agronomy

MDPI

Article

Metabolomic Profile of *Citrus limon* Leaves ('Verna' Variety) by 1H-NMR and Multivariate Analysis Technique

Pablo Melgarejo [1], Dámaris Núñez-Gómez [1], Juan José Martínez-Nicolás [1,*], Francisca Hernández [1], Rafael Martínez-Font [1], Vicente Lidón [1], Francisco García-Sánchez [2] and Pilar Legua [1]

[1] Centro de Investigación e Innovacion Agroalimentaria y Agroambiental (CIAGRO-UMH), Miguel Hernandez University, Ctra. Beniel km 3.2, 03312 Orihuela, Spain; pablo.melgarejo@umh.es (P.M.); dnunez@umh.es (D.N.-G.); francisca.hernandez@umh.es (F.H.); rafa.martinez@umh.es (R.M.-F.); vicente.lidon@umh.es (V.L.); p.legua@umh.es (P.L.)

[2] Centro de Edafologia y Biologia Aplicada del Segura, Consejo Superior de Investigaciones Científicas, Campus Universitario de Espinardo, Espinardo, 30100 Murcia, Spain; fgs@cebas.csic.es

* Correspondence: juanjose.martinez@umh.es

Abstract: The elaboration and definition of "metabolic fingerprints" can subsidize both the identification and determination of plant varieties, as well as the increase in knowledge about the responses and adaptations of plants to external and/or internal factors. The lemon tree (*Citrus limon* Burm.) is one of the most important crops in the Spanish southeast and is often consumed around the world. Although the study and characterization of its fruits are common due to its economic interest, its leaves are limited to specific functionalized studies related to the objective of the work (extraction of essential oils, stabilizing agent, aromatic extracts, etc.). So, this study aimed to identify the primary and secondary metabolites of *Citrus limon* Burm. ('Verna' variety) leaf samples cultivated under different conditions (three rootstocks and three culture media). In total, 19 metabolites were identified for all samples, of which 9 were amino acids, 5 organic acids, 3 sugars and 2 intermediate metabolites. The results pointed to a limited influence, both of the substrate and of the crop rootstock, on the metabolomic differentiation of lemon leaves. Knowledge and foliar metabolomic differentiation can offer important information that supports the application of crop foliar treatments but also helps in the management of diseases and pests.

Keywords: *Citrus limon* Burm.; citrus rootstock; culture media; metabolomic differentiation; lemon leaves

Citation: Melgarejo, P.; Núñez-Gómez, D.; Martínez-Nicolás, J.J.; Hernández, F.; Martínez-Font, R.; Lidón, V.; García-Sánchez, F.; Legua, P. Metabolomic Profile of *Citrus limon* Leaves ('Verna' Variety) by 1H-NMR and Multivariate Analysis Technique. *Agronomy* **2022**, *12*, 1060. https://doi.org/10.3390/agronomy12051060

Academic Editor: María Ángeles Ferrer Ayala

Received: 28 March 2022
Accepted: 25 April 2022
Published: 28 April 2022

1. Introduction

The advances and improvement of laboratory techniques, as well as the increase in accumulated knowledge about plant metabolism, have allowed the scientific community to achieve great advances in the identification of the metabolites present and/or absent, as well as in the identification of their impact or role in the different metabolic pathways. This knowledge has been applicable not only at the agronomic level for the control and management of crops, but also at the pharmaceutical, food, medical and industrial level, among others [1–3].

The metabolomic study, defined as the identification and quantification of all the metabolites within an organism under a given set of conditions, has been providing a qualitative and quantitative, objective and complete description of the metabolites present in a plant. The elaboration of specific metabolomic profiles allows researchers to elaborate and define the "*metabolic fingerprints*" that can contribute to the identification and determination of plant varieties, to the knowledge about the responses and adaptations of the plants to the external and/or internal factors and to the potentiation of its functionalization [4,5].

On the other hand, although it is true that the lemon (*Citrus limon* Burm.) is one of the most important crops in the Spanish southeast, and therefore its organoleptic characteristics are well known and studied [6,7], the study and characterization of its leaves are limited to

specific functionalized studies related to the objective of the work (extraction of essential oils, stabilizing agent, aromatic extracts, etc.) [7–10].

In this context, this study aims to identify the primary and secondary metabolites of foliar samples of *Citrus limon* Burm. ('Verna' variety) cultivated under different conditions. The differences between the culture conditions are related to the substrate and the rootstock used. The results of the study will allow for the correlation of the presence/absence of the metabolites depending on the sources of variability (substrate and rootstock) on the crop and its foliar development. NMR spectroscopy was used in conjunction with multivariate data analysis to identify metabolites directly from leaf extracts, without any chromatographic separation.

2. Materials and Methods

2.1. Plant Material and Sampling Preparation

For the foliar metabolomic study of lemon leaves (*Citrus limon* Burm. f. var. "Verna"), two independent variables were considered in this work: (i) the culture substrate (n = 3), and (ii) the rootstock used (n = 3). In relation to the culture medium, three substrates composed of phytoremediated port sediment and commercial substrate (peat) mixed in different proportions were tested. The phytoremediated port sediment comes from Livorno port (Italy) and has already been previously studied and applied, by the same research group, for other food crops, such as strawberries, pomegranates and lettuce [11–13]. Related to the rootstock, the most common rootstocks in citriculture were used, these being *Citrus macrophylla*, *Citrus aurantium* and Sweet/Sour Orange.

For the present work, 9 treatments (3 rootstock × 3 substrates) were considered. For each rootstock/substrate combination studied, a total of 10 trees were evaluated. The experimental design adopted was random distribution by blocks (n = 5), with two repetitions of each combination per block. In total, 90 lemon trees (3 substrates × 3 rootstocks × 2 replicates × 5 blocks) of 2 years of age, grown in polypropylene pots with a maximum capacity of 40 L, were evaluated in an experimental plot of the Miguel Hernandez University (Orihuela, Spain). Both the cultivation conditions and the management of the plantation were kept homogeneous throughout the trial, in order to minimize external influences on the parameters evaluated. In all cases, leaf samples were collected at the beginning of September before the plant entered winter dormancy. For each combination studied, one leaf was taken per cardinal point at two plant heights; in total, 16 leaves per sample were collected (1 leaf × 4 orientations × 2 heights × 2 repetitions). All trees were in good health and vegetative status at the time of the test.

In order to guarantee the non-degradation of the samples, immediately after collection, the leaves were taken to the laboratory and their preparation began the same day. For all the samples, manual cleaning of the surface of the leaves with distilled water was carried out, in order to eliminate possible dust and dirt residues. Subsequently, they were submerged in liquid nitrogen for 30 s, cut into smaller pieces and stored in sterile polypropylene containers with a 50 mL maximum capacity and a screw cap (Deltalab, Barcelona, Spain). The samples were stored at −80 °C for 48 h until lyophilization (Christ Alpha 2-4, LSCplus, Martin Christ). Finally, the lyophilized samples were crushed (TSM6A013, Taurus, Spain) and sieved (20 Mesh, stainless steel. Cymax Group Ltd., Burnaby, BC, Canada), guaranteeing the homogeneity of the samples in a fine powder, and preserved in sterile polypropylene tubes with a 12 mL maximum capacity (Deltalab, Barcelona, Spain) at −20 °C until used in metabolomic analyses.

2.2. Leaves Metabolomic Profile by ^{1}H-NMR

Prior to the metabolite determination by nuclear magnetic resonance (^{1}H-NMR), the lyophilized samples underwent an extraction process following the methodology described by Van der Sar et al. [14] with some modifications; briefly: In Eppendorf tubes with a 2 mL maximum capacity, 0.5 mg of the lyophilized sample was mixed with 1200 µL of a hydromethanolic mixture (1:1, MeOH: H_2O). The Eppendorf tubes were sonicated for

3 min at 1 min intervals and left at 4 °C for 30 min. Once this time had elapsed, the samples were centrifuged at 11,000 rpm for 20 min at 4 °C. The recovered supernatant was subjected to Speed-Vacuum, with a maximum temperature of 27 °C, until all the liquid phase had evaporated (overnight). Subsequently, the soluble solid obtained was resuspended by adding 800 µL of 100 mM potassium phosphate buffer (KH_2PO_4) at pH = 6.0 (dissolved in 100% D_2O) + 0.58 mM of TPS (internal standard) and filtered with 0.45 µm nylon filters. Finally, aliquots of 600 µL of the filtered volume were placed in 5 mm NMR tubes for quantification by ^{1}H-NMR.

2.3. Statistical Analysis

The spectra resulting from the ^{1}H-NMR analysis of the foliar samples were processed and compared with the MestReNova Software (Mestrelab Research, Spain). Spectral intensities were pooled (δ 0.04) considering the region of δ 0.5–9.0. The regions corresponding to the solvent D_2O (δ 4.70–4.9) and water (δ 3.09–3.15) were not considered in the analysis [15]. The subsequent statistical analysis was performed with the specific software for metabolomic data processing, MetaboAnalyst 5.0 (Wishart Research Group, University of Alberta, Canada), which allowed the identification and definition of spectral intensities, as well as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Loading plots, Variable Importance in Projection (VIP) scores, and *t*-tests (p values < 0.05) were used to determine metabolites contributing to significant between-group differences in PLS-DA score plots [16].

3. Results and Discussion

3.1. Metabolomic Fingerprint

The ^{1}H-NMR spectra or general "*metabolomic fingerprint*" of *Citrus limon* (var. "Verna") leaves are shown in Figure 1. As a result of the NHR analysis of the 45 citrus foliar samples (3 treatments × 3 rootstocks × 5 repetitions), 19 metabolites were identified for all the samples, these being: 9 amino acids, 5 organic acids, 3 sugars and 2 intermediate metabolites, a considerably lower amount if compared to the 35 metabolites identified for the lemon fruit and/or its parts [3,17]. The ^{1}H-NMR chemical shifts of the identified metabolites are listed in Table 1.

All ^{1}H-NMR spectra of the samples revealed by visual inspection a similar peak distribution between δ 0.8 and 8.5 ppm. The main signals were detected in the high field or aliphatic (δ 0.5–3.0 ppm) and midfield or sugars (δ 3.0–5.5 ppm) regions, but signals were also identified in the low field or aromatic region (δ 5.5–9.0 ppm), as shown in Figure 2.

The correlated peaks for organic acids and amino acids were identified in the high field or aliphatic region. In relation to the organic acids identified in this region, the signals corresponded to malic acid, quinic acid and citric acid as the most abundant organic acids and, to a lesser extent, formic acid and succinic acid. It should be noted that regardless of the rootstock and/or treatment used, for all the foliar samples, quinic acid ($C_7H_{12}O_6$) was the dominant organic acid followed by malic > citric > formic = succinic acids. That quinic acid is the majority in the leaves may be related to the influence of this organic acid in the development and maintenance of leaf structures, mainly during the warm season, and therefore during its maximum development [18].

Signals for valine, threonine and alanine amino acids were detected in the range between δ 0.9 and 1.5 ppm, while arginine, proline and aspartate were observed as a doublet between δ 1.5–2.8 ppm. GABA (γ-aminobutyrate) and glutamate were also identified in the high field region (Figure 3A). Amino acids have several important functions in plants; therefore, it is not surprising that they are the majority group (n = 9) of the metabolites identified in lemon leaves. In addition to their use during protein biosynthesis, amino acids also represent building blocks for other biosynthetic pathways such as plant growth and development, control of intracellular pH, generation of metabolic energy or redox power, and resistance to stress abiotic and/or biotic [19–23].

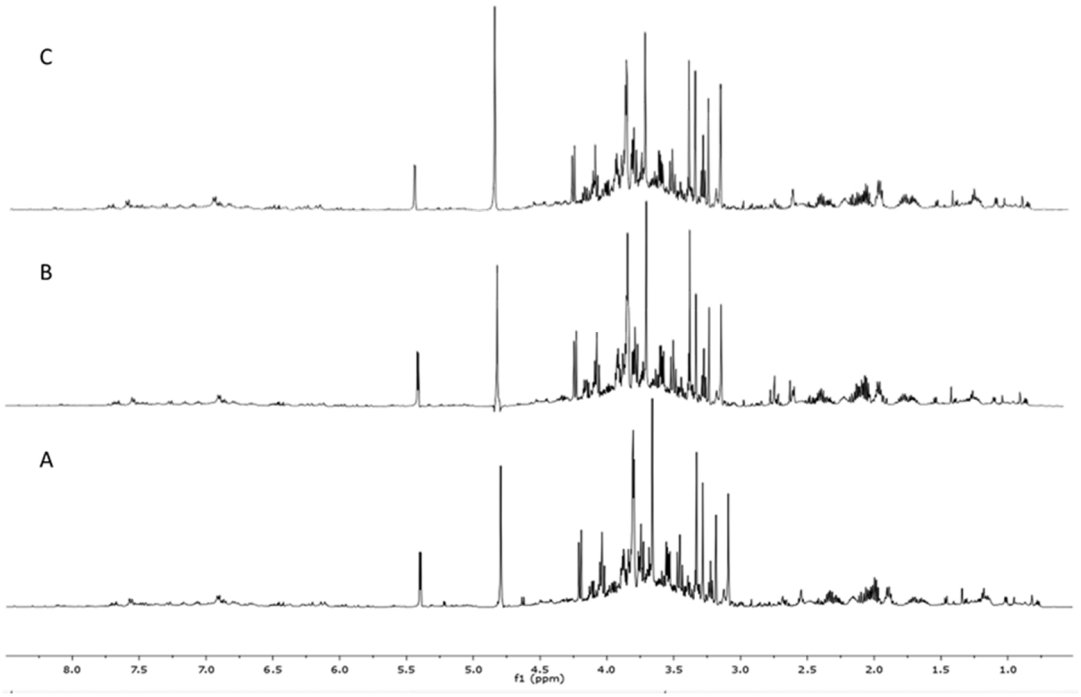


Figure 1. Simplified comparison of the complete spectra of *Citrus limon* leaves cultivated with different culture media, where (**A**) 25% remediated port sediment +75% universal substrate; (**B**) 50% remediated port sediment +50% universal substrate; and (**C**) 75% remediated port sediment +25% universal substrate.

Table 1. [1]H-NMR chemical shifts (ppm) used to quantify and identify metabolites in citrus leaf samples.

Compound	Chemical Shift (ppm) [1]
Amino acids	
GABA	3.01 (t)
Alanine	1.46 (d)
Arginine	1.6 and 1.7 (m)
Asparagine	2.94 (dd)
Aspartate	2.81 (dd)
Glutamate	2.3 (m)
Proline	2.00 (m)
Threonine	1.32 (d)
Valine	1.02 (d)
Organic acids	
Citrate	2.79 (d)
Formate	8.43 (s)
Malate	2.7 (dd)
Quinate	1.86 (dd)
Succinate	2.39 (s)
Sugars	
Glucose (α and β forms)	5.22 (d)
Maltose	5.42 (d)
Sucrose	5.40 (d)
Other metabolites	
Choline	3.19 (s)
Trigonelline	9.11 (s)

[1] Where the letter represents the multiplicity: s, singlet; d, doublet; t, triplet; dd, double of doublets; and m, multiplet.

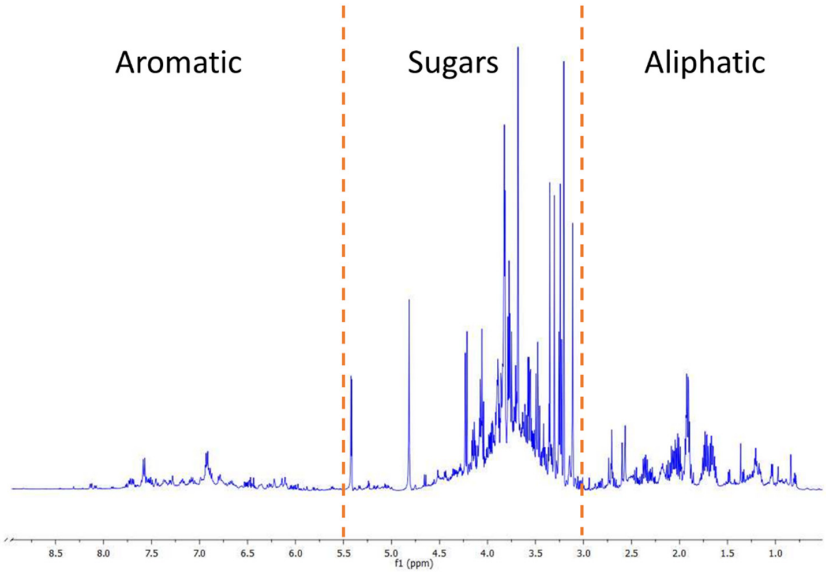


Figure 2. Representative ^{1}H-NMR spectrum of lemon leaf extracts (*Citrus limon*, 'Verna' variety) grown in 9 different treatments (3 rootstocks × 3 substrates), highlighting the main regions studied.

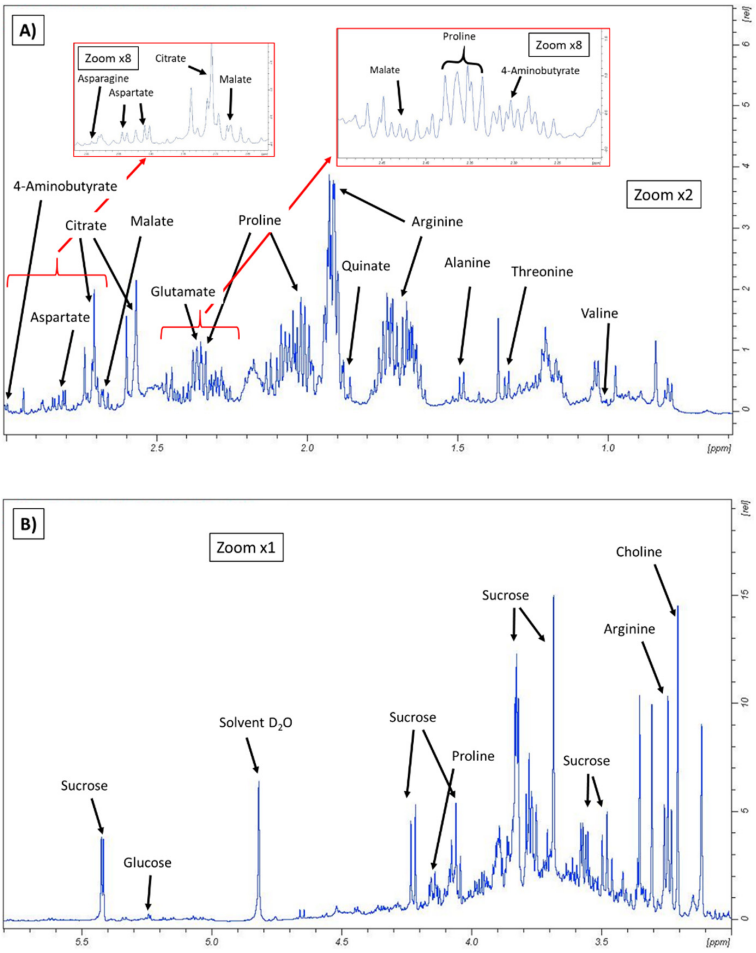


Figure 3. Representative ^{1}H-NMR spectrum of lemon leaf extracts (*Citrus limon* var. 'Verna') grown in 9 different treatments (3 rootstocks × 3 substrates) corresponding to: (**A**) the aliphatic region (δ 0.5–3.0 ppm); (**B**) the region of sugars (δ 3.0–5.5 ppm) with emphasis on the identification of the most representative compounds.

In the midfield or sugar region (δ 3.0–5.5 ppm), corresponding peaks associated with sucrose and glucose as dominant sugars were identified. Low-intensity signals visible around δ 4.5 ppm indicated the presence of malatose. The identification of the sugars would confirm the degree of maturity of the studied leaves, since it is the mature leaves that are responsible for carrying out photosynthesis and providing energy and carbon sources (mainly sugars such as glucose) to the other parts of the plant, such as roots, young growing leaves, etc. [24,25]. The choline metabolite, a basic constituent of lecithin and an important osmoregulator [26], was also identified in this region (Figure 3B).

Already in the low field or aromatic region (δ 5.5–8.5 ppm), normally associated with aromatic groups of amino acids and phenolic compounds, were low-intensity signals associated with formic acid (δ 8.56 ppm) and with trigonelline (as a doublet at δ 8.85 and 9.16 ppm). Although to the knowledge of the authors no specific bibliographical references have been found on the metabolomic study of foliar samples of *C. limon* var. 'Verna', the results obtained are consistent with the available literature on leaf samples [27,28].

3.2. Multivariate Analysis

In general, the analysis of variance (ANOVA) determined that, for the lemon tree foliar samples, the metabolites that showed significant differences according to the Tukey test ($p < 0.05$) were arginine, malate, quinate and sucrose, both as a function of the substrate as rootstock; aspartate, formate, malate and choline only showed differences depending on the substrate used, while alanine, aspargine, threonine, citrate and succinate only in relation to the rootstock. The rest of the identified metabolites did not show significant differences between the treatments studied.

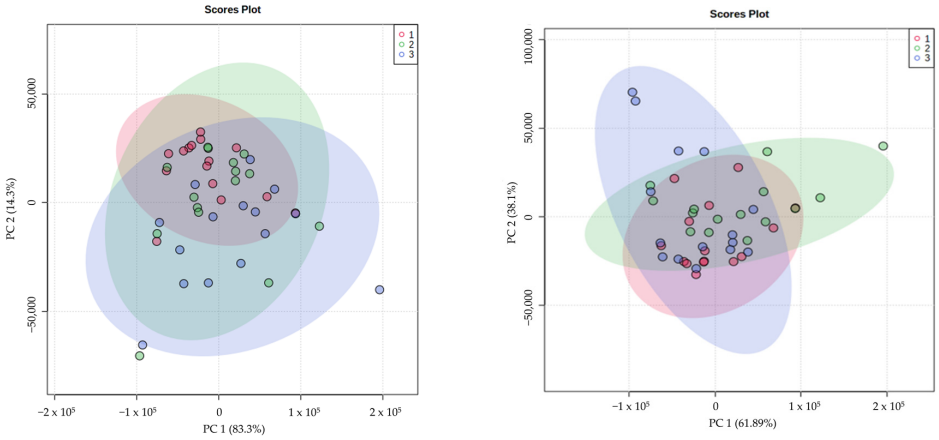
With more detail, and because the spectra showed an obvious visual similarity, a multivariate analysis of the PCA and PLS-DA results was performed to help identify the spectral changes in the *C. limon* var. 'Verna' cultivated in three rootstocks (*C. macrophylla*, *C. aurantium* and Sweet/Sour Orange) and on three substrates with different proportions of marine sediment and universal substrate (25%, 50% and 75% port sediment content), i.e., to determine the impact of rootstock and substrate on the lemon's foliar metabolic profile.

The 45 foliar samples analyzed (3 substrates × 3 rootstocks × 5 repetitions) were included in the PCA and PLS-DA analysis, classifying them differently according to the substrate and the rootstock, with the aim of identifying the intrinsic differences, avoiding data duplication.

In this sense, the PCA results for the classification by substrates showed that the first two principal components (PC) explained 97.6% of the total variation. PC1, related to 83.3% of the total variance, was related to the variables alanine, arginine, aspargine, aspartate, proline valine, citrate, formate, malate, quinate, succinate and choline, which corresponds, for the most part, to the main amino acids. PC2, which accounted for 14.3% of the total variance, correlated with GABA, glutamate, threonine, glucose, sucrose and trigonelline content (Figure 4a). Regarding the type of rootstock, the PCA analysis concluded that 100% of the total variance corresponded to the first two main components, with PC1 (61.89%) related to asparagine, glutamate, citrate, formate, malate, quinate, succinate, glucose and trigonelline, while PC2 (38.11%) correlated with GABA, alanine, arginine, aspartate, proline, threonine, valine, sucrose and choline content (Figure 4b).

In the same way, a PLS-DA regression was carried out to investigate the correlations between the treatments. In the PLS-DA model generated for the substrates, the first and second PLS-DA components explained 73.3% and 24.3% of the total variance, respectively (Figure 5a), while, for the rootstocks, the first and second PLS-DA components were related to 78.7% and 18.9% of the total variance, respectively (Figure 5b). Additionally, the study of the variable importance projection (VIP), derived from the PLS-DA analysis, confirmed the identification of the quinate, arginine and malate metabolites as significant and differentiating between the substrates, and the arginine, quinate and sucrose metabolites between the standards. Considering that quinate is a derivative of quinic acid and is therefore responsible for the development and maintenance of foliar structures, and that arginine participates

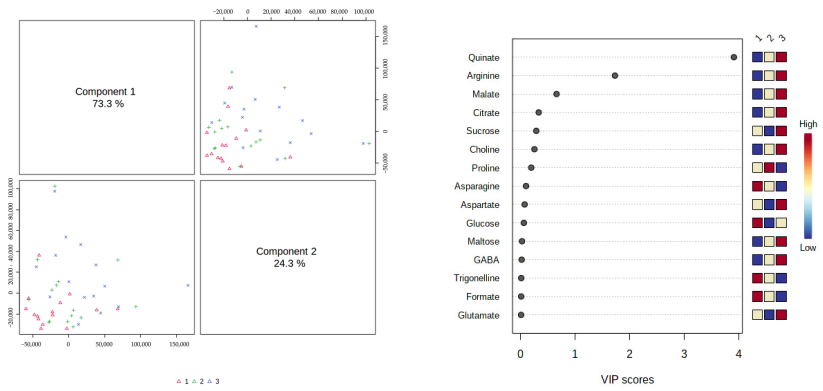
in the physiological processes of the plant as an important nitrogen reserve [18,29], their identification as significant metabolites in foliar samples is consistent. The differences between sucrose and malate could be related to the time the leaves were harvested since both metabolites would be related to the mitochondrial respiratory metabolite of the plant [30].



(**a**) (**b**)

Figure 4. Principal component analysis (PCA) graphs of the metabolomic study of lemon leaves (*Citrus limon* var. 'Verna') cultivated in 9 different treatments (3 rootstocks × 3 substrates) differentiating (**a**) according to the substrate used, and (**b**) depending on the rootstock.

(**a**)



(**b**)

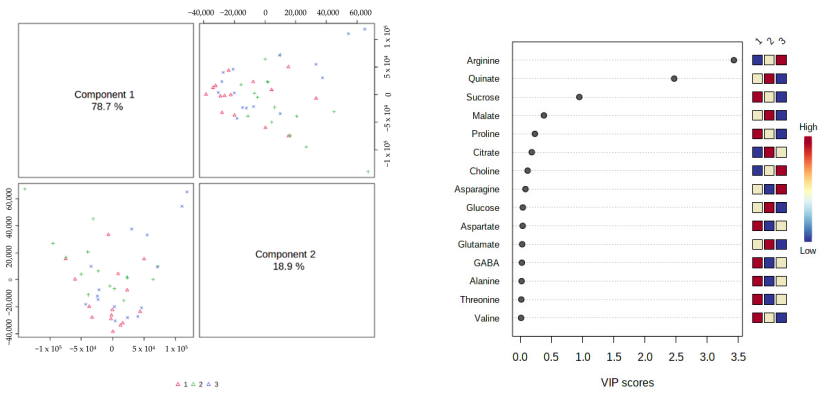


Figure 5. PLS-DA plot for lemon tree leaves grown with 9 different treatments (3 substrates × 3 standards) and VIP scores with the corresponding heat map where red and blue indicate the level of metabolites. The results are analyzed according to (**a**) the substrate, and (**b**) the rootstock used.

3.3. Metabolomic Pathway

For the metabolic pathway analysis, the results were analyzed and processed with the specific software for metabolomic data processing, Metaboanalyst, using the KEGG HMDB database. As shown in Figure 6, the relevant metabolic nodes identified were independent of both the substrate and the rootstock used. Thus, in both cases, the route with the greatest impact is the one related to the metabolism of alanine, aspartate and glutamate, followed by the nodes related to the citrate cycle (TCA cycle or Krebs cycle), arginine biosynthesis and the metabolism of arginine and proline. This last metabolic route presented significant differences between the variables studied; its impact is greater when evaluating the rootstock than the substrate.

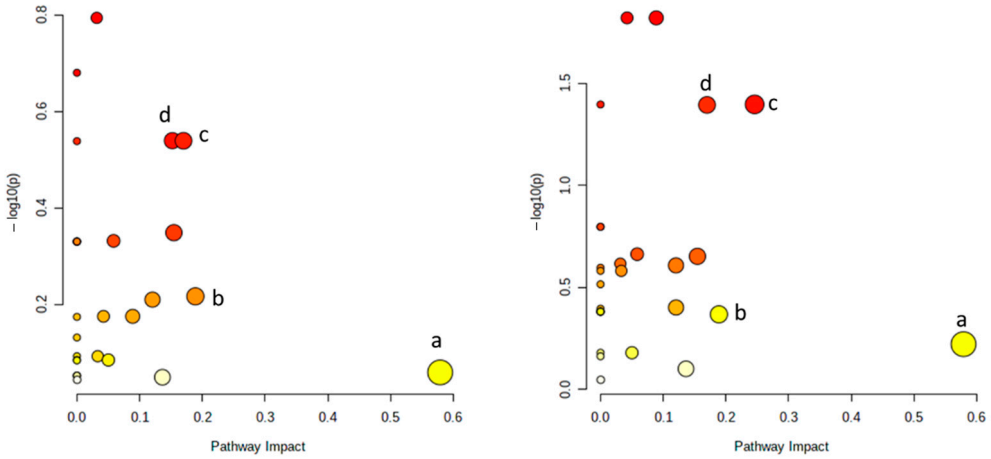


Figure 6. Comparison of the results of the metabolic pathway for the lemon leaves obtained as a function of the substrate (**A**) and the rootstock (**B**) where, in both cases, node (a) represents the metabolism of alanine, aspartate and glutamate; (b) the pathway of the citrate cycle (TCA cycle or Krebs cycle); (c) the biosynthesis of arginine; and (d) the metabolism of arginine and proline.

The presence of alanine and glutamate in plant leaves has been previously reported. Ivanov et al. [31] identified alanine in pea leaves and catalogued it as a metabolite resulting from biosynthetic reactions of oxalyl-CoA incorporated through glyoxylate. Aspartate is already related to the carbon fixation process considered an important photosynthetic metabolite [32].

The TCA cycle is an important aerobic pathway for the final steps of carbohydrate and fatty acid oxidation, as well as supplying important precursor metabolites, including 2-oxoglutarate. The variation or impact on the metabolic pathway may be associated with the ripening state of the fruits, since other studies indicate that, during the ripening of lemons, there may be a net disassimilation or synthesis of the stored tricarboxylic acid (TCA) cycle, which directly impacts both the content and concentration of organic acids [32].

Finally, the results obtained were confirmed through the enrichment analysis calculated by the relationship between the compounds detected and those expected based on the metabolic pathways/nodes identified regardless of the variable considered. The high enrichment ratio (>3.5) for carbohydrates and organic acids confirms the suitability of the routes evaluated (Figure 7).

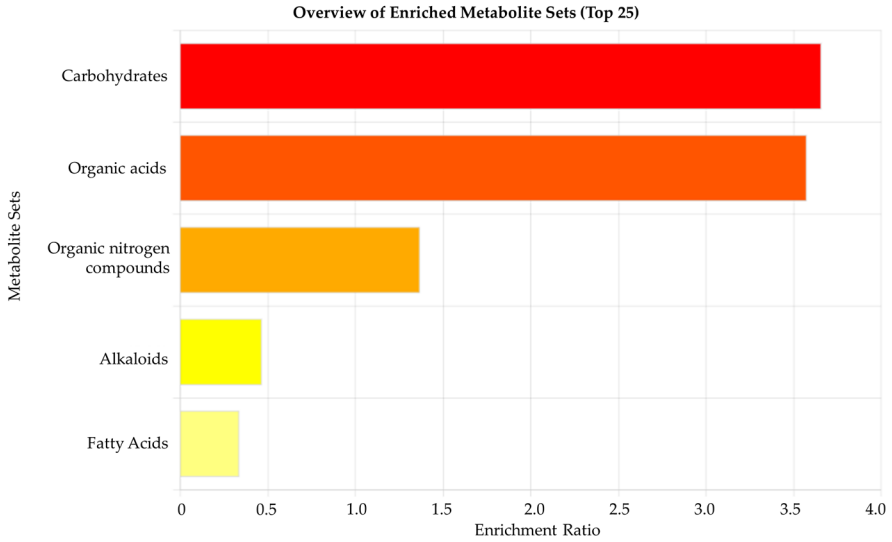


Figure 7. Bar chart result of the enrichment analysis of metabolites identified from the lemon leaf samples.

4. Conclusions

The present study focused on knowing and determining the metabolomic configuration of lemon leaves (*C. limon* var. 'Verna') cultivated in nine different treatments (three substrates × three rootstocks) with the aim of identifying the differences and impacts on the metabolic pathways not dependent on the cultivated variety. In total, 19 metabolites were identified for all samples, of which 9 were amino acids, 5 organic acids, 3 sugars and 2 intermediate metabolites. The results pointed to a limited influence, both of the substrate and of the crop rootstock, on the metabolomic differentiation of lemon leaves. More detailed complementary studies must be carried out both at other times of the year, to identify possible seasonal metabolomic variations, and in other varieties to determine their real impact. Knowledge and foliar metabolomic differentiation can offer important information that supports the application of foliar treatments but also helps in the management of diseases and pests.

Author Contributions: Conceptualization, P.M., J.J.M.-N. and P.L.; Data curation, D.N.-G., R.M.-F., V.L. and P.L.; Formal analysis, D.N.-G. and F.G.-S.; Investigation, D.N.-G., J.J.M.-N., F.H. and P.L.; Methodology, P.M., D.N.-G., J.J.M.-N., V.L. and F.G.-S.; Project administration, P.L.; Software, D.N.-G.; Supervision, P.L.; Validation, P.M., J.J.M.-N. and P.L.; Writing—original draft, P.M., D.N.-G. and J.J.M.-N.; Writing—review and editing, P.M., D.N.-G., J.J.M.-N., F.H., R.M.-F., V.L. and P.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Commission with the LIFE Project SUBSED 'Sustainable substrates for agriculture from dredged remediated marine sediments: from ports to pots' (LIFE17/ENV/IT/000347).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors gratefully acknowledge the administrative management and support of María Dolores Torregrosa as LIFE SUBSED project staff.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, L.; Ma, K.B.; Lu, Z.G.; Ren, S.X.; Jiang, H.R.; Cui, J.W.; Chen, G.; Teng, N.J.; Lam, H.M.; Jin, B. Differential physiological, transcriptomic and metabolomic responses of Arabidopsis leaves under prolonged warming and heat shock. *BMC Plant Biol.* **2020**, *20*, 1–15. [CrossRef]

2. Dean, L.L. Targeted and Non-Targeted Analyses of Secondary Metabolites in Nut and Seed Processing. *Eur. J. Lipid Sci. Technol.* **2018**, *120*, 1700479. [CrossRef]
3. Mucci, A.; Parenti, F.; Righi, V.; Schenetti, L. Citron and lemon under the lens of HR-MAS NMR spectroscopy. *Food Chem.* **2013**, *141*, 3167–3176. [CrossRef]
4. Kang, Z.; Babar, M.A.; Khan, N.; Guo, J.; Khan, J.; Islam, S.; Shrestha, S.; Shahi, D. Comparative metabolomic profiling in the roots and leaves in contrasting genotypes reveals complex mechanisms involved in post-anthesis drought tolerance in wheat. *PLoS ONE* **2019**, *14*, e0213502. [CrossRef]
5. Sung, J.; Lee, S.; Lee, Y.; Ha, S.; Song, B.; Kim, T.; Waters, B.M.; Krishnan, H.B. Metabolomic profiling from leaves and roots of tomato (*Solanum lycopersicum* L.) plants grown under nitrogen, phosphorus or potassium-deficient condition. *Plant Sci.* **2015**, *241*, 55–64. [CrossRef] [PubMed]
6. Aguilar-Hernández, M.; Núñez-Gómez, D.; Forner-Giner, M.-Á.; Hernández, F.; Pastor-Pérez, J.J.; Legua, P. Quality Parameters of Spanish Lemons with Commercial Interest. *Foods* **2021**, *10*, 62. [CrossRef] [PubMed]
7. Hojjati, M.; Barzegar, H. Chemical Composition and Biological Activities of Lemon (*Citrus limon*) Leaf Essential Oil. *Nutr. Food Sci. Res.* **2017**, *4*, 15–24. [CrossRef]
8. Kamiyama, S. Studies on the Leaf Oils of Citrus Species. *Biosci. Biotechnol. Biochem.* **2014**, *31*, 1091–1096. [CrossRef]
9. Jha, M.; Ansari, S.; Shimpi, N.G. Ultrasonic assisted green synthesis of Ag:CdO nanocubes and nanospheres using *Citrus limon* leaves for efficient degradation of organic dyes. *J. Ind. Eng. Chem.* **2019**, *69*, 269–284. [CrossRef]
10. Vankar, P.S.; Shukla, D. Biosynthesis of silver nanoparticles using lemon leaves extract and its application for antimicrobial finish on fabric. *Appl. Nanosci.* **2012**, *2*, 163–168. [CrossRef]
11. Melgarejo, P.; Legua, P.; Pérez-Sarmiento, F.; Martínez-Font, R.; José Martínez-Nicolás, J.; Hernández, F. Effect of a New Remediated Substrate on Fruit Quality and Bioactive Compounds in Two Strawberry Cultivars Effect of a New Remediated Substrate on Fruit Quality and Bioactive Compounds in Two Strawberry Cultivars. *J. Food Nutr. Res.* **2017**, *5*, 579–586. [CrossRef]
12. Martínez-Nicolás, J.J.; Legua, P.; Núñez-Gómez, D.; Martínez-Font, R.; Hernández, F.; Giordani, E.; Melgarejo, P. Potential of dredged bioremediated marine sediment for strawberry cultivation. *Sci. Rep.* **2020**, *10*, 1–11. [CrossRef]
13. Tozzi, F.; Pecchioli, S.; Renella, G.; Melgarejo, P.; Legua, P.; Macci, C.; Doni, S.; Masciandaro, G.; Giordani, E.; Lenzi, A. Remediated marine sediment as growing medium for lettuce production: Assessment of agronomic performance and food safety in a pilot experiment. *J. Sci. Food Agric.* **2019**, *99*, 5624–5630. [CrossRef]
14. Van der Sar, S.; Kim, H.K.; Meissner, A.; Verpoorte, R.; Choi, Y.H. Nuclear Magnetic Resonance Spectroscopy for Plant Metabolite Profiling. *Handb. Plant Metab.* **2013**, 57–76. [CrossRef]
15. Berrueta, L.A.; Alonso-Salces, R.M.; Héberger, K. Supervised pattern recognition in food analysis. *J. Chromatogr. A* **2007**, *1158*, 196–214. [CrossRef]
16. Broadhurst, D.I.; Kell, D.B. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics* **2006**, *2*, 171–196. [CrossRef]
17. Arbona, V.; Iglesias, D.J.; Gómez-Cadenas, A. Non-targeted metabolite profiling of citrus juices as a tool for variety discrimination and metabolite flow analysis. *BMC Plant Biol.* **2015**, *15*, 1–16. [CrossRef]
18. Passarinho, J.A.P.; Lamosa, P.; Baeta, J.P.; Santos, H.; Ricardo, C.P.P. Annual changes in the concentration of minerals and organic compounds of Quercus suber leaves. *Physiol. Plant.* **2006**, *127*, 100–110. [CrossRef]
19. Pratelli, R.; Pilot, G. Regulation of amino acid metabolic enzymes and transporters in plants. *J. Exp. Bot.* **2014**, *65*, 5535–5556. [CrossRef]
20. Galili, G.; Amir, R.; Fernie, A.R. The Regulation of Essential Amino Acid Synthesis and Accumulation in Plants. *Annu. Rev. Plant Biol.* **2016**, *67*, 153–178. [CrossRef]
21. Hildebrandt, T.M. Synthesis versus degradation: Directions of amino acid metabolism during Arabidopsis abiotic stress response. *Plant Mol. Biol.* **2018**, *98*, 121–135. [CrossRef] [PubMed]
22. Binder, S.; Knill, T.; Schuster, J. Branched-chain amino acid metabolism in higher plants. *Physiol. Plant.* **2007**, *129*, 68–78. [CrossRef]
23. Fowden, L. Aspects of Amino Acid Metabolism in Plants. *Annu. Rev. Plant Physiol.* **1967**, *18*, 85–106. [CrossRef]
24. Wingler, A.; Roitsch, T. Metabolic regulation of leaf senescence: Interactions of sugar signalling with biotic and abiotic stress responses. *Plant Biol.* **2008**, *10*, 50–62. [CrossRef]
25. Van Dingenen, J.; de Milde, L.; Vermeersch, M.; Maleux, K.; de Rycke, R.; de Bruyne, M.; Storme, V.; Gonzalez, N.; Dhondt, S.; Inzé, D. Chloroplasts Are Central Players in Sugar-Induced Leaf Growth. *Plant Physiol.* **2016**, *171*, 590–605. [CrossRef]
26. Zhang, J.; Okubo, A.; Yamazaki, S. Measurement of Free Choline in Plant Leaves by Capillary Electrophoresis. *Biosci. Biotechnol. Biochem.* **2014**, *65*, 2573–2576. [CrossRef]
27. Achituv, M.; Bar-Akiva, A. Metabolic Pathway of a-Ketoglutarate in Citrus Leaves as Affected by Phosphorus Nutrition. *Plant Physiol.* **1978**, *61*, 703–705. [CrossRef]
28. Gogna, N.; Hamid, N.; Dorai, K. Metabolomic profiling of the phytomedicinal constituents of *Carica papaya* L. leaves and seeds by 1H NMR spectroscopy and multivariate statistical analysis. *J. Pharm. Biomed. Anal.* **2015**, *115*, 74–85. [CrossRef]
29. Gao, H.J.; Yang, H.Q.; Wang, J.X. Arginine metabolism in roots and leaves of apple (Malus domestica Borkh.): The tissue-specific formation of both nitric oxide and polyamines. *Sci. Hortic. Amsterdam* **2009**, *119*, 147–152. [CrossRef]
30. Lee, C.P.; Elsässer, M.; Fuchs, P.; Fenske, R.; Schwarzländer, M.; Millar, A.H. The versatility of plant organic acid metabolism in leaves is underpinned by mitochondrial malate–citrate exchange. *Plant Cell* **2021**, *33*, 3700–3720. [CrossRef]

31. Ivanov, B.; Igamberdiev, A.; Zemlyanukhin, A. Nonphotosynthetic Utilization of Oxogenous C-14 Oxalate by Pea and Rice Seedlings Exposed in the Helium and Carbon-Dioxide Media. *Fiziol. Biokhimiya Kult. Rastenii.* **1989**, *21*, 329–333.
32. Vallarino, J.G.; Osorio, S. Organic Acids. Postharvest Physiol. *Biochem. Fruits Veg.* **2019**, 207–224. [CrossRef]