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Impact of Different Amounts of Biochar as Growth Media on Macronutrient Transport Systems of Carrizo Citrange Rootstocks and Related Expression Analysis

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Abstract: Citrus nurseries significantly increase production costs due to the application of strictly technical and sanitary protocols. The growth media used are generally based on peat, a limited resource that is becoming increasingly scarce and consequently more expensive. Among the alternatives to peat is biochar, which could constitute a valid growing medium component for citrus seedling production. Three growth media were compared, each containing 50% sandy volcanic soil and the remaining 50% being: (i) biochar 50%; (ii) black peat 25% + biochar 25%; and (iii) black peat 25% + lapillus 25% as the control. The impact on the agronomic performance of citrus seedlings was assessed, and the involvement of specific genes in macronutrient uptake was evaluated. Destructive and molecular analyses were performed on leaves and roots during two different periods of the year: February and April. Based on physicochemical parameters and seedling growth, it can be assumed that peat can be partially substituted by conifer wood biochar in a total amount of 25 or 50%. A general comparison of the averages from the sampling and the various analyzed substrates revealed that in February, the evaluated genes involved in the absorption and transport of nutrients were differentially expressed in both leaves and roots, while in April, the expression was not consistent. Additionally, a general comparison between the analyzed tissues showed that, in most cases, expression was higher in the roots than in the leaves. Overall, a comparison among plants grown in different substrates indicated that the medium with 50% biochar displayed the highest expression levels.

Keywords: nursery; citrus; macronutrient absorption; rootstock; nutrient transport genes

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1. Introduction

The citrus nursery industry is highly significant in the Mediterranean Basin, with Italy ranking third in production. Approximately 1000 farms produce citrus plants across 16,000 hectares, employing 80,000 workers and generating around 600 million euros, of which 50% are destined for export. On average, the cycle from sowing to a marketable potted grafted plant takes 36 months. During this period, the main costs for producers arise from the economic depreciation of protective structures, purchase of seeds and pots, growth media, nutrition (both as solid and fertigation), and training management over

three years. Regarding growth media and nutrition, peat is recognized as the primary organic material, while plant nutrition relies mainly on mineral fertilizers. Although these technical means ensure high drainage and aeration, thereby enhancing the suitable environment for root development, nurserymen face two fundamental challenges: (1) the need to replace peat, as it is an expansive, scarce, and non-renewable resource; and (2) the necessity to improve nutritional efficiency to reduce costs of production and minimize groundwater contamination. Moreover, in the context of climate change, there is a growing global interest in sustainable agriculture, which aims to mitigate CO_2 emissions from conventional practices by increasing carbon input to the soil [1] and replacing mineral fertilizers with organic alternatives [2]. Among the potential alternative substrates, biochar shows promising results as a high-performance growth medium. Biochar is a solid material obtained from the thermochemical conversion of various biomasses in the absence of oxygen [3,4].

The role of biochar as a soil amendment or growth medium component in nurseries has been extensively investigated [5,6]. Its impact on plant nutrition is considered an indirect effect due to its carbon content, capacity to reduce leaching, ability to mobilize nutrients, and modification of the physical and chemical properties of the soil and/or growth medium [7]. However, based on its composition, it is also possible to hypothesize that it plays a direct role in plant nutrition by influencing the nutrient transport systems under genetic control. To achieve adequate growth, plants need a contribution of sixteen essential mineral elements, which are divided into three classes: macronutrients (N, P, K), absorbed in relatively high concentrations, and 11 meso- and micronutrients (C, H, O, S, Ca, Mg, Cl, Fe, Mn, Zn, Cu, B, Mo), needed in much lower amounts. Over the past twenty years, significant advancements have been made in identifying the transporter genes of mineral elements across various plant species, particularly in the regulation and use within model plants like Arabidopsis and rice [8]. Plant transporters are designed to relocate mineral elements to different organs, and numerous types of transporters and transport systems have been identified in various plant species [9].

Although rapid progress in genome sequencing has provided a lot of information on transporters through homology searches, the role of most transporters in plants has not been thoroughly investigated. Nitrogen, phosphorus, and potassium are the most important macronutrients and are closely related to crop productivity. Several studies have focused especially on model plants [9–15]. In citrus, some studies have focused on nitrogen transporters [16–22], while a few investigations have focused on phosphorus and potassium [23–26].

This research aimed to investigate the impact of biochar on agronomical performance of Carrizo citrange rootstock (*Citrus sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.) seedlings grown in pots using media containing different amounts of biochar, as well as the expression levels of some genes involved in the transport of macronutrients.

This research aimed to evaluate the expression levels of some genes involved in the transport of macronutrients as well as the impact of biochar on agronomical performance of Carrizo citrange rootstock (*Citrus sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.) seedlings grown in pots using media containing different amounts of biochar.

2. Materials and Methods

2.1. Plant Material and Nursery Growing Conditions

This research was carried out in Mascali (Catania), southern Italy, in a commercial nursery that produces certified citrus trees. Seedlings of the Carrizo citrange were used and managed in the nursery, as described by Ferlito and coworkers [1]. After replanting, the seedlings were fertilized every six months with a slow-release fertilizer containing

nitrogen (N), phosphorus (P), potassium (K), and magnesium (Mg) in the ratios 16:8:10:2. The fertilizer also contained the main micronutrients. The sampling of leaves and roots was carried out 14 to 16 months after transplanting (in February and April, respectively), which corresponds to 24 to 26 months after sowing.

2.2. Experimental Design

Three growth media were prepared, each containing 50% sandy volcanic soil and the remaining 50%: biochar 50% (B50), black peat 25% + biochar 25% (BP25B25), and black peat 25% + lapillus 25% (BP25L25). BP25L25 was the medium commonly used in the nursery that hosted the trial and served as the control. The biochar was obtained from conifer forest wood and produced by pyrolysis at 400 °C. The experiment was performed in a randomized complete-block design with three replicates, with each replicate comprising 20 plants for a total of 180 plants (3 media × 3 reps × 20 plants per rep).

2.3. Physical and Chemical Properties of Growing Media

The principal physical-chemical characteristics of the sandy volcanic soil, black peat, and biochar used as components for the studied growth media are shown in Table 1.

Table 1. The main physical−chemical characteristics of the sandy volcanic soil, black peat, and biochar used as components for the studied growth media.

Parameter	Volcanic Soil	Black Peat	Biochar
pH	6.8	5.7	8.5
EC (dS m^{-1})	0.04	2.1	11.5
Organic matter (%)	3.4	55.3	75.6
Total nitrogen (%)	0.34	1.2	0.45
P (g/kg dw)	0.048	29.3	4.0
K (g/kg dw)	0.288	102.5	138.7
Ca (g/kg dw)	0.001	104.0	16.1
Mg (g/kg dw)	0.05	37.5	5.8
Na (g/kg dw)	0.018	10.2	7.0

For each growth medium used in this work, the total available water (TAW), water at field saturation (WFS), water content at field capacity (WCFC) and at permanent wilting point (WCPWP), water-filled porosity (WFP), total porosity (TP), and air space of the mixtures (AS) were determined. All the applied methods used for the above-mentioned analysis are described by Ferlito and coworkers [1]. The chemical parameters evaluated were nitrogen (g kg^{-1}), organic matter (OM), total organic carbon (TOC, mg kg^{-1}), pH, and EC [27].

2.4. Macro- and Micronutrients Analysis

Leaves and roots collected in February and April were chemically characterized to determine macronutrient (N, P, K, and Ca) and micronutrient (Fe, Zn, Mn, and Cu) concentrations. Following the experimental protocol, leaf and root samples were oven-dried at 60 °C and ground into a fine powder (Ika-M 20 Universal mill, 20.000 rpm). A 0.5 g aliquot of the powder was analyzed for total nitrogen (%N) using the micro-Kjeldahl method [28] with a Büchi Digest Automat K-438, Büchi Scrubber B-414, and Büchi Distillation Unit K370 (Büchi, Switzerland). For P, K, Ca, Mg, Fe, Zn, Mn, and Cu concentrations, a separate 0.5 g aliquot was incinerated in a muffle furnace at 600 °C for

12 h. The resulting ash was dissolved in 0.5 mL of 69.5% nitric acid (Superpure; Merck, Darmstadt, Germany), transferred to a 50 mL volumetric flask, and diluted with deionized water to achieve a final nitric acid concentration of 1/100 v/v. The solution was analyzed using Inductively Coupled Plasma–Optical Emission Spectrometry (ICP-OES, Optima 2000 DV, PerkinElmer).

2.5. Seedling Agronomical Behavior and Biomass Partitioning

Seedling growth was observed every 60 days, starting one month before transplanting (December) until April of the following year, by measuring the stem height and basal diameter of the plants. Seedling growth was determined by destructive measurements, carried out on six seedlings per growing medium, to determine the dry matter partitioning in the root system and leaves. Measurements were carried out two times, in winter (February) and in spring (April), corresponding to the 14th and 16th month after seedling transfer into the pot, respectively.

2.6. Gene Selection and Primer Design

All genes involved in N, P, and K transport were selected from the NCBI [29] and *C. sinensis* Annotation Project databases (CAP) [30], including the name "transporter" as a keyword. We retrieved 24 sequences coding for the Ammonium Transporter (AMT) gene family, 77 for nitrate belonging to the nitrate transporter (NRT) and nitrate transporter/peptide transporter (NRT/PTR) gene families, 38 for phosphorus belonging to the Phosphate Transporter genes (PHTs) family and PHO family (previously identified in Arabidopsis as a protein involved in loading inorganic phosphate (Pi) into the xylem of roots), and 36 for potassium belonging to K^+ uptake permeases the KT (K^+ transporter)/HAK (high-affinity K^+)/KUP (K^+ uptake) family (HAK/KUP/KT).

We aligned the sequences of each gene family using Clustal Omega [31], and a corresponding cladogram was constructed using the Molecular Evolutionary Genetics Analysis (MEGA X) software v10.0;5 [32,33] using the Maximum Likelihood method and Tamura-Nei model [34] (Figure S1). This alignment allowed us to select unique sequences for each gene family, producing a consensus sequence useful for designing forward and reverse primers for quantitative real-time PCR (qRT-PCR). We designed three (AAm-CA1, AAm-CA2, AAm-CB7-9) and six (An-C31-101, An-C32-98, An-C63-81, An-C7-21, An-C1-27, An-C48-84) primer pairs for ammonium (NH_4^+) and nitrate nitrogen (NO_3^-) transporter sequences, respectively; three (Ph_C1, Ph_C2, Ph_CD) primer pairs were drawn for phosphorous (P) and two (Pot-CH, Pot-CE) for potassium (K) (Table 2).

Table 2. Primer sequences.

Nutrient	Primer Sequence (5′-3′)
NH_4^+	AAm-CA1 Fw 5′-GGGAACACTACATTGGCCTTG-3′ AAm-CA1 Rev 5′-CCCATTTGCCTGAATCCTGC-3′
	AAm-CA2-6 Fw 5′-GAAACCTTCTGTTATTGGC-3′ AAm-CA2-6 Rev 5′-TAGCAGCCCATCCTTGAACA-3′
	AAm-CB7-9 Fw 5′-TCTCCGCTTACCTCGTCTTC-3′ AAm-CB7-9 Rev 5′-TGTTCATGGTGTTCTTGGCG-3′

Table 2. *Cont.*

Nutrient	Primer Sequence (5′-3′)
NO_3^-	An-C31-101 Fw 5′-TGTAATCTTCTGGGCTCCTGT-3′ An-C31-101 Rev 5′-CAATGCCCATCCGTTGAAGTTG-3′
	An-C32-98 Fw 5′-TGGGATATCGTCAAATTTGGTGA-3′ An-C32-98 Rev 5′-GGTGACATTGTTAGCTGACTTCA-3′
	An-C63-81 Fw 5′-CAACTTTCTTGCTTTGCCTCG-3′ An-C63-81 Rev 5′-ACTTCGAGTGTTCCAAAGATCA-3′
	An-C7-21 Fw 5′-GCTGACTCATTTCTAGGCCG-3′ An-C7-21 Rev 5′-GCTAACAAGCCAGTCCCAAA-3′
	An-C1-27 Fw 5′-TCATCCGCGACAATCTCAATC-3′ An-C1-27 Rev 5′-ACCCATTACGAGCCTTGAGAATA-3′
	An-C48-84 Fw 5′-ATCATTACCTCAGGCTGCGG-3′ An-C48-84 Rev 5′-TTTCACTTGGCACGAGACG-3′
P	Ph_C1 Fw 5′-TTGCTCTCGCTATCCCTTA-3′ Ph_C1 Rev 5′-AAATGTTGTGGCATTTGGTCC-3′
	Ph_C2 Fw 5′-TACACAGCCTTGGTCGAG-3′ Ph_C2 Rev 5′-ACTTTGGCCATATCAACTGCA-3′
	Ph_CD Fw 5′-TGGAATTTCTACAGGTTA-3′ Ph_ CD Rev 5′-CTGCCTTCACAGCCCTGAA-3′
K	Pot-CH Fw 5′-ACTGACGAGGAGCTTACGAC-3′ Pot-CH Rev 5′-CTCCAACCATCTCTTAGTCT-3′
	Pot-CE Fw 5′-ACCAACCTTCCAGCTTTTCA-3′ Pot-CE Rev 5′-ACATGTGGCACTGGAACTGA-3′

Exemplificative sequence for each cluster was blasted against the most recent *C. sinensis* genome (Citrus Genome Database: https://www.citrusgenomedb.org/, accessed on 4 November 2024) to confirm the differences among clusters. BLAST analysis was carried out to identify orthologous sequences to *C. sinensis* in *Arabidopsis thaliana* (*At*) and *Populus trichocarpa* (*Pt*) to deduce a putative similar function (Table S1).

2.7. RNA Extraction, cDNA Synthesis, and qRT-PCR

Total RNA was extracted from leaves and roots at two different periods of the year, February and April, using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA). The total RNA amount was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the quality was verified by gel electrophoresis (agarose 0.8% in TAE 1×). cDNA was synthesized using 1 µg of total DNase-free RNA, according to the manufacturer's instructions (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). Three biological replicates for each tissue (leaves and roots) from potted plants grown in the three different substrates were used to perform qRT-PCR with 100 ng cDNA. The analyses were conducted using a 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR® Master Mix (Applied Biosystems, Foster City, CA, USA), according to the protocol provided by the manufacturer. Elongation factor 1α (EF; AY498567) was used as the reference gene [35]. Relative quantification was normalized to the EF reference gene, and the fold increase was calculated using the standard curve method.

2.8. Statistical Analysis

Statistical analysis employed the application StatSoft 6.0 (Statistic for Windows. Statsoft, Tulsa, OK, USA, 2001) for analysis of variance (ANOVA), and mean separation by the Tukey HSD test was performed at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

Principal component analysis (PCA) was performed using data collected in February and April, consisting of the physical and chemical characteristics of three growing media (B50, BP25B25, and BP25L25), physicochemical properties, biomass partitioning of Carrizo citrange seedlings, and expression values of the different genes analyzed. PCA was conducted using the PAleontological STatistics (PAST) software package, version 2011 [36].

3. Results and Discussion

This research evaluated the effects of growth media containing different amounts of biochar using a multidisciplinary approach. Specifically, both the agronomical and genetic behaviors of Carrizo seedlings were considered. Regarding the agronomical effects of biochar monitoring of seedling growth, genetic analyses were performed to better understand the involvement of genes belonging to different families in macronutrient transport and uptake.

The main growing season for plants typically spans from April to October. However, February and April were selected for sampling because vegetative growth begins in spring at the latitude where the study was conducted. Approximately 30–40 days before this growth starts, the activation of genes related to this process already occurs, utilizing the first available nutrient resources. During this period, the response of the genes responsible for nutrient uptake and transport is likely to be more immediate, and their expression is more pronounced.

3.1. Seedling Morphological Behavior and Nutrient Content

During the first six months, no significant differences were observed in the seedlings' height and stem diameter; on the month 8th (June), a significant increase in both parameters was recorded for the seedlings growing in the BP25L25 medium, while no differences were observed in seedlings grown in the substrates containing biochar (B50, BP25B25). After the eighth month, seedling performance was similar across the three growth media, as well as during the graft season (April) (Figure 1A,B). Destructive analyses in winter (February) and spring (April) (Figure 2A–D) showed that the partitioning of dry matter accumulated in leaves and roots was similar for all growth media. However, between the two sampling periods (February and April) and during the first period of vegetative regrowth, a different behavior was detected among the different growth media. Specifically, for leaves collected from seedlings grown in BP25L25, we observed a decrease (−25%) in biomass allocation, while in B50, we registered an increase (+22%). This trend was also observed in the roots. Particularly, seedlings grown in BP25L25 showed a decrease (−20%) between February and April, whereas those collected from BP25B25 and B50 registered an increase, of +27% and +8%, respectively.

Data on seedling morphology (seedling increase in height, diameter, and dry matter partitioning) in the leaves and root systems showed that dry matter accumulation was most evident during the first stages of seedling management; specifically, those grown in BP25L25 were most reactive only during the mid-phase (June). However, the absence of above- and below-ground biomass growth for leaves and roots in both February and April indicated that the root system efficiency was satisfactory. Most importantly, the seedlings in growing media containing biochar, mostly in B50, showed the best performance during the first period of vegetative growth (interval February–April). Moreover, during the destructive measurements, a reduction in root growth in the lower container layers was

observed, indicating that the substrate quality was compromised at this level. This was attributed to the accumulation of small particles at the container ground level, which reduced the porosity (not shown). It has been reported that when bulk density increases, the number of larger pores decreases, making it more difficult for roots to deform and displace substrate particles, thereby limiting root elongation rates [37] and affecting root development and morphology. Specifically, when the substrate was more compact, the roots were concentrated in the upper part of the pot to ensure the development of above-ground biomass and adequate root aeration.

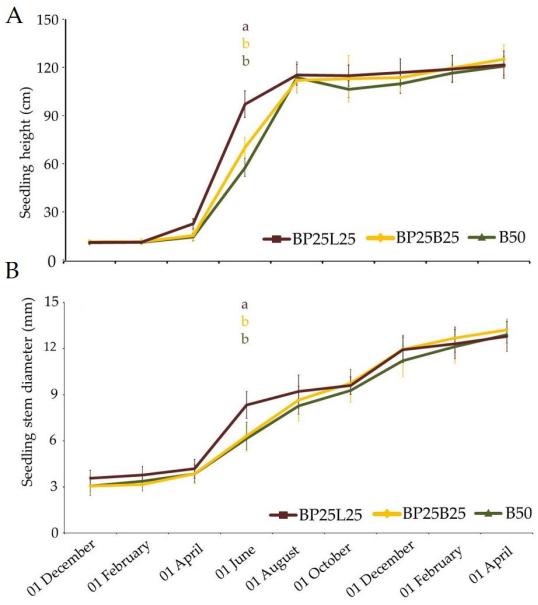

Figure 1. Seedling height (**A**) and stem (**B**) diameter measured at the soil level on Carrizo citrange seedlings potted in different growing media right after transplanting until the next season. Mean values are reported for each data point (different letters indicate a significant difference at $p \leq 0.05$ based on Tukey's HSD test, bars indicate the standard deviation).

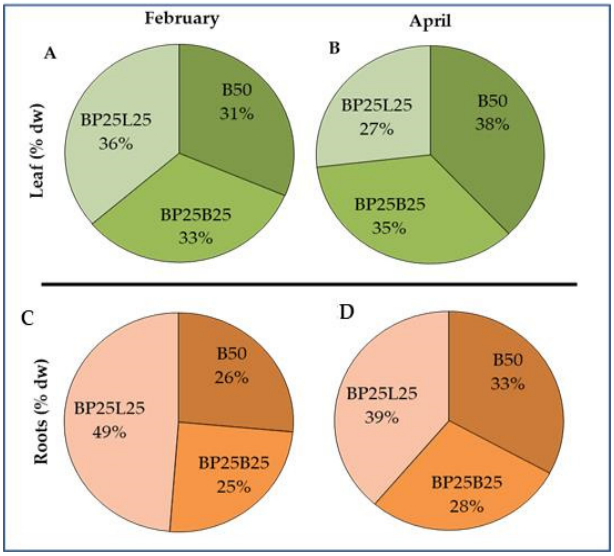

Figure 2. Dry matter distribution recorded in winter (February) (**A**,**C**) and spring (April) (**B**,**D**) of Carrizo citrange seedlings potted in different growing media.

No significant differences were found in the nitrogen content of leaves and roots on both sampling dates. Statistically significant differences in the leaves of the B50 substrate

and roots of BP25B25 were found for Ca in February. In April, the Ca content of both growth media was significantly higher than that of BP25L25 in leaves and roots. In February, the BP25L25 medium showed significantly higher values of magnesium and manganese in the roots compared to the other two substrates (Tables 3 and 4).

Table 3. Macro-and micronutrient content registered during winter (February) on Carrizo citrange seedlings potted in different growing media. Values are presented as mean (±standard deviation). Values in a column indicated by different letters are significantly different (lowercase, $p \leq 0.05$; uppercase, $p \leq 0.001$) based on Tukey's HSD test; the absence of letters indicates the absence of significance.

Growth Medium	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Calcium (%)	Magnesium (%)	Iron (mg kg^{-1})	Zinc (mg kg^{-1})	Manganese (mg kg^{-1})	Copper (mg kg^{-1})
					Leaf				
B50	3.03 ± 0.43	0.34 ± 0.09	1.23 ± 0.23	3.19 ± 0.29 a	0.19 ± 0.04	180.23 ± 17.57	44.04 ± 1.22 AB	37.71 ± 18.61	143.33 ± 33.78
BP25B25	3.11 ± 0.21	0.32 ± 0.04	1.26 ± 0.10	3.25 ± 0.36 a	0.17 ± 0.02	248.13 ± 85.88	56.12 ± 4.84 A	45.94 ± 12.15	143.50 ± 48.68
BP25L25	2.88 ± 0.26	0.29 ± 0.01	1.27 ± 0.09	2.32 ± 0.12 b	0.17 ± 0.03	246.77 ± 16.37	36.52 ± 5.38 B	47.37 ± 3.74	142.47 ± 47.17
					Root				
B50	2.26 ± 0.20	0.18 ± 0.03	1.97 ± 0.12	1.04 ± 0.04 A	0.22 ± 0.02	1002 ± 344.18	211.67 ± 92.87	66.26 ± 10.37	26.03 ± 5.25
BP25B25	2.16 ± 0.23	0.18 ± 0.02	1.74 ± 0.34	0.93 ± 0.04 A	0.26 ± 0.05	1221 ± 276.49	260.80 ± 135.97	84.66 ± 38.90 b	25.04 ± 4.60
BP25L25	1.88 ± 0.07	0.17 ± 0.01	1.72 ± 0.15	0.67 ± 0.01 B	0.29 ± 0.01	1318 ± 74.91	155.5 ± 13.95	429.67 ± 38.20	14.29 ± 5.53

Table 4. Macro-and micronutrient content registered during spring (April) on Carrizo citrange seedlings potted in different growing media. Values are means (±standard deviation). Values in a column indicated by different letters are significantly different (lowercase, $p \leq 0.05$; uppercase, $p \leq 0.001$) based on Tukey's HSD test; the absence of letters indicates the absence of significance.

Growth Medium	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Calcium (%)	Magnesium (%)	Iron (mg kg^{-1})	Zinc (mg kg^{-1})	Manganese (mg kg^{-1})	Copper (mg kg^{-1})
					Leaf				
B50	2.76 ± 0.13	0.18 ± 0.00	1.11 ± 0.13	3.05 ± 0.41 a	0.16 ± 0.02	335 ± 310	33.31 ± 7.19	43.86 ± 12.64	49.38 ± 10.43
BP25B25	2.68 ± 0.10	0.15 ± 0.01	1.05 ± 0.22	2.65 ± 0.25 ab	0.19 ± 0.01	309 ± 73	48.77 ± 21.63	81.80 ± 31.01	64.6 ± 10.85
BP25L25	2.95 ± 0.28	0.18 ± 0.02	0.98 ± 0.23	2.17 ± 0.17 b	0.22 ± 0.04	748 ± 420	25.66 ± 3.16	80.95 ± 30.57	46.58 ± 13.00
					Root				
B50	2.24 ± 0.17	0.17 ± 0.01	1.02 ± 0.10 b	0.89 ± 0.09 ab	0.22 ± 0.01b	1276 ± 234	72.35 ± 18.99	72.10 ± 14.49 B	13.45 ± 7.07
BP25B25	2.42 ± 0.27	0.17 ± 0.04	1.67 ± 0.36 a	1.00 ± 0.07 a	0.22 ± 0.03b	1378 ± 293	10.77 ± 17.45	109.41 ± 27.09 B	31.79 ± 6.84 B
BP25L25	2.34 ± 0.33	0.18 ± 0.02	1.69 ± 0.23 a	0.69 ± 0.10 b	0.28 ± 0.01a	1186 ± 255	115.29 ± 14.95	401.57 ± 25.71 A	30.51 ± 10.84 B

3.2. Gene Selection

From the alignment of the sequences identified for each gene family, a cladogram was obtained from which it was possible to highlight a different number of clusters for each nutrient under investigation (3 for NH_4^+, 6 for NO_3^-, 3 for P, and 2 for K) (Figure S1).

3.3. Expression Analysis

Genetic analysis of genes involved in nutrient transport and uptake was performed to compare the expression levels in the leaves and roots of seedlings grown on the three different media (B50, BP25B25, and BP25L25) at two different sampling times, February and April.

The data show the expression of genes involved in the transport of nutrients: (1) comparing their behavior in February and April (Figure 3), (2) evaluating the differences depending on the tissue (leaves and roots) (Figure 4), and (3) considering their effects depending on the growth media (Figure 5).

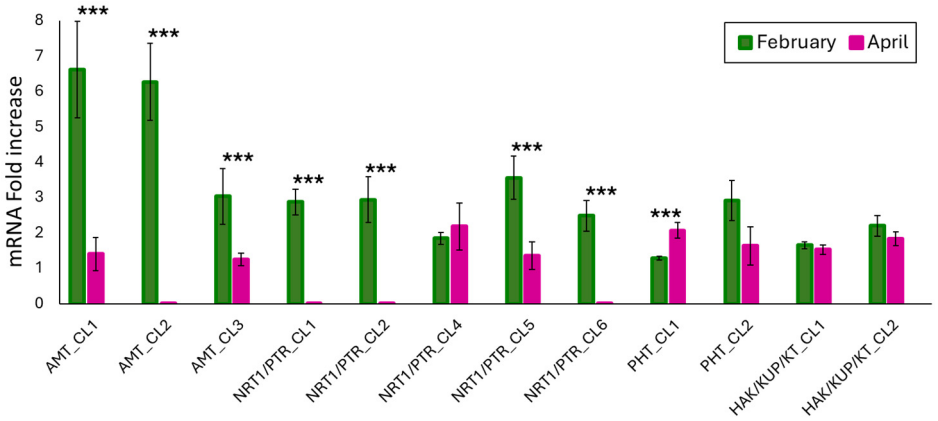

Figure 3. Comparison between the gene expression of Carrizo citrange seedlings in all growing media, including both roots and leaves. Mean values ± S.E. of at least three replicates obtained for each gene are reported. *** = significantly different at $p \leq 0.001$; based on Tukey's HSD test, bars indicate the standard error within each data sampling (February and April).

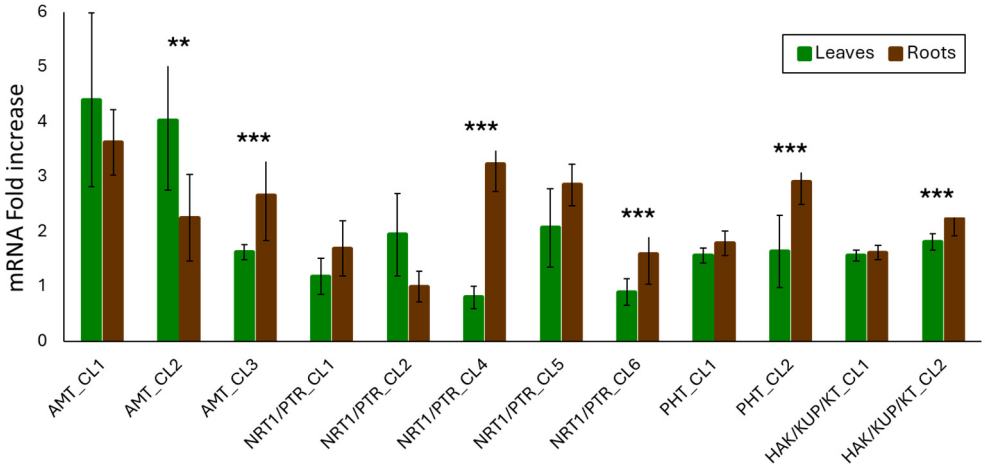

Figure 4. Comparison between the gene expression of Carrizo citrange seedlings in all the growing media, including both the sampling data (February and April). Mean values ± S.E. of at least three replicates obtained for each gene are reported. ** and *** = significantly different at $p \leq 0.01$ and $p \leq 0.001$, respectively; based on Tukey's HSD test, bars indicate the standard error within each tissue (leaves and roots).

In February, all the analyzed genes included in the previously described clusters were expressed in both leaves and roots. The level of many transcripts was statistically different compared to April, except for *NRT1/PTR_CL4*, *PHT_CL2*, *HAK/KUP/KT_CL2*, and *HAK/KUP/KT_CL1* genes. In April, in contrast, we did not observe expression of *AMT_CL2*, *NRT1/PTR_CL1*, *NRT1/PTR_CL2*, and *NRT1/PTR_CL6* in both tissues (Figure 3).

Gene expression averages for each sampling are consistent, at least, with what is already known about nitrogen uptake in many plant species, including citrus fruits. In fact, the absorption of N varies during the life cycle of plants, decreasing normally during flowering [38]. The higher expression of almost all genes involved in nitrate uptake in February may be due to the lower availability of this nutrient. Furthermore, to better understand this dynamic, it is important to consider the roles of nitrate and nitrite reductase, which are also influenced by the presence of potassium [39].

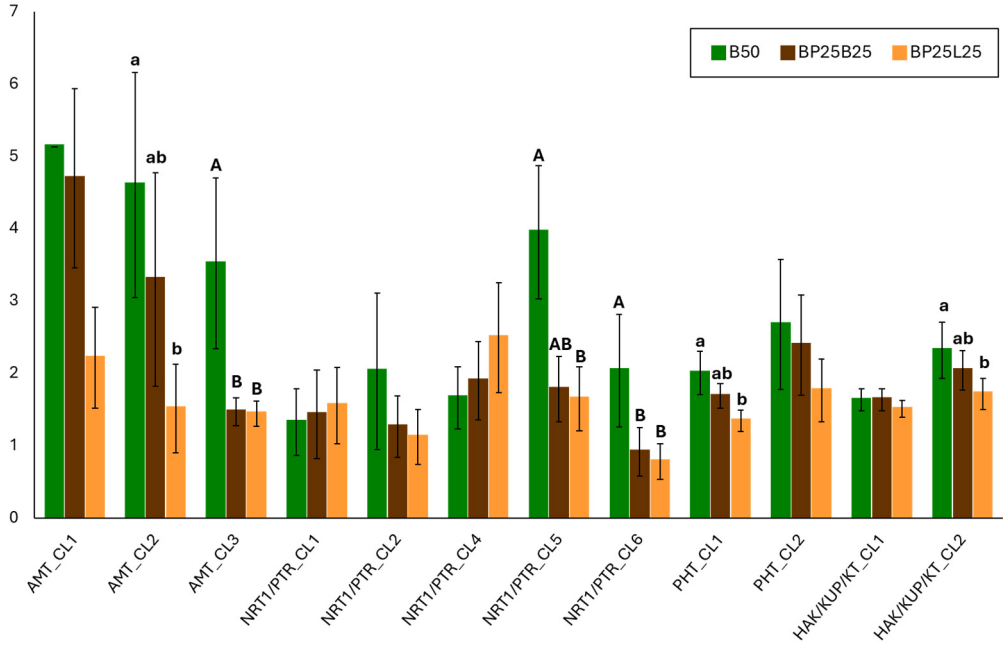

Figure 5. Comparison between the gene expression of seedlings in both tissues (leaves and roots) and both data sampling (February and April) within the tested growth media. Mean values ± S.E. of at least three replicates obtained for each gene are reported. Different letters indicate a significant difference (capital letter = significantly different at $p \leq 0.01$ and $p \leq 0.001$, lowercase letter = significantly different at $p \leq 0.05$; based on Tukey's HSD test, bars indicate the standard error) within each growth medium.

When we pointed the attention to the two tissues, we observed that the expression of genes coding for *AMT_CL3*, *NRT1/PTR_CL4*, *NRT1/PTR_CL6*, *PHT_CL2*, and *HAK/KUP/KT_CL2* was statistically significant in the roots compared to that in the leaves (Figure 4). On the contrary, genes coding for *AMT_CL2* and *NRT1/PTR_CL2* were highly expressed in the leaves compared to the roots (Figure 4). This remark was also evidenced by Wang et coworkers [10], who highlighted the importance of the roots in the physiological behavior of the plant. This could be due to the modification and availability of ammonium in the soil compared to that in the leaves. However, these results could be because of the influence of the biochar component on the substrate.

When we evaluated gene expression in the tested growing media, the most evident differences were observed for the genes involved in ammonium transport (*AMT_CL1*, *AMT_CL2*, and *AMT_CL3*) between B50 and BP25L25 (Figure 5). Indeed, the highest amount of transcripts for the B50 medium agrees with the findings of previous studies obtained with biochar [40], where the induced changes in the soil and its influence on plant growth were reported in consideration of nitrogen availability. Even in the cycle of P and K, the presence of biochar determines an increase in these two nutrients in the soil and an enhancement of the concentration of K in the plant tissue (roots and leaves) [40–42]. Moreover, it can be assumed that the NH_4^+ content in the growth media increased due to a reduction in the loss of nutrients by leaching [43,44]. This result confirms the role of biochar in improving NH_4^+ uptake by plants; this growth media component would reduce the volatilization of ammonia, an important atmospheric pollutant [45]. All that could improve the nutrition efficiency in the nursery is by reducing the use of N fertilizers and pot volume.

Results similar to those detected for the genes involved in ammonium transport were observed for *NRT1/PTR_CL5*, *NRT1/PTR_CL6* (nitrate), *PHT_CL1* (phosphorus), and *HAK/KUP/KT_CL2* (potassium) genes. Instead, the *NRT1/PTR_CL4* gene showed increased

expression in BP25L25 compared to the B50 growing medium, while the *PHT_CL2* gene exhibited higher expression in BP25B25 compared to BP25L25 (Figure 5).

3.3.1. Nitrogen

The major sources of nitrogen for plants are NH_4^+ and NO_3^-. In the soil, NO_3^- is generally present in higher concentrations and is also more mobile and available to plants than NH_4^+ [12]. Plants have developed various nitrogen acquisition mechanisms, which include transport systems with different affinities for nitrate and ammonium (HATS and LATS) [18,20,46].

Regarding genes involved in NH_4^+ transport, the genetic sequences of *C. sinensis* from cluster 1 were orthologous to *Pt* uncharacterized protein. Cluster 2 included members of *C. sinensis* orthologs of the AMT2 family member of *At* and members of *C. sinensis* orthologs of the AMT2 and AMT3 family of *Pt*. Cluster 3 enclosed AMT1 family members from *C. sinensis*, *Poncirus trifoliata*, and Carrizo citrange (Table S1, Figure S1A).

Concerning NH_4^+, almost all studied genes exhibited a high expression level in February, both in leaves and roots (Figure 6A). In April, the *AMT_CL3* gene was expressed in both tissues; on the contrary, *AMT_CL1* was expressed only in the roots, while no expression was detected for *AMT_CL2* (Figure 6B). Specifically, the greatest expression among the growing media was found in those containing 50% (B50) and 25% (BP25B25) of biochar, which was statistically significant only in the roots (Figure 6A). Recently, Ferlito and coworkers [1] observed a reduction in growth for both aerial and hypogean parts of the seedlings, primarily with the medium containing a high percentage of biochar (B50).

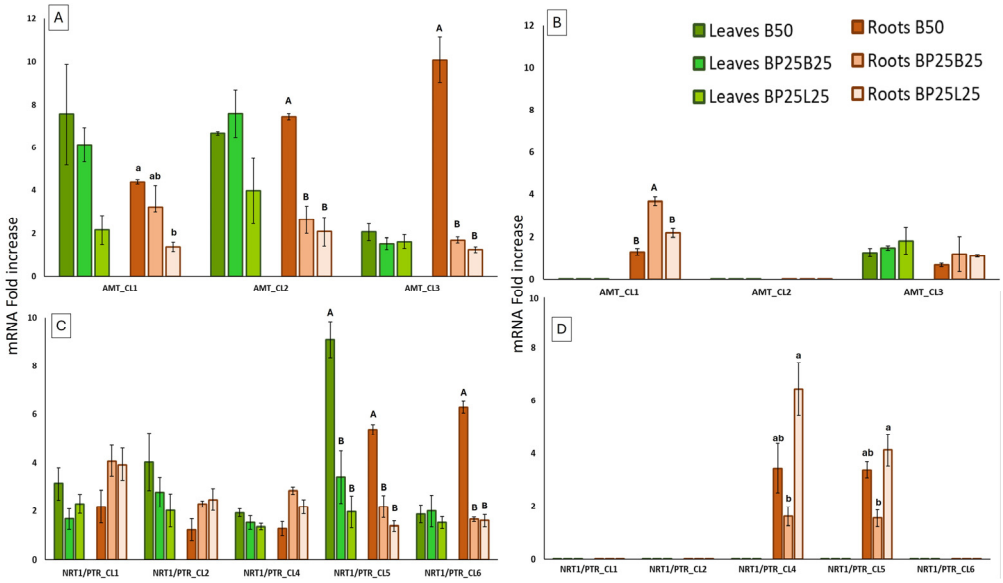

Figure 6. Real-time expression data of ammoniacal nitrogen in February (**A**) and in April (**B**) Real-time expression data of nitrate nitrogen in February (**C**) and in April (**D**). The data are presented as the mean ± S.E. of at least three replicates. For each gene, different letters indicate a significant difference (capital letter = significantly different at $p \leq 0.01$ and $p \leq 0.001$, lowercase letter = significantly different at $p \leq 0.05$; based on Tukey's HSD test, bars indicate the standard error).

The sequences selected for nitrate nitrogen were found to be orthologous to the NRT and NRT/PTR families in both *At* and *Pt*. Clusters 1 to 6 were mainly orthologous to the NRT and NRT/PTR family members 8, 5, 4, 6, 2, and 1, respectively (Table S1, Figure S1B). A different behavior was observed for the studied genes between winter and spring. In February, transcripts were obtained in both roots and leaves, while in April, the genes *NRT1/PTR_CL1*, *NRT1/PTR_CL2*, and *NRT1/PTR_CL6* showed no expression, and

NRT1/PTR_CL5 and *NRT1/PTR_CL4* genes were expressed only in the roots (Figure 6C,D). In February, the level of NO_3^- transcripts on average was lower than that of NH_4^+ transcripts (Figure 6A,C); moreover, for each gene and tissue investigated, always in NO_3^-, the significant differences between growth media were similar to those observed for NH_4^+. In February, the *NRT1/PTR_CL5* gene showed a statistically significant expression level in the B50 medium compared with the other two growing media in both leaves and roots. Conversely, the *NRT1/PTR_CL6* gene produced a transcript level statistically significant among the studied media, which was observed only in the roots (Figure 6C). In the April sampling, only *NRT1/PTR_CL4* and *NRT1/PTR_CL5* were similarly expressed, exclusively in the roots for all growing media; moreover, the expression found in B50 was greater compared to that observed in BP25B25 (Figure 6D).

In winter, low temperatures are responsible for reducing nitrate and nitrite reductase enzymatic activity, decreasing the availability of nitric nitrogen [47]. This deficiency may trigger an increase in the expression of NH_4^+ and NO_3^- transporter genes. We hypothesize that seedlings grown in substrates containing biochar, thanks to the ability of this additive to mobilize nutrients, can promote earlier gene activation and accumulate higher levels of nitrogen than the control in February. The absence of transcripts of the other analyzed genes in April could be expected because, in this season, the plant should have already accumulated the amount of nitrogen necessary for its development (Figure 6D).

3.3.2. Phosphorus

Phosphorus is one of the main limiting factors for plant growth. In the soil, this nutrient is generally present in forms that are not available to plants and has a very low solubility [48]. Only the inorganic form (Pi) is truly available for root absorption. Moreover, its content in soils is relatively low, with only a marginal portion present in the solution, such as the phosphate ion ($H_2PO_4^-$; HPO_4^{2-}). This amount is very low compared to the optimal needs of plants [48].

Similar to nitrogen, phosphorus has two different transport systems with varying affinities for Pi [49]. The high-affinity transporter is activated when Pi in the soil is in a low range in terms of micromoles (μM) [50], while the low-affinity transport system is triggered when the concentration range of Pi in the soil is in the order of millimoles (mM) [51]. Furthermore, some authors suggest that a low-affinity transport system is constitutively active in plants [52].

Data regarding phosphorus gene expression are shown in Figure 7.

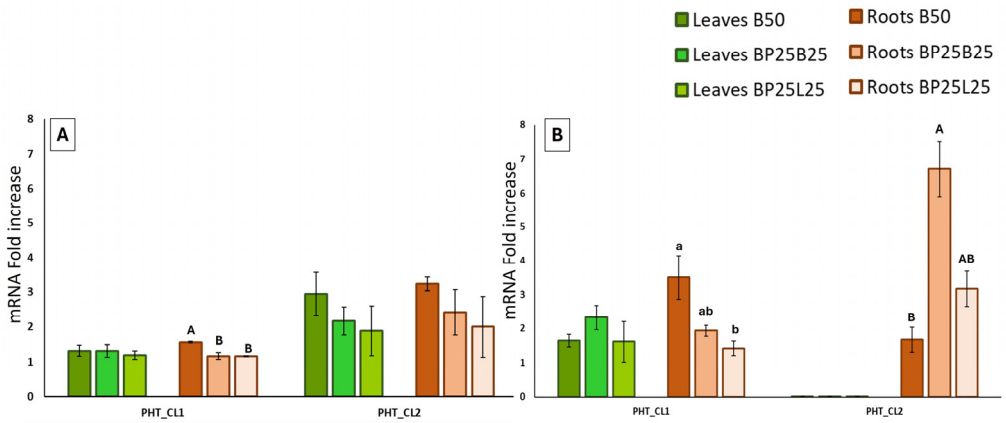

Figure 7. Real-time expression data of phosphorus in February (**A**) and in April (**B**). The data are the mean ± S.E. of at least three replicates. For each gene, different letters indicate significant differences (capital letter = significantly different at $p \leq 0.01$ and $p \leq 0.001$, lowercase letter = significantly different at $p \leq 0.05$; based on Tukey's HSD test, bars indicate the standard error).

No expression has been reported for the *PHO1_CL3* gene. No significant difference was observed among the growing media in both sampling data in the leaves for the *PHT_CL1* gene. In contrast, a statistically significant difference was detected in the roots between B50 and the other two growing media in February and between B50 and BP25L25 in April (Figure 7A,B). The *PHT_CL1* gene is included among the sequences of cluster 1, among which the ortholog of *At* and *Pt* is PHT 1-4 described mostly expressed in roots [51] (Table S1, Figure S1C).

Unlike the *PHT_CL1* gene, transcripts of *PHT_CL2* were produced in both tissues only in February and only in roots in April (Figure 7A,B). The expression level of *PHT_CL2* was, in general, higher than that of *PHT_CL1*. In April, the abundance of transcripts in the roots peaked in the BP25B25 growth medium, showing a statistically significant difference compared to B50.

Our work highlighted a certain expression of the *PHT_CL1* gene in both the analyzed tissues and sampling data (February and April). The absence of differences in both sampling data at the leaf level suggests that the activation of the genes in this cluster begins at the root system, possibly because sampling and analysis were performed too early to the peak of the leaves' vegetative growth.

The gene expression of *PHT_CL1* could be explained by the fact that cluster 1 includes the *PHT1* gene family (1;1 and 1;4). As described for genes *AtPHT1;1* and *AtPHT1;4* in *Arabidopsis* [53,54], we assume that these could play a major role in phosphate uptake, especially since many members of this family are generally modulated by low Pi content [55,56]. This agrees with Deb and coworkers [57], who reported how biochar increased the production of Pi solubilizing organic acid in nutrient-poor soils such as volcanic soil, which was used in this research [58]. Previous research has highlighted that in P-deficient soils, biochar actively influences the growth and yield of some species by increasing microbial biomass [59]. Therefore, we can hypothesize that our findings related to the expression of genes responsible for P uptake and transport are influenced by the biochar as well.

Furthermore, cluster 1 includes members of the PHT2 family, who generally have low-affinity Pi transporters. The *PHT2* gene family is predominantly expressed in green tissue [60,61], although Guo and coworkers [62] described the presence of transcripts in the roots, as shown in Figure 7A,B. The slight discrepancy in root transcripts highlighted in the two sampling data (high value in April compared to February; Figure 7A,B) could be due to the maintenance of P in its two forms, Pi and PO, after uptake. When phosphate is translocated into the xylem, only the Pi fraction is incorporated, while the PO fraction is released back as Pi, largely confined to the root xylem, from which it moves to the newly formed organs (leaves) in spring [12]. In February, Pi and its esterified fraction (such as PO) have not yet been mobilized to the leaves, which could justify the gene expression in this tissue (Figure 7A). As the PO begins to be mobilized to the leaves, the roots become deficient in P; this could explain the gene expression in April in the roots observed for both the *PHT_CL1* and *PHT_CL1* genes (Figure 7A,B).

3.3.3. Potassium

Potassium is necessary for all plant activities that require water transport, including the opening and closing of stomata [63]. In addition, potassium ensures the stability and quality of the plant and regulates many other processes [64,65]. In soil, potassium is generally divided into four categories: water-soluble, exchangeable, slowly available, and structural potassium [66]. The latter constitutes the largest amount in the environment, even though it is difficult to use directly by plants [67].

Potassium transporters can be attributed to four families of membrane proteins: K^+ uptake permeates the KT (K^+ transporter)/HAK (high-affinity K^+)/KUP (K^+ uptake) family,

the K^+ transporter Trk/Ktr/HKT family, the KEA (K^+ efflux antiporter) family, and the CHX (cation/hydrogen exchanger) family [68]. The HAK/KUP/KT family is the largest K^+ transporter family in plants. It holds high-affinity K^+ transport carriers and is mainly involved in plant growth and development [69].

From the BLAST analysis, we found that the sequences of *C. sinensis* are orthologous to members of the HAK/KUP/KT family and correspond to the HAK/KUP/KT 11, 3, 2, and 6 of *At* and *Pt*, which are involved in the development and morphology of roots and leaves [68,70,71]. Additionally, six *C. sinensis* sequences were found to be "Probable potassium transporter 11-like isoform X1" in *At*.

HAK/KUP/KT_CL1 gene expression did not show any statistically significant difference either between the tissues analyzed or between the sampling periods (February and April) (Figure 8A,B).

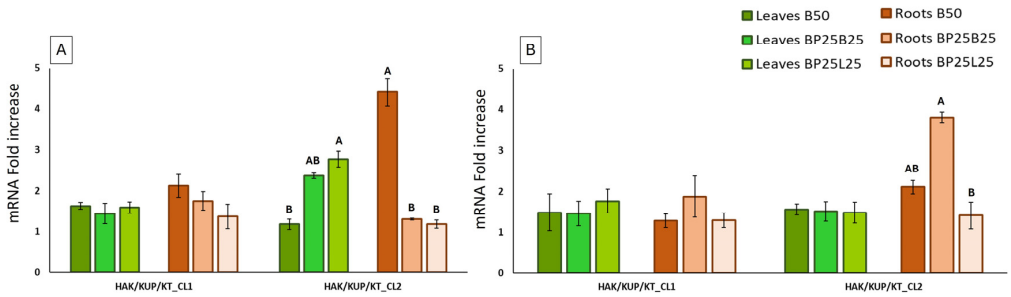

Figure 8. Real-time expression data of potassium in February (**A**) and in April (**B**). The data are the mean ± S.E. of at least three replicates. For each gene, different letters indicate significant differences (capital letter = significantly different at $p \leq 0.01$ and $p \leq 0.001$; based on Tukey's HSD test, bars indicate the standard error).

In contrast, the expression of HAK/KUP/KT_CL2 showed statistically significant differences between the roots (Figure 8A,B) in February and April. In February, roots showed higher values with the growth medium containing 50% biochar (B50), while in April, higher expression was detected in the BP25B25 medium. In February, the leaves showed statistically significant differences between B50 and the other two growth media (Figure 8A).

It can be hypothesized that since the root system begins its biological and morphological growth one month earlier than the aerial part of the plant, growth in B50 medium allows for advantageous K uptake that promotes significant root system growth already in February. The findings from April are consistent with previous studies on root system morphology using the same substrate compositions evaluated in this work [1], which showed a significant reduction in the root system of plants grown in the BP25B25 substrate compared to the other substrates (Figure 9). Biochar seems to be an effective material that can improve the availability of potassium in the soil [26]; therefore, a substrate with a greater quantity of biochar (B50) facilitates greater absorption of K compared to BP25B25. In April, the gene expression of *HAK/KUP/KT_CL2* was less pronounced in the B50 medium in the roots because the plants had already absorbed the necessary amount of K. Conversely, the plants grown in the BP25B25 medium, which facilitates absorption to a lesser extent, showed an increased expression in April, probably because it still needs the nutrient absorbed in relative quantity in February [72,73]. It could be speculated that in April, the plant, grown in BP25B25, tries to absorb as much potassium as possible to restore the proper morphology of the root system, as already described; some members of the family *HAK/KUP/KT* genes plants affect not only K^+ acquisition and transportation, but also the root and shoot morphology and elongation [68,74,75].

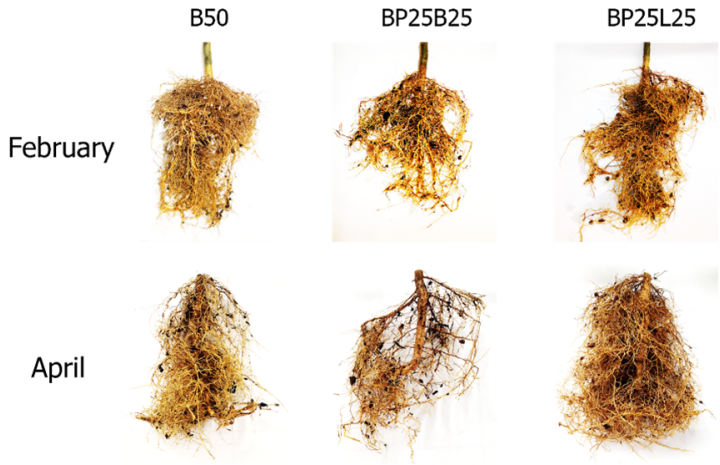

Figure 9. Root systems of seedlings grown on the three different growing media.

4. Statistical Analysis

To evaluate the effect of all the analyses performed in this work and deduce a putative relationship among them, PCA was performed, which revealed that the first two components accounted for 50.1% of the total explained variance (PC1 = 33.2%; PC2 = 16.9%) (Table 5). Growth media B50, BP25B25, and BP25L25 were clearly distinguished in a multidimensional space (Figure 10).

Table 5. Principal Component Analysis (PCA). Eigenvalue and proportion of variance explained by each principal component.

PC	Eigenvalue	% Variance	Cumulative
1	26.8866	32.008	32.008
2	12.6607	15.072	47.08
3	7.89338	9.3969	
4	6.80928	8.1063	
5	4.67556	5.5661	
6	3.99779	4.7593	
7	3.54255	4.2173	
8	3.02139	3.5969	
9	2.72536	3.2445	
10	2.33653	2.7816	
11	1.82232	2.1694	
12	1.79565	2.1377	
13	1.65289	1.9677	
14	1.32044	1.5719	
15	1.23239	1.4671	
16	1.01022	1.2026	
17	0.616928	0.73444	

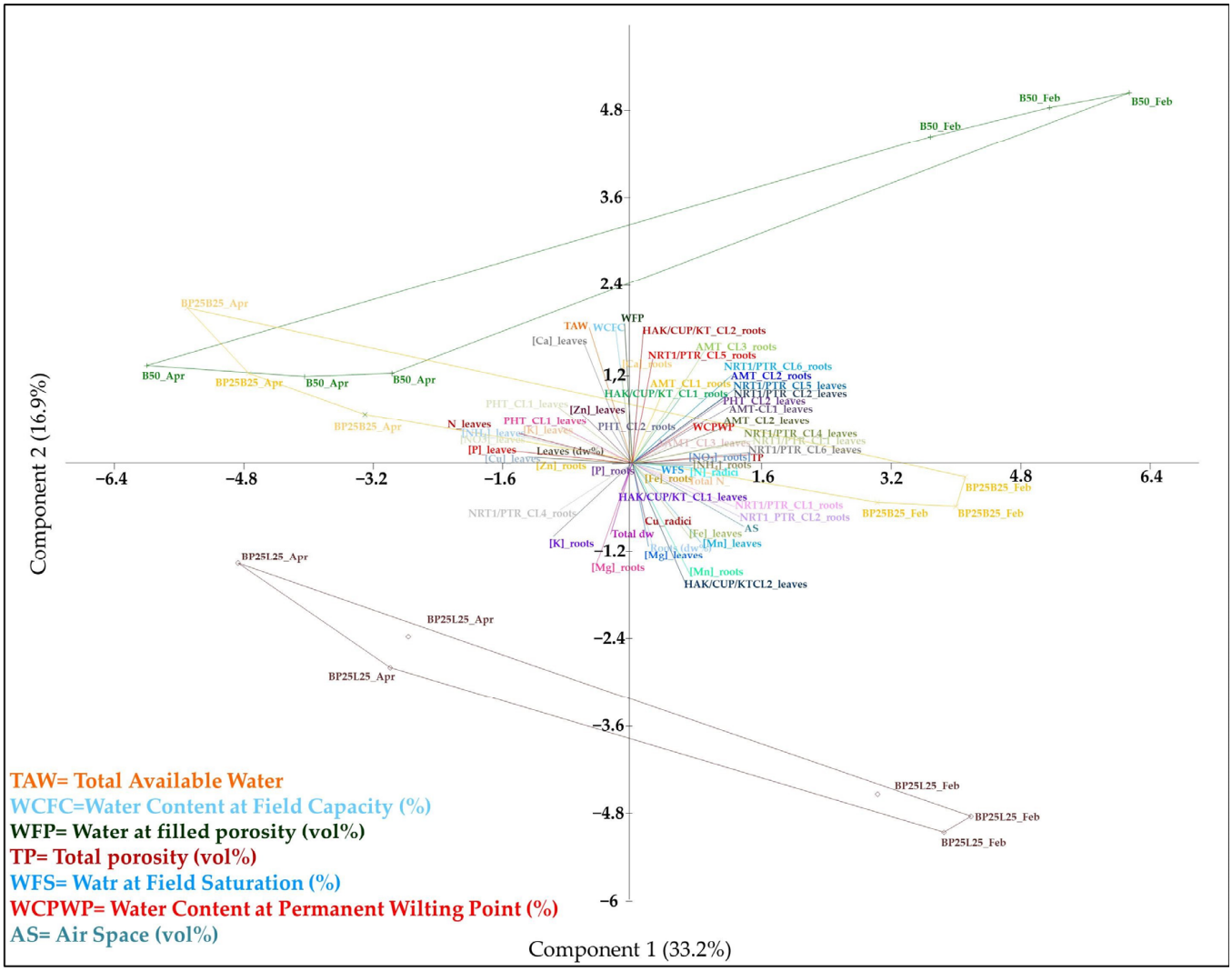

Figure 10. Principal component analysis (PCA) biplot defined by the first two principal components. Vectors represent the loadings of the physical and chemical characteristics of three growing media, each containing 50% sandy volcanic soil and the remaining 50% being: biochar 50% (B50); black peat 25% + biochar 25% (BP25B25); black peat 25% + lapillus 25% (BP25L25), the physical and chemical properties, the biomass partitioning of Carrizo citrange seedlings, and the expression of genes involved in nitrogen, phosphorus, and potassium transport in leaves and roots. Data were collected in February (winter) and April (spring).

The transcripts of NRT1/PTR_CL1, NRT1/PTR_CL2, NRT1/PTR_CL4, NRT1/PTR_CL5, NRT1/PTR_CL6, AMT_CL1, AMT_CL2, and PHT_CL2 in leaves, NRT1/PTR_CL1, NRT1/PTR_CL2, NRT1/PTR_CL6, and AMT_CL2 in roots, along with total_porosity and air_space of the growing media, showed a strong positive correlation (>70.0%) with PC1 (Table S2). All samples collected in February, positioned in the positive portion of PC1, were characterized by high values for these parameters. In contrast, P, N, Cu, NO_3^-, NH_4^+, and N_minerale_tot_mg/Kg in leaves, along with Zn in roots, showed a strong negative correlation (>−70.0%), representing the samples collected in April, positioned in the negative section of PC1 (Table S2, Figure 10).

Cation exchange capacity (CIC), available water capacity AWC, and water-filled porosity (WFS), as well as HAK/KUP/KT_CL2 in roots and Ca in leaves, exhibited a strong positive correlation with PC2, which represented B50 growing medium in both February and April, along with BP25B25 collected in April, all positioned in the positive portion of PC2. In contrast, a strong negative correlation with PC2 was noted for HAK/KUP/KT_CL2

in the leaves, which primarily characterized the BP25L25 growing medium positioned in the negative portion of PC2 (Table S2, Figure 10).

5. Conclusions

Based on physicochemical parameters and seedling growth, we conclude that peat can be partially substituted with conifer wood biochar for a total amount of 25–50%. The new growth medium for citrus nurseries allows growth rates to be comparable to those recorded for plants grown in conventional peat mixtures.

Further, we observed that the comparison between different sampling data (February and April) and tissues highlighted that all analyzed genes were highly expressed in leaves and roots in February. Conversely, in April, some genes were not expressed in both tissues. Generally, transcription levels were greater in the roots than in the leaves. Furthermore, comparing plants grown on different substrates, it is evident that the B50 growth medium has the highest expression.

The higher expression of ammonia and nitric nitrogen transporter genes suggests that substrates containing biochar compensate for nitrogen scarcity during the cold periods, owing to the earliest gene activation in February.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture15010113/s1, Figure S1: Phylogenetic analysis based on multiple sequence alignment. The evolutionary history was inferred using the Maximum Likelihood method and the Tamura−Nei model (56). Analyses were conducted using MEGA X (24). For each tree obtained (one for each macronutrient), it was possible to divide the analyzed sequences into clusters. The species is shown next to its accession number. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree with the highest log likelihood is shown. (A) Twenty-three sequences of ammonium (NH_4^+) transporters; (B) Forty sequences of nitrate (NO_3^-) transporters; (C) Twenty-six sequences of phosphorus (P) transporters; (D) Twenty sequences of potassium (K) transporters. Table S1: Nutrient name, accession numbers, cluster, Gene family, Orthologs from OrthoDB, function, ID *C. sinensis* DHSO v3.0 genome CDS of all genic sequences used in this study. Table S2: Principal component analysis (PCA) loadings expression values of the different genes analyzed and physical and chemical characteristics of three growing media, each containing 50% sandy volcanic soil and the remaining 50% being: biochar 50% (B50); black peat 25% + biochar 25% (BP25B25); black peat 25% + lapillus 25% (BP25L25), the physical and chemical properties, the biomass partitioning of Carrizo citrange seedlings, and the expression of genes involved in nitrogen, phosphorus, and potassium transport in leaves and roots. Data were collected in February (winter) and April (spring).

Author Contributions: Conceptualization, supervision, writing—original draft, P.C.; investigation, writing—review and editing, data curation, M.P.R.; formal analysis, writing—review and editing, M.A.; formal analysis, writing—review and editing, B.T.; resources, funding acquisition, writing—review and editing, G.F.; Investigation, writing—review and editing, G.L.C.; Writing—Review and Editing, C.L.; Writing—Review and Editing M.C.; visualization, A.C.; Conceptualization, supervision, writing—original draft, F.F. All authors have read and agreed to the published version of the manuscript.

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