Agricultural Water Management 228 (2020) 105897

Contents lists available at ScienceDirect

Agricultural Water Management

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

Rootstocks modulate the physiology and growth responses to water deficit and long-term recovery in grafted stone fruit trees

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ARTICLE INFO

Keywords:
Prunus
Relative transpiration
Aquaporin
Drought
Water-use efficiency
Rootstock/scion interaction

ABSTRACT

The effects of climate change have forced to search quickly new strategies to achieve sustainable agriculture in the context of a growing demand for food. Rootstocks have shown to have a key role in the resistance and tolerance to biotic and abiotic stresses in horticultural and fruit trees species, being a way to incorporate these strengths to the cultivated varieties. Here, we report the assessment of physiological parameters and growth responses of two commercial interspecific hybrid *Prunus* rootstocks contrasting in their tolerance to water deficit: 'ROOTPAC®40' (tolerant) and 'ROOTPAC®20' (sensitive); grafted with the almond cultivar Non Pareil or the Japanese plum cultivar Angeleno. Plants were subjected during 35 days to two irrigation treatments, well-watered and water deficit (drought period), followed by a recovery period of 44 days. On each period, biomass accumulation, stomatal density, water-use efficiency at the whole plant level and root hydraulic conductivity were determined in both irrigation treatments. Also, in the drought period, the fraction of transpirable soil water when the relative transpiration drops and the expression of seven aquaporins belonging to plasma membrane intrinsic proteins subfamily was assessed. In the drought period, 'ROOTPAC®20' had a decrease in the aquaporin gene expression in roots and also had an early decline in transpiration, independent of the grafted scion. Also, on this rootstock, the biomass was more severely affected. On the other hand, 'ROOTPAC®40' induced a late decline in transpiration and increased the water-use efficiency, keeping less affected the biomass accumulation. Contrasting with the above, in the recovery period, 'ROOTPAC®20' improved the water-use efficiency, reaching higher biomass accumulation in the grafted plants. Also, we observed some interesting interactions between rootstocks and scions. 'ROOTPAC®20' induced a higher stomatal density in both, almond and plum scions, and 'Angeleno' plum induced a bigger root hydraulic conductivity in both rootstocks, in comparison to almond 'Non Pareil', in the drought period.

1. Introduction

Water availability is the most determinant environmental factor in plant evolution (Zhu, 2002; Xoconostle-Cazares et al., 2010) and, among the abiotic stresses, water stress has one of the largest impacts on agricultural productivity (Reddy et al., 2004). In the context of global climatic change, a reduction in annual rainfall and an increase in mean temperatures is expected (Li et al., 2009), a condition leading to an increase in crop's water demand, which can significantly affect agricultural worldwide. Water deficit affects the plant metabolism at several levels (Ingram and Bartels, 1996; Valliyodan and Nguyen, 2006), including gene expression, physiology, metabolomics, growth and development (Yordanov et al., 2000; Reddy et al., 2004; Chaves et al., 2009). In many cases, moderate water deficit increases water-use efficiency (WUE) because of a partial closing of the stomata, which allows the maintenance of the CO_2 influx into the mesophyll but reducing transpiration (Chaves et al., 2009).

In plants and other organisms, aquaporins are proteins that allow the transport of water and small molecules across the plasma membrane (Kaldenhoff and Fischer, 2006; Maurel et al., 2015). Aquaporins are classified into five subfamilies, where those known as Plasma Membrane Intrinsic Proteins (PIPs) have been widely studied for their role under water deficit (Chaumont et al., 2005; Maurel et al., 2008) and might be involved in the control of plant transpiration under water deficit (Shekoofa and Sinclair, 2018). However, there is still debate on the relation between aquaporin activity and the hydraulic conductance in plants, and the effect that may have on the transpiration rate under water deficit conditions (Shekoofa and Sinclair, 2018).

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https://doi.org/10.1016/j.agwat.2019.105897
Received 9 August 2019; Received in revised form 1 November 2019; Accepted 4 November 2019

The technique of grafting has been known from the beginning of the first millennium BC, mainly for propagating woody species difficult to root from cuttings, such as apples, pears, and plums (Mudge et al., 2009). Only in the last two centuries, the use of rootstocks has been rapidly increased to enhance productivity, disease resistance, adaptability to unfavorable soil conditions and to reduce productive costs of intensive high-value agricultural species (Albacete et al., 2015). Although the key role played by both rootstock and scion is acknowledged, their relative contribution on specific combinations of genotypes is not yet well understood (Tramontini et al., 2013). It has been suggested that rootstocks can contribute to food security by increasing yield potential of elite varieties, closing the yield gap under suboptimal growing conditions and increasing the efficiency of water and soil use, among others (Albacete et al., 2015). For this reason, some researchers have highlighted the importance of the study of roots as the key factor to achieve a second green revolution in the Agriculture (Gewin, 2010). In the global context of climate change, the selection of rootstocks as a mean for improving the water use efficiency has been proposed as an essential strategy to face this scenario (Berdeja et al., 2015).

In the last years, the importance of rootstocks has been understood as a key component to face water deficit conditions. Rootstocks could strongly impact the gene expression in grapevine (*Vitis vinifera* L.) berries under water deficit (Berdeja et al., 2015). It has been observed that the drought-tolerant rootstock 4X of Rangpur lime (*Citrus limonia* Osbeck) grafted with Valencia Delta sweet orange (*Citrus sinensis* L.) modified the gene expression patterns in Rangpur lime citrus roots to regulate the adaptation to water defict (Allario et al., 2013). The recent discovery of a small peptide synthesized in the root vascular tissues of *Arabidopsis thaliana* L. under drought conditions, capable of moving from roots to the leaves and induced stomatal closure, highlights the molecular root control on plant transpiration under drought condition (Takahashi et al., 2018). The control of the threshold at which the plant transpiration drops in a drying soil, determines the adaptive strategy used by a genotype (more or less conservative) and has been suggested as a key trait for predicting the success of a crop under water deficit condition (Belko et al., 2012; Vadez et al., 2013; Berger et al., 2016; Shekoofa and Sinclair, 2018). On this regard, the control of the scion transpiration under WD has been observed to be determined by the rootstock in grapevines (Marguerit et al., 2012).

Stone fruit crops (*Prunus* spp.) are the seventh largest group of fruit trees produced worldwide (FAOSTAT, 2017) and, in the present study, we propose the genus *Prunus* as a model system for assessing the interaction between rootstock and scion. On *Prunus* species, it is possible to graft different species of high economic importance with different degrees of drought tolerance like plum (*P. salicina* and *P. domestica*), peach (*P. persica*), almond (*P. dulcis*), cherry (*P. avium*) and apricot (*P. armeniaca*) directly or using an interstock, taking into account the wide genetic diversity of interspecific rootstocks currently available. Additionally, in general terms, these *Prunus* rootstocks are easy to propagate clonally and count with six full genomes availabile: [*P. persica* (Verde et al., 2017*)*, *P. avium* (Hirakawa et al., 2017), *P. mume* (Zhang et al., 2012), *P. yedoensis* (Baek et al., 2018), *P. domestica (*Zhebentyayeva et al., 2019*)* and *P. dulcis* (Sánchez-Pérez et al., 2019)], which is advantageous for comparative molecular studies. Species in the subgenus *Amygdalus* (especially almond tress) are recognized as water deficit-tolerant within genus *Prunus* (Fußeder et al., 1992; Camposeo et al., 2011). Consequently, the use of almond x peach hybrid rootstocks increases tolerance to water deficit in peach (Jiménez et al., 2013) and almond trees (Ben Yahmed et al., 2016). In contrast, species in the subgenus *Prunophora*, such as plums and *P. cerasifera*, have lower tolerance to water deficit (Duval, 2015).

In this study we aimed to determine the influence of rootstocks on physiological performance and growth parameters to water deficit on grafted plants. For this purpose, we have assessed the responses of almond and Japanese plum, grafted onto two contrasting rootstock genotypes, under well-watered and water deficit conditions. In addition, we evaluated such responses under a post-stress long-term recovery period.

2. Materials and methods

2.1. Plant material and experimental conditions

This study was conducted at the Centro de Estudios Avanzados en Fruticultura (CEAF), Rengo, Chile (34°19′S 70°50′W). 'Non Pareil' (Np) almond (*Prunus dulcis* [Mill.] DA Webb) and ‘Angeleno’ (An) Japanese plum (*Prunus salicina* L.) were selected as scion, because they are genetically distant, differing in leaf morphology, size (individual leaf area ˜ 12 vs 25 cm^2 in ‘Np’ and ‘An’, respectively. Supplementary Table 1) and stomatal density (˜230 vs 820 stomata per mm^2 in ‘Np’ and ‘An’, respectively. Supplementary Table 1). During winter of 2016, ‘Np’ and ‘An’ scions were grafted onto two one-year old interspecific *Prunus* hybrid rootstocks, which were selected based on their contrasting responses to water deficit as ungrafted rootstocks (Opazo et al., accepted) and differences in their genetic composition: ROOTPAC®40 [(*P. dulcis* x *P. persica* Batsch) x (*P. dulcis* x *P. persica*)] (‘R40’) as a water-deficit tolerant genotype. On the other hand, ROOTPAC®20 (*Prunus besseyi* Bailey × *Prunus cerasifera* Ehrh) (‘R20’) was used as a sensitive genotype (Jiménez et al., 2013) (Table 1). Both, ‘R20’ and ‘R40’ rootstocks, are considered as semi-dwarfing rootstocks (Scalisi et al., 2018). Thereby, four rootstock/scion combinations were established: 'R40/Np', 'R40/An', 'R20/Np' and 'R20/An'. In spring, plants ca. 40 cm tall were transferred to 20-L containers filled with a mixture of 1:1 peat/perlite supplemented with Basacote Plus 9 M at a 6 g L^{-1} as a controlled release fertilizer (BASF, Limburgerhof, Germany). Prior to transplant, each container was saturated with tap water, allowed to drain and covered with plastic bags to avoid evaporation during 24 h. After that, similar initial weight of each container was reached (ca. 11 kg) and recorded. This was established as a 100% of substrate water content (SWC) and considered as field capacity. Plants were grown for 30 days in a shade house (50% sunlight) and then were transferred to field condition (full sunlight). Plants were acclimatized to this condition for two weeks prior to the start of the experiment. Containers were covered with plastic bags and irrigated using two drippers per plant with a flow rate of 2 L h^{-1} for 15 days. After that, 26 uniform and healthy plants, with ca. 60 cm tall, of each rootstock/scion combination were selected for all evaluations. Four plants were used to determine the initial biomass, eight plants for final biomass and root hydraulic conductance at the end of the drought period, eight plants for final biomass at the end of recovery period and physiological evaluations (net photosynthesis, stomatal conductance, midday water potential and root hydraulic conductance) along the drought and recovery periods, and six plants were used for

Table 1
List of the studied rootstocks (*Prunus* spp.), almond (*Prunus dulcis*) and plum (*Prunus salicina*) cultivars.

	Genotype	Species	Origin
Rootstock	Rootpac®20	*P. besseyi* x *P. cerasifera* (plum hybrid)	AI, Spain
	Rootpac®40	(*P. dulcis x P. persica*) x (*P. dulcis x P. persica*) (almond-peach hybrid)	AI, Spain
Cultivar	Non Pareil	*P. dulcis* (almond)	A.T Hatch, USA
	Angeleno	*P. salicina* (plum)	J.M Garabedian, USA

gene expression analysis. In the drought period (from December 28, 2016 to February 1, 2017), average day- and night-time temperature were 33 and 13 °C and relative humidity ranged between 31 and 68%, respectively. During recovery period (full irrigation in water deficit plants; from February 2, 2017 to March 18, 2017), average day- and night-time temperature were 31 and 11 °C and relative humidity ranged between 35 and 83%, respectively.

2.2. Irrigation treatments

Plants were subjected to two irrigation treatments: well-watered (WW) and water deficit (WD), in a completely randomized experimental design. Plants under WW condition were irrigated three times per week adding as much water as to reach the corresponding 100% of the SWC for each pot. In WD condition, on the other hand, irrigation of plants was withheld for 33 days, weighting each pot three times per week in order to determine the water consumption of every single plant. Then, all the pots were filled with the volume of water necessary to reach the same water content of the plant with the lowest transpiration rate which, in turn, was not irrigated. Therefore, after the irrigation, all the WD pots contained the same soil water % of the SWC. This irrigation schedule was maintained for 35 days. The minimum water content in the pot (0% SWC) was determined at the end of the drought period using the average of 10 containers with the lowest substrate weight (maximum water extraction) on independent plants. The SWC (Supplementary Fig. 1) was calculated for each container as Eq.1.

$$\mathrm{SWC}\ (\%) = (\mathrm{SW} - \mathrm{AMSW})/(\mathrm{MSW} - \mathrm{AMSW}) \qquad (1)$$

where SW is the daily substrate weight, AMSW is the average minimum substrate weight of 10 containers with the lowest substrate weight and MSW is the maximum substrate weight at field capacity. After the drought period, the recovery period was maintained for 44 days and all plants were allowed to reach the 100% of SWC in each irrigation cycle.

The plant water uptake capacity under WD was calculated as the relationship between the relative transpiration rate (RT) and the fraction of transpirable substrate water (FTSW) for each container (Sinclair and Ludlow, 1986). RT corresponds to the daily amount of transpired water under WD, divided by the average daily transpiration of the WW for each rootstock/scion combination. FTSW correspond to the fraction of water inside the container that plants can use to transpiration. The RT of each plant was divided by the mean relative transpiration rate for that plant during the period when the soil was still well-watered in order to normalize the initial values (Sinclair and Ludlow, 1986). According to Bindi et al. (2005) the initial point for stress ($\mathrm{FTSW}_{\mathrm{threshold}}$) is around to a RT value of 0.9. Then, RT was adjusted to a logistic equation as in Eq.2.

$$\mathrm{RT} = 1/(1 + \alpha\cdot\exp^{(-\beta*\mathrm{FTSW})}) \qquad (2)$$

where α and β are constants to be determined for each plant related to the curvature of a logistic regression.

2.3. Stomatal density

The stomatal density was determined at three different times on different leaves developed during each period: before of the imposition of drought, at the end of the drought and recovery periods. Four mature leaves were selected from each treatment and combination in order to assess the stomatal density. Stomatal imprint in attached leaves were made by applying a nail varnish on the abaxial surface of the leaves, avoiding the midrib and the leaf margin. After drying, the nail varnish film was gently peeled off using transparent tape and was fixed on a clean labelled microscope slide (Kardel et al., 2010). The stomatal imprints were analyzed with a light microscope (Olympus BX43, Olympus, Hamburg, Germany). For each imprint, two images were taken in different zones. Stomatal density (number of stomata per mm^2) was made counting all the stomata of the image (known area) and extrapolating to 1 mm^2. In total, 8 images (4 leaves and 2 zones per

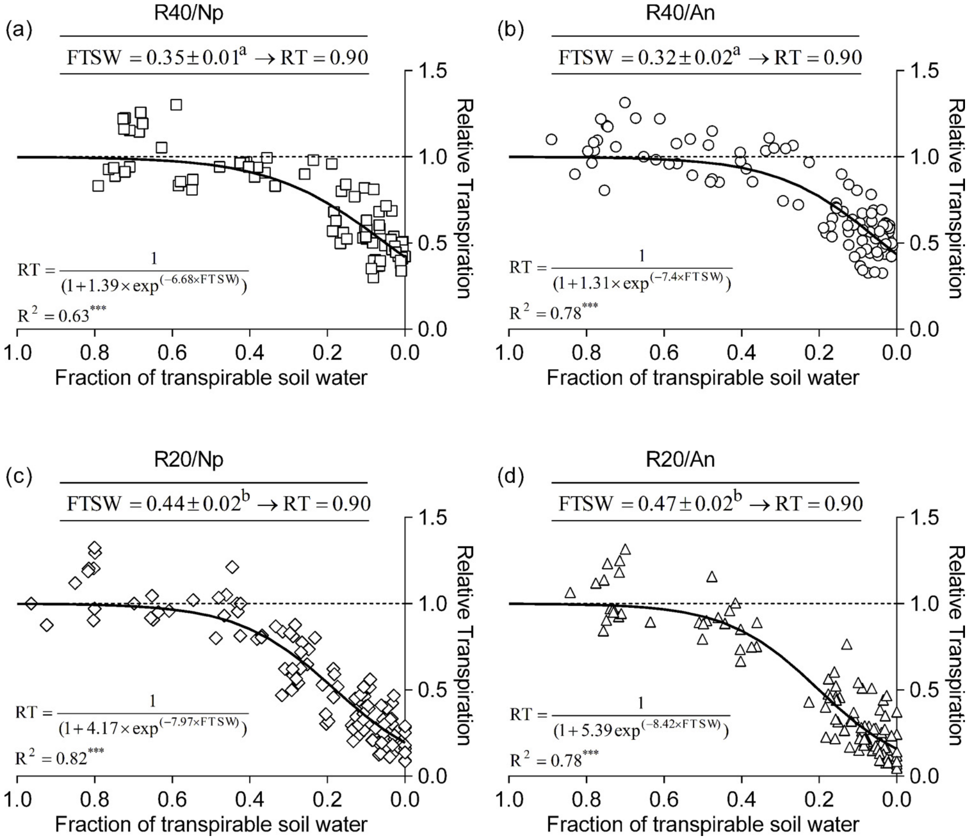

Fig. 1. Relationship between the fraction of transpirable substrate water (FTSW) and relative transpiration (RT) of (a) R40/Np (squares), (b) R40/An (circles), (c) R20/Np (diamonds) and (d) R20/An (triangles). Dotted lines indicate RT equal to 1. Logistic lines were fitted using all available replicates and the equation is presented. *** indicated significant regression ($p < 0.001$). On top of each graph, the value of initial point for stress ($\mathrm{FTSW}_{\mathrm{threshold}}$), which corresponds to a RT value of 0.90, is indicated using the logistic curves derived from all the replicates within a genotype. Different letters denote significant differences ($p < 0.05$) between genotypes according to Fisher's LSD test.

leaf) were analyzed to obtain an average stomatal density per plant in each experimental period, drought and recovery.

2.4. Root hydraulic conductivity

The root hydraulic conductivity (L_p) was determined by a High Pressure Flow Meter (HPFM, Dynamax, Houston, TX, USA) according to Tyree et al. (1995). The night before measurements, WD plants were fully irrigated in order to recover the water columns, reducing artifacts due to cavitation (Alsina et al., 2011). The L_p measurements were made in the whole root system below the rootstock/scion junction at the end of the periods of drought and recovery, twice per plant. Subsequently, this value was normalized by the dry weight of the complete root system (Vandeleur et al., 2014).

2.5. Stomatal conductance, net photosynthesis and stem water potential

Stomatal conductance (g_s) and net photosynthesis (P_n) were measured after 1, 8, 15, 22, 29 and 35 days of water deficit, and after 1, 7 and 13 days of recovery. These measurements were conducted on mature fully expanded leaves using a portable photosynthesis equipment (model CIRAS-2, PPSystem, Hitchin, UK) equipped with a 2.5 cm^2 LED lighting cuvette (model CIRAS PLC, PPSystem). The temperature within the cuvette was maintained at 25 °C, with a radiation level of 1000 $\mu mol\ m^{-2}\ s^{-1}$, 400 $\mu moL\ CO_2\ mol^{-1}$ air and relative humidity of. Both g_s and P_n measurements were performed between 9:30 and 11:00 am on the day before irrigation. Midday stem water potential (Ψ_{stem}) was measured on a mature leaf (fifth leaf expanded from the apex) in the same days that g_s and P_n measurements. The leaves were covered with plastic bags coated with aluminum foil to stop transpiration and allow it to balance with the stem potential at least 2 h before measuring. The measurement was made at solar noon (between 13:00 and 15:00 pm) with a Schölander pressure chamber (Schölander et al., 1965).

2.6. Whole-plant water use efficiency and growth

At the beginning and at the end of the periods of drought and recovery, plants were harvested and split into leaves, stem and roots. The dry weight of each plant part was determined after placing the samples in an oven at 70 °C until reaching a constant weight. Growth for each plant part was calculated by means of the difference between the final biomass of leaves, stems and roots of each plant and the average initial biomass of each scion/rootstock combination. In the case of the drought period this difference was between the biomass at the end of the drought period and the average initial biomass, and in the recovery period the difference was between the final biomass with the average biomass at the end of the drought period, per treatment and plant part. The leaf area, stomatal density and stomata length of different commercial *Prunus* leaves were analyzed prior to the experiment in order to select the two more contrasting scions (Supplementary Table 1). Leaf area and stomata length were obtained by scanned leaves and processing stomatal imprints, respectively, using the ImageJ software (version 1.51j8 NIH) with a reference area (O'Neal et al., 2002).

Whole-plant water use efficiency (WUE_{wp}) was calculated as the difference between the total biomass at the end of drought and recovery periods minus the average biomass at the beginning of drought and recovery periods, respectively, both divided by water consumption of the respective period, as in Eq. 3.

$$WUE_{wp} = (\text{Final dry biomass} - \text{Initial dry biomass})/(\text{Total water consumption}) \quad (3)$$

2.7. Transcriptional analysis

At the end of the drought period, plants of all the rootstock/scion combination and irrigation treatments were selected for gene expression. Three root samples were collected from three different plants in order to assess the expression level of genes encoding for seven PIPs (PIP1;1, PIP1;2, PIP1*;3,* PIP2;1, PIP2;2, PIP2*;3*, and, PIP2*;4*). Total RNA was extracted following the CTAB methodology described by Chang et al. (1993). DNAse I (Ambion, Austin, USA) was used to remove genomic DNA contamination. RNA quality was tested using electrophoresis in agarose gel stained with RedGel. Total RNA concentration was determined by spectrophotometry (InfiniteR M200 Pro NanoQuant, Tecan Group Ltd., Switzerland). Then, cDNA was synthesized from 2 μg RNA using the Maxima First Strand cDNA Synthesis kit (Thermo Scientific, USA). The obtained cDNA was diluted in a 1:4 relation and, using 2 μL of the solution, qRT-PCR reactions were run using specific primers designed with the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA; Supplementary table 2) and synthesized by IDT Integrated DNA Technologies, INC (Fermelo Biotec). All reactions were run in an MX3000 P QPCR System (Agilent Technologies, USA) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) as the fluorescent detector. Two technical replicates were run for each biological sample. The specificity of the amplified products was confirmed by the registration of a single peak in the melting curves and the visualization of a single band in the agarose gels. Data was normalized using *RPII* (*RNA Polymerase subunit 2*; GenBank accession Prupe.8G132000.1) (Tong et al., 2009). Well-watered plants from each rootstock/scion combination, were used as calibration samples, and the normalized values were used for the determination of the relative quantification (Pfaffl, 2001).

2.8. Experimental design and statistical analysis

Differences in $FTSW_{threshold}$, stomatal density, L_p, biomass, WUE_{wp}, and gene relative expression were tested using irrigation, rootstock, scion and their interactions as fixed factors. Stomatal conductance, net photosynthesis and midday stem water potential were tested using repeated measure ANOVA where leaves measured over time are the repeated measures. Heteroscedastic variance models were used when necessary, followed by LSD mean separation when appropriate. All the statistical analyses were made using InfoStat (version 2016e, Universidad de Córdoba, Córdoba, Argentina) statistical software (Di Rienzo et al., 2011).

3. Results

3.1. Water consumption and stomatal density

Two different RT vs. FTSW relationship patterns were observed (Fig. 1). The first, with a late reduction of RT, occurring when the FTSW reached values of 0.35 and 0.32 in 'R40/Np' and 'R40/An', respectively (Fig. 1a and b). The second, experiencing an earlier RT reduction, when the FTSW values reached down to 0.44 and 0.47 in 'R20/Np' and 'R20/An', respectively (Fig. 1c and d). The WD affected the internal plant water status in the different rootstock/scion combinations being evidenced by time-course Ψ_{stem} measurements. From 15 at 35 days of drought, plants under WD condition had a lower Ψ_{stem} value than the WW counterpart, and these values were decreasing along this period in WD plants (Supplementary table 5).

Under WW condition, the combinations using 'R20' rootstock reached higher values of g_s and P_n during the most part of the whole experiment in comparison to 'R40' grafted plants (Supplementary table 3 and 4). Under WD condition, the g_s was similar between plants using the same scion (Supplementary table 3). However, P_n was higher in 'R40/An' respect to 'R20/An' (Supplementary table 4). The stomatal density, on the other hand, was always higher in plums as compared to almonds, regardless of the rootstock (Fig. 2). At the beginning of the experiment, and before the drought period, 'An' and 'Np' grafted on 'R20' rootstock evidenced a higher stomatal density than those grafted

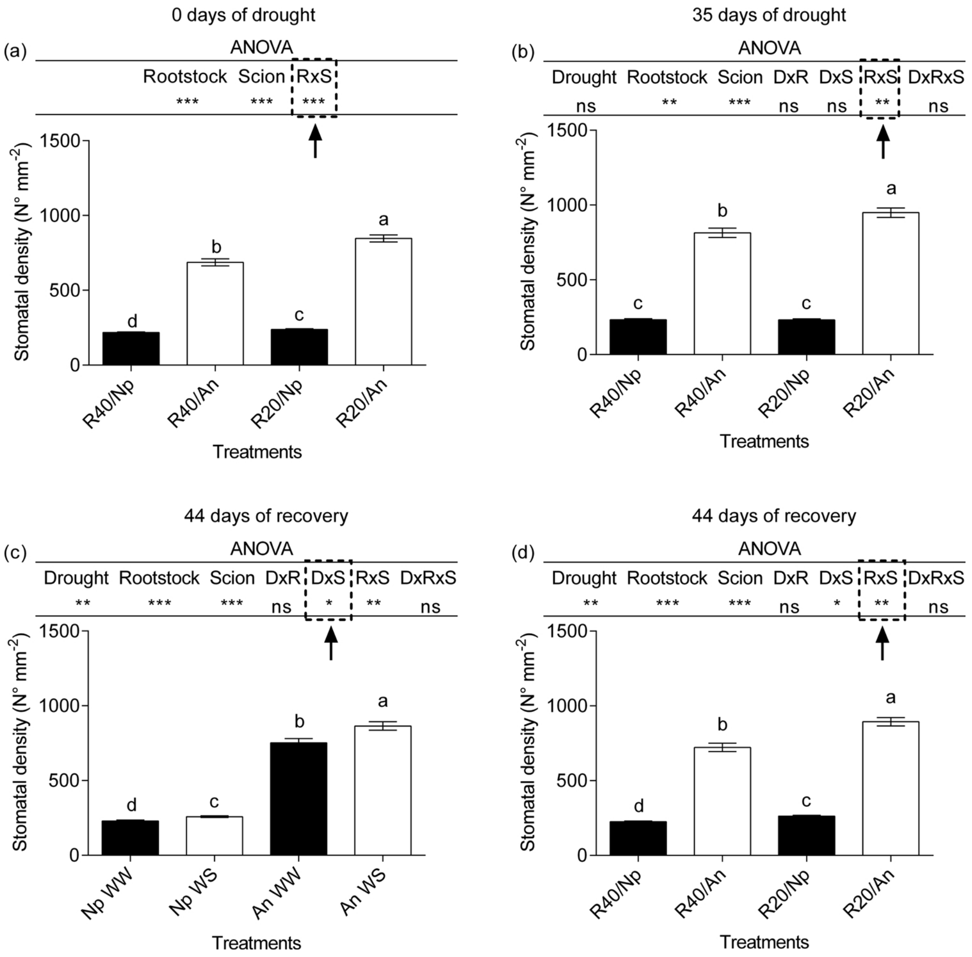

Fig. 2. Stomatal density (number per mm^2) at the beginning of the experiment (a), after 35 days of drought (b) and after 44 days of recovery (c and d) on leaves developed during these periods. Black and white bars represent ‘Np’ and ‘An’ scions (a, b and d) or well-watered (WW) and water deficit (WD) treatments, respectively (c). Different letters denote significant differences ($p < 0.05$) within the same factor or interaction according to Fisher's LSD test. Top tables indicates the statistical significance of each factor from the analysis of variance. ns = non-significant factor. *, **, *** indicates $p < 0.05$, 0.01 or 0.001 respectively. Mean ± SE (n = 4). Top arrow indicate the factor or interaction plotted.

onto ‘R40’ (Fig. 2a). After 35 days of drought, a higher stomatal density was observed in 'An' when grafted on 'R20' in comparison to ‘R40/An’ (Fig. 2b), with no differences in ‘Np’ regardless the rootstock used. After 44 days of post-drought recovery, ‘Np’ and ‘An’ resulted with higher stomatal densities compared to those plants with continuous irrigation (Fig. 2c). Also, at the end of the recovery period, a higher stomatal density was observed in ‘R20’ than ‘R40’ grafted with ‘Np’ and ‘An’ (Fig. 2d).

3.2. Root hydraulic conductivity and PIP aquaporin genes expression

After 35 days of drought, a significant difference in L_p was observed between the ‘R40’ and ‘R20’ rootstocks, with a lower value for the former compared to the latter, but with no differences in WW conditions between both rootstocks (Fig. 3a). Also, compared to their respective WW counterpart, only ‘R40’ reduced L_p under WD. In relation with the the interaction within the scion/rootstock combination, it is clear in the Fig. 3b that the scion affects the rootstock L_p. In particular, both ‘R40’ and ‘R20’ grafted with ‘An’ resulted in a significantly higher L_p compared to those with ‘Np’. On the other hand, at the end of the recovery period, the values of L_p were equal in all the irrigation and grafting treatments involving ‘R40’ (Fig. 3c). However, the ‘R20/Np’ plants reached significantly lower L_p values compared to both WW ‘R20/Np’ and WW ‘R20/An’ plants (Fig. 3c).

Regarding the seven aquaporin genes assessed in the present study, in general, the ‘R40’ rootstock did not change their transcript abundance as a response to WD, except for the *PIP 2;3* and *PIP 2;4*, increasing the former and decreasing the later for the ‘R40/Np’ combination, and also the *PIP 2;2* and *PIP 2;3*, both increasing, in the ‘R40/An’ combination (Fig. 4a and b). In contrast, a general repression of *PIP* genes in WD ‘R20’ rootstocks were observed, except for *PIP 2;1*, *PIP 2;2* and *PIP 2;3* with similar transcript abundance in WD compared to WW condition in the ‘R20/Np’ combination and the *PIP 2;3*, which increased under water deficit in the ‘R20/An’ combination (Fig. 4c and d). In fact, it is noteworthy that nearly all the *PIP* genes assessed in the present study were repressed under WD conditions in the ‘R20/An’ combination, with the exception of *PIP 2;3* which increased its mRNA levels under water deficit in all rootstock/scion combination.

3.3. Biomass accumulation and water use efficiency

During the 35 days of drought, the dry matter accumulation in leaves was significantly lower in all the rootstock/scion combinations under WD compared to their WW counterparts, except for the ‘R40/An’ combination, with no significant differences between the irrigation regimes (Fig. 5a). At the end of the recovery period, a greater leaf biomass accumulation was evidenced in ‘An’ scion, regardless of the rootstocks (Fig. 5b). In contrast, after the recovery period, the ‘R40/Np’ combination did not accumulate leaf biomass (Fig. 5b). As for the stems after the drought period, plants grafted on the ‘R40’ rootstock maintained the biomass accumulation similarly in WD and WW conditions (Fig. 5c). In the ‘R20’ rootstock, on the other hand, the stem biomass accumulation was significantly lower in both, the ‘Np’ and ‘An’ under WD condition compared to WW (Fig. 5c). After the 44 days of post-drought recovery, the stem biomass accumulation in the ‘R40/Np’ combination, were similar in the WD and WW treatments, opposite to all the remaining treatments in which the stem biomass was significantly lower in plants previously exposed to WD, than those well irrigated (Fig. 5d). The lowest stem biomass accumulation as compared to their well irrigated counterpart was observed in the WD ‘R20/Np’

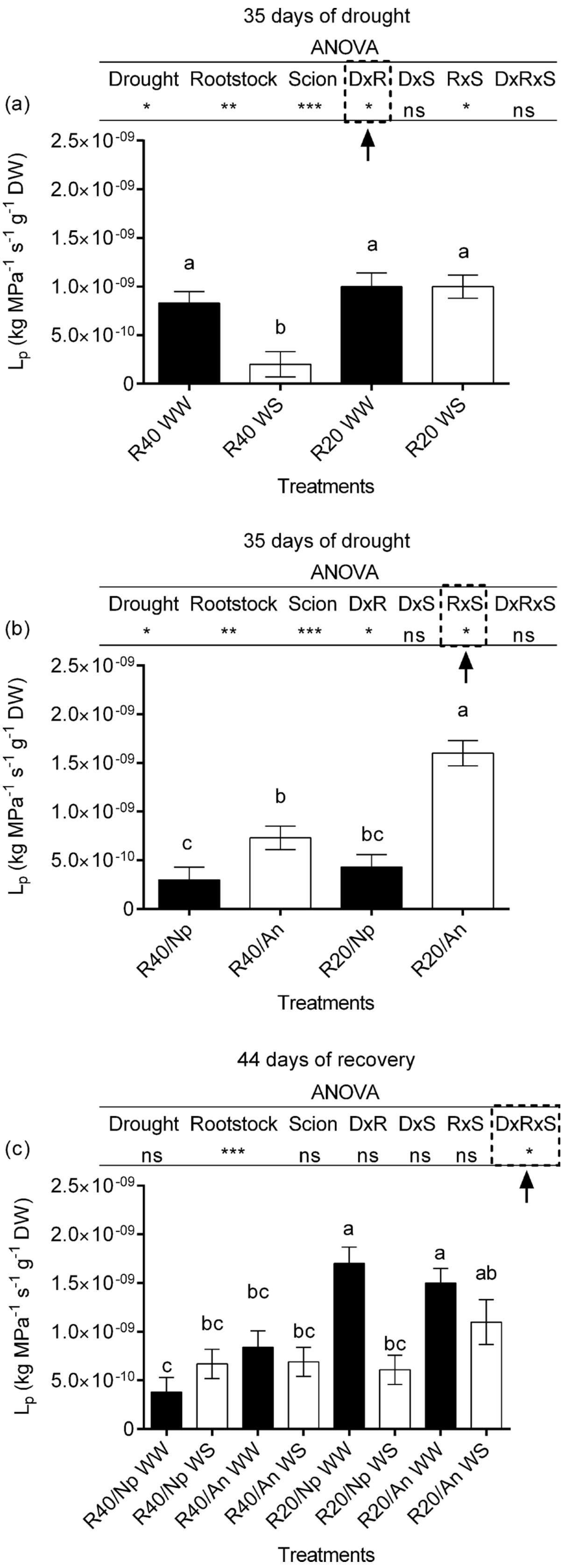

Fig. 3. Root hydraulic conductivity (L_p) after 35 days of drought (a and b) and after 44 days of recovery (c). Black and white bars represent well-watered (WW) and water deficit (WD) treatments (a and c) or 'Np' and 'An' scions (b), respectively. Different letters denote significant differences ($p < 0.05$) within the same factor or interaction according to Fisher's LSD test. Top tables indicates the statistical significance of each factor from the analysis of variance. ns = non-significant factor. *, **, *** indicates $p < 0.05$, 0.01 or 0.001 respectively. Mean ± SE (n = 4). Top arrow indicate the factor or interaction plotted.

plants (Fig. 5d). Regarding the root biomass accumulation during drought, higher values were observed in the 'R40' plants in the WD condition compared to their 'R20' counterparts (Fig. 5e). Also, and during this time, the 'R20/Np' plants had the lowest root biomass accumulation (Fig. 5f). In general, after the recovery period, the plants that were under WD condition accumulated a lower root biomass compared to the WW plants (Fig. 5g) and, in particular, the 'R40/Np' plants accumulated a lower root biomass compared to the remaining combinations (Fig. 5h).

As for the WUE_{wp}, at the end of the drought period, the plants grafted on the 'R40' rootstock increased their water use efficiency at the whole plant level up to significantly higher values compared to the WW condition, contrary to that observed in plants grafted on 'R20', with similar WUE_{wp} values between the irrigation treatments (Fig. 6a). Also, at the end of the drought period, the 'R20/Np' combination resulted in the lowest WUE_{wp} values (Fig. 6b). When comparing the WUE_{wp} values for all the rootstock/scion combinations, as shown in Fig. 6c, all the combinations previously exposed to drought reached lower values compared to the WW condition, except for the 'R20/An' combination, which evidenced no significant differences between irrigation regimes.

4. Discussion

Studies regarding the root system have been rising in recent years due to their fundamental role in water deficit scenarios (Webster, 1995; Isaakidis et al., 2004). Also, the use of drought-tolerant rootstocks has been proposed as a convenient way to provide tolerance to the cultivated fruit trees varieties (Isaakidis et al., 2004; Marguerit et al., 2012; Cantero-Navarro et al., 2016). Several studies relate drought-tolerance with the vigor induced by rootstocks. In this sense, vigorous plants are usually more tolerant due to a bigger root system (Atkinson et al., 1998; Serra et al., 2014; Zhang et al., 2016) but, at the same time, with a higher consumption of water per plant (Atkinson et al., 2000). However, this trend has shown exceptions, since some dwarfing rootstock have been reported as more WD tolerant (Di Vaio et al., 2012; Tworkoski et al., 2016). Regarding to 'R40' and 'R20' rootstocks, it has been observed in almond that 'R40' induced a higher vigor in in comparison to 'R20' rootstock (Ben Yahmed et al., 2016). In our experiment, the ranking from the more to less vigorous combination under WW conditions in the drought period was: 'R20/An' (average total DW = 529 g), 'R40/Np' (average total DW = 500 g), 'R20/Np' (average total DW = 274 g) and 'R40/An' (average total DW = 250 g). According to these results, an influence of the rootstocks in the production of biomass in the different combinations is not evident. Thus, such production under well-watered conditions seems to be defined by the compatibility of the rootstock and graft since those combinations that involve more closely related taxonomically species accumulated more biomass. However, when the water deficit was imposed, the influence of the rootstocks becomes clearly decisive in terms of biomass production. In this regard, the combinations involving the 'R40' rootstock (an almond-peach hybrid) showed a lower decrease in their biomass (Fig. 5). It is interesting to note that 'R40' and 'R20' rootstocks are considered as semi-dwarfing *Prunus* rootstocks, therefore the differences of the biomass production reported should be explained by other traits distinct to plant vigor.

Tolerance, however, is not necessarily related to a more conservative use of water. For instance, the grapevine tolerant rootstock 'M4' [(*V. vinifera* × *V. berlandieri*) × *V. berlandieri* cv Resseguier] had a higher transpiration rate, under severe WD condition, compared to the

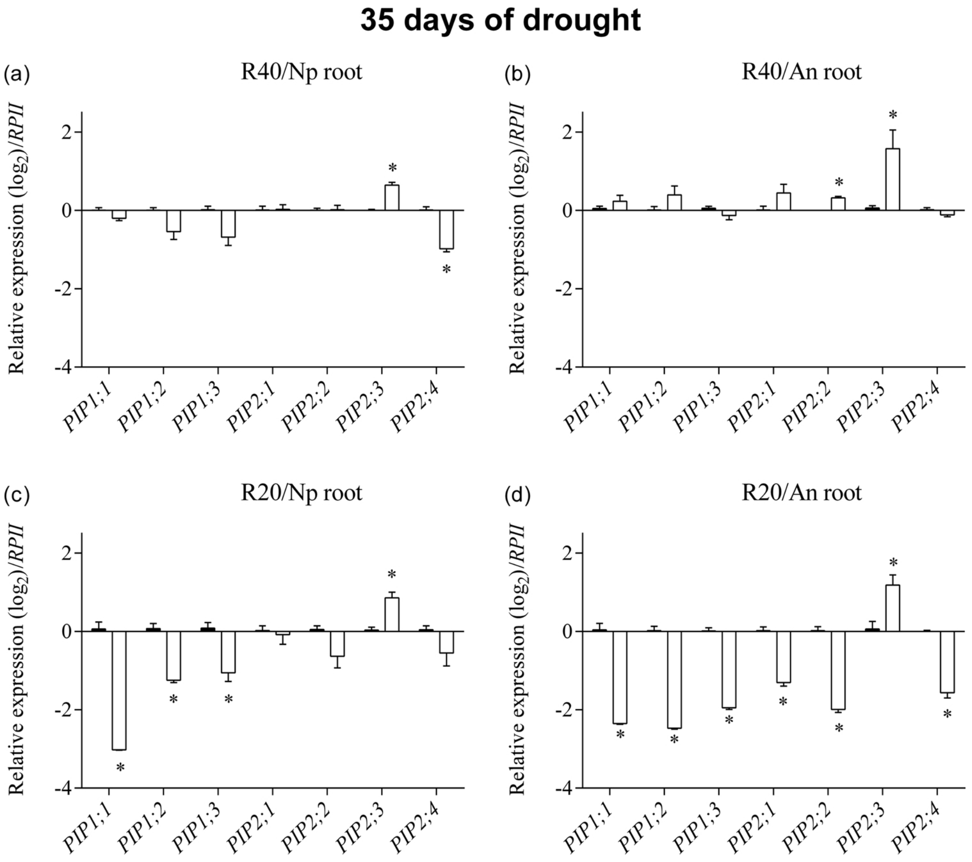

Fig. 4. Relative expression of aquaporin *PIPs* genes in roots of *Prunus* rootstocks 'ROOTPAC®40' grafted with 'Non Pareil' almond (a) and 'Angeleno' Japanese plum (b) and 'ROOTPAC®20' grafted with 'Non Pareil' almond (c) and 'Angeleno' Japanese plum (d) at the end of the drought period under well-watered (WW, black bars) and water deficit (WD, white bars) conditions. Asterisks indicate significant differences between WW and WD of the same rootstock/scion combination ($p < 0.05$) according to Fisher's LSD test. Bars indicate mean relative expression values ± SE (n = 3), normalized with *RPII* as a constitutive expressed gene.

sensitive '101.14' (*V. riparia* × *V. rupestris*) rootstock (Corso et al., 2015). Similarly, the 'R40' grafted plants, which proved to be more tolerant than 'R20' (Fig. 5 and 6), are also those with higher transpiration rates at a low FTSW values, as observed by the late RT reduction under WD (Fig. 1). The $\text{FTSW}_{\text{threshold}}$, that is the FTSW value resulting in a RT value of 0.9, as proposed by Bindi et al. (2005), is clearly dependent of the rootstock, but not of the grafted scion (Fig. 1), despite the great morphological differences existing between almond and plums (Supplemental Table 1). 'R40' rootstock had a lower $\text{FTSW}_{\text{threshold}}$, maintaining a higher RT at low SWC in comparison to 'R20' (Fig. 1). Noteworthy, in grapevine rootstocks, the QTLs suggested as having control on the scion transpiration were colocalized, among others, with aquaporin genes (Marguerit et al., 2012). In these regard, we observed a higher $\text{FTSW}_{\text{threshold}}$ for the RT drop in 'R20' grafted plants (Fig. 1) and, at the same time, a reduction on the expression of several *PIP* genes in roots under WD (Fig. 4c and d), but further studies are needed in order to prove a functional relationship between both traits. The overexpression of *PIP* genes seems to be involved in increments of root hydraulic conductance (Gambetta et al., 2012), leading to the regulation of water flow at a whole-plant level (Grondin et al., 2016) and could be involved in the control of plant's transpiration under WD condition (Shekoofa and Sinclair, 2018). In the present study, however, no clear relationship between *PIP* transcript abundance and L_p was observed. Several *PIP* genes were down-regulated in 'R20' roots under WD condition (Fig. 4c and d) but no differences in L_p between WW and WD conditions in the drought period were observed (Fig. 3a). In contrast to 'R20' genotype, 'R40' roots showed few differences on *PIP* genes expression between WW and WD conditions (Fig. 4a and b), but reduced L_p values under WD were evident (Fig. 3a). The research on plant aquaporins has yielded contrasting results in terms of tolerance to water deficit (Pou et al., 2013; Xu et al., 2014; Brunner et al., 2015; Li et al., 2015; Reddy et al., 2017). In this sense, studies focused on their activity, functional regulation and localization will be helpful to a better understanding of the relationship between PIP aquaporins and L_p in *Prunus* species.

Alsina et al. (2011) reported that the tolerant grapevine rootstock '1103P' (*Vitis berlandieri* x *V. rupestris*) has a higher root hydraulic conductance compared to sensitive genotypes under WD in field conditions by mean of the generation of deep roots that explore more soil layers to find water. As for potted peach (*P. persica*), olive (*Olea europea* L.), citrumelo (*Poncirus trifoliata* Raf. × *Citrus paradisi* Macf.) and pistachio (*Pistachia integerrima* L.) (Rieger, 1995), plants of these species have been observed to have a reduced L_p when grown under WD. The fact that 'R40' rootstocks reduce their L_p under WD conditions (Fig. 3a) might be related to the volume of the container where the roots were confined in contrast to field conditions. However, no relationship has been observed between root hydraulic conductivity and WD tolerance in *Prunus* genotypes with different levels of drought adaptation in pots assays (Rieger and Duemmel, 1992). It is possible that the container might modify the relationship between L_p and drought tolerance, but further studies are needed in order to clarify the extent of such effect.

Tolerant WD cowpea (*Vigna unguiculate* L.) genotypes have been reported to have a rather late decline in plant transpiration and higher WUE under WD conditions in comparison to sensitive genotypes (Belko et al., 2012). Similarly, the low $\text{FTSW}_{\text{threshold}}$ induced by the 'R40' rootstock in the grafted 'An' and 'Np' (Fig. 1a and b) is associated with an increase in the WUE_{wp} in WD plants (Fig. 6a). Consequently, during the drought period, WD 'An' plants grafted on 'R40' reached a leaf biomass equal to WW plants (Fig. 5a), an equal stem biomass in 'Np' and 'An' scions (Fig. 5c) and an equal root biomass between WW and WD plants (Fig. 5g). In contrast, the higher $\text{FTSW}_{\text{threshold}}$ induced by 'R20' rootstock (Fig. 1 c and d), but with similar WUE_{wp} between WW and WD plants (Fig. 6a), together with a lower WUE_{wp} on the 'R20/Np' plants (Fig. 6b) in the drought period, implied a reduction in biomass accumulation. Additionally, leaf and stem biomass in the 'Np' and 'An' scions were reduced (Fig. 5a and c, respectively) and, also, a low root

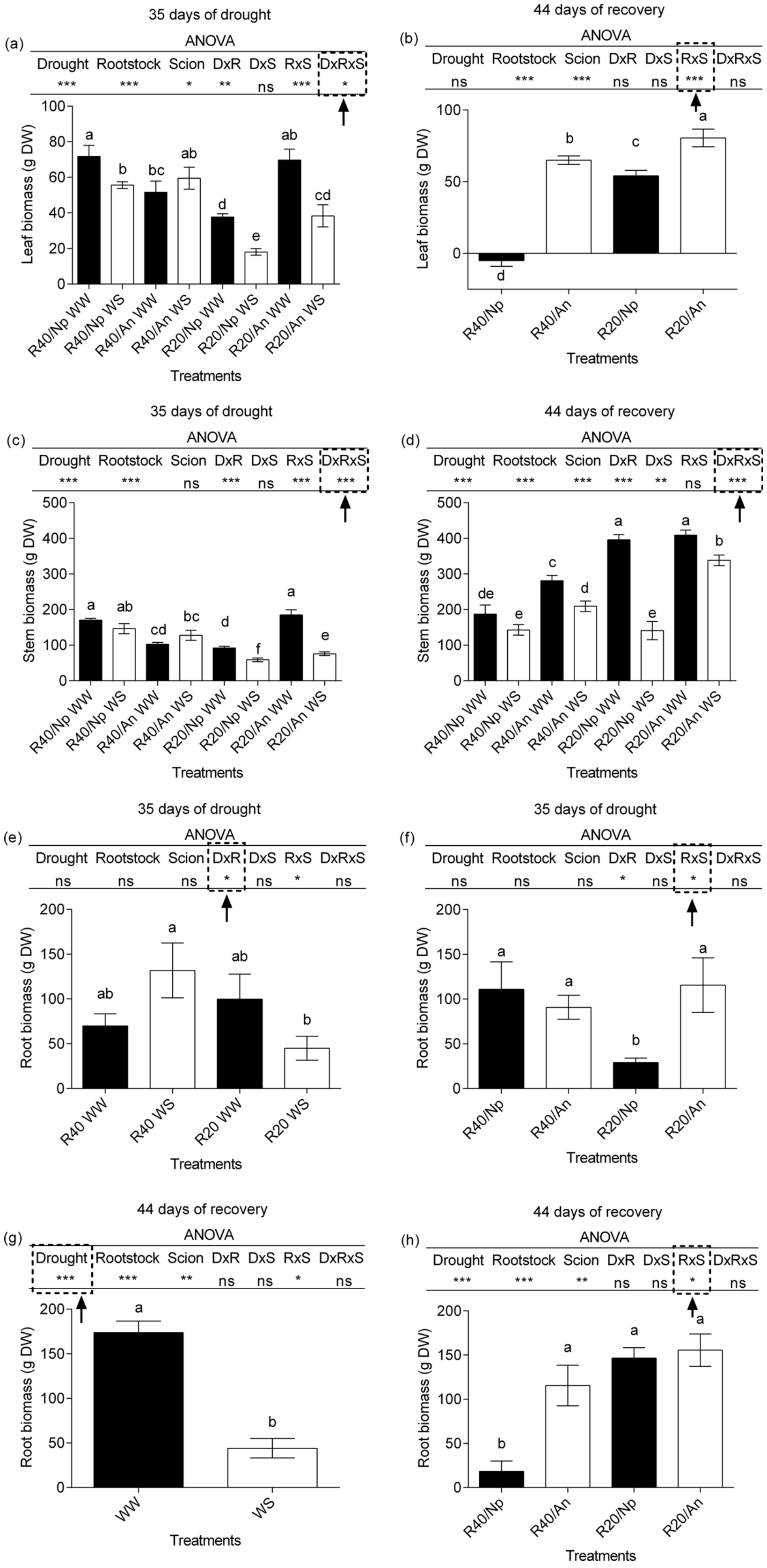

Fig. 5. Accumulation of dry leaf biomass during drought (a) and recovery (b) periods, stem biomass during drought (c) and recovery (d) periods and root biomass during drought (e and g) and recovery (f and h) periods. Black and white bars represent well-watered (WW) and water deficit (WD) treatments (a, c, d, e and g) or 'Np' and 'An' scions (b, f and h), respectively. Different letters denote significant differences ($p < 0.05$) within the same factor or interaction according to Fisher's LSD test. Top tables indicates the statistical significance of each factor from the analysis of variance. ns = non-significant factor. *, **, *** indicates $p < 0.05$, 0.01 or 0.001 respectively. Mean ± SE (n = 4). Top arrow indicate the factor or interaction plotted.

biomass with 'Np' scion was observed using 'R20' rootstock (Fig. 5g). It has been suggested that a higher $\text{FTSW}_{\text{threshold}}$ for transpiration decline would have a negative consequence under water deficit conditions, because of the negative effect on stomatal conductance and photosynthesis (Vadez et al., 2013). This fact might explain the lower biomass accumulation in 'R20' grafted plants under WD. The genetic background of 'R40' is composed by species from the *Amygdalus* subgenus which have been described as drought-tolerant species (Fußeder

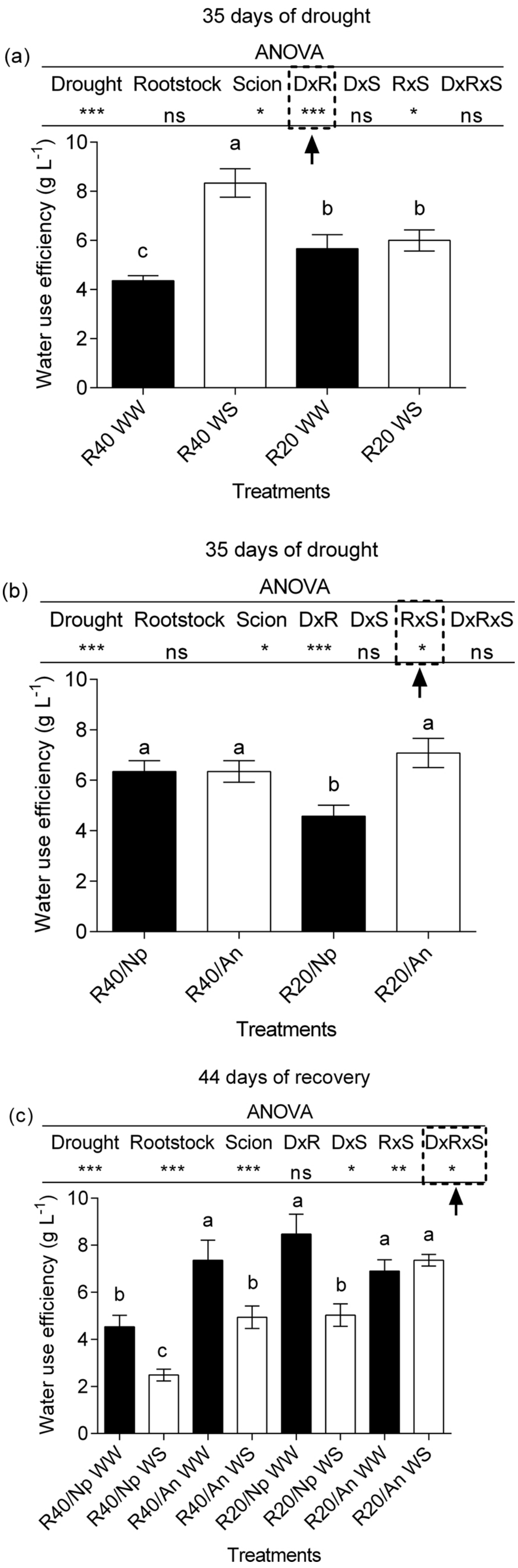

Fig. 6. Whole-plant water-use efficiency after 35 days of drought (a and b) and after 44 days of recovery (c). Black and white bars represent well-watered (WW) and water deficit (WD) treatments (a and c) or 'Np' and 'An' scions (b), respectively. Different letters denote significant differences ($p < 0.05$) within the same factor or interaction according to Fisher's LSD test. Top tables indicates the statistical significance of each factor from the analysis of variance. ns = non-significant factor. *, **, *** indicates $p < 0.05$, 0.01 or 0.001 respectively. Mean ± SE (n = 4). Top arrow indicate the factor or interaction plotted.

et al., 1992; Camposeo et al., 2011). Wild *Prunus* species from arid regions like *Prunus ferganensis* Kost. & Riab, under water deficit conditions, are characterized by a rapid use of all the available water in soil with a high efficiency, in order to maintain their biomass (Rieger et al., 2003). On the other hand, one of the parents of 'R20' is composed by a specie of the *Prunophora* subgenus (*P. cerasifera*) which might reduce the tolerance to water deficit (Duval, 2015). It is noteworthy that similar responses were observed in ungrafted 'R40' and 'R20' plants under WD condition in terms of $FTSW_{threshold}$, biomass accumulation, WUE_{wp} and root *PIP* aquaporin genes expression (article under revision), suggesting that rootstocks responses to WD might be a valuable prognostic for the performance of a grafted tree. It is interesting to note that even though 'R40' grafted plants were more tolerant to WD, they do not perform as good as 'R20' grafted plants after a post stress recovery period. Indeed, 'R40' grafted plants previously water stressed, maintained a significantly lower WUE_{wp} compared to WW plants (Fig. 6c). 'R20/Np' also reduced the WUE_{wp}, but not as much as the 'R40/Np' plants (Fig. 6c). Consequently, the leaf biomass accumulation at the end of the recovery period was larger on 'R20/Np' and 'R20/An' in comparison to 'R40/Np' and 'R40/An' plants, respectively (Fig. 5b). The stem biomass accumulation, on the other hand, was also larger on 'R20/An' in comparison to 'R40/An' in both, WW and WD conditions (Fig. 5d). 'R20/Np' under WW condition had more stem biomass that 'R40/Np' under WW and WD, but 'R20/Np' with a previous WD condition did not increased the stem accumulation in the recovery period (Fig. 5d). Finally, root biomass accumulation was reduced on 'R40/Np' plants in WW and WD condition (Fig. 5h). These contrasting effects induced by the rootstocks between drought and recovery periods highlights the importance of studying the recovery period in the long term.

A close interaction between shoot and roots L_p have been observed in grapevine, soybean (*Glycine max* L.) and maize (*Zea mays* L.) (Vandeleur et al., 2009). From our results, during the drought period, 'An' scion induced a high L_p value on roots of both rootstocks in comparison to 'Np' scion (Fig. 3b). A possible explanation might be that plums might induce greater internal tension in the transpiratory stream, given by a bigger transpiratory surface, as a result of their high stomatal density as compared to almond leaves (Fig. 2). In fact, it has been reported before that L_p in roots is likely to be increased under a high hydrostatic gradient (Steudle, 2000). On the other hand, 'R20' rootstock induced a greater stomatal density on 'An' and 'Np' scions in comparison to 'R40' rootstock (Fig. 2). Besides, both scions resulted in frequent higher stomatal conductance and photosynthetic CO_2 assimilation under WW condition when grafted onto 'R20' rootstock (Supplementary table 3 and 4). At the end of the experiment, 'R20' grafted plants under WW condition were the more vigorous plants in comparison to 'R40' grafted plants. These differences might be the consequence of a higher stomatal density induced by 'R20' rootstock, implying an increase of the gas exchange surface, increasing net photosynthesis. In poplar (Populus trichocarpa Torrey and Gray × P. deltoides Bartram) stomatal density of new leaves were positively correlated with stomatal conductance of old leaves (Turpin et al., 2005). In our study, a higher stomatal density was observed on leaves developed on the recovery period in plants that had a previous WD condition (Fig. 2c). However, in the case of our study, mature leaves on WD plants had a lower stomatal conductance (Supplementary table 3). Camposeo et al. (2011) reported, in almond, that spring-leaves had a higher stomatal density in comparison to leaves developed in summer. Additionally, in *Prunus serotina* Ehrh. a reduction in stomatal density was observed in response to drier environments

(Abrams, 1994). In both cases, this reduction in stomatal density is a consequence of environmental water stress. Our results show that after a water deficit period, new leaves developed on well-watered conditions (at the end of summer) increase the stomatal density, probably by the new optimum water status condition.

5. Conclusions

The use of rootstocks as a mean for increasing the tolerance of species of agricultural importance to constraining environments is becoming a general trend worldwide, especially in fruit tree orchards. As for water deficit, some genotypes have proven to be potentially useful because of their tolerance to drought and, therefore, their potential for conferring tolerance to grafted species. Indeed, from our results, plants grafted onto the 'R40' rootstock resulted in better physiological performance, including a late reduction on RT, an increase on WUE_{wp} and with lesser detrimental effects on the biomass, as compared to those grafted on 'R20'. However, such response is not necessarily conserved after a recovery period, when irrigation is resumed. In this case, plants grafted on the 'R20' resulted in a higher WUE_{wp} and growth. This is relevant since in the productive context of commercial orchards in arid and semiarid zones, plants are not necessarily at the risk of terminal water scarcity, but to transient water shortages, where the capacity of plants to recover is of great importance.

Up to now, it is clear that rootstocks affects morpho-physiological traits on the grafted scions. For instance, the 'R20' rootstock induced a greater stomatal density in both, 'Np' and 'An' scion. Also, 'R40' induced a low $FTSW_{threshold}$ together with an increased WUE_{wp} in WD condition, both advantageous traits under controlled irrigation systems such as that practiced in fruit production in central Chile. Interestingly, our results suggest that there is a scion effect over the rootstocks also. 'Angeleno' plum, for instance, induced a greater L_p in comparison to 'Non Pareil' almond in drought periods regardless of the rootstock used.

Recently, the principles underlying water movement and hydraulic responses under stress has become clearer. The importance of the responses at the molecular level, however, is still under debate. Aquaporins have been argued to play a critical role, at the whole plant level, by allowing water re-allocation and to sustain transpiration under stress (Vitali et al., 2016). We have assessed the responses of seven *PIP* genes on the root system of the 'R20' and 'R40' rootstock in combination with grafted 'An' and 'Np'. Some trends are clear, for instance, such as the general reduction of the transcript abundance of *PIP´s* under stress when grafted onto 'R20'. However, the linkage of the comparative responses in 'R20' and 'R40' to the physiological changes found in terms of $FTSW_{threshold}$, WUE_{wp}, biomass, etc. is rather elusive and further studies are needed in order to better understand the relevance of the function of aquaporins at the whole plant level.

Finally, we demonstrate that stone fruits fit well with a novel model to study the interaction between rootstocks and scions, focused on physiological and molecular parameters to conduct research on drought tolerance. Future researches considering the use of interstocks, needed to combine rootstocks and scions of *Prunus* species usually incompatible, will wide this type of studies to other stone fruits species such as sweet cherry, apricot or peach which would help to improve our understanding about the adaption of grafted stone fruit trees to water deficit, likely unraveling further interactions between rootstocks and scions.

6. Funding

This work was supported by grant from project FONDECYT Nº 1150853, CONICYT Regional/CEAF/R08I1001, CONICYT scholarship PFCHA/Doctorado Nacional 2018/Folio 21180766 and Programa de Doctorado en Ciencias Silvoagropecuarias y Veterinarias de la Universidad de Chile.

7. 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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Acknowledgments

We gratefully acknowledge the special contribution of the late Professor Dr. Nicolás Franck Berger to this work.

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.agwat.2019.105897.

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