Plant Science 312 (2021) 111048

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

It takes two: Reciprocal scion-rootstock relationships enable salt tolerance in 'Hass' avocado

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ARTICLE INFO

Keywords:
Grafting
Ionome
Lipidome
Metabolome
Persea americana

ABSTRACT

Commercial avocado orchards typically consist of composite trees. Avocado is salt-sensitive, suffering from substantial growth and production depreciation when exposed to high sodium and chloride levels. Salt ions penetrate the roots and are subsequently transferred to the foliage. Hence, understanding distinct physiological responses of grafted avocado plant organs to salinity is of great interest. We compared the ion, metabolite and lipid profiles of leaves, roots and trunk drillings of mature 'Hass' scion grafted onto two different rootstocks during gradual exposure to salinity. We found that one rootstock, VC840, did not restrict the transport of irrigation solution components to the scion, leading to salt accumulation in the trunk and leaves. The other rootstock, VC152, functioned selectively, moderating the movement of toxic ions to the scion organs by accumulating them in the roots. The leaves of the scion grafted on the selective rootstock acquired the standard level of essential minerals without being exposed to excessive salt concentrations. However, this came with an energetic cost as the leaves transferred carbohydrates and storage lipids downward to the rootstock organs, which became a strong sink. We conclude that mutual scion–rootstock relationships enable marked tolerance to salt stress through selective ion transport and metabolic modifications.

1. Introduction

Avocado (*Persea americana* Mill.) is a highly valuable crop, originating in Mesoamerica [1], with rapidly increasing global popularity [2]. Salt stress severely affects growth and productivity of avocado trees, being one of the most limiting factors for its cultivation through both osmotic and toxic mechanisms [3,4]. Similarly to other fruit trees, salinity damage in avocado, which is generally characterized by high Cl concentrations in leaves, includes growth retardation and yield reduction [5]. However, the threshold levels of Na and Cl in the soil solution that cause leaf necrosis are markedly lower in avocado compared to most other crops [6].

The response of avocado to conditions of increased salinity is becoming more relevant due to the expansion of its cultivation areas and the concomitant shortage of freshwater resources [7]. Reclaimed or recycled water, which tends to contain relatively high levels of salts, is becoming an important source for irrigation in many crops worldwide [8], including avocado.

Commercial avocado trees are produced by grafting the shoot of a fruit-bearing variety (scion) onto a rootstock with superior soil-related characteristics such as water use efficiency or stress tolerance [9]. To merge the transport systems of the two components of the trees, expertise and prior knowledge of their characteristics are required [10]. In order to thrive, the two components of a composite tree must cooperate, as the rootstock supplies nutrients and water from the soil via the roots, while the scion assimilates carbon and produces energy [11]. There are reports of mutual influences in avocado trees—scion on rootstock and vice versa [12,13].

Avocado rootstocks are known to differentially influence crop tolerance to salt stress [9,14,15]. There are three main genetic sources for avocado rootstocks, West India, Guatemala and Mexico, which have been reported to respond differently to salinity. Rootstocks originating from West India are most tolerant and those from Mexican origin most susceptible so salinity [16]. Salinity tolerance of avocado rootstocks involves the improvement of water use efficiency [17] and is related to differential translocation of chloride and other salts from roots to shoots

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https://doi.org/10.1016/j.plantsci.2021.111048
Received 4 April 2021; Received in revised form 24 August 2021; Accepted 28 August 2021
Available online 1 September 2021

[18,19]. Therefore, leaf nutrient content and salt accumulation in leaves are clearly influenced by the rootstock [20] and are expected to differ accordingly to their degree of salinity tolerance. Some non-tolerant rootstocks have been found to influence not only foliar concentration of toxic salts, but also the concentration of all nutrients in the leaves, therefore critically affecting tree growth rate and yield [21]. In previous studies, leaves from avocados growing on Mexican rootstocks were found to have higher concentrations of nitrogen (N) than those growing on Guatemalan rootstocks, but lower concentrations of magnesium (Mg) and calcium (Ca) [22]. Similar results were observed by Willingham et al. (2006) [71] who found higher N and potassium (K), and lower Ca and Mg in trees with Mexican compared to West Indian rootstocks. Chloride concentration was generally highest in the leaves of avocado trees grafted on Mexican rootstocks, compared with Guatemalan and West Indian [20]. Although seedling rootstocks still dominate commercial avocado production, vegetatively cloned (VC) rootstocks are gaining popularity, due to greater uniformity in tree development and performance, hence supporting superior expression of scion characteristics [23]. Commercial breeding programs of avocado are introducing new varieties for already a century [24]. Long-term programs exist in California [25], Australia [26], Israel [27] and other countries. Vegetative cloning improves breeding programs as it facilitates the selection and propagation of stress-tolerant rootstocks [28]. Rootstock–scion interactions broaden the genetic basis of avocado breeding, as some rootstock-dependent traits were found to be inherited to the tree progeny [29]. Metabolomics is an innovative methodology, in which the metabolic modifications of an organism are characterized relatively to its environment, specifying its stress responses [30]. Metabolite profiling of plants under salt stress has revealed an accumulation of known osmolytes, such as proline and glycine, as well as other primary and secondary metabolites [31,32]. Salt stress has been found to affect lipid peroxidation [33,34], and salt tolerance is highly correlated to alterations in membrane-lipid composition [35,36].

To the best of our knowledge, no metabolite or lipid profiling is available in the context of avocado's response to salinity, and the interaction between the physiological and metabolic mechanisms of avocado scion and rootstock have not previously been published. In fact, metabolomics has been applied mostly to avocado fruit [37–40], rather than its other organs. Our hypothesis was that salt tolerance mechanisms are a consequence of cooperation between the shoot and the root, and therefore should be reflected in the metabolic and nutritional status of the separate organs. The aim of our work was to trace the physiological relationships and interactions of avocado tree organs exposed to root zone salinity, by comparing the salt response of a single scion (Hass) grafted on either a relatively salt-tolerant or a salt-sensitive rootstock.

2. Materials and methods

2.1. Plant material and growing conditions

We studied two rootstocks, VC840 and VC152, onto which 'Hass' scions were grafted in 2011 and planted in 2013 at the Gilat Research Center, Israel (31°20′08.6″N 34°39′57.0″E). This area is defined with a semi-arid climate, with mean annual temperature of 20.9 °C and annual precipitations of 113 mm (according to Gilat agrometeorological station, The Ministry of Agriculture, Israel). While VC840 is of Mexican origin, VC152 is a West Indian rootstock.

2.2. Experimental design

The trees were planted at 4 × 3 m tree spacing with a plant density of 800 trees per hectare. The soil was sandy loam, with 15 % clay, 50 % sand, 35 % silt, 0.5 organic matter (0–5 cm), 0.73 dS/m EC, 11.5 % active CO3Ca, pH of 7.79 (saturated soil extract in 1:2.5 soil:H_2O). Na and Cl concentrations in the saturated soil extract were 22.27 and 17.62 mg/L, respectively, and sodium adsorption ratio (SAR) was 0.9. The trees were drip irrigated using emitters with flow rate of 1.2 L/h spaced every 0.25 m in a single lateral per tree row. During the eight weeks of the experiment, each tree was irrigated daily with 76.6 L. The orchard was fertigated daily according to local commercial practice with liquid fertilizer (Shefer™+3, Fertilizers & Chemicals Ltd., Israel), from March to October. The fertilizer solution contained 7 % N, 2 % P_2O_5 and 7 % K_2O, 300 mg/kg iron (Fe), 150 mg/kg manganese (Mn), 75 mg/kg zinc (Zn), 11 mg/kg copper (Cu), and 8 mg/kg molybdenum (Mo). Annual irrigation was 14,508 m^3/ha, and annual liquid fertilization was 2940 kg/ha. In 2019, an experiment was performed to test the response of the trees to continuous salinity exposure. From March 2019 and thereafter, NaCl was added to the irrigation solution to reach 280−300 mg/L, which resulted in an EC of irrigation water ranging from 1.25 to 1.52 dS/m throughout the experiment (Table S1). This level of salinity is considered harmful but not lethal to avocado [41,42]. Variation of the environmental conditions during the experiment (maximal and minimal air and soil temperatures) registered by the nearby agrometeorological station is shown in Fig. S1.

Fifteen 'Hass' trees grafted on VC152 and 15 additional trees grafted on VC840 rootstock were divided into five repetitions with three trees each, from which we sampled the plant material. The trees in each repetition were scattered throughout the orchard, following a randomized block design. On each sampling date, the composed pool of each repetition (15 leaves or 150 g roots each) was of the same three trees as on the other sampling dates. Diagnostic leaves (youngest fully expanded leaves) and last-order roots (the thinnest roots, located near drip irrigation emitters) were sampled from the trees at six dates: a day before the initiation of salinity treatment (0 days of salinity – 0D), three days after initiation of the salinity treatment (3D), and four more times at two-week intervals (10D, 24D, 38D, 52D. Table S1). This experimental set-up allowed us to track the gradual response to cumulated salt exposure of individual trees. All sampled organs were equal in age and visual conditions, to be comparable between the rootstocks and the sampling dates. Damaged leaves were not sampled. Sixty days after the initiation of the salinity treatment, on a bright sunny day, midday net assimilation rates (A) and stomatal conductance (gs) were measured by a CIRAS-3 portable photosynthesis system (PP Systems) in intact mature leaves of one tree per repetition to assess the photosynthetic performance of each rootstock. The average intercellular CO_2 concentration (Ci) was 246 μmol mol^{-1} and the average VPD was 4.9 kPa. To evaluate the scion-rootstock compatibility, trunk drillings, 3 cm deep, were taken five months after the onset of the salinity treatment, 5 cm below the graft union and 15 cm above it. During August 2019, leaf area index (LAI) was measured by a portable ceptometer (AccuPAR LP-80). To estimate the vegetative development of the tree, the Photosynthetic Active Radiation (PAR) sensor was placed in a sunny point, one meter height, and then inserted into the canopy of each tree, close to the trunk, at the same height. LAI value was calculated by the ceptometer, as the ratio between PAR measurements in both places. Simultaneously to LAI measurements, a salt damage survey was conducted, ranking each tree from 0 to 3 accordingly to the predominant visual symptoms of the leaves in response to salinity. 0: no visual symptoms (Fig. 1A), 1: moderate salt damages (Fig. 1B), 2: severe salt damages (Fig. 1C) and 3: defoliation of most leaves."

2.3. Ionome profile

The samples of leaves, roots and trunk drillings (mostly xylem, as the bark was removed) were dried at 80 °C for 48 h, ground and analyzed for mineral concentration. Chloride concentration was determined based on water extraction (0.1 g dry matter in 10 mL deionized water), using an MKII chloride analyzer 926 (Sherwood). Nitrogen and P were analyzed using Gallery Plus Discrete Analyzer (Thermo Scientific) after digesting the powdered material with sulfuric acid and hydrogen peroxide (Snell and Cornelia Snell 1954). Other nutrients (boron (B), Na, Fe, K, Mn, Ca, Mg and Cu) were determined by digesting the powdered material with

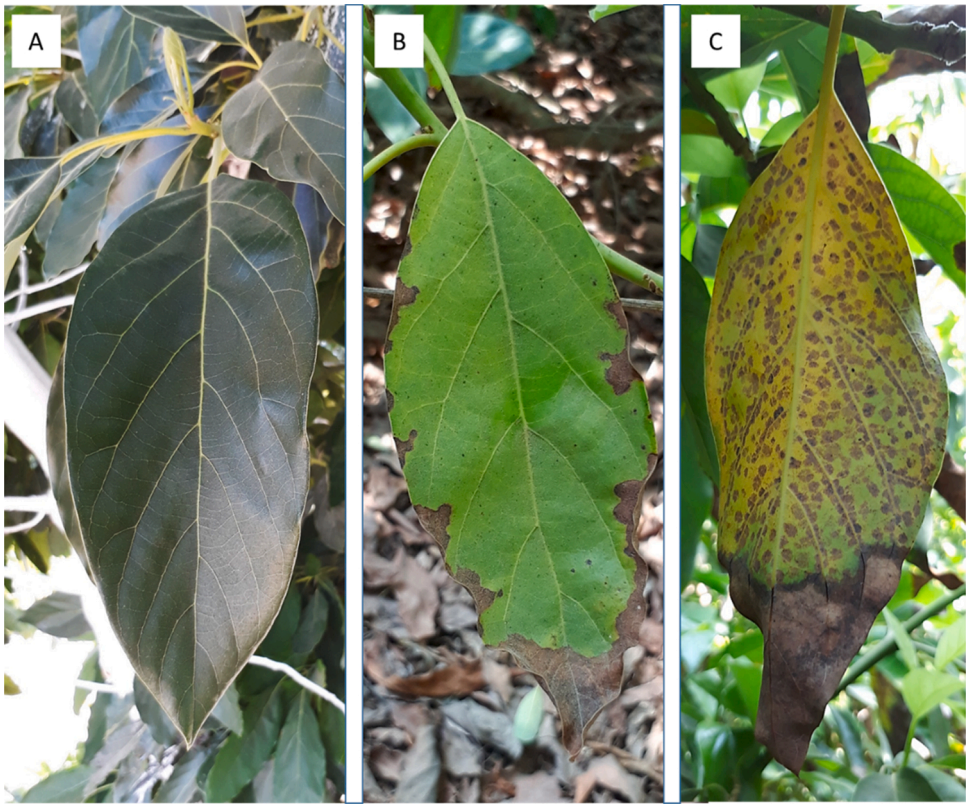

Fig. 1. 'Hass' avocado leaves. A: a healthy leaf. B: a moderate salt damage. C: a severe salt damage.

nitric acid and hydrogen peroxide and analyzing in an ICP-OES 5100 (Agilent Technologies).

2.4. Metabolome and lipidome profiles

On each sampling date, five samples per rootstock (one per block), containing 15 leaves or 150 g roots each, were immediately frozen in liquid nitrogen, then ground and stored at -80 °C. Metabolites and lipids were extracted using the MTBE method described by [43]. The organic phase run through a GC–MS or UPLC−MS C-18 column for organic acid and lipid detection, respectively. The polar phase was submitted to GC–MS for primary metabolite (PM) detection. Following MSTFA derivatization, GC–MS analysis was performed using an Agilent 6850 gas chromatograph coupled with a 5975C mass spectrometer. GC was performed in a 30 m x0.25 mm x0.25 μm HP-5 MS column (J&W Scientific) and the samples were analyzed using split mode 1:50. MassHunter software (Agilent) was used for compound analysis, by comparing to the NIST 14 library or authentic standards. The metabolite response values were normalized to the internal standard ribitol. For lipids, positive and negative ionization modes were used to acquire the mass spectra, in an Exactive mass spectrometer (Thermo-Fisher, http://www.thermofisher.com). Chromatogram peak detection and integration were processed by REFINER MS 10 (Genedata, http://www.genedata.com). Selected features were annotated using an in-house lipid database.

2.5. Statistical analysis

To evaluate the cumulated effect of the salinity treatment on each rootstock, repeated measures ANOVA and hierarchical clustering analysis were carried out by the JMP®14.0.0 software (SAS Institute Inc.). The mineral concentrations, sugar ratios, photosynthetic indices, salt damage ranks and LAI were analyzed by one-way ANOVA. Homogeneity of variance was checked by the Levene's test, and Tukey-Kramer test was used to compare all treatments by rootstocks at $p \leq 0.05$. For metabolome and lipidome analyses, false discovery rate (FDR) method was used to calculate significance, with separation between metabolic groups (e.g. sugars, storage lipids etc.).

Please note: Throughout the manuscript, we use the rootstock names - VC152 and VC840 - when referring to either roots, trunks or leaves. However, the leaves and the upper part of the trunk were genetically identical and belong to the 'Hass' scions grafted above the rootstocks. Using the rootstock names is for distinguishing purposes only.

3. Results

Two-factorial repeated measures analyses revealed significant interaction between the rootstocks and salinity treatment, therefore we proceeded to separate analysis of each factor.

3.1. Ionome profile

Root Na levels in both rootstocks increased gradually during the salinity treatment (Fig. 2A). A significant difference in Na root levels was found between the rootstocks at 0, 3, 10 and 52 days of salinity application, higher for VC152 than for VC840 (Fig. 2A). Leaf Na levels did not change significantly in VC152 during the experiment, whereas in VC840, a significant increase was found 52 days into the salinity treatment, with higher levels compared to VC152 (Fig. 2C). Leaf Na levels were 10–100 times lower than in the roots, in both rootstocks. Chloride levels in roots increased gradually in VC152, but did not change significantly in VC840 (Fig. 2B). Before initiation of salinity treatment, root Cl level was significantly higher in VC840 than in VC152. Three days into the salinity treatment, the opposite was observed (Fig. 2B). At later points in time, no significant differences were found between the rootstocks. Leaf Cl level in VC152 increased significantly after 52 days of salinity. In VC840, the level increased gradually and was significantly

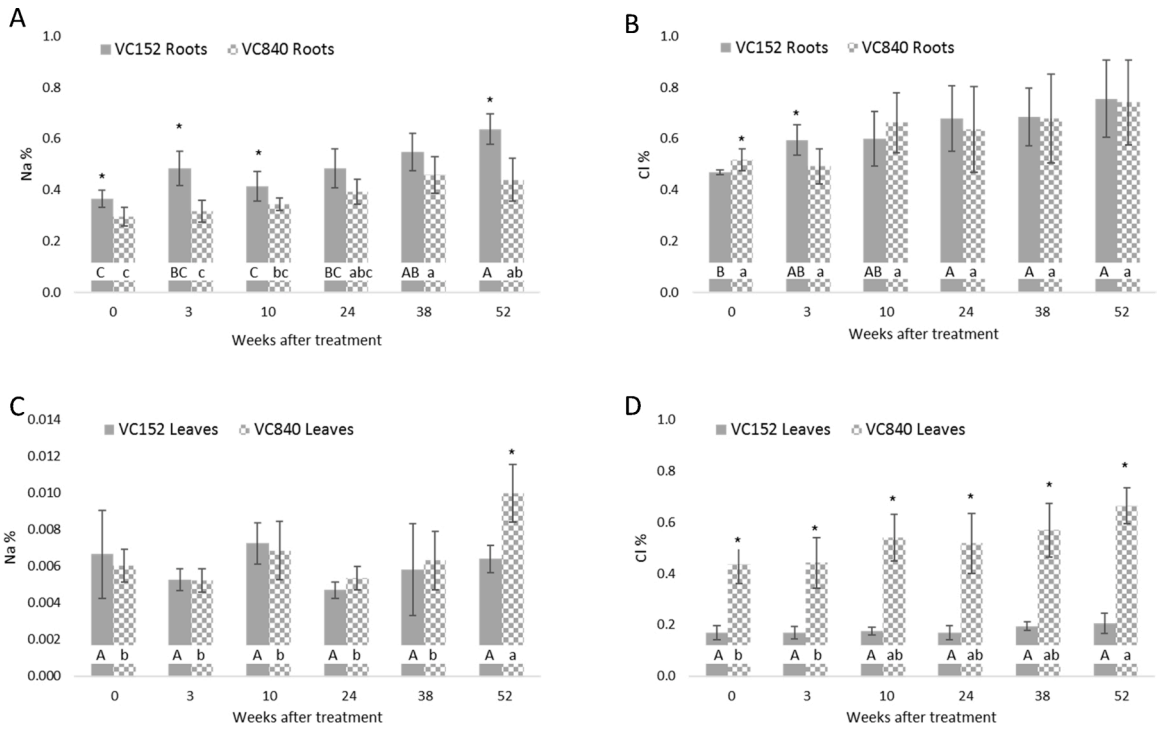

Fig. 2. The concentration of Na and Cl, before and during salinity treatment. (A, B) Last-order roots. (C, D) Mature diagnostic leaves. 0–52: days of saline irrigation. Bars represent SD. Asterisk (*) indicates a significant ($p < 0.05$) difference between the rootstocks on a specific sampling date. Different letters indicate significant differences between treatments for the same rootstock. n=5.

Table 1
Mean values (n = 5) of mineral concentrations in leaves and roots of avocado trees at the beginning of the experiment (day 0) and percentages of increase or decrease after the exposure to salinity conditions from 0–52 days.

Rootstock	VC152											
Organ	Leaves						Roots					
Salinity days	0	3	10	24	38	52	0	3	10	24	38	52
Cl, %	0.17	101	103	100	115	122	0.47	**127**	**128**	**145**	**146**	**161**
Na, %	0.01	79	109	71	88	96	0.37	**132**	113	**132**	**150**	**174**
N, %	1.31	95	94	96	98	100	1.06	98	**89**	91	102	104
P, %	0.11	101	94	97	96	89	0.30	116	**83**	**71**	**73**	87
K, %	0.87	110	103	95	103	**91**	1.06	103	100	101	92	**87**
Ca, %	2.25	100	112	**128**	**138**	**134**	0.93	99	117	**87**	120	**143**
Mg, %	0.68	94	104	**115**	**116**	**112**	0.28	**116**	98	83	93	102
S, %	0.24	98	105	**115**	**115**	113	0.18	**123**	95	98	105	**111**
B, ppm	32.53	102	100	105	103	**131**	44.73	97	90	89	96	92
Cu, ppm	7.76	103	105	**117**	–	**135**	22.04	103	104	89	126	**162**
Fe, ppm	169.5	90	101	**121**	–	115	681.7	70	144	59	105	137
Mn, ppm	235.2	91	109	131	**136**	**138**	46.12	110	118	105	120	**127**
Zn, ppm	14.87	100	105	106	111	100	29.99	105	94	**72**	100	99
Rootstock	VC840											
Organ	Leaves						Roots					
Salinity days	0	3	10	24	38	52	0	3	10	24	38	52
Cl, %	0.44	101	**124**	**119**	**131**	**153**	0.52	95	**128**	123	**132**	**144**
Na, %	0.01	87	114	89	105	**166**	0.30	107	**116**	**133**	**155**	**149**
N, %	1.60	89	101	92	94	**81**	0.91	95	93	94	104	101
P, %	0.13	100	103	102	**89**	**77**	0.20	98	91	90	89	76
K, %	0.85	100	98	86	83	**77**	1.07	**84**	104	94	96	**73**
Ca, %	2.14	103	106	**119**	**137**	**126**	1.06	109	111	95	109	122
Mg, %	0.56	101	103	**109**	**113**	107	0.20	92	109	91	93	94
S, %	0.26	106	**112**	**119**	**126**	**121**	0.13	108	103	114	**122**	104
B, ppm	34.09	103	111	118	123	121	42.17	86	102	99	99	94
Cu, ppm	8.74	90	103	111	–	**123**	18.17	115	117	120	140	115
Fe, ppm	165.6	94	108	**131**	–	**132**	565.7	96	**207**	108	139	175
Mn, ppm	130.8	105	106	114	125	124	39.23	106	**137**	104	117	137
Zn, ppm	18.52	103	102	106	109	84	21.65	100	91	92	112	89

Significant differences ($p < 0.05$) between day 0 and a given day are in bold. Values underlined indicate significant differences between rootstocks on the same treatment date and in the same organ. Leaf Cu and Fe for 38 days are not shown due to technical problems.

higher (2–3 times) than in VC152 throughout the experiment (Fig. 2D).

Ionome analysis of leaves and roots of both rootstocks revealed significant changes in the levels of several minerals other than Na and Cl during the experiment (Table 1). Unlike Fig. 2, in Table 1 each sampling date is compared with the "0 days" treatment. Nitrogen, P and K decreased significantly in VC840 leaves; in VC152 leaves, only K decreased significantly, after 52 days. Fe, Cu, sulfur (S), Mg and Ca increased significantly in the leaves of both rootstocks; B and Mn only increased significantly in VC152 leaves. There was no significant change in root N levels for either rootstock, except a temporary decrease in VC152 roots, 10 days into the treatment. Phosphorus only decreased significantly in VC152 roots. Potassium decreased significantly in roots of both rootstocks. Sulfur and Mn increased significantly in the roots of both rootstocks. In VC152, Ca and Cu also increased. A comparison of mineral levels between rootstocks revealed that roots of VC152 had significantly higher levels of most minerals, regardless of the salinity treatment. The leaves showed the opposite pattern, where most minerals were higher in VC840, with the exception of Mg and Mn, which were higher in VC152 leaves during most of the experiment, and Ca and K, which were higher at the end of the experiment.

The mineral concentration in the avocado trunk below and above the graft union was determined under the salinity treatment for both rootstocks. A few significant differences were found between trunk locations within each rootstock (Table 2). In VC152, Na, Cu and Fe were significantly higher below the graft union (rootstock), whereas Ca and Mn concentration were higher above the graft union (scion). In VC840, only Na showed a significant difference between the trunk areas, being significantly higher above the graft union. Sodium concentration was markedly higher in VC840 than in VC152, both in the scion and the rootstock. In VC152, K and Mn were higher in both trunk parts. Phosphorus and Ca were higher in the scion part but not below the graft union.

3.2. Metabolome profile

Hierarchical clustering of all annotated primary metabolites (PM) of leaves and roots revealed a clear distinction between samples that were taken before and after the salinity treatment (Fig. 3). Among the leaves, second-degree clustering was observed between the rootstocks. Among the roots, the distinction between rootstocks was present only after the salinity treatment.

The salinity treatment did not similarly affect the metabolite levels in the leaves and roots of each rootstock (Table 3). Thus, before and after the salinity treatment, perseitol in the roots was higher in VC152 than in VC840. However, under non-saline irrigation (0 days) fructose and glucose levels were higher in the leaves of VC152, but after 52 days of saline irrigation these levels were substantially higher in VC840's leaves. Ethylene glycol, myo-inositol and mannoheptulose were higher in VC840 leaves after the salinity treatment as well, compared with VC152. TCA cycle elements were higher in both the leaves and roots of VC152.

These results evidence that metabolite's level of the leaves and roots of each rootstock are affected by salinity treatments in a different manner, which translate into significant differences among rootstocks in the levels of specific metabolites after 52 days of salinity exposure (Table 3). Changes in metabolite profile of both rootstocks due to salt exposure are detailed in Fig. 4, with the background of major metabolic pathways in plants. TCA cycle elements showed the most contradictory response to salinity of the rootstocks, both in roots and leaves. In both rootstocks, most of the sugars decreased in the leaves in response to salt exposure. However, sucrose did not change in VC152's leaves. Most amino and organic acids increased. Pyruvate decreased in leaves of both rootstocks. Citrate decreased in VC840's leaves but remained stable in the leaves of VC152. Succinate decreased in VC152's leaves and increased in VC840's leaves. Fumarate increased only in VC840's leaves. Malate decreased only in VC152's leaves. The osmoprotectant hydroquinone [44] increased in the leaves of both rootstocks after salt exposure, and this was also the case for most phenols. Cumarate and ferulate decreased in leaves of VC152 but increased in leaves of VC840. The roots hardly responded to the salt by metabolic modifications. There was a decrease in most sugars in both rootstocks, an increase in aspartate, succinate and pyroglutamate in VC152, and a decrease in malate in VC840. Succinate increased in VC152's roots and did not change in VC840's. Malate decreased in the roots of VC840.

When the leaf/root ratio of specific sugars was considered under non-saline irrigation (0 days of salinity), in both rootstocks fructose, glucose and sucrose were higher in leaves compared to roots (Table 4). In VC152, perseitol, the major carbohydrate in avocado, and mannoheptulose, the perseitol's precursor, were evenly distributed between the two organs. However, in VC840 mannoheptulose was higher in the roots but perseitol did not differ between both parts of the tree. After 52 days of salinity exposure, the leaf/root ratio of these carbohydrates changed considerably in comparison with the non-saline conditions. In VC152, relative proportions of fructose, sucrose and perseitol between tissues were barely affected by 52 days of irrigation with saline water, but glucose showed a greater decrease in the leaves than in the roots which lowered their ratio significantly (Table 4). In VC840, fructose, glucose and perseitol significantly decreased more in the roots compared with the leaves. The ratio change in sucrose was significant at $p = 0.08$ but not 0.05. The changes in mannoheptulose levels following the salinity exposure were not significant in both rootstocks. After 52 salinity days, the leaf/root ratio in VC840 of fructose, glucose, sucrose and perseitol was significantly higher than in VC152 (Table 4).

Table 2
Mean values (n = 5) of the mineral concentration of avocado trunks 5 cm below (RS) and 15 cm above (scion) the graft union, under salinity treatment.

	VC152		VC840	
	RS	Scion	RS	Scion
Cl, %	0.08	0.06	0.07	0.07
Na, %	**0.04**	0.01	0.19	**0.26**
N, %	0.30	0.25	0.22	0.24
P, %	0.04	0.05	0.04	0.04
K, %	0.47	0.49	0.29	0.34
Ca, %	0.34	**0.39**	0.32	0.35
Mg, %	0.05	0.05	0.05	0.05
S, %	0.03	0.05	0.04	0.05
B, ppm	143.74	144.80	144.88	147.40
Cu, ppm	**3.96**	3.40	3.89	3.99
Fe, ppm	**114.62**	80.55	111.60	85.03
Mn, ppm	17.09	**20.60**	13.75	15.47
Zn, ppm	7.78	7.46	6.39	7.33

Significant ($p < 0.05$) differences between trunk locations within each rootstock (VC152 or VC840) are in bold. Significant differences between rootstocks for the same trunk location are underlined.

3.3. Lipidome profile

Table 5 presents the lipid profiles in our experiment. Lipids were manually clustered by their functional identity: storage, plastids, and other membrane lipids. Most plastid lipids quantified in the leaves of both rootstocks were chloroplast related. 28.6 % of chloroplast-related lipids increased significantly in VC152 leaves upon salinity exposure, while 20 % decreased in VC840 leaves. In VC152 leaves, there was a significant increase in 97 % of storage lipids after three days of saline irrigation, but later in the experiment, almost 70 % of them decreased significantly. In VC840 leaves, 72 % of the storage lipids increased after the early exposure period, and only 13–15 % decreased during the salinity treatment. Almost 30 % of membrane lipids decreased in VC840 leaves. In the roots of VC152, 72 % of storage lipids decreased under the salinity treatment.

Several lipids were significantly higher in the leaves or roots of one rootstock compared to the other, after 52 days of salinity treatment. At

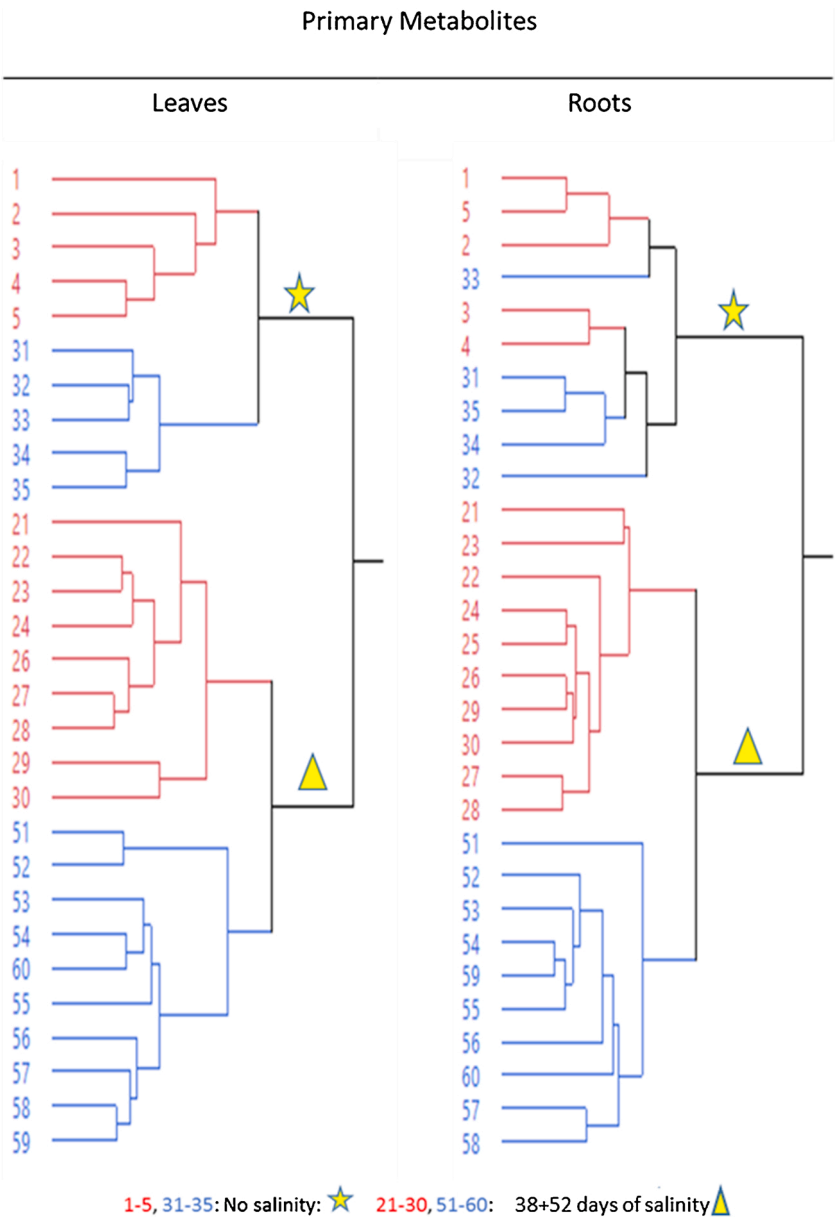

Fig. 3. Hierarchical clustering of primary metabolites in leaves and roots of 'Hass' avocado trees, grafted on two different rootstocks. Red: VC152, blue: VC840. 1–60: all samples from the experiment (biological repetitions) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the end of the experiment, 14.3 % of chloroplast-related lipids and 28.6 % of membrane lipids were higher in VC152' leaves, while 46.2 % of storage lipids were higher in VC840's leaves. In the roots, after 52 days of salinity VC152 had significantly higher lipid level compared with VC840, in all three classes.

After 60 days of salinity exposure, stomatal conductance and carbon assimilation were significantly higher in VC152's leaves, compared with VC840 (Fig. 5A, B). After five months of salinity exposure (during August 2019), a visual salt damage survey in the leaves revealed significantly higher level of damage symptoms (Fig. 1) in VC840's leaves compared with VC152 (Fig. 5C). The LAI in VC152 trees was significantly higher than in VC840 (Fig. 5D).

4. Discussion

This study provides new insights on the mechanisms operating in avocado salt tolerance by integrating, for the first time, metabolic approaches and physiological mechanisms with scion-rootstock relations.

In non-tolerant avocado plants, salt stress causes visual damage to the leaves and physiological and growth impairments [45], which are associated with nutritional disorders stemming from the salinity effects on nutrient uptake, availability, transport and distribution within the plants [46]. In this sense, it is known that the rootstock is a key factor influencing avocado response to salinity by affecting scion physiology [4,14] and leaf ionome [20]. The comparative analysis of the salinity response of 'Hass' avocado grafted on two selected rootstocks, VC840 and VC152, showed great differences in their susceptibility to salt stress, as revealed the appearance of visual damage in the leaves of VC840 compared to VC152 and the lower LAI in VC840 trees. This different susceptibility could be associated to: i) a different ability for excluding or including selectively Na and Cl ions as well as other nutrients from or in the roots; ii) a different capacity for nutrient distribution within the tree organs; iii) a distinct metabolite profile under non-saline conditions

Table 3
Metabolites that were significantly ($p < 0.05$) higher in one rootstock compared to the other, in leaves or roots, under either non-saline irrigation (0D) or 52 days of salinity (52D). Metabolites are ordered alphabetically.

Leaves			Roots		
Metabolite	0 Days	52 Days	Metabolite	0 Days	52 Days
Citric acid		VC152	3-Hydroxy-3-methylglutaric acid	VC152	VC152
Erythrono-1,4-lactone		VC840	Catechin	VC152	VC152
Ethanolamine	VC840	VC840	Cyanamide		VC152
Ethylene glycol		VC840	Fumaric acid		VC152
Fructose	VC152	VC840	Malic acid		VC152
Fumaric acid		VC152	Monomethyl phosphate		VC152
Galacturonic acid		VC152	Perseitol	VC152	VC152
Gluconic acid	VC152	VC152	Threonic acid		VC152
Glucose	VC152	VC840			
Glycerol	VC840				
Malic acid	VC152	VC152			
Mannoheptulose		VC840			
Methyl galactoside		VC840			
Myo-inositol		VC840			
Quininic acid	VC152	VC152			
Serine	VC840	VC840			
Threonic acid	VC152	VC152			

and/or in response to salt stress.

Prior to exposure to salinity, VC840, a Mexican rootstock, showed higher levels of Cl in its roots and leaves. Other minerals that were higher in the leaves were N, P, S, Cu and Zn. In contrast, VC152, a West Indian rootstock, had higher levels of Na, N, P, Mg, S and Zn in its roots, and higher levels of Mg and Mn in its leaves. This is in consonance with the different origin of the rootstocks [20]. The lower concentration of 46 % of the tested minerals in VC152's leaves could be attributed to greater scion-rootstock incompatibility, which could affect transport and nutrient distribution in the tree resulting in dissimilar accumulation above and below the graft union. However, the analyses of the trunk drill mineral concentrations revealed that this is not the case in VC152, as we did not find significant accumulation of most minerals below the graft union. Moreover, all minerals reached the standard threshold as suggested by the University of California (http://ucavo.ucr.edu/General/LeafAnalysis), and no deficiency was detected in VC152's leaves.

Under saline conditions there was a gradual accumulation of Na and Cl in both rootstocks' roots, and after 52 days of salinity there were higher levels of Na, Ca, S, Cu and Mn in VC152's roots. The leaves of VC840 had higher levels of Cl, Na and S, and suffered from visual salt damage, while those of VC152 had higher levels of K, Ca, Mg and Mn and had a healthy appearance. It is remarkable that foliar application of K, Ca and Mg, the same minerals that were higher in VC152's leaves, was found to mitigate salt stress in strawberry [47]. Mg and Mn were higher in VC152's leaves even before the onset of salinity treatment. Mg is an essential part of the chlorophyll molecule [48], and its high level in VC152's leaves is in line with their higher photosynthesis rates. Mn is an activator and cofactor of several enzymes [49], and its salt-induced deficiency negatively affects plant development [50,51]. As minerals are absorbed by the roots and transported upward, the change in mineral concentrations between the rootstocks' leaves is a reflection of the root response to the salinity exposure. The gradual increase in S concentration within VC840's leaves is of a specific interest, as S application

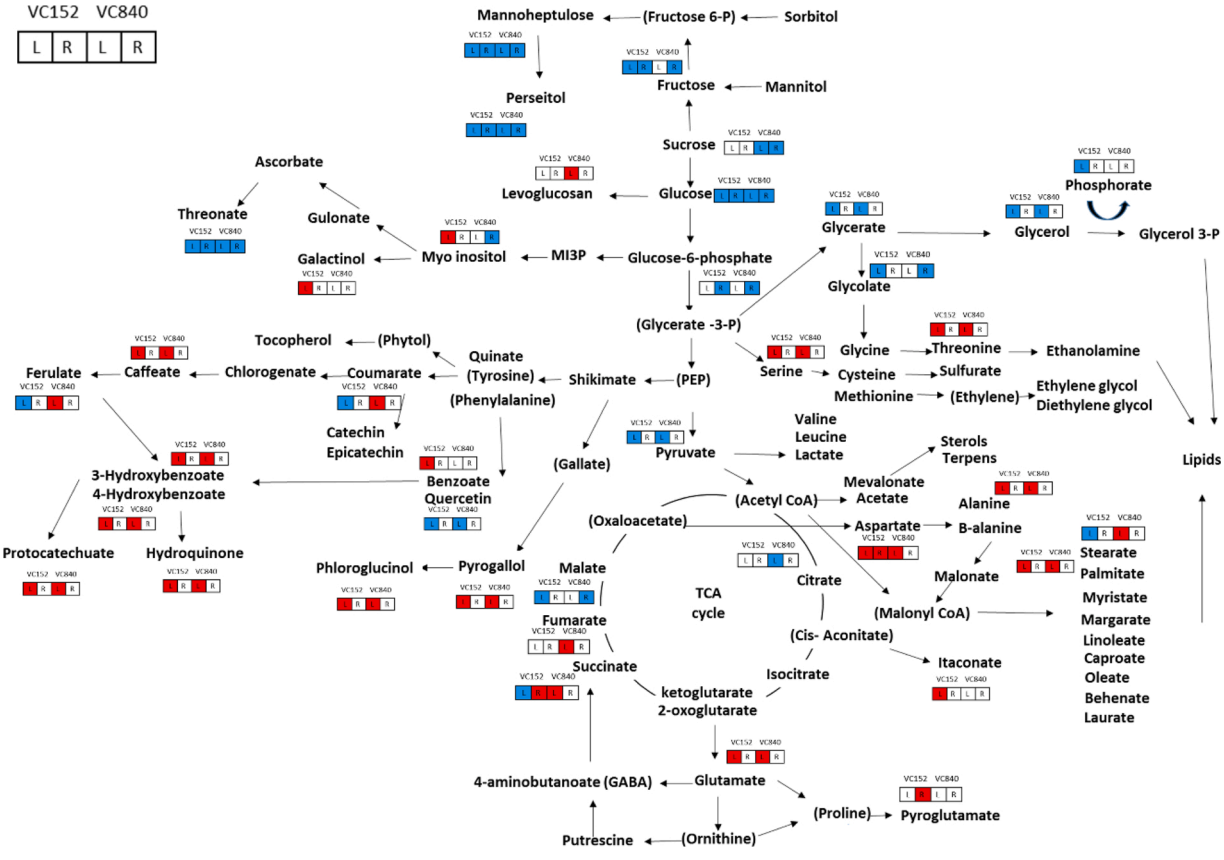

Fig. 4. Metabolic pathways in the leaves and roots of 'Hass' avocado trees grafted on two different rootstocks: VC152 and VC840. Metabolites which were significantly ($p < 0.05$) influenced by the salinity treatment (38-52 days of salt treatment) are marked with boxes panel. L: leaf. R: root. Red: increased; blue: decreased; empty: did not change significantly. Metabolite name between parentheses: was not detected (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4
The leaf/root ratio (mean ± SD; n = 5) of sugar intensity in each rootstock at 0 days and 52 days after salinity exposure. Within the same rootstock, significant ($p < 0.05$) differences between salinity treatments are in bold. Significant differences between rootstocks under the same salinity treatment are underlined.

Rootstock	Salinity days	Fructose	Glucose	Sucrose	Mannoheptulose	Perseitol
VC152	0	4.3 ± 2.8	**35.9**±17.5	33.4 ± 10.4	0.9 ± 0.6	1.1 ± 0.4
	52	5.4 ± 1.7	4.5 ± 3.1	38.4 ± 16.5	0.4 ± 0.2	1.2 ± 0.5
VC840	0	2.4 ± 0.5	34.3 ± 16.6	61.6 ± 28.0	0.4 ± 0.1	1.2 ± 0.4
	52	**10.2**±5.5	**84.9**±41.0	114.5±51	0.9 ± 0.9	**2.2**±0.4

Table 5
Variation in lipid profiling in leaves and roots of 'Hass' avocado grafted onto VC152 or VC840 during the salinity exposure.

		Leaves						Roots					
		0	3	10	24	38	52	0	3	10	24	38	52
Plastid-related lipids[1]													
VC152	↑		88.6	0	0	11.4	28.6		17.6	2.9	0	0	5.9
	↓		0	0	0	0	0		11.8	35.3	52.9	32.4	41.2
								(32)	*(15)*	*(6)*	*(12)*	*(21)*	*(32)*
VC840	↑		25.7	0	2.8	2.8	8.6		35.3	2.9	2.9	0	0
	↓		0	0	0	14.3	20		8.8	8.8	14.7	29.4	47.1
Storage lipids[2]													
VC152	↑		97.4	0	0	0	0		6.2	0	0	3.1	4.6
	↓		0	0	0	66.6	69.2		0	4.6	66.2	66.2	72.3
		(3)						*(23)*	*(22)*	*(6)*	*(5)*	*(3)*	*(22)*
VC840	↑		71.8	0	2.5	5.1	7.7		29.2	0	0	1.5	1.5
	↓		0	0	0	15.4	12.8		0	0	0	0	24.6
			(3)	*(8)*	*(56)*	*(28)*	*(46)*		*(5)*	*(5)*	*(3)*		
Membrane lipids[3]													
VC152	↑		92.8	0	0	21.4	21.4		6.7	0	0	10	13.3
	↓		0	0	0	0	25		6.7	0	23.3	13.3	20
			(11)	*(4)*		*(4)*	*(29)*	*(10)*	*(3)*			*(3)*	*(50)*
VC840	↑		10.7	0	0	3.6	0		3.3	0	0	3.3	3.3
	↓		0	0	0	3.6	28.6		3.3	0	6.7	13.3	43.3
		(4)			*(18)*								

Each value represents the percentage of lipids that were significantly ($p < 0.05$) higher (↑) or lower (↓) than the value of non-salinized trees (0 days). Values within parenthesis indicate the percentage of lipids that were significantly higher in one rootstock compared to the other on each sampling time. The lipids were clustered by their functional identity: plastid-related, storage and other membranes. In leaves, plastid lipids were mostly chloroplast-related.

[1] Classes: Monogalactosyldiacylglycerol (MGDG), Digalactosyldiacylglycerol (DGDG), Sulfoquinovosyl diacylglycerol (SQDG).

[2] Classes: Diacylglycerol (DAG), Triacylglycerol (TAG).

[3] Classes: Phosphatidylglycerol (PG), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE).

was found to improve the growth of several crops under salt stress [52, 53]. Sulphur-containing compounds such as sulfolipids and the amino acids methionine and cysteine are known to promote or modify physiological processes under abiotic stress conditions, and specifically salinity [54]. However, the sulpholipids decreased in VC840's leaves under salinity, these amino acids' levels were stable, and no salt-tolerance mechanism was detected in this rootstock [15]. The fact that Na accumulated in the trunk drillings (below the graft union in VC152 and above it in VC840) is in line with results from grapevine, where Na was found to be accumulated mostly in woody tissues [55]. Sodium was also found to be high in *Pistacia* tree wood compared to its bark [56]. It is worth noting that K was significantly higher in VC152's trunk and leaves, whereas Na was higher in VC840's trunk and leaves. This result reflects the known competition among K and Na ions for the same root transporter [57].

As salt exposure has long been known to affect metabolism in plants [58], and several studies have revealed the involvement of specific metabolites in salt tolerance [3] and references therein), the next step was to analyze the root and leaf metabolomes of both rootstocks, before and after salt exposure. Under non saline conditions there was almost no difference in the root metabolite profile of the rootstocks, as was reflected in the clustering presented in Fig. 3. Out of all PMs that were detected, only three (3-Hydroxy-3-methylglutaric acid, catechin and perseitol) were significantly higher in VC152's roots under non-saline conditions. This result indicates that the metabolic difference in the roots PM levels stems from the salt stress and not because they belong to different rootstocks. However, in the leaves there were several significant differences between the rootstocks and they clustered by the rootstock identity even under non-saline conditions, which is interesting as in both cases the leaves belonged to 'Hass'. This finding suggests that there is a constant effect of the rootstocks over the metabolic function of the leaves. In response to salt stress, there were metabolite shifts in the roots and leaves of both rootstocks. The general response to salinity exposure had a similar pattern in both rootstocks; e.g. accumulation of phenols or degradation of sugars. However, VC152's roots exhibited higher level of such shifts than VC840's, which might reflect higher metabolic activity or metabolite mobilization from other organs. The ratio between the plant organs' carbohydrates was significantly different between the rootstocks only under salinity conditions. Sugar accumulation serves for osmotic adjustment in plants exposed to salinity [59]. In the absence of salt, VC152 maintained higher levels of leaf osmolytes such as fructose, glucose and malate [60], displaying a constant state of stress-preparedness. A similar anticipatory situation was reported in a comparison of *Arabidopsis* with its halophytic relative *Thellungiella halophila*, the latter maintaining high levels of osmolytes even when no salt was present [61]. After salt exposure, leaves of VC840

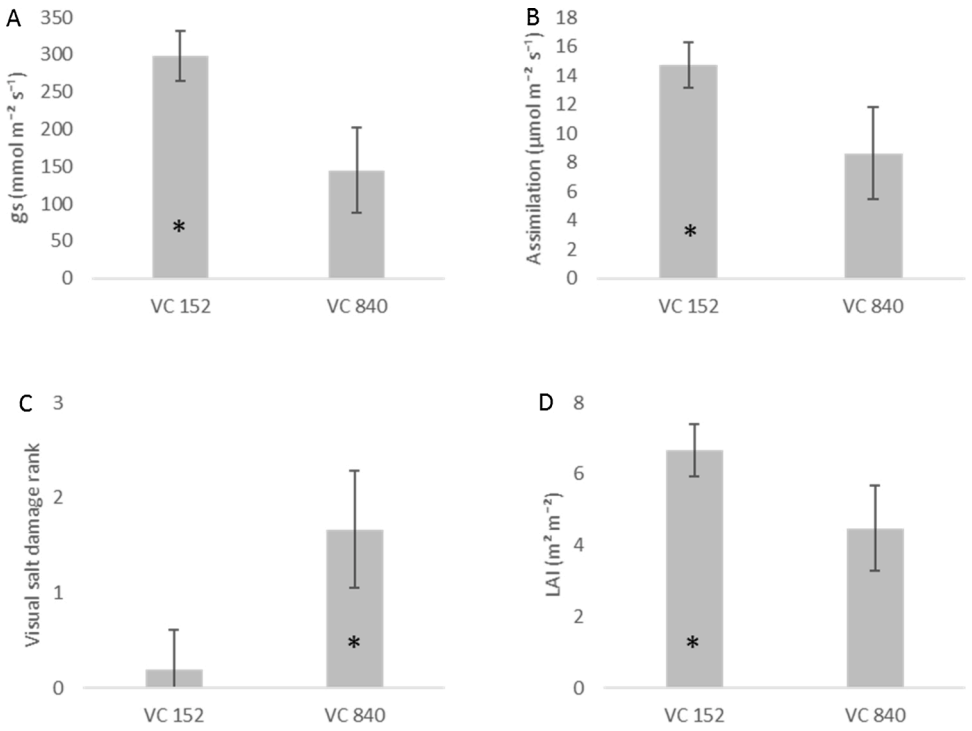

Fig. 5. Salinity effects on avocado 'Hass' trees grafted onto VC152 or VC840 after 60 days of salinity exposure. (A) Leaf stomatal conductance, (B) Leaf carbon assimilation rate (C) Index of visual damage, (d) Leaf area index (LAI). Each bar is the mean ± SD (n = 5). The asterisk (*) indicates significant differences between rootstocks ($p < 0.05$).

had higher levels of glucose and fructose than those of VC152, changing the leaf/root sugar ratio. It is interesting to notice that although perseitol's leaf level was not significantly different between rootstocks, in VC840's leaves there was a significant increase in its leaf/root ratio under salt stress. Combined with the general decrease in perseitol's level under salinity in both rootstocks, this difference in its leaf/root ratio reflects greater decrease of perseitol in VC840's roots, compared with VC152's. We found that the glucose leaf/root ratio significantly decreased in VC152 after the salt exposure. This change can be explained either by lower sugar production, carbohydrate transportation from the leaves to the roots or metabolism within the roots. As the carbon assimilation rates were higher in VC152 leaves, and the fructose leaf/root ratio was not changed, we assume that glucose specifically was transferred to the roots. This assumption is supported by the fact that the main photosynthetic sugars in avocado that are phloem-mobile are mannoheptulose and perseitol [62], which are broken into glucose for energy generation [63]. In VC840, however, the leaf/root proportions of most sugars increased after salt exposure, signifying greater sugar decrease in its roots.

Transcriptomic analyses have suggested that the metabolism of specific lipids is enhanced by salt stress [64,65]. We found a significant increase in chloroplast-related lipids in VC152's leaves and a decrease in their level in VC840 under salt stress. This result is in line with the higher photosynthesis rates in VC152's leaves and strengthens the hypothesis that the decrease in sugar levels is due to carbohydrates transport, rather than an inhibition of sugar biosynthesis. A decrease in chloroplast quantity and disorganization of the chloroplast membranes are known salinity responses in plants [66]. Therefore, the difference in chloroplast-related lipids' levels between the rootstocks supports the opposing Cl levels in their leaves. The storage lipids, which are energy reserves [67], decreased in the leaves of both rootstocks after salt exposure, similarly to the carbohydrates discussed previously. Lipids were found to be translocated from the leaves by the phloem sap in Arabidopsis [68] and canola [69], and we suggest that this was the pathway through which the lipids were transferred from VC152's leaves, leading to higher lipid levels in the roots compared with VC840. The membrane lipids in VC152's leaves were divided by subclass; encouraging higher membrane fluidity through an increase in the non-saturated forms of the subclass and a decrease in the saturated forms. An enhancement in membrane fluidity enables modification and repair of the physiological damage caused by abiotic stress conditions [70].

When comparing PM and lipid levels between the rootstocks, the metabolic difference that might explain the distinct responses to salt is better exposed. Without salinity exposure, leaves and roots of VC152 had higher levels of energy resources including carbohydrates, TCA cycle elements and storage lipids compared to VC840. Upon salt exposure, the leaves of VC152 seemed to transfer these energy reserves, which became higher in its roots compared with VC840. These energy reserves enabled higher metabolism in VC152's roots and consequently, greater salt-tolerance.

In conclusion, although salt ions accumulate mainly in the roots, the primary metabolic response of a relative salt-tolerant avocado tree to salinity is observed at the scion rather than at the root level. We suggest that the difference in salinity responses between the two investigated rootstocks stems from enhanced energy production in 'Hass' leaves when grafted onto VC152, combined with transport of assimilates and other reserves to the roots. This transport supplies the resources to prevent Cl and Na ions from being transported to the aboveground parts of the tree. With this energetic support and osmotic adjustment, roots can continue their normal function, absorbing minerals from the soil solution, simultaneously with toxic ion accumulation. Nutrients continue moving through the trunk to the leaves, and no salt damage or nutrient deficiency are detected. We found that despite its high susceptibility to salinity-induced damage, physiological and metabolic mechanisms of salt tolerance are indeed present in avocado. We suggest that reciprocal cooperation between the scion and rootstock is essential in this context, as the roots become a strong sink for the carbohydrates and storage lipids produced in the leaves. Such a relationship requires communication between the grafted tree's components and warrants

further investigation.

Author contributions

Conceptualization: S.L., A.D.
Methodology: S.L., H.Y., U.Y., Y.B., A.D.
Data curation: S.L.
Formal analysis: S.L., A.K.
Visualization: S.L.
Writing - original draft: S.L.
Writing - review & editing: H.Y., U.Y., A.K., Y.Y., A.B., A.D

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Dan Hamus Cohen, Yulia Subbotin, Talal Hawashla, Yonatan Ron, Gregory Mishiev, Inna Faingold, Riva Gavrilov and Simmi Wile for technical support in the field and laboratory.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2021.111048. Other data that support the findings of this study are available on request from the corresponding author.

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