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Research Article

Establishment and optimization of a hydroponic system for root morphological and nutritional analysis of citrus

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ABSTRACT: The hydroponic growth system is a convenient platform for studying whole plant physiology, especially for root morphological and nutritional analysis. However, we found that most hydroponic systems described in the literature are not suitable for citrus plants. In this study, a hydroponic system for citrus was designed, comprising three principal components: power and time switch, aeration and hydroponic culture. Herein, details of the protocol were described, including equipment setup, seed pregermination and cultivation, together with preparation and transfer of nutrient solution into hydroponics. In order to demonstrate the adaptability of the trifoliate orange plant to our hydroponic system, comparative tests between soil- and hydroponically-grown plants were carried out. The results showed that the plants grew normally and there were no obvious differences between soil- and hydroponically-grown plants. In addition, nutrient deficiency and transcriptional analysis were carried out to test the efficiency, functionality and suitability of our hydroponic system for the application of physiological and molecular analysis. The results, compared with previous studies, showed that our hydroponic system delivered superior performance as regards the physiological and molecular analysis. Taken together, we established the culture system which is best suited for the growth of trifoliate oranges under hydroponic conditions. The hydroponic system described in this paper is easily constructed and controlled at a low cost. It may serve a wide gamut of experimental purposes, especially root morphological and nutritional analysis of trifoliate oranges and the system is also adaptable to other citrus plants by varying the device size.

Keywords: cultivation, plant nutrition, trifoliate orange, root morphology

Introduction

Hydroponics, as a convenient method for studying plants in the laboratory and for growing commercial crops, was a term first coined by William F. Gericke in 1929 (Hershey, 1994). It is an extremely useful technique for growing plants under controlled nutrient conditions, particularly where clean roots are needed for physiological or microscopic analysis or for RNA extraction. The detailed protocol set up in our study should make it straightforward for other laboratories to adopt the technique rapidly and successfully. For higher plants, studies relevant to nutrients (deficiency or toxicity) on root growth and development are usually carried out under hydroponic conditions because of the wide availability and easy controlability of nutrient concentration. Over the last ten years, several different types of hydroponic systems have been established and developed for *Arabidopsis thaliana* (Conn et al., 2013; Alatorre-Cobos et al., 2014) or model plants of other species (Kim et al., 2005; Stefanelli et al., 2013).

As one of the important rootstocks for the majority of the citrus species, trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is found and utilized in citrus-cultivated regions in China and other countries. Thus, *Poncirus trifoliata* could serve as a model plant in citrus (just like *Arabidopsis thaliana* in plant), and an excellent tool for the investigation of the molecular and physiological mechanisms of citrus. Recently, more and more studies have been reported on *Poncirus trifoliata*, but soil-grown *Poncirus trifoliate* plants take a long time to be cultivated and their growing conditions are difficult to control. Therefore, standardization of growing conditions is essential to the obtaining of experimental plant materials with high reproducibility. This may be solved by establishing a hydroponic system which is suitable for *Poncirus trifoliata*. However, most hydroponic systems reported are used to cultivate herbaceous rather than woody plants, such as citrus plants. The main reasons are not only that woody plant is too difficult to cultivate, but also that citrus plants depend mainly on AM (arbuscular mycorrhizas) for absorption nutrients (Wu and Xia, 2006), which also poses problems of cultivation under hydroponic conditions, and nutrient distribution throughout the root and enhance plant growth.

Thus, this study aimed to establish a hydroponic system for growing *Poncirus trifoliata* plants, which is convenient and also offers the following advantages: (i) optimization and synchronization of plant growth; (ii) easy monitoring and manipulation of mineral nutrition; and (iii) observation and sampling of roots without damage.

Materials and Methods

Plant materials

Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seeds were used in this experiment.

Equipment

The following equipment were used in the present study: 15 L soil-growth pot (with 30 cm height and 25 cm internal diameter) for culturing plant seedlings, black, plastic, 8; 15 L opaque container (25 cm × 20 cm × 28 cm), measuring 2 cm from the top to board so that each container holds 14 L of solution, four in each group; plant holder, KT board for supporting the plant in hydroponic tanks (30 cm × 25 cm, 50 mm thick) with 8 wells (15 mm diameter), one for each tank; Leather punch (5 mm and 20 mm external diameter), one of each type; Black sponge strip (15 mm × 15 mm × 30 mm), 32 in each group; micro-computer time switch, one; Aquarium air pump 58 W/220 V (e.g. Risheng, ACO-003), output: 50 L min^{-1}, pressure: 0.028 MPa, one in each group; Aquarium air stone (15 mm diameter), two in each container; aquarium air tubing (3.8 mm internal diameter and 5.0 mm external diameter), silicone flexible tube, 0.5 m × 2 in each container and 1.0 m × 4 in each group; plastic Y-shape connector, two in each container; 50 L nutrient solution stock container.

Equipment setup

As shown in Figure 1A, the KT board (30 cm × 25 cm, 50 mm thick) was drilled with 8 wells (20 mm diameter) with a leather punch (20 mm external diameter) to make the plant holder. Additionally, 2 wells (5 mm diameter) for aquarium air tubing (3.8 mm internal diameter and 5.0 mm external diameter) were also drilled with the leather punch (5 mm external diameter).

Aeration of each hydroponics tank was provided via a single aquarium air tube (3.8 mm internal diameter and 5.0 mm external diameter) from a 4-outlet aquarium air pump (58 W, 50 L min^{-1} maximum), with a plastic Y-type connector fitted inline to permit the use of 2 aquarium air stones (15 mm diameter) in each tank. A micro-computer time switch (HHQ 4) was used to control the aeration time (Figure 1B).

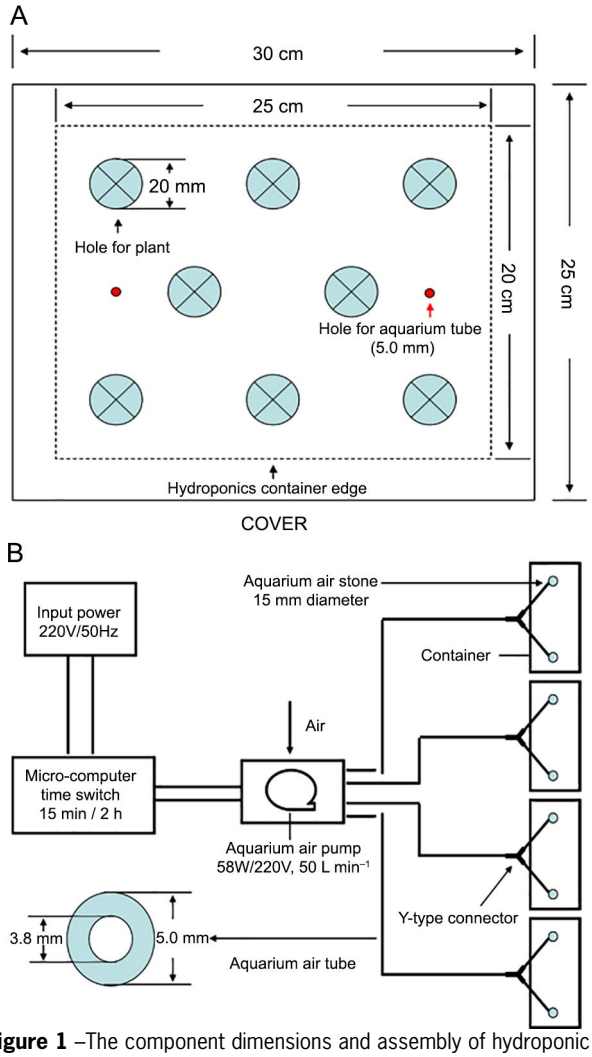

Figure 1 –The component dimensions and assembly of hydroponic system. A) The dimensions of plant holder. The numbers indicate the dimensions of holes and edges. B) The assembly and dimensions of aeration system.

Protocol

The general workflow for the trifoliate orange hydroponic system is shown in Figure 2. Step by step instructions for setting up our hydroponic system are described in the following sections. Critical points and important notes are also annotated where appropriate.

Seed germination

1) The seeds of trifoliate orange were selected and then rinsed repeatedly in distilled water to remove sediment and floating seeds. Then the seeds were soaked in distilled water for 30 min.

NOTE (for future application): If the storage time of seeds are too long, the seeds have to be soaked in 35-40 °C warm distilled water for 1 h, then soaked in cold distilled water overnight.

2) Selected trifoliate orange seeds were surface-sterilized in a 5 % sodium hypochlorite solution for 15 min and 70 % ethanol for 1.5 min, followed by thorough washing in distilled water.

3) The seeds were placed on a porcelain tray with moistened gauze and transferred to an incubator at 30 °C in the dark with 75 % relative humidity. Then they were moistened every day with distilled water till seed germination.

NOTE (for future application): The incubator should be cleaned up and surface-sterilized with 70 % ethanol before seed pregermination. The porcelain tray and gauze should also be sterilized. Before moistening the seeds, distilled water must be preheated in an incubator for at least 1 h.

Seedling culture

1) After germination, the seeds were transferred into 14 L plastic soil-growth pots (20 plants per pot) filled with vermiculite.

NOTE (CRITICAL POINT): Often, trifoliate orange seeds will germinate in one week, but most seeds will germinate 10-15 days after pre-germination. Therefore, in order to obtain more uniform seedlings, those seeds which fail to germinate within 10-15 days after pre-germination should be abandoned.

NOTE: Vermiculite should be sterilized in 121 °C for 30 min before being used to germinate the seeds. Vermiculite was selected as the seedling media because it is easy to clean up. Thus, there was no contamination of the nutrient solution when the seedlings were transferred to a hydroponics container.

NOTE: The plastic pot must be deep since the taproot of trifoliate oranges is long.
In order to preserve the humidity and temperature of the pot, all the pots were covered with plastic mulch. The plastic mulch applied in this step required a fine light transmission property, and were removed once the seedlings had two leaves.

3) The plants were transferred to a growth chamber and submitted to the following conditions (28 °C day and 22 °C night with 75 % relative humidity, and light intensity of 800 µmol m^{-2} s^{-1} of photosynthetically active radiation) and 14/10 h photoperiod. They were then irrigated twice a week, until the plants had 4 leaves (approximately 3 weeks later).

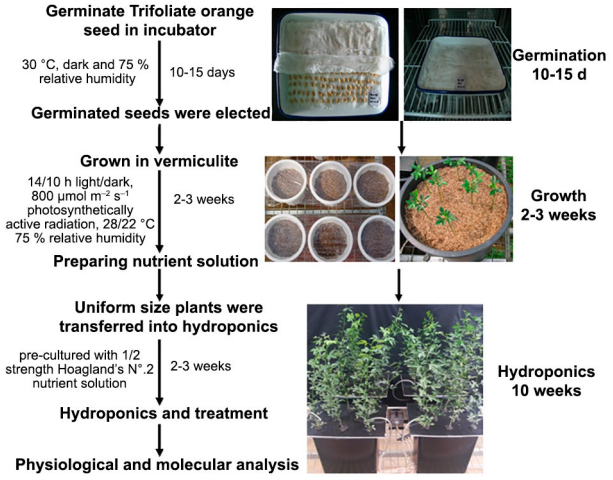

Figure 2 – Simplified trifoliate orange hydroponics growth method. Flow chart outlining the timeline and the key steps in the process. Timing (in bold) on right of arrows indicate time between steps (d = days). Images on right-hand panel show setup of seed germination and representative images of seedling plants.

Preparing nutrient solution

1) The modified Hoagland's N°.2 nutrient solution was used in this protocol, which contained 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4$, 9 µM $MnCl_2$, 0.8 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.01 µM H_2MoO_4 15 µM H_3BO_3 and 50 µM Fe-EDTA (Hoagland and Arnon, 1950). The detailed nutrient solution formulae are shown in Table 1.

2) The modified Hoagland's N°. 2 nutrient solution used in this protocol was divided into two stock solutions, one with six macronutrients and the other with seven micronutrients.

CRITICAL POINT: Na_2Fe-EDTA solution is difficult to prepare, thus the 1000X Na_2Fe-EDTA stock solution should be prepared according to the following procedure: 1) 0.1 mol EDTA-Na_2 solution: 37.7 g EDTA-Na_2 + 600 mL H_2O should be heated to dissolve and set the volume to 1 L; 2) 0.1 mol $FeSO_4$ solution: 27.8 g $FeSO_4$ $7H_20$ + 600 mL H_2O constant volume to 1 L; 3) 0.05 mol Na_2Fe-EDTA solution: mixed in equal volumes of the two solutions.

NOTE: All the reagents used to make the nutrient solutions should be guaranteed reagent (GR) with a green label.

NOTE: Nutrient solution level should be 2 cm below the container rim.

NOTE: All the prepared stock nutrient solutions and the working nutrient solutions must be kept in opaque containers to exclude light to prevent moss breeding.

Transferring to hydroponics

1) When the plants had 4 leaves (about 3 weeks), 32 seedlings from each group were selected by uniform size and transferred into hydroponics. The seedlings were

Table 1 – The modified Hoagland's N°. 2 nutrient solution formula.

Nutrients	FW	Final conc.	Stock conc.	To make 1 L stock	Vol of stock for 1 L
		mg L^{-1}	g L^{-1}	g	mL
Macronutrients			100X stock	100X stock	
$Ca(NO_3)_2 \cdot 4H_2O$	236.1	945	94.5	945	10
KNO_3	101.1	607	60.7	607	10
$NH_4H_2PO_4$	115.0	115	11.5	115	10
$MgSO_4 \cdot 7H_2O$	246.5	493	49.3	493	10
Micronutrients			1000X stock	1000X stock	
Na_2Fe-EDTA	390.0	19.50	19.50	19.50	1
H_3BO_3	61.8	1.43	1.43	1.43	1
$MnCl_2 \cdot 4H_2O$	197.9	1.81	1.81	1.81	1
$ZnSO_4 \cdot 7H_2O$	287.5	0.22	0.22	0.22	1
$CuSO_4 \cdot 5H_2O$	249.7	0.08	0.08	0.08	1
H_2MoO_4	180.0	0.02	0.02	0.02	1

Note: The pH of the nutrient solution was adjusted to 6.0 with 0.1 M KOH.

fixed on a black plant holder and then transferred to a growth chamber under the following conditions (28 °C day and 22 °C night with 75 % relative humidity, and light intensity of 800 µmol m^{-2} s^{-1} of photosynthetically active radiation) and 14/10 h photoperiod.

2) Plants were pre-cultured with 1/2 Hoagland's N°.2 nutrient solution for 2-3 weeks until the new white root appeared, and then the solution was replaced for experimentation. The solution was ventilated for 20 min every 2 h and changed twice a week until analysis. The pH of the nutrient solution was adjusted to 6.0 with 0.1 M NaOH.

CRITICAL POINT: Citrus seedlings grown in nutrient solutions consume a lot of water through transpiration. Thus, the nutrient solution level decreases and a large number of roots are exposed to the air. Moreover, concentration of the nutrient solutions also increase as a result of plant transpiration, and plant growth and development will be influenced by the high nutrient solution concentration. Therefore, the nutrient solution level should be checked every day, and the hydroponic container filled with water, not with nutrient solution.

NOTE: If not all the 32 plant wells (each group) are filled with plants, or plants are sampled during the experiment, unused holes must be covered with black sponge strip (15 mm × 15 mm × 30 mm) to exclude light from the growth solution.

NOTE: The plant holder provides a useful handling tool for transferring the seedlings to experimental chambers or different nutrient solutions.

Nutrient deficiency treatments and sampling

The 2-month-old trifoliate orange seedlings of uniform size were selected and transferred into our hydroponic culture system as described above. These plants were precultured for 2-3 weeks until the new white root appeared, and then transferred into new hydroponic containers (easily transferred from one container to another new one) with 0 µM Fe-EDTA for Fe-deficiency treatment and 0 µM H_3BO_3 for B-deficiency treatment, respectively. For investigating the physiological and root morphological changes of trifoliate orange seedlings in response to Fe- and B-deficiency, the samples were harvested randomly after ten weeks of treatment. For the microarray analysis of B-deficient experiment, the root tissue samples were taken at 3 h, 6 h and 12 h after treatment and frozen immediately at each prescribed point in time.

Plant-growth parameters measurement

After ten weeks of treatment, 32 plants per group were harvested randomly and the pant-growth parameters including leaf area, dry weights and seedling height were determined using the method previously reported (Zhou et al., 2014).

Root morphology analysis

32 seedlings (8 plants in each hydroponic container or soil pot, 4 replicates) were randomly sampled in each group, and the total root length, root surface area, root volume and root number were then analyzed as described by Zhou et al. (2014). Length of primary root (cm) was measured using a scaled ruler, and then the root density (number/cm) was calculated.

Determination of mineral nutrients

The mineral concentration of P, K, Ca, Mg, Fe, Mn, Zn, B, Cu, Na, Al, Ni, Cr and Co in the different plant tissues were determined using the method described by Storey and Treeby (Storey and Treeby, 2000). Briefly, 0.50 g of each sample was dry-ashed in a muffle furnace at 500 °C for 6 h, followed by dissolution in 0.1 N HCl, and then the mineral nutrients concentration were determined using ICP-MS (Inductively Coupled Plasma Mass Spectrometry).

Microarray analysis

The gene expression profiles in root tissue were investigated using microarray analysis after 3, 6 and 12 h B-deficient stress and the corresponding non-stress controls were investigated by microarray analysis. Fluorescent dye-labeled cDNA and hybridization were prepared according to a previous published protocol (Guo et al., 2005). Microarray data and EST sequence analysis had been carried out during our previously published study (Zhou et al., 2015).

Results

Comparison of soil-grown and solution-grown plants

In order to compare the growth and development of citrus between soil-grown and hydroponic, a number of growth indicators were identified. Under both soil and hydroponic conditions, the plants can grow normally, with no obvious difference in biomass and plant height (Table 2). However, the leaf area and number of leaves in hydroponic cultured plants was much higher than in soil cultured plants (Table 2). These results indicated that the growth and development of trifoliate or-

Table 2 – Comparative growth indicators of soil-grown and hydroponically-grown plants.

Indicator	Soil-grown	Hydroponics	Ratio
Leaf dry weight (g per plant DW)	0.65 ± 0.04^b	0.74 ± 0.04^a	0.88
Stem dry weight (g per plant DW)	1.13 ± 0.05^a	1.24 ± 0.06^a	0.91
Root dry weight (g per plant DW)	0.62 ± 0.03^a	0.54 ± 0.03^b	1.15
Total dry weight (g per plant DW)	2.50 ± 0.11^a	2.62 ± 0.12^a	0.96
Seedling height (cm)	50.32 ± 1.51^a	54.31 ± 1.46^a	0.93
Number of leaf (number per plant)	27.44 ± 0.63^b	30.67 ± 0.91^a	0.89
Leaf area (cm^2)	117.72 ± 5.73^b	130.00 ± 4.61^a	0.91

All the plants were grown in hydroponics or soil for 10 weeks. Means ± SE of 32 seedlings (8 plants in each container or pot, 4 repeats) are shown. Different small letters behind the mean values indicate significant differences ($p < 0.05$) between the different growth conditions. The same below: Ratio which compares growth indicators of soil-grown plants to hydroponically-grown plants.

ange seedlings were not influenced by nutrients in our hydroponics system.

The effects of hydroponics on the root-morphological traits of trifoliate orange seedlings were also determined. As shown in Table 3, the length of primary root and root total length (cm) were higher in hydroponics than in soil, but there was no remarkable difference in root surface area and root volume. In contrast, the number of lateral root and root density were lower in hydroponics compared to that in soil.

We tested a number of plant growth solutions and found that a modified 1/2 Hoagland's solution was a simple, defined and affordable media, which supported fine plant growth as described above. To further investigate the effects of our hydroponic solution on plant nutrient status, the plant ionome of trifoliate orange seedlings were determined and compared to plants grown in soil. The ionomic analysis results after ten weeks growth are shown in Table 4. A ratio was drawn up to compare the ionome of soil-grown plants to hydroponics plants which showed lines are similar in nutrient content for most essential nutrients, except for Mn and Cu in root. As for non-essential nutrients, there were no significant differences between Na, Al and Co, since they are not essential nutrients required for plant growth and thus they were not added to the hydroponic solutions.

Applications of our hydroponic system

1) Plant nutrient deficiency experiments

In order to demonstrate the efficiency and functionality of our hydroponics system, micro-mineral nutrient deficiency (Fe- and B-deficiency) experiments were performed on trifoliate orange seedlings, respectively. After ten weeks of nutrient-deficient treatments, typical symptoms in leaf and root architecture of trifoliate orange seedlings (Figure 3A-C and 4A-F) began to appear. As shown in Figure 3C, the serious vein swelling and cracking of the leaves were observed under B-deficient conditions. In the case of Fe-deficiency, there

Figure 3 – Hydroponically cultured trifoliate orange seedlings and morphological symptoms caused by Fe and B-deficiency treatment. All the trifoliate orange seedlings were grown under hydroponic conditions and treated for 10 weeks. A) Control; B) Fe-deficiency; C) B-deficiency.

Table 3 – Root morphology of trifoliate orange seedlings grown in both soil and hydroponics.

Root morphology	Soil-grown	Hydroponics
Length of primary root (cm)	26.01 ± 0.48[b]	32.43 ± 0.57[a]
Root total length (cm)	894.14 ± 18.74[b]	1008.78 ± 15.17[a]
Root surface area (cm^2)	161.75 ± 4.22[a]	154.11 ± 6.53[a]
Root volume (cm^3)	2.26 ± 0.07[a]	2.32 ± 0.09[a]
Number of lateral root (number per plant)	441.28 ± 11.92[a]	371.00 ± 7.73[b]
Root density (number cm^{-1})	0.47 ± 0.01[a]	0.36 ± 0.02[b]

Table 4 – Comparative ionomics of soil-grown and hydroponically-grown plants in the leaf and root.

Element	Soil-grown		Hydroponics		Ratio	
	Leaf	Root	Leaf	Root	Leaf	Root
			Macronutrients (g kg^{-1} DW)			
P	3.24 ± 0.18	5.47 ± 0.40	3.76 ± 0.08	5.19 ± 0.24	0.86	1.05
K	15.52 ± 0.35	13.32 ± 0.54	17.95 ± 0.42	14.88 ± 0.27	0.87	0.90
Ca	12.42 ± 0.73	8.68 ± 0.13	12.74 ± 1.01	8.47 ± 0.19	0.97	1.02
Mg	5.03 ± 0.37	3.24 ± 0.05	4.99 ± 0.40	3.36 ± 0.17	1.01	0.96
			Micronutrients (mg kg^{-1} DW)			
Fe	146.22 ± 17.28	1138.0 ± 79.71	141.67 ± 1.15	1144.0 ± 10.13	1.03	0.99
Mn	99.57 ± 2.23	595.83 ± 4.53	107.83 ± 3.41	712.33 ± 27.46	0.92	0.83
B	111.73 ± 3.20	25.91 ± 1.00	106.57 ± 4.67	25.12 ± 0.84	1.05	1.03
Cu	1.89 ± 0.21	4.95 ± 0.18	1.66 ± 0.24	4.01 ± 0.24	1.13	1.23
Zn	25.01 ± 1.48	37.37 ± 1.53	28.78 ± 0.34	39.03 ± 1.09	0.87	0.96
			Other nutrients (mg kg^{-1} DW)			
Na	478.89 ± 10.72	1056.8 ± 95.72	138.80 ± 8.57	523.90 ± 12.17	3.45	2.02
Al	577.44 ± 18.68	1414.8 ± 49.06	27.61 ± 0.46	55.08 ± 1.08	20.1	25.7
Ni	3.48 ± 0.80	11.45 ± 0.72	2.09 ± 0.55	11.31 ± 0.21	1.67	1.01
Cr	< 0.00	0.46 ± 0.00	< 0.00	< 0.00	n.d.	n.d
Co	< 0.00	0.63 ± 0.01	< 0.00	1.21 ± 0.18	n.d.	0.52

Note: DW = dry weight; n.d. = not determined as one or both readings were given as a detection limit.

was leaf etiolation in the young leaves at the top of the plant (Figure 3B). In contrast, there was no change in the control plants (Figure 3A).

Root morphological traits under these nutrient deficiencies were also examined in this experiment (Figure 4A-F). Results showed that the primary root length, root total length, root surface area, root volume and root number were decreased significantly by B-, and Fe-deficiency treatments (Figure 4A, B, C, D and E). In contrast, the root density increased markedly in all three nutrient deficiency treatments compared with control plants (Figure 4F). In the case of B-deficiency treatment, the root morphological traits were inhibited or improved more seriously than those under Fe-deficiency conditions.

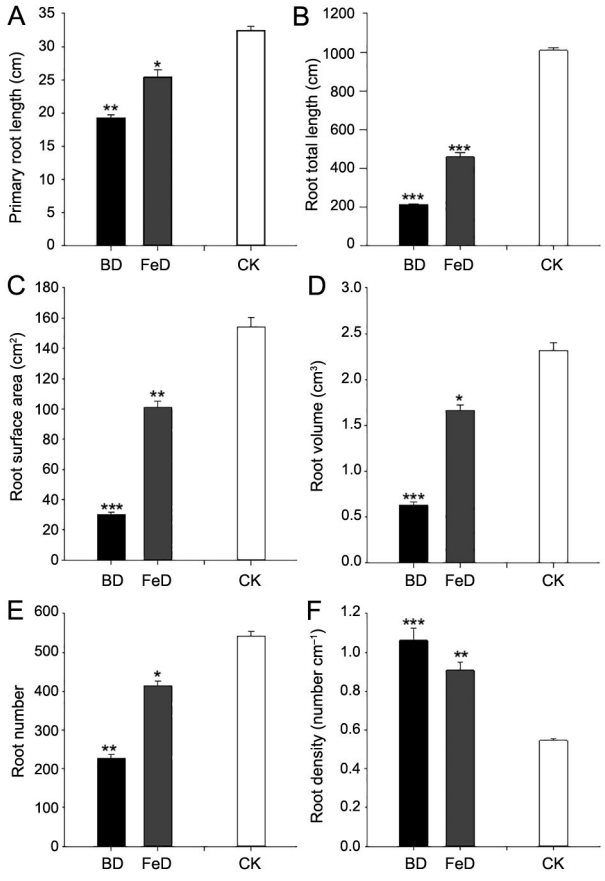

Figure 4 – Effects of Fe and B-deficiency on root morphology of trifoliate orange seedlings under hydroponic conditions. A) primary root length; B) root total length; C) root surface area; D) root volume; E) root number; F) root density. All the trifoliate orange seedlings were grown under hydroponic conditions and treated for 10 weeks. Data are presented as means ± SE of 8 replicates (n = 8, one plant for each replicate). Significance of ANOVA: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. BD = B-deficiency; FeD = Fe-deficiency; CK = Control.

2) Transcriptional responses to B-deficiency

In order to demonstrate the application of our hydroponic system on the molecular level, transcription analysis was performed on the root of trifoliate orange under B-deficiency conditions. In this study, two-month-old trifoliate orange seedlings were grown in our hydroponic system with 0 μM H_3BO_3 and the root tissue samples were harvested at three prescribed points in time (3 h, 6 h and 12 h). After microarray analysis, a total of 63 differentially expressed genes (FDR < 0.01 and fold change > = 2) from trifoliate orange were identified under B-deficiency conditions. As shown in Figure 5A, the expression patterns of trifoliate orange showed that the largest number of up-regulated genes appeared at 6 h, but, in contrast, the down-regulated genes appeared at 6 h after B-deficient treatment. Out of these genes, there were 6, 43 and 20 genes up-regulated and 13, 3, and 7 down-regulated by B-deficient stress at 3 h, 6 h and 12 h, respectively (Figure 5B). However, only one gene out of the up-regulated genes and no genes from amongst the down-regulated genes were common to all three prescribed points in time.

Distribution of differentially expressed genes of trifoliate orange root by B-deficiency stress were shown in Figure 5C, a total of 63 unique genes were grouped into ten functional categories based on the Munich Information Center for Protein Sequences' (MIPS) functional categories. The majority of these differentially expressed genes in trifoliate orange are involved in metabolism (11 %), subcellular localization (10 %), cell transport, transport facilitation and transport routes (11 %), and cell rescue, defense and virulence (13 %). Interestingly, there is a large number of genes regulated by B-deficiency in this study encoding unclassified (10 %) and unknown (22 %) proteins, and these genes may be responsible for the responsive mechanism of trifoliate orange to B-deficiency.

In this study, we focused on genes involved in plant metabolism, subcellular localization, and cellular transport related genes (Table 5). In the metabolism category, there were four gene encoding key enzymes in the lignin biosynthesis metabolism which were significantly up-regulated under B-deficiency, including phenylalanine ammonia-lyase (*PAL*; JK817683), 4-coumarate: CoA ligase (*4CL*; *JK817661*), cinnamoyl-CoA reductase 4 (*CCR4*; KJ817664) and peroxidase (*POD*; JK817712). There are another six genes involved in the cell wall metabolism (belonging to the subcellular localization category) which were significantly affected under B-deficiency. These six genes are xyloglucan endotransglycosylase/hydrolase 9 genes (*XTH9*; JK817615), proline-rich cell wall protein 2 (*PRP2*; JK817604), glucan endo-1.3-beta-glucosidase (JK817631), polygalacturonases (*PG*; JK817590), expansion (*EXP*; JK817639), and pectin methylesterase (*PME*; JK817660). As for the cellular transport category, genes involved in transmembrance transport were also identified, amongst which there were three aquaporin genes [*PIP1;3* (JK817607); *TIP2;2* (JK817649) and *NIP5;1* (JK81752)] that were highly regulated under B-deficiency stress.

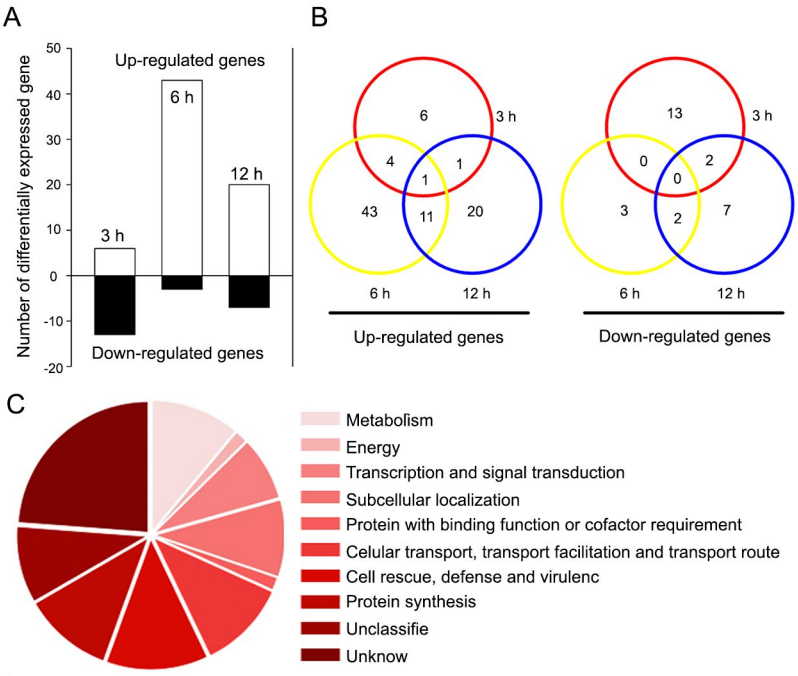

Figure 5 – The transcriptome change in the root of trifoliate orange seedling under B-deficiency hydroponic conditions. A) Number of differentially expressed genes significantly up- or down-regulated in trifoliate orange root in response to boron deficiency stress at various time points. B) Venn diagram illustrates the number of common or distinct regulated genes up- or down-regulated by B-deficiency stress over the sample time points. C) Distribution of differentially expressed genes of trifoliate orange root under B-deficiency conditions based on MIPS functional categories.

Table 5 – A list of some important differentially expressed genes in the root of trifoliate orange under hydroponic boron deficiency stress. Focused on genes involved in plant metabolism, subcellular localization and cellular transport, transport facilitation and transport route related genes.

Gene ID	Putative function	e-value	Treatment time		
			3 h	6 h	12 h
	Metabolism				
JK817683	Phenylalanine ammonia-lyase	8e-57	**2.24**	**2.44**	**2.46**
JK817661	4-coumarate:*CoA ligase*	1e-123	1.23	**2.09**	**2.69**
JK817644	Cinnamoyl-CoA reductase4	1e-23	1.28	**7.10**	**2.56**
JK817712	Peroxidase	6e-44	1.43	**3.15**	1.42
JK817680	2-phospho-D-glyceratehydrolase	6e-56	1.17	**2.36**	1.36
JK817717	Glyceraldehyde-3-phosphate dehydrogenase	3e-76	1.56	**2.86**	1.49
JK817711	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	1e-107	1.18	**2.24**	**2.41**
	Subcellular localization				
JK817615	Xyloglucan endotransglucosylase/hydrolase protein 9	2e-50	1.07	**–2.38**	**–4.17**
JK817604	Proline-rich cell wall protein 2	2e-13	**–2.08**	–1.41	**–2.04**
JK817639	Expansin	6e-41	1.03	–1.14	**–2.44**
JK817590	polygalacturonase	2e-28	1.28	**2.02**	1.64
JK817660	Pectin methylesterase	1e-39	–1.73	**–2.92**	–1.15
JK817631	Glucan endo-1,3-beta-glucosidase	4e-80	**2.19**	**7.47**	1.43
	Cellular transport, transport facilitation and transport routes				
JK817607	Aquaporin PIP1;3	1e-60	1.4	1.81	**2.25**
JK817649	Aquaporin TIP2;2	1e-102	1.26	**2.56**	1.43
JK817582	Aquaporin NIP5;1	3e-47	1.24	**3.81**	**2.35**
JK817627	Phosphate transporter	3e-84	1.59	**2.09**	1.22
JK817610	Ammonium transporter	4e-53	**–2.08**	1.22	–1.04
JK817688	Annexin D1	6e-19	1.81	1.57	**3.84**
JK817587	Voltage-dependent anion-selective channel	1e-45	**–2.7**	**2.04**	1.23

Different expression levels based on fold change (FC, signal from B deficient roots/signal from B sufficient roots; '–' means the value of the signal from B sufficient roots/signal from B deficient roots) is indicated. Significant difference (FDR test, $p < 0.01$) in relative level is shown in bold.

In addition, the differentially expressed genes in trifoliate orange roots were also clustered using the hierarchical correlation and average linkage clustering in the TreeView 3.0 software program (Figure 6). The clustering analysis of expression patterns showed that most of these genes were up-regulated at various prescribed points in time, especially at the 6 h point.

Discussion

The establishment and advantages of our hydroponic system for trifoliate orange seedlings

In this study, we established a culture system which is best suited to the growth of trifoliate orange seedling in hydroponics, based on a hydroponic device which can be quickly and cheaply constructed, and is easy to control (Figure 2). This hydroponic system consists of three principal sections: power and time switch section, aeration section and plant hydroponic culture section. We described step by step a detailed protocol for setting up this hydroponic system, including equipment setup, seed pregermination and culture, nutrient solution preparing and transferring into hydroponics. As we know, hydroponics was a term first coined by William F. Gericke in 1929 (Hershey, 1994). Increasingly, hydroponic culture systems were established and optimized for Arabidopsis (Conn et al., 2013; Alatorre-Cobos et al., 2014), rice (Kim et al., 2005), barley (Battke et al., 2003), lettuce (Kratky, 1993), tomato (Stefanelli et al., 2013) amongst other crops. However, all these hydroponic culture systems were for herbaceous plants rather than for woody plants, (not to mention citrus plants). Thus, our culture system represents the first hydroponic culture system for woody plants, especially for citrus plants.

As a model plant in citrus (just like *Arabidopsis thaliana* in plant), *Poncirus trifoliata* is an excellent tool for the investigation of the molecular and physiological mechanisms of citrus. In this study, we compared plant growth and development between soil-grown and hydroponics, and a number of growth indicators were determined. Under both soil and hydroponic conditions, the plants can grow normally, and no obvious difference was found in biomass nor plant height (Table 2). These results indicated that our our hydroponic culture system is best suited to the cultivation of trifoliate orange seedling.

In addition to the general advantages of the hydroponic system, such as control of mineral nutrition and access to the root system, there are the many other advantages to our hydroponic system described above. First, our hydroponic system is a simple, quickly and cheaply constructed system. It consists of three principal sections: power and time switch section, aeration section and plant hydroponic culture section. Details of the protocol of the system are presented below, including equipment setup, seed pregermination and culture, nutrient solution preparing and transferring into hydroponics (Figure 1A and B; Figure 2). Second, our hydroponic system is convenient and quick for transferring the plants from one hydroponic solution to another in new nutrient solutions. In this study, nutrient deficiency (Fe- and B-deficiency) experiments were performed on trifoliate orange seedlings (Figure 3B and C; Figure 4A-F). At the beginning of these experiments, the plant materials were grown in normal nutrient hydroponic solution for 2-3 weeks, and were then transferred to Fe- and B-deficient nutrient solutions without changing the plant holder for ten weeks, respectively. Results revealed that our hydroponic system is convenient and quick in plant

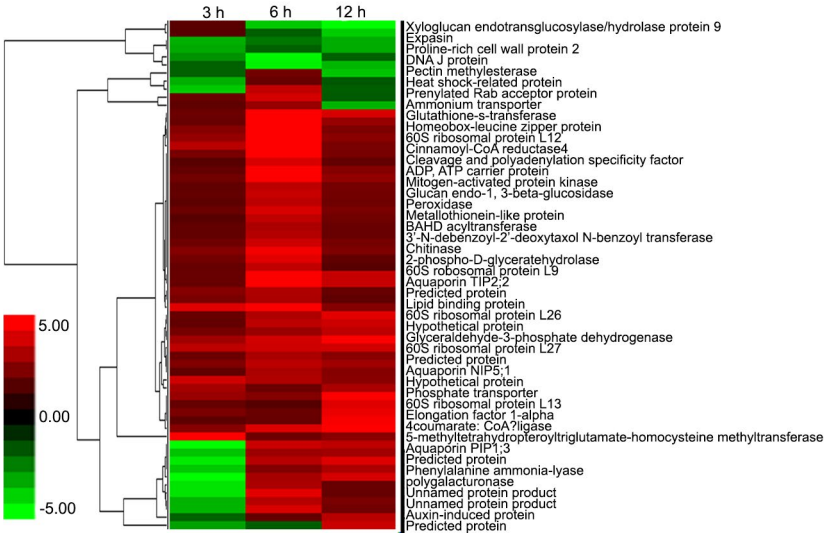

Figure 6 – TreeView representation of the differentially expressed genes of trifoliate orange root by B-deficient stress. Hierarchical clustering of 63 differentially expressed genes that showed a fold change of at least ± 2 and FDR-corrected P values < 0.01 at any time point. The signals are shown on a red-green color scale, where red represents higher expression and green represents lower expression.

material treatment. Third, the tissue samples cultured in our hydroponic system can be harvested quickly and without damage. The tissue samples are clean for RNA isolation, especially for root.

In addition, our hydroponic system can be easily adapted for other citrus plants, such as for Carrizo citrange (*C. sinensis* L. × *P.* trifoliata L.), Red tangerine (*C. Reticulata* B.), Cleopatra mandarin (*C. reshni* Hort.), Fragrant citrus (*C. junos* Sieb.), Sour orange (*C. aurantium* L.) and others by changing the hole size and other conditions (Mei et al., 2011; Zhou et al., 2014). Furthermore, we also adapted our system to investigate the salt stress on trifoliate orange on the addition of excessive sodium (Na^+) and (or) chloride (Cl^-) to the standard nutrient solution (Wei et al., 2013). This is an additional advantage of our hydroponic system.

Performance of our hydroponic system on root morphological and nutritional analysis

The way roots develop in soil can have a critical effect on plant growth and impact crop yield (de Dorlodot et al., 2007; Postma and Lynch, 2011). Root washing is the most common method used to study the root system of plants grown in their natural soil environment (Gregory, 2006; Smit et al., 2000). However, this method often leads to the underestimation of fine root through breakage during the washing process. Recently, a new method has been developed that can recover the complete structure of the plant root system from soil via X-ray μ-Computed Tomography, but this method process is too complex and the equipment too expensive (Mairhofer et al., 2013). In contrast, using our hydroponic system to investigate the plant root system architecture is easy and cheap. Moreover, our hydroponic system can be used to investigate the dynamic of plant root system development without root breakage and damage. In our method, as shown in Table 3, the length of primary root and root total length were higher in hydroponics than in soil, but there was no noticeable difference in root surface area and root volume. However, the number of lateral root and root density were lower in hydroponics compared to that in soil. These results are similar to a previous study on the root of honey locust, whose results showed the root systems grown in solution had longer primary roots, fewer lateral roots and root hairs, and a greater distance between the tip of the primary root and the junction of the youngest secondary root and the primary root than root systems grown in sand (Graves, 1992).

In this study, in order to demonstrate the performance of our hydroponic system on root morphologic analysis, we also examined critically the root morphology of trifoliate orange under Fe- and B-deficiency stress. Fe and B were selected in this verification experiment, and Fe- and B-deficiency are frequently observed in the citrus growing regions of China and other countries (Forner-Giner et al., 2010; Peng et al., 2010; Chen et al., 2012; Liu et al., 2012). After ten weeks of Fe- and B-deficiency stress, typical changes in root morphology in trifoliate orange. As shown in Figure 4A-F, the root morphology of trifoliate orange was influenced significantly, in comparison with the control plants, and all the results are in agreement with previous studies (Mei et al., 2011; Han et al., 2012; Cao et al., 2013; Zhou et al., 2014). In sum, all the results indicated that the root growth and development of trifoliate orange seedlings were not significantly influenced by the nutrient solution in our system and the typical changes in root morphology can be found under nutrient deficiency conditions using our hydroponic system.

The efficiency and functionality of our proposed hydroponic system is demonstrated by the nutrient deficiency experiments. It is known that B is essential for the growth and development of higher plants (Warington, 1923). Although citrus plants are not classified as the species the most sensitive species to B-deficiency, the occurrence of B-deficiency has been reported in major citrus producing countries of the world, such as Spain, the United States, Brazil and China (Shorrocks, 1997; Han et al., 2008; Chen et al., 2012; Liu et al., 2013). Moreover, it is hard to create B-deficiency growth conditions for the culturing of plant materials because B is a micro-nutrient for plants. In this study, a complete set of B-deficiency growth conditions was established using our hydroponic system. To demonstrate the application of our hydroponic system under B-deficiency conditions, physiological and molecular experiments were carried out on trifoliate orange seedlings. As shown in Figures 3A-C and 4A-F, after ten weeks of B-deficient treatment the typical symptoms of B-deficiency were observed in the leaves and root of trifoliate orange.

Performance of our hydroponic system on molecular level

To demonstrate the application of our hydroponic system on the molecular level, microarray analysis was carried out on trifoliate orange root under B-deficiency conditions. According to the results of our transcription analysis, a total of 63 unique genes significantly changed by B-deficiency stress in the root of trifoliate orange (Figure 5A and B). Among these genes, there are several gene encoded key enzymes involved in the cell wall metabolism, such as *XTH9*, *PRP2*, glucan endo-1.3-beta-glucosidase, *PG*, *EXP*, and *PME* (Figure 5C and Table 5). Except for PG and glucan endo-1.3-beta-glucosidase gene, which were up-regulated under B-deficiency, the other four genes were down-regulated markedly. It is known that the plant cell wall plays a very important role in plant growth and development, and the cell wall mediates the responses of plants to environment and pathogen-induced stress (Farrokhi et al., 2006). On the other hand, B has been established as an essential element for the structure and functions of the cell wall (O'Neill et al., 2004). Taken together, our results demonstrate that the expression of several enzyme genes involved in cell wall metabolism were significantly changed in trifoliate orange roots under B-deficiency conditions. Similar re-

sults were also reported in Arabidopsis (Camacho-Cristóbal et al., 2008).

There are three aquaporin genes [*PIP1;3* (JK817607); *TIP2;2* (JK817649) and *NIP5;1* (JK81752)] were highly up-regulated under B-deficiency stress (Table 5). As we know, aquaporins are water channel proteins of intercellular and plasma membranes which are involved in many functions of plants, such as nutrient acquisition, carbon fixation, cell signaling and stress responses (Maurel, 2007). To date, it has been reported that three subgroups (NIPs, PIPs and TIPs) of the major intrinsic protein (MIP) family were involved in B transmembrane transport (Takano et al., 2006; Tanaka et al., 2008; Fitzpatrick and Reid, 2009; Pang et al., 2010). In this study, it was also found that the expression levels of three genes matched previous reports, including the higher expression of *TIP2*; *2*, *NIP5*; *1* and *PIP1*; *3* in the trifoliate orange root.

Taken together, all these results described above demonstrated that our hydroponic system delivered a more than satisfactory performance for studying plant responses to B-deficiency (or other nutrient deficiency or toxicity) at the molecular level.

Conclusions

In this study we have described, step by step, a protocol for setting up a simple, quickly and cheaply constructed hydroponic system which has standardized growth conditions for growing trifoliate orange and other citrus plants. Quality and versatility of our hydroponic system are demonstrated by profiling and comparing with soil-grown trifoliate orange seedlings, including biomass, ionomics, and root morphology. The results showed that our hydroponic system is best suited to the growth of trifoliate orange seedling. In order to test the performance of our hydroponic system on mineral nutrient deficiency, Fe- and B-deficiency treatment experiments were carried out on trifoliate orange seedlings, respectively. Moreover, to further demonstrate the application of our hydroponic system on the molecular level, transcriptional analysis was also carried out on the root of trifoliate orange under B-deficiency conditions. According to our results and previous reports, our hydroponic system performed better in terms of a physiological and molecular analysis of trifoliate orange. In conclusion, the system should be suitable for many experimental purposes, but especially for root morphologic and nutritional analysis of trifoliate orange, and it can also be adapted to other citrus plants by varying the device number and (or) size.

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Authors' Contributions

Experimental design: Zhou, G.; Liu, G. Writing and editing: Zhou, G. Data analysis: Wei, Q.; Li, B.; Zeng, X. Proposed and supervised the overall project: Liu, G.

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