the most important metabolites across the five theses [54]. For instance, quinic acid has the highest concentration in T0 at t_1, while tyrosine is the most concentrated metabolite in T4 at t_2.

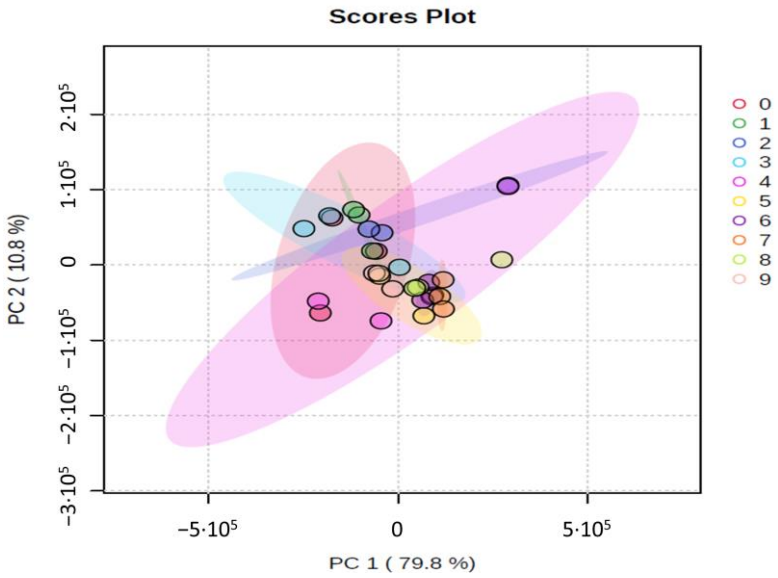

Figure 1. Principal component analysis (PCA) graph of all one-factor theses (0.'T0_t_1'; 1.'T1_t_1'; 2.'T2_t_1'; 3.'T3_t_1'; 4.'T4_t_1'; 5.'T0_t_2'; 6.'T1_t_2'; 7.'T2_t_2'; 8.'T3_t_2'; 9.'T4_t_2').

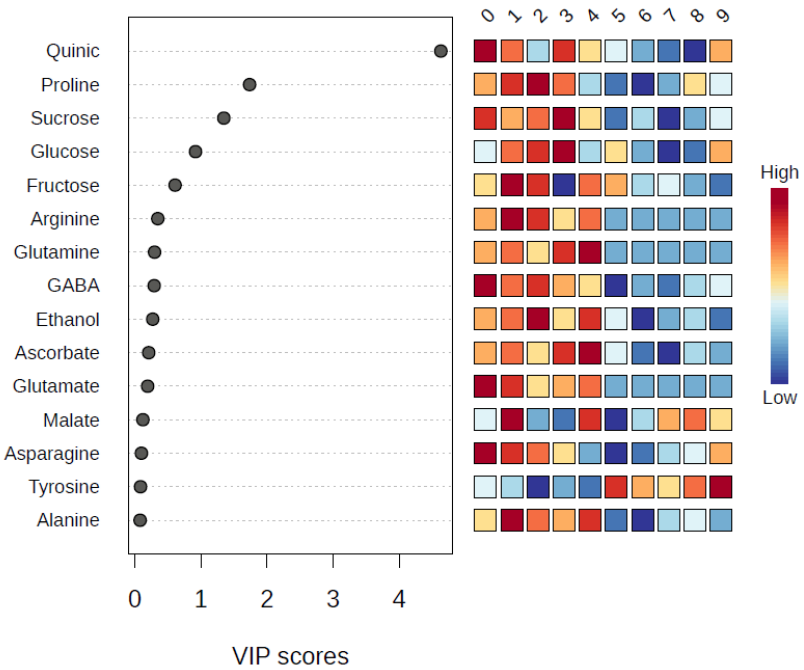

Figure 2. Graphical representation of partial least squares discriminant analysis (PLSD-DA) using a variable importance projection (VIP) plot of the set of most significant metabolites detected in all study theses of a factor. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each study thesis, with red being the color representing the highest concentration of each metabolite (0.'T0-t_1'; 1.'T1-t_1'; 2.'T2-t_1'; 3.'T3-t_1'; 4.'T4-t_1'; 5.'T0-t_2'; 6.'T1-t_2'; 7.'T2-t_2'; 8.'T3-t_2'; 9.'T4-t_2').

The heatmap of hierarchical clustering displays the concentrations of all detected metabolites using a color scale [55]. This heatmap is often presented alongside the dendrogram as a visual aid. In the heatmap shown in Figure 3, we observe that the red color, representing higher metabolite concentrations, is concentrated on the right side of the map, indicating that most metabolites had their highest concentrations at t_1. In contrast, cooler colors (blues) are predominantly located on the left side of the map, showing that the lowest metabolite concentrations were mostly detected at t_2. The samples are correctly clustered in the dendrogram of the heatmap, with the samples from t_1 on the right and those from t_2 on the left of the dendrogram [26].

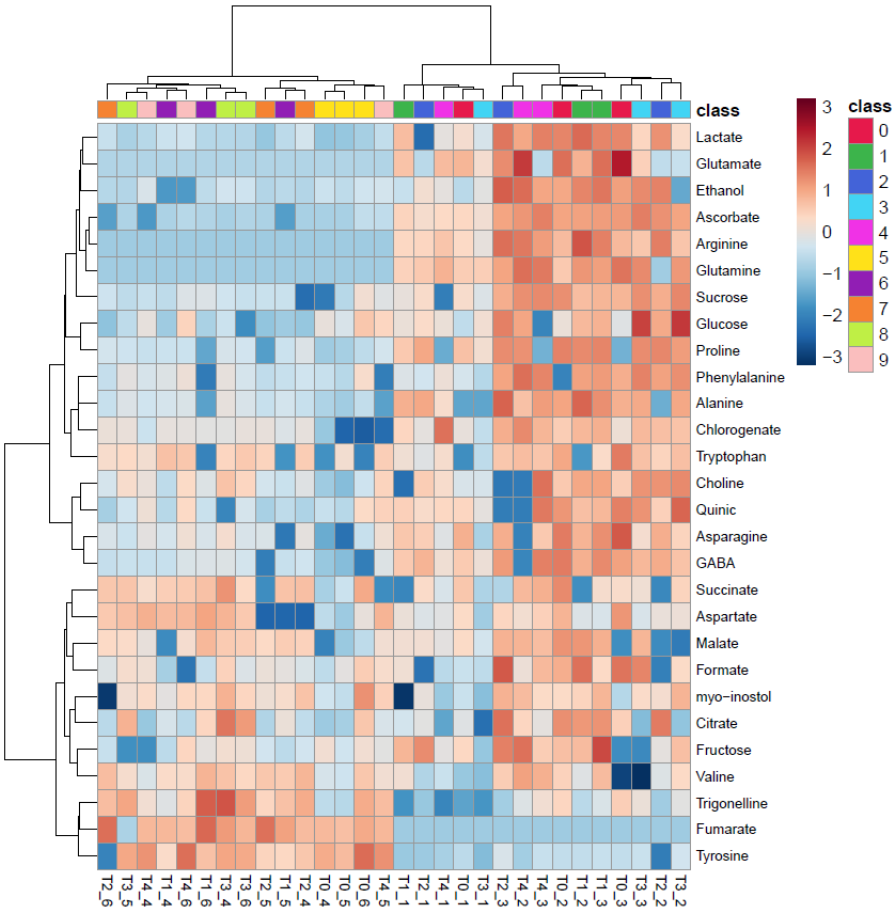

Figure 3. Hierarchical clustering heat map of metabolites detected in leaves from all experimental theses [T0-t_1 (T0_1, T0_2, T0_3), T1-t_1 (T1_1, T1_2, T1_3), T2-t_1 (T2_1, T2_2, T2_3), T3-t_1 (T3_1, T3_2, T3_3), T4-t_1 (T4_1, T4_2, T4_3), T0-t_2 (T0_4, T0_5, T0_6), T1-t_2 (T1_4, T1_5, T1_6), T2-t_2 (T2_4, T2_5, T2_6), T3-t_2 (T3_4, T3_5, T3_6), T4-t_2 (T4_4, T4_5, T4_6)]. Warm colors (red) indicate a higher concentration of metabolites and cold colors (blue) indicate a lower concentration.

To analyze metabolic pathways, the partial correlation deviation algorithm [56] was applied to the metabolites detected at t_1 and t_2. The size of the connection nodes is directly related to a weighting that represents the partial correlation coefficients and *p*-values of each pair of metabolites correlated through metabolic pathways [57]. Two nodes connected by a red line indicate a positive relationship, while those connected by a blue line represent a negative relationship [58].

In Figure 4, we observe that ethanol has a degree of 11 and an interrelation value of 29.04, indicating that it is a major metabolite, acting as both an intermediate and precursor for several metabolic pathways involved in maintaining cellular functions under stress conditions. Ethanol shows positive correlations with myo-inositol, succinate, arginine,

phenylalanine, quinic acid, lactate, tyrosine, and glucose, and negative correlations with fumarate, GABA, and trigonelline.

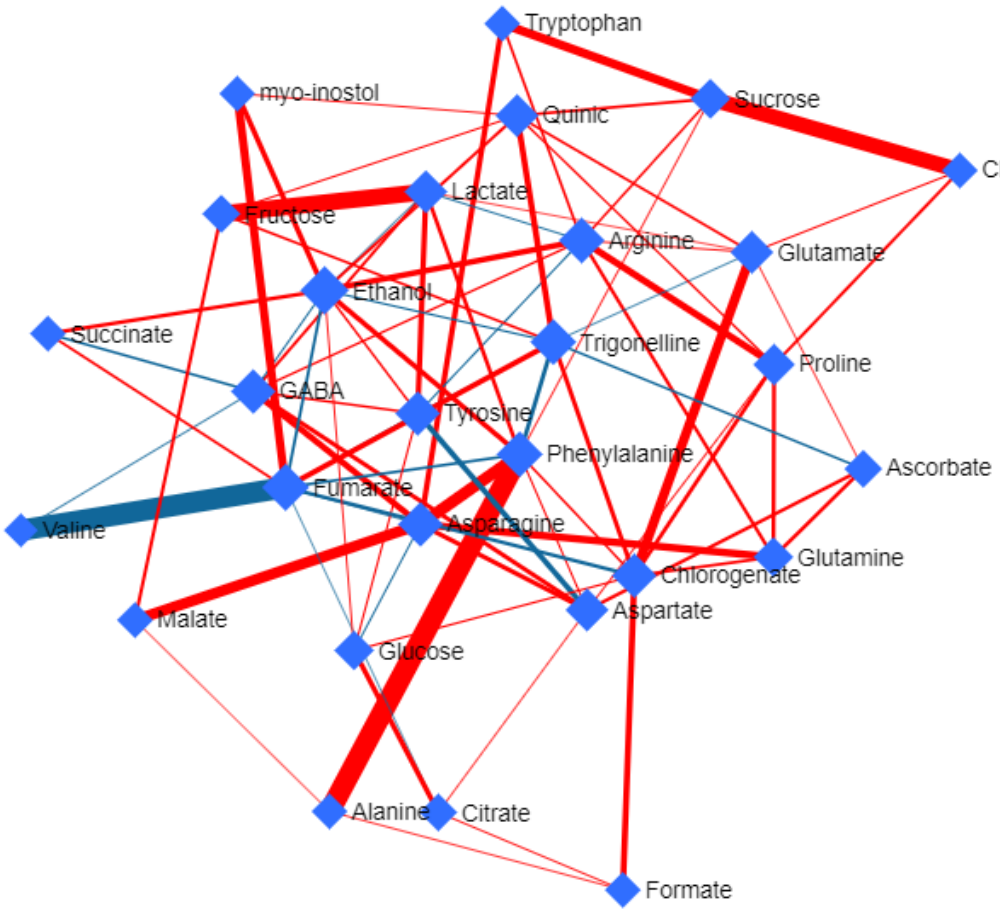

Figure 4. Correlation network formed using the unbiased sparse partial correlation (DSPC) algorithm of the metabolites detected in the orange leaves of the five experimental theses. Red lines express positive correlations, while blue lines express negative correlations. The size of the nodes shows the direction of change. The color of the lines is adjusted to a p-value < 0.05 and the false discovery rate (FDR) is adjusted to a p-value < 0.2.

Following this, fumarate appears with a degree of 10 and an interrelation value of 42.35. Fumarate is an important organic acid, participating in the tricarboxylic acid (TCA) cycle and possibly involved in the transport of carbon chains through the phloem. Fumarate exhibits positive relationships with tyrosine, myo-inositol, and succinate, while it is negatively related to valine, asparagine, phenylalanine, and ethanol.

4. Conclusions

This study demonstrates that the use of anti-weed mesh and a drainage system in the cultivation of Citrus sinensis 'Navelina' provides significant benefits in terms of growth, development, and yield compared to traditional cultivation systems. The experimental plots with mesh (T1, T2, T3, and T4) consistently outperformed the control plot (T0), showing greater height, crown diameter, and trunk diameter, as well as higher flower production. Additionally, the use of anti-weed mesh contributed to a reduction in water consumption, herbicide use, and associated labor costs.

At the metabolomic level, the plots that best maintained "metabolomic homeostasis" between the two seasons (pre-winter and post-winter) were T3 and T4, which showed the greatest vegetative growth, highest flower production, and fewer significant changes in their metabolite profile. These plots demonstrated a balanced allocation of metabolic

resources between growth and flowering, suggesting that these management practices can improve resource efficiency and stability in citrus development.

In the long term, the use of anti-weed mesh not only promotes an earlier entry into full production but also shows promise as a beneficial practice for other citrus crops. The results suggest that this cultivation technique can be recommended for commercial citrus plantations, especially in water-scarce regions such as southeastern Spain, promoting a sustainable and profitable agricultural approach.

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