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RESEARCH PAPER

Phenotypic plasticity and water flux rates of *Citrus* root orders under salinity

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Abstract

Knowledge about the root system structure and the uptake efficiency of root orders is critical to understand the adaptive plasticity of plants towards salt stress. Thus, this study describes the phenological and physiological plasticity of *Citrus volkameriana* rootstocks under severe NaCl stress on the level of root orders. Phenotypic root traits known to influence uptake processes, for example frequency of root orders, specific root area, cortical thickness, and xylem traits, did not change homogeneously throughout the root system, but changes after 6 months under 90 mM NaCl stress were root order specific. Chloride accumulation significantly increased with decreasing root order, and the Cl^- concentration in lower root orders exceeded those in leaves. Water flux densities of first-order roots decreased to <20% under salinity and did not recover after stress release. The water flux densities of higher root orders changed marginally under salinity and increased 2- to 6-fold in second and third root orders after short-term stress release. Changes in root order frequency, morphology, and anatomy indicate rapid and major modification of *C. volkameriana* root systems under salt stress. Reduced water uptake under salinity was related to changes of water flux densities among root orders and to reduced root surface areas. The importance of root orders for water uptake changed under salinity from root tips towards higher root orders. The root order-specific changes reflect differences in vulnerability (indicated by the salt accumulation) and ontogenetic status, and point to functional differences among root orders under high salinity.

Key words: Anatomy, biomass, *Citrus volkameriana*, miniature depletion chambers, NaCl accumulation, phenotypic plasticity, root architecture, root order, salt stress, water flux density.

Introduction

Salinity is a major concern for agriculture worldwide; at least 20% of all irrigated lands are salt affected, with some estimates being as high as 50% (Pitman and Läuchli, 2004). Secondary salinization is particularly widespread in arid and semi-arid environments whose agricultural systems are often associated with cultivation of one of the various *Citrus* varieties. With global food production having to meet the demands of a growing world population, understanding plant responses to salinity is decisive to improve the salt tolerance of crops.

Phenotypical or physiological changes in response to environmental conditions often enhance the fitness of plants (Sultan, 2000). Root systems can exhibit enormous plasticity on the level of biomass, morphology, and/or physiology in response to different environmental parameters such as water and nutrient availability (e.g. Sorgonà *et al.*, 2007; Wang *et al.*, 2009; Gruber *et al.*, 2011) or excess ions (Deak and Malamy, 2005; Zolla *et al.*, 2010; Rewald *et al.*, 2011*b*, *c*). Previous studies addressing salinity effects on tree crops such as *Citrus* spp. and *Olea europaea* found, for example, increased root:shoot ratios (Zekri and Parsons, 1989), reduced root branching (Gucci and Tattini, 1997), modified axial root conductivity (Rewald *et al.*, 2011*c*), and a well-developed Casparian strip closer to the root apex (Walker *et al.*, 1984). While salt exclusion, compartmentation, and osmoregulation are the mechanisms particularly considered

to increase the salt tolerance of *Citrus* spp. and other woody glycophytes, adaptation to salinity is determined by the integrating effects of several mechanisms (Zekri and Parsons, 1992; Maas, 1993; Kozlowski, 1997; Munns, 2002). Thus, it is reasonable to speculate that root system modifications under salinity are a trade-off between the capacity to exclude excess ions and sustained water or nutrient uptake. However, studies on the (structural) differences among salt-stressed root systems that may partially underlie uptake capacities have received less attention (Vadez *et al.*, 2007; Rewald *et al.*, 2011*b*).

Because root system traits, such as water uptake rates per surface area, are defined by the properties of individual root segments (see Rewald *et al.*, 2011*a*, and references within), detailed studies about the abundance, morphology, anatomy, and physiology of individual roots are needed. Due to the fact that traits often vary according to the position of individual root segments among the root branching hierarchy (i.e. 'root order'; Pagès and Kervella, 1990; Pregitzer *et al.*, 2002; Valenzuela-Estrada *et al.*, 2008), analysis by root order is a powerful approach to understand complex woody root systems under stress. However, the morphological/anatomical properties and frequencies of the most distal root orders have been determined to date on <40 woody species world-wide (e.g. Pregitzer *et al.*, 2002; Wang *et al.*, 2006; Guo *et al.*, 2008*a*); even fewer studies have quantified total number, biomass, and/or surface area of root orders (Valenzuela-Estrada *et al.*, 2008; Rewald *et al.*, 2011*a*).

Most previous studies have used indirect, specifically morphological and anatomical, analyses to estimate differences in root order functionalities (e.g. Valenzuela-Estrada *et al.*, 2008; Huang *et al.*, 2010). Direct hydraulic measurements on certain root orders were restricted for a long time to abscised (e.g. Schulte, 2006; Bramley *et al.*, 2007) or distal (Zwieniecki and Boersma, 1997) root segments. However, Rewald *et al.* (2011*a*) have recently developed a method to determine water fluxes among root orders. It was shown that water flux densities under homogeneous, non-stressed conditions are determined by root order but not by root diameter or the position of a root segment within a root branch or the whole root system. Because water uptake is reduced under salinity and during periods of salt stress release (Cimato *et al.*, 2010; Rewald *et al.*, 2011*b*), detailed knowledge on water uptake capacities within the root branching system is key to understanding plant functioning under salinity.

To understand the adaptive response of *Citrus volkameriana* rootstocks under severe NaCl stress, the present work studies root traits and water uptake on the level of root orders. Two questions are addressed in detail. (i) Which architectural, morphological, and anatomical changes occur in salt-stressed *C. volkameriana* rootstocks? (2) What contribution do specific root orders make to water uptake under salinity and after a rapid release of salt stress? It is hypothesized that the type of plasticity (e.g. architectural, morphological, and anatomical) differs among root orders and that the water uptake by salt-stressed root systems is highly influenced by changes in abundance and water flux density among specific root orders.

Materials and methods

Plant material and growth conditions

Citrus volkameriana Ten. & Pasq. rootstocks are of economic importance because of their resistance to the *Citrus tristeza virus* and as medium salt excluders (Levy and Shalhevet, 1990; Ramin and Alirhezanezhad, 2005). In 2006, 1-year-old *Citrus sinensis* Osbeck var. Newhall shoots were grafted on adequately sized *C. volkameriana* rootstocks. The plants were grown in fertigated, soil-filled 10 litre pots in a greenhouse at ambient temperature until October 2009. As of this time, eight equal sized plants were selected, roots were rinsed, and plants were moved to constantly aerated hydroponics (Supplementary Fig. S1 available at *JXB* online). Plants were placed into opaque 20 litre pots filled with ~17 litres of either 1.0 strength Long Ashton (LA; Ottow, 2005) solution (control treatment) or 1.0 strength LA plus 90 mM NaCl (salt treatment). The osmolalities of the solutions were 24±1 mmol kg^{-1} and 162±1 mmol kg^{-1}, respectively (mean ±SE, n=10; Vapro 5520, Wescor, Logan, UT, USA). The pots, tightly covered to prevent light penetration and evaporation, were placed in a controlled growth room [air temperature ⩽28 °C (day), 20 °C (night); relative humidity 30–40% (day), 70% (night); photosynthetic photon flux density (PPFD) 300–400 µmol m^{-2} s^{-1} (day-length 12.5 h)]. The temperature of the hydroponic system was kept at 20±0.1 °C (BL-30, MRC, Holon, Israel); transpired water was refilled every other day and the entire solution was exchanged every other week. After 5 months, leaf stomatal conductance between 12:00 h and 13:00 h was 81.6±7.4 mmol m^{-2} s^{-1} (control) and 35.5±3.5 mmol m^{-2} s^{-1} in plants under salinity (mean ±SE, n=22–24; SC-1 Porometer, Decagon, Pullmann, WA, USA).

Analysis of root morphology, surface area, and biomass

After ~6 months in hydroponics, the rootstocks of three *Citrus* plants per treatment were severed from the stem above the highest root. Aboveground biomass was separated into leaves and branches, dried (70 °C, 48 h), and weighed. Subsequently 12 'large root branches' (i.e. branches with 5–6 root orders) and four 'small root branches' (⩽4 root orders present) attached to the tap root were randomly severed per individual. This ratio was chosen according to a visual pre-examination of the control rootstocks. The 16 root branches per plant were dissected into root orders and were kept moist constantly. The architectural classification follows the stream classification approach (Pregitzer *et al.*, 2002), allowing most distal root segments (root tips) to be defined persistently as first-order roots even if the total number of orders is subject to change or unknown. Roots that possess only first-order side roots were named second-order roots, root segments bearing exclusively first- and second-order side roots were named third-order roots (starting at the most distal point along the root axis where two second-order roots met), and so on (see Supplementary Fig. S1A at *JXB* online).

The remaining roots (i.e. roots beside the 16 root branches analysed in detail) were separated into fourth root order and higher, while the biomass of the root orders 1–3 was later divided by the ratios calculated from the detailed dissection of root branches. Finally, ~60–70% of the root systems were analysed in detail. Live roots were distinguished from dead roots (Rewald and Leuschner, 2009); dead roots and any adhering particles were discarded.

After dissection, root segments were stored in closed Petri dishes with small amounts of tap water to keep the roots hydrated (4 °C, <5 d), separated by treatment, plant, root branch, and order, until digital images capturing of root orders 1–6 took place on a flatbed scanner (grey scale, 400 dpi). To determine root diameters and surface areas, images were analysed with the software WinRhizo 2005c PRO (Régent, Quebec, Canada). Finally, root samples were dried (70 °C, 48 h) and weighed to a precision of ±0.1 mg using an analytical scale (CP225D, Sartorius, Göttingen, Germany). The

specific root area [SRA, cm^2 g dry weight $(d.wt)^{-1}$] and the root diameter were calculated per root order using data of the 16 dissected root branches per plant. The relative biomass and surface area of root orders 1–6 within root branches was calculated, using the 12 'large root branches' per plant. The root to leaf biomass ratio ('root:leaf ratio'), the total plant biomass (DM_T), and the rootstock biomass were calculated from dry weights per plant individual.

Analysis of root anatomy

Tissue sections from root order 1 were obtained close to the basal site of the root segments. Root orders 2–4 were sampled for tissue sections in the middle between two side branches. Eight randomly selected tissue sections of root orders 1–4 in each treatment were studied; root orders 5 and 6 were not analysed due to the difficult preparation of heterogeneously dense samples. The sections were fixed for 48 h by immersion [5% formaldehyde, 5% acetic acid, 90% ethanol (70%)]. Dehydration of the tissue sections was accomplished in a graded ethanol series (50, 70, 95, and 100%, 30 min each) followed by immersion in tert-butanol (8 h) and embedding in Paraplast Plus. After hardening, 12 μm thick cross-sections were cut with a rotation microtome (RM2235, Leica, Nussloch, Germany). Cross-sections were collected on glass slides and placed on a warming tray (40 °C, 3 h). The tissue sections were deparaffinized in xylene (3× 10 min) and rehydrated (ethanol 100, 95, 70, and 50%, 5 min each). The washed sections (H_2O, 1 min) were successively stained with safranin (0.5%) and fast green (0.5%; Ruzin, 1999, and references within), cleared with 100% xylene (3× 10 min), and air dried. Digital images were taken (Zeiss AxioImager A1 microscope) and cross-sections were analysed (AxioVision 4.6, Carl Zeiss, Wetzlar, Germany). Measured parameters included the number of exodermal layers, and area and diameter of the root cross-section, the cortex, the stele, and the xylem. Relative proportions of the cortex diameter and the xylem area were calculated. The xylem was analysed in detail by quantifying the number, radii, and areas of xylem vessels; the hydraulically weighted average conduit diameter [HWCD, i.e. $2(\Sigma r^5\ (\Sigma r^4)^{-1})$] was calculated (Lewis and Boose, 1995). Furthermore the cross-sections were analysed for differences in the suberization of endodermis and peri-/exodermis after staining with aniline blue using fluorescence microscopy (Brundrett *et al.*, 1988).

Plant chloride and sodium analysis

Dry materials of leaves and root orders 1–6, separated by plant individual, were ground to a powder and extracted overnight with distilled water (0.1 g of dry material in 10 ml of double-distilled water). Chloride (Cl^-) concentration was determined by silver ion titration (Chloride Analyzer 926, Corning, MA, USA), while sodium (Na^+) analyses were carried out using a Corning Flame Photometer 410 (Raveh, 2005).

Miniature depletion chamber set-up

Rewald *et al.* (2011*a*) constructed 'miniature depletion chambers' to measure the water fluxes of *C. volkameriana* root orders under fresh water supply ('control' treatment). The current study measured the water flux rates under salinity and after released salt stress. The measurements took place after the plants were growing under salinity for >4 months and in parallel to the measurements on fresh water-grown plants.

In brief, the chambers were manufactured from small plastic tubes (diameter=15 mm) which were shortened to 15 mm in length (Supplementary Fig. S1 at *JXB* online). Septa (IceBlue, Restek, Bellefonte, PA, USA) were glued in place (LocTite Super Glue-3, Henkel, Boulogne, France) on both ends. Both septa and plastic tubes were cut open on one side, allowing for root insertion by spreading the chamber open along the section. Septa were pre-drilled (diameter=0.3–3.5 mm) to enable sealing of inserted roots (diamter=0.5–4.1 mm) while preventing excessive squeezing. For measuring water fluxes of the first-root order, chambers with only one pre-drilled septum were used, with the root tip ending within the chamber.

For chamber placement, the root systems were lifted out of the hydroponics and fixed in mid-air for <10 min. A root segment was chosen by the following criteria: lack of side braches on a length of ⩾17 mm, no signs of decay (e.g. dark-coloured, shrivelled), and undamaged epi-/peridermis. The segment was gently blotted dry using a paper towel, placed in the septa holes, and the chamber was closed by a clamp. Cuts were sealed with either hot glue (plastic tube) or superglue (septa); the root–septa interfaces were sealed by the pressure of the septa and a small amount of superglue.

Two different measurements were performed on four salt-stressed rootstocks and the first four root orders: (i) 0.5 strength LA plus 90 mM NaCl (osmolality: 156±2 mM kg^{-1}; mean ±SE, n=10; Vapro 5520, Wescor) was inserted into the chamber to measure the water fluxes under salt stress ('salt' treatment) or (ii) 0.5 strength LA (osmolality: 15±1 mM kg^{-1}) was used to measure the flux rates after rapid release of salt stress ('stress release' treatment). In each case, 1.3 ml of aerated (>18 h) solution was injected into the chamber at 9:00 h to allow for 2 h of equilibration before measurement started at 11:00 h.

Determination of water flux rates per root order

In brief, a thin plastic tube attached to a hollow needle was used to connect the 'miniature depletion chambers' to a storage container placed on an analytical scale (see above; Supplementary Fig. S1 at *JXB* online). Both the tube and the storage container were filled with the type of solution added to the chamber. To prevent bias by gravimetric force, solution levels in the storage container and the hydroponic pot were brought to the same height. The weight of the storage container was recorded every minute (Sartorius Connect 1.0; Sartorius, Göttingen, Germany). To induce high, measurable mass flux rates (F_m; g h^{-1}) the period between 11:00 h and 14:00 h was chosen because transpiration maxima (related to temperature maxima and relative humidity minima) were expected during this time.

Five to 11 flux measurements were performed per solution type and per root order 1–4; higher replicate numbers were used if the first five measurements were very heterogeneous. Linear regressions (R^2=0.43–0.99, $P < 0.01$) were performed to determine F_m from the 3 h measurement period and it was correlated with the surface area (cm^2) of segments to calculate the water flux density (J_s; g cm^{-2} h^{-1}).

Water flux rates on the level of root branches

The 12 large root branches per plant were used to calculate 'standardized' *Citrus* branches under fresh water and salinity. The biomass and surface area proportions of root orders 1–4 were used to determine their absolute surface areas (SAs; cm^2) in a root branch of 1 g dry weight. Root orders 5 and 6 were excluded from estimates of biomass and surface area proportions owing to their small surface area (<4% and <6% SA of large root branches under control treatment and salt stress, respectively) and because they were not measured for water flux.

To determine the mean water flux densities ($J_{\bar{s}}$) of the standardized root branches, the water flux densities (J_s) under fresh water, salinity, or stress release conditions were weighted by the surface area of the respective root order (A) under fresh water or salt stress, respectively. By setting the total flux rates per branch as 100% and dividing them by the flux rates of the four root orders, the relative proportion of different root orders on the total flux rates of the root branch was calculated.

Statistics

Statistical calculations were conducted with SAS version 9.2 (SAS Institute, Cary, NC, USA). Data sets were tested for Gaussian distribution with the Shapiro–Wilk test and for homogeneity of variances with the Levene test. Because of unbalanced data, a general linear model (PROC GLM) was used to test for significant influences of treatment, order, and interactive effect on root traits and for root order-specific changes in traits between treatments. For traits expressed as percentages, the Bliss angular transformation was applied. To test for salt effect on the root biomass and the root:leaf ratio, the DM_T was used as a covariate in PROC GLM. Parametric Tukey test was used for examination of tissue NaCl concentrations. Analyses of variance comparing root order, treatment, and their interaction were performed by the PROC ANOVA procedure for Na^+ and Cl^- contents in roots; the interaction was removed later because of non-significance. Critical α for all tests was set at ≤ 0.05.

Results

Plant biomass, total root biomass, and root:leaf biomass ratio

Total plant biomass was reduced by 27% under salinity (Fig. 1A), caused by a major reduction in leaf biomass, some dead branches, and a minor reduction in total root biomass (~2%; Fig. 1B inset, Table 1). While the reduction in root biomass was marginally significant (P=0.09), the root:leaf biomass ratio increased significantly ($P < 0.03$) from 0.65 under fresh water to 2.96 after 6 months under high salinity (Fig. 1B, Table 1).

Root architecture

After 6 months in hydroponics, the *C. volkameriana* rootstocks had eight root orders in total under fresh water (control) and seven root orders when grown under salt stress (data not shown). In both treatments, the highest root order formed the tap root; the first root orders were clearly distinguishable as root tips. The architecture of *C. volkameriana* rootstocks; that is, the proportion of root orders within the branching root system, changed under salinity in respect to both biomass and surface area (Fig. 2A, B). Both biomass and surface area frequencies changed significantly among root orders 1–6 ($P < 0.001$) and as an interactive effect of root order and salinity ($P < 0.5$; Table 2).

Table 1. GLM results for the effect of salt stress on the root:leaf biomass ratio and root biomass (n=3)

The total plant dry mass (DM_T) was used as covariate for root:leaf ratio and root biomass.

Parameter		Class	Covariate
		Salinity	DM_T
Root:leaf biomass ratio	*F*	16.24	8.62
	P	**0.026**	0.061
Root biomass	*F*	6.34	13.12
	P	0.086	**0.036**

First-order roots (root tips) provided 25±2% of the biomass under fresh water; under salinity this amount was significantly ($P < 0.001$) reduced to 17±2% (mean ±SE; Fig. 2A, Table 2). The abundance of the root order-specific biomass declined markedly with increasing root order under fresh water supply; biomass was more homogeneously distributed among root orders 1–5 under salinity. No significant changes were found in biomass frequencies of root orders 2–6 under salt stress (Table 2).

The surface area shares of root orders (SA%) varied from the biomass distribution due to differences in SRAs (see below). Root orders 1 and 2 showed contrasting changes under salinity; the relative SA provided by the first-root order (root tips) decreased significantly ($P < 0.05$) from 42±2% (Ctrl) to 35±2% (Salt), while the second-order roots accounted for a significantly ($P < 0.05$) higher percentage (3%) of root branch SA under salinity (29±1%; mean ±SE; Fig. 2B, Table 2). The SA% of root orders 3–6 did not change significantly between treatments.

Morphology of root orders

Root diameter increased significantly under salinity ($P < 0.01$) and with increasing root order ($P < 0.001$; Fig. 2C, Table 2). However, when separately analysed by root order, only the diameter increases in root orders 2, 3, and 4 were significant ($P < 0.05$). For example, the root diameter of third order roots increased from 0.86±0.02 mm (control) to 0.98±0.04 mm under salinity (mean ±SE; Fig. 2C).

The SRA decreased significantly ($P < 0.001$) with increasing root order under both treatments and was significantly lower ($P < 0.001$) under salinity (Table 2). The interaction effect between treatment and root order was found to be significant ($P < 0.01$). Analysed by order, the SRAs of root orders 1–4 were significantly ($P < 0.05$) reduced under salinity while the SRA of root orders 5 and 6 did not change significantly (Fig. 2D). For example, the SRAs of root orders 1 and 4 were 382±10 $cm^2\ g^{-1}$ and 101±4 $cm^2\ g^{-1}$ under fresh water and 338±6 $cm^2\ g^{-1}$ and 87±4 $cm^2\ g^{-1}$ under salt stress. respectively (mean ±SE).

Anatomy of root orders

The cortex thickness increased significantly ($P < 0.01$) in higher root orders but showed no homogeneous change under salinity (Fig. 2E, Table 2; Supplementary Fig. S2 at *JXB* online). Split up into root orders, the relative cortex thickness decreased significantly in root order 3 and increased significantly in root order 1 (root tips) and 4 under salinity ($P < 0.05$).

The proportion of the stele increased significantly ($P < 0.001$) in higher root orders (Table 2). However, the direction of change differed among orders under salinity: stele proportions were unchanged in first-order roots, increased significantly in third-order roots (~39%; $P < 0.01$), and decreased significantly in fourth-order roots (~32%; $P < 0.05$; Fig. 2F, Table 2).

Table 2. Frequency of root orders (in respect to biomass and surface area; n=36), root diameter, specific root area (n=36–197), cortex and stele dimensions, xylem density, and the hydraulically weighed conduit diameter (HWCD; n=8) were analysed by two-way GLM either pooled or separated by root order

Parameter		Salinity effect	Root order effect	Salinity × root order	Salinity effect by root order					
					1	2	3	4	5	6
Frequency of root order (biomass)[a]	*F*	0.76	135.65	10.31	14.08	0.01	0.68	2.29	3.16	0.00
	P	0.385	**<0.001**	**0.001**	**<0.001**	0.925	0.412	0.134	0.079	0.954
Frequency of root order (surface area)[a]	*F*	1.47	1780.67	4.47	6.16	4.58	0.02	0.90	3.49	0.64
	P	0.226	**<0.001**	**0.035**	**0.015**	**0.035**	0.089	0.345	0.065	0.424
Root diameter	*F*	12.21	1194.64	0.20	2.01	6.39	6.34	6.45	0.25	2.33
	P	**0.001**	**<0.001**	0.652	0.157	**0.012**	**0.012**	**0.012**	0.621	0.125
Specific root area	*F*	62.54	2247.65	10.76	13.63	28.75	26.55	4.62	0.41	1.31
	P	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**0.032**	0.524	0.260
Cortex diameter:root diameter ratio	*F*	0.67	41.14	0.81	5.23	0.20	5.10	10.44	n.d.	n.d.
	P	0.416	**<0.001**	0.372	**0.026**	0.664	**0.041**	**0.006**		
Stele area : root area ratio	*F*	0.89	115.36	2.63	0.44	0.12	9.78	6.49	n.d.	n.d.
	P	0.349	**<0.001**	0.110	0.519	0.731	**0.007**	**0.023**		
Xylem density	*F*	0.23	33.03	5.23	1.34	0.23	4.38	11.70	n.d.	n.d.
	P	0.636	**<0.001**	0.026	0.267	0.636	0.055	**0.004**		
HWCD	*F*	4.57	86.00	6.73	0.00	1.38	11.56	3.40	n.d.	n.d.
	P	**0.037**	**<0.001**	**0.012**	0.976	0.300	**0.004**	0.086		

[a] Bliss angular transformed. n.d., not determined.

The xylem density was significantly lower in higher root orders ($P < 0.001$) and a significant interaction effect between salinity and root order was found ($P < 0.05$; Table 2). Fourth-order roots of salt-stressed plants had a significantly ($P < 0.01$) lower xylem vessel density (2.1±0.2 n mm^{-2}) than control plants (3.3±0.5 n mm^{-2}; mean ±SE, Fig. 2G). No significant changes in xylem density associated with salinity were found for root orders 1–3 (Table 2); however, the xylem vessel density tended to decrease (P=0.06) with salinity in third-order roots.

HWCD increased significantly ($P < 0.001$) in higher root orders (Fig. 2H; Table 2). Changes in HWCD were also significant ($P < 0.05$) between treatments and a significant ($P < 0.05$) interaction effect between salt stress and root order was found. Analysed per root order, the HWCD of root order 3 increased significantly ($P < 0.01$) from 11.4±0.7 μm under control treatment to 14.8±0.7 μm (mean ±SE) under salt stress, and the HWCD of root order 4 tended to increase (P=0.09) by the same magnitude (Table 2).

No differences in the number of peri- and exodermal layers and the suberinization of the endodermis and the peri-/exodermis were found between treatments (data not shown). However, in higher root orders, larger areas of both endo- and exodermis were suberized compared with low root orders (data not shown).

Na and Cl ion accumulation in leaves and roots

Sodium and chloride ion concentrations increased significantly ($P < 0.01$) in salt-stressed leaves (7- and 4-fold, respectively; Table 3). In roots, the concentration of both ions differed significantly ($P < 0.001$) between root orders, with lower concentrations in higher root orders (Table 3, Supplementary Table S1 at *JXB* online). For example, the Cl^- concentration in salt-stressed root tips (root order 1) was 13.65±4.08 mg g d.wt^{-1}, while sixth-order roots had a Cl^- concentration of 3.68±0.27 mg g d.wt^{-1} (mean ±SE). Ion concentrations in low root orders were often significantly ($P < 0.05$) higher than in leaves (Table 3). Between treatments, concentrations of both ions increased in roots under salinity; while the increase was marginally significant ($P = 0.07$) for sodium, possibly due to low Na^+ concentrations in higher root orders, the increase in chloride concentrations was highly significant (P <0.001; Supplementary Table S1).

Surface areas of root orders within root branches

When root systems were analysed on the level of root orders 1–4 (which were measured for water flux density, see below), 1 g of root biomass (d.wt) built 261 cm^2 root SA under control conditions and 201 cm^2 under salt stress. Approximateloy 39–50% of the SA was provided by root order 1 (root tips); that is, a SA of 122±5 cm^2 under fresh water in contrast to 78±5 cm^2 under salinity (mean±SE; Fig. 3). Root order 2 accounted for 28–32%, root order 3 for 18%, and the fourth root order for 7–12% of root branch SA. While the SA generally decreased with increasing root order under both control and salinity, no significant difference was found between SA of first and second root orders under salinity. Significant differences were found between the root SA built by root orders 1 and 3 between the fresh water and saline treatment, with significantly lower SA in first- and third-order roots under salinity ($P < 0.05$).

Table 3. Sodium (Na^+) and chloride (Cl^-) ion concentration in Citrus spp. leaf and root (root orders 1–6) tissues under fresh water (Control) and salinity (90 mM NaCl)

Organ	Ion concentration in tissues (mg g d.wt^{-1})			
	Control		Salt	
	Na^+	Cl^-	Na^+	Cl^-
Leaves	1.02±0.10 a	1.82±0.10 a	7.49±0.09 a,b	6.04±0.03 b,c
First-order roots	7.95±1.35 b	6.84±2.07 b	10.86±2.98 a	13.65±4.08 a
Second-order roots	8.19±1.64 b	7.14±1.22 b	9.39±1.92 a	12.84±2.48 a,b
Third-order roots	5.79±1.31 b	6.21±0.38 b	6.08±1.48 a,b	10.38±1.70 a,b,c
Fouth-order roots	2.12±0.39 a	3.86±0.36 ab	3.36±0.22 b	6.45±0.28 a,b
Fifth-order roots	1.08±0.32 a	2.19±0.22 a	2.51±0.04 b	4.62±0.31 c
Sixth-order roots	0.99±0.18 a	2.00±0.16 a	2.12±0.03 b	3.68±0.27 c

Different lower letters denote differences within columns (mean ±SE; Tukey, $P < 0.05$, n=3–10); see Supplementary Table S1 at *JXB* online for ANOVA results on the influence of treatment and root order on the ion concentrations.

Water flux density

Water flux densities (J_s) differed significantly between *C. volkameriana* root orders and treatments (Fig. 4). Water uptake under fresh water (control) declined significantly ($P < 0.05$) from root order 1 (root tips) to root orders 2 and 3 (0.71±0.15, 0.16±0.04, and 0.11±0.02 g cm^{-2} h^{-1}, respectively; mean ±SE); fourth root orders possessed water excess (–2.49±0.69 g cm^{-2} h^{-1}; Fig. 4A). Under salinity and after stress release, J_s of first-order roots was significantly reduced by >80% to 0.12±0.25 g cm^{-2} h^{-1} and 0.13±0.09 g cm^{-2} h^{-1}, respectively (Fig. 4B, C). Mean water flux densities ($J_{\bar{s}}$) in root orders 1–4 were generally low under salinity (–0.07 g cm^{-2} h^{-1} to 0.18 g cm^{-2} h^{-1}) while the variability in water flux densities increased significantly as compared with the control (data not shown). Under stress release conditions (i.e. 0.5 LA solution in the miniature depletion chamber, root system placed in 1.0 LA + 90 mM NaCl) all root orders took up water (Fig. 4C). The highest J_s values under stress release conditions were found in second- (0.31±0.06 g cm^{-2} h^{-1}) and third-order roots (0.67±0.49 g cm^{-2} h^{-1}); second-order roots had significantly ($P < 0.05$) higher uptake rates of fresh water after stress release than under continuous fresh water treatment (control). The fluxes of stress-released third-order roots were significantly higher than under salinity ($P < 0.05$; Fig. 4B, C).

Up-scaled, using the surface area and J_s of root orders, a root branch with four root orders and 1 g d.wt (Fig. 3) had mean water flux densities ($J_{\bar{s}}$) of 0.208, 0.099, and 0.283 g cm^{-2} h^{-1} under control conditions, salinity, and stress release, respectively (Fig. 4, insets).

The relative contribution of root orders to the branch water flux density differed among treatments (see Supplementary Fig. S3 at *JXB* online). For example, 57% of the water fluxes were mediated by first-order roots under fresh water (control), in contrast to 38% under salinity and 18% after stress release conditions. Second-order roots mediated 48% of the water fluxes under salinity and 35% after salt release in contrast to 7% under fresh water supply (Supplementary Fig. S3).

Discussion

Changes in phenotype in response to salinity are often adaptive by enhancing the fitness of plants. For example, increased root:shoot ratios are thought to improve the 'source:sink ratio' for water and nutrients under salinity (Zekri and Parsons, 1989). In this study, the root:leaf ratio of *Citrus* increased significantly while the rootstock biomass was marginally reduced under salinity (Fig. 1, Table 1). However, because root functions, such as water uptake, are strongly related to root tissue differentiation (Doussan *et al.*, 1998) and root order (Pregitzer *et al.*, 2002; Comas and Eissenstat, 2009; Rewald *et al.*, 2011*a*), total root mass and root:shoot ratios cannot effectively determine the functionality of woody root systems under stress. Thus, to predict uptake, knowledge of the active root surface area and the flux density is needed (Hinsinger *et al.*, 2011)

Structural, morphological, and anatomical changes under salinity

Several previous studies on woody species found reduced numbers of lateral roots under salinity (e.g. Reinhard and Rost, 1995; Eshel and Waisel, 1996; Croser *et al.*, 2001); similarly, this study provides evidence that severe NaCl stress reduces the number of *Citrus* root orders from eight to seven. However, more importantly, the current study shows that the frequency of root orders and morphological and anatomical traits known to influence uptake processes, for example root system branching (Dunabin *et al.*, 2004), SRA (Trubat *et al.*, 2006), cortex thickness (Rieger and Litvin, 1999), and xylem traits (Rodríguez-Gamir *et al.*, 2010), do not change homogenously throughout *C. volkameriana* root systems under salinity but that changes are often root order specific (Table 2).

Changes among order frequencies of *Citrus* roots were previously reported under altered nitrate supply (Sorgonà *et al.*, 2007, 2011). Similarly, in this study, lower root orders, especially root tips, were most plastic in frequency, expressed as both relative biomass and surface area per root

branch (Fig. 2A, B, Table 2). Because low root orders have a high SRA, the 3% loss in total root system biomass under salinity caused a major reduction of active root surface area by ~23% (Figs 1–3). Because uptake is strongly coupled to root SA (e.g. Korn, 2004), the reduced SA provided by root tips under salinity is indicative of a decrease in functionality, in terms of uptake, of this order. In contrast, the relative importance of root orders ⩾2 for water and/or nutrient uptake should increase accordingly. The high plasticity of first (second) root orders in respect of biomass (SA) frequency and morphology was anticipated as lower root orders have relatively high turnover rates (Guo *et al.*, 2008*b*, and references within) and are considered most vulnerable to environmental stresses. However, in addition to results from *Citrus* spp., seedlings under varied nitrate supply, in which the morphology of second and third (i.e. tap roots of these seedlings) root orders were considerably less plastic than those of root tips (Sorgonà *et al.*, 2007, 2011), the present result showed that even intermediate, third and fourth, root orders of more mature plants are able to undergo significant morphological changes within 6 months (Table 2).

Similar to earlier reports, the cortex diameter decreased and the proportion of the stele increased in higher root orders under fresh water supply (Fig. 2E, F; Guo *et al.*, 2008*a*). The increase in root diameter under salinity was expected to be caused by increasing cortex dimensions as reported, for example, for cotton roots (Casenave *et al.*, 1999). However, under salinity, significant changes of cortex and stele dimensions were found in third and fourth root orders only, and reaction norms differed in direction (Fig. 2E, F, Table 2). It is hypothesized that the contrasting changes are related to the different functions of these two root orders for water uptake under fresh water; in brief, third-order roots of *C. volkameriana* were found to perform water uptake under fresh water supply, while fourth-order roots showed water excess (Fig. 4A). The outflow of water in fourth-order roots was related to changes in chamber solute osmolalities, possible caused by exudation (for details, see Rewald *et al.*, 2011*a*).

The lack of significant changes in gross root anatomy (i.e. cortex and stele dimension) and xylem traits in first and second root orders was surprising because these root orders have a larger number of passage cells (Eissenstat and Achor, 1999), and are the preferred sites of water and nutrient uptake under fresh water (Fig. 4; Supplementary S2 at *JXB* online; Peterson and Enstone, 1996; Rewald *et al.*, 2011*a*). Previous studies on cotton radicles and roots of herbaceous plants found smaller xylem vessels at higher frequencies under salinity, probably caused by a repression in the development of metaxylem vessels and altered cambial activity (Reinhardt and Rost, 1995; Casenave *et al.*, 1999; Boughalleb *et al.*, 2009). However, wider and

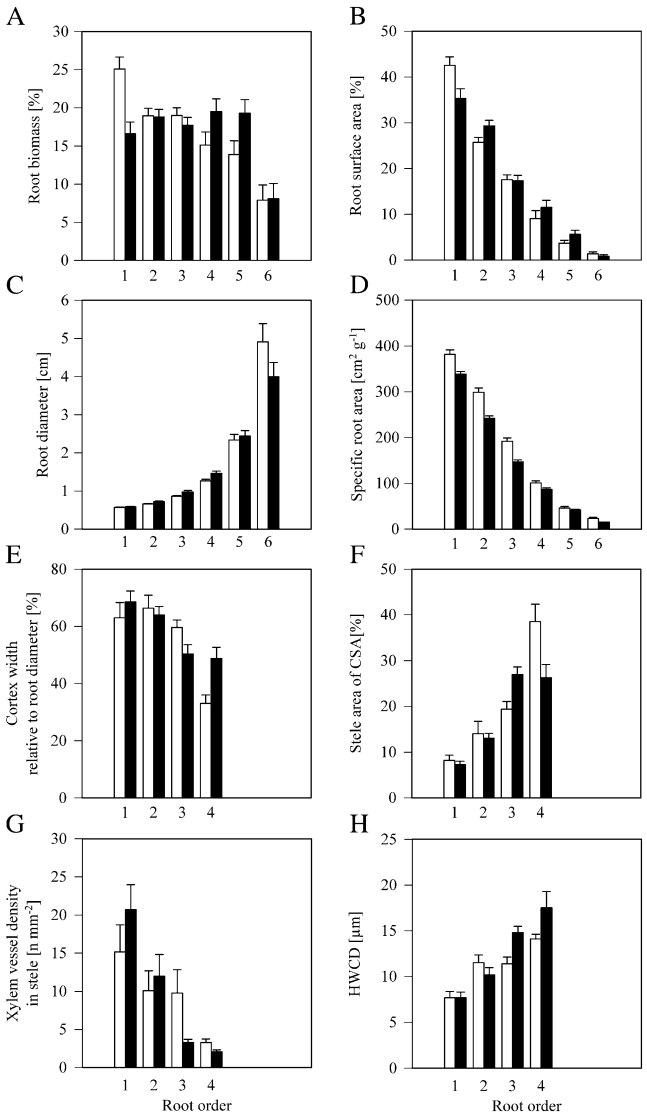

Fig. 2. Structural, morphological, and anatomical traits of *Citrus volkameriana* root orders 1–4/6 after 6 months under fresh water (ctrl, open bars) or salt stress (salt, filled bars). (A and B) Relative root biomass and root surface area of orders 1–6 in root branches (mean ±SE, *n*=36). (C and D) Root diameter and specific root area (mean ±SE, *n*=36–197). (E–H) Cortex diameter relative to root diameter, percentage of the stele on the root cross-section area (CSA), xylem vessel density related to the area of the stele, and hydraulically weighed conduit diameter (HWCD; mean ±SE, *n*=8). See Table 2 for statistics.

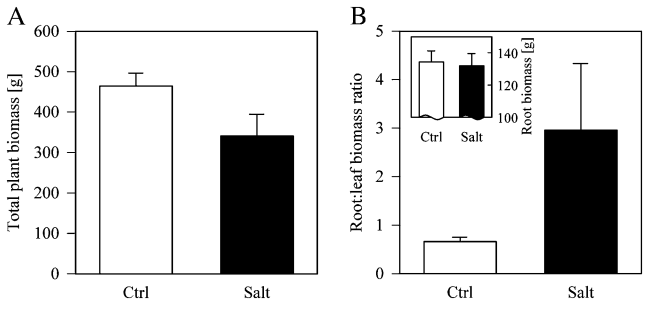

Fig. 1. (A) Total plant biomass and (B) root:leaf biomass ratio of *Citrus* spp. after 6 months under fresh water (Ctrl, open bars) and salinity (Salt, filled bars). The inset in B shows the root biomass per treatment (mean ±SE, *n*=3). See Table 1 for statistics.

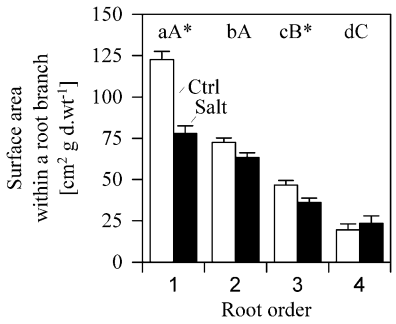

Fig. 3. Surface area of root orders 1–4 under fresh water (Ctrl, open bars) and salinity (Salt, filled bars) in a standardized root branch of 1 g d.wt. Different lower/upper case letters denote significant differences between root orders within control and saline treatments respectively; Asterisks denote significant differences between treatments (mean ±SE; Mann–Whitney U-test, $P < 0.05$, n=189–214).

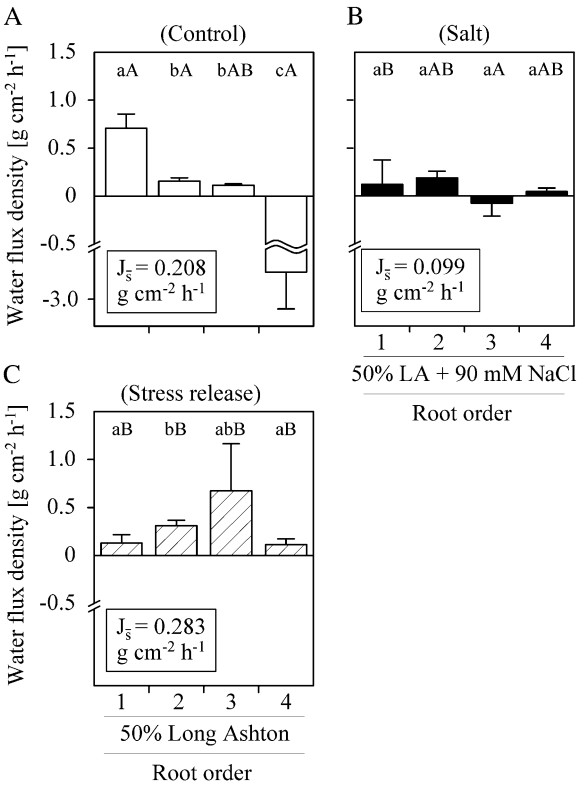

Fig. 4. Water flux density (J_s) of *Citrus volkameriana* root orders 1–4 under (A) fresh water (control), (B) salinity, and (C) stress release conditions. Plants were placed either in 1.0 strength LA (A) or 1.0 strength LA + 90 mM NaCl (B, C). The miniature depletion chambers were filled with either 0.5 strength LA (A, C) or 0.5 LA + 90 mM NaCl (B), respectively. Different lower case letters denote significant differences between root order-specific flux densities; different upper case letters denote significant differences between treatments (mean ±SE; Mann–Whitney U-test, $P < 0.05$, n=5–11). Mean water flux densities (J_s) of root branches of 1 g d.wt. are given.

fewer xylem vessels have been found in both stems and some coarse roots after exposure to salinity (e.g. Eckstein *et al.*, 1978, Rewald *et al.*, 2011*c*), thus the present results might indicate a different reaction norm in ephemeral roots (such as root order 1 and 2) and more persistent woody roots (such as root order 3 and 4) under salinity. While the underlying molecular mechanisms and the functional significance of these changes in the xylem structure remain open, the differences between root orders under salt stress are probably related to the varied accumulation of sodium and chloride ions (Table 3). Lower salt concentrations are suggested to impair the cambial activity and metaxylem differentiation in higher root orders to a lesser extent. Suberization of the endo- and exodermis increased in older (higher) *Citrus* root orders (data not shown) as found elsewhere (e.g. Eissenstat and Volder, 2005) and is likely to be one factor underlying the significantly lower Na^+ and Cl^- accumulation in higher root orders of *C. volkameriana* (Table 3, Supplementary Table S1 at *JXB* online; Krishnamurthy *et al.*, 2009). In contrast to findings in herbaceous plants, which often have lower Na^+ and Cl^- concentrations in roots than the external solution (Munns, 2002), salt accumulation in *Citrus* root orders 1–5 was explicitly higher than those of the surrounding solution and partially higher than those in leaves (see also Arbona *et al.*, 2005). Because physiological damage in *Citrus* spp. is associated with tissue chloride build-up rather than with sodium accumulation (Romero-Aranda *et al.*, 1998), the high Cl^- concentration in lower root orders backs up the hypothesis that many structural, morphological, and anatomical changes were driven by accumulating salt.

Effect of salinity on root water uptake

It was hypothesized that changes in water flux rates in salt-stressed *Citrus* rootstocks are related to changes in both root surface area and root anatomy/physiology, as originally suggested by Storey and Walker (1999). As mentioned above, changes were observed in abundance and SRA of root orders 1–4 that resulted in a 23% reduction of root branch surface area under salinity compared with fresh water (Fig. 3). Changes among anatomical (see above) and physiological parameters (e.g. membrane properties) are also known to modify root hydraulic conductivity (Lp_r; Peterson and Enstone, 1996; Steudle, 2000; Vandeleur *et al.*, 2009). In the present study we did not find major changes in gross root anatomy; thus, besides the lower water potential of the salt solution, fine-scale anatomical or physiological changes and damage caused by high salt accumulation (see above) have probably contributed to the 50% reduction of root branch mean water flux densities (J_s) in this study (Fig. 4A, B). This is supported by the finding that J_s did not increase in first-order roots after release of the salt stress (Fig. 4C) as expected if only temporarily caused by the lower water potential of the salt solution. Because water flow rates among *Citrus* root systems follow the same trend as root conductivities (Zekri and Parsons, 1989), it is valid to hypothesize that the reduction of J_s in salt-stressed *Citrus* rootstocks is related to changes in both root surface area and root physiology.

Interestingly, the water flow densities (J_s) of root orders changed differently under salinity (Fig. 4A, B). The decrease in J_s under salinity was mainly caused by a significant reduction (>80%) of the water uptake by first-order roots. The degree of J_s reduction is in accordance with measurements on apical segments of corn roots, among which Lp_r was reduced by 80% under salinity (Evlagon *et al.*, 1990). The water flux densities of the other three *Citrus* root orders did not change significantly under salinity (Fig. 4B). Together with the different reduction in surface areas (see above), the varying water flux densities are changing the contribution of root orders to the overall water flux density of the root system (Fig. 4, Supplementary Fig. S3A, B at *JXB* online). For example, second-order roots contributed only 7% to the water fluxes under fresh water but nearly 50% under salinity. The increased importance of higher root orders for water uptake under salinity may help to explain previous results of Zekri and Parsons (1989) who found the highest reductions of root length under salinity in *Citrus* species which were most tolerant to salinity in terms of water flow rate or root conductivity. This has been thought to be in contrast to studies which found that *Citrus* rootstocks with high specific root lengths tend to exhibit high hydraulic conductivities under fresh water supply (Graham and Syvertsen, 1985; Eissenstat, 1997). However, the overall root length density might cause an overestimation of the water uptake capacity under salt stress if thin, first-order roots contribute less to water uptake (as seen in the current study). This might be true as well if parts of the root system are temporarily released from salt stress; in this study, second-order and third-orders roots contributed more to water uptake under stress relief than under both control and salt treatments (Fig. 4, Supplementary Fig. S3C). A temporal and spatial release of roots from salinity might occur *in situ*, for example in saline water-irrigated orchards (during 'salt leaching') or after rainfall events (Cimato *et al.*, 2010).

While Sorgonà *et al.* (2007) stated that more distal root orders have a prominent adaptive significance in *Citrus*, possibly due to their high number of passage cells (Eissenstat and Achor, 1999), this study demonstrated that the importance of specific root orders for water uptake and tolerance of the whole root system is subject to changes in response to environmental conditions. The underlying anatomical and physiological factors still remain open but root order-specific changes in the development of Casparian bands, suberin lamellae, passage cells (Peterson *et al.*, 1993; Peterson and Enstone, 1996), aquaporin expression (Vandeleur *et al.*, 2009), or a different susceptibility to reactive oxygen species (Li *et al.*, 2009) or salt accumulation (this study) might have resulted in the observed differences. Further investigation is needed to determine the parameters underlying the (i) different susceptibility of root tissues to salt stress and ion accumulation and (ii) the different water flux densities among root orders, and should seek to compare the function of different salt-tolerant rootstocks on the level of root orders.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Schematic side view of a 'miniature depletion chamber' (A), attached to a second-order root, and a drawing of the experimental set-up (B).

Figure S2. Photographs of *Citrus volkameriana* root orders 1–4 after 6 months under fresh water (A–D) and salinity (E–H).

Figure S3. Relative contribution of root orders 1–4 to the total water flux (100%) under (A) fresh water (control), (B) salt stress, and (C) after stress release.

Table S1. Influence of treatment and root order on the Na^+ and Cl^- concentrations in *Citrus volkameriana* roots.

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