

Exploring the use of scions and rootstocks from xeric areas to improve drought tolerance in *Castanea sativa* Miller

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ABSTRACT

Nut production by the European sweet chestnut (*Castanea sativa* Mill.) in grafted orchards is under threat of increased drought stress associated with current global warming. To explore whether drought tolerance in *C. sativa* could be improved using drought tolerant scions and rootstocks, trees from humid (H) and xeric (X) populations in Spain were used to generate intra-family (H/H and X/X) and reciprocal inter-location (X/H and H/X) grafts. We studied the effects of the (i) scion, (ii) rootstock and (iii) the grafting itself as a wounding stress on the vegetative budbreak, secondary growth and drought tolerance of *C. sativa* trees. Drought tolerance was assessed by measuring leaf gas exchange, chlorophyll fluorescence, water status and leaf wilting two weeks after water deprivation. Hormone (ABA, SA, JA and JA-Ile) and proline quantification in tree leaves and fine roots were assessed, as well as tree and scion mortality. Rootstocks and scions of xeric origin induced earlier flushing and improved drought tolerance of scions and rootstocks of humid origin. Tree mortality after drought was 57 % lower in H/X than in H/H trees, and scion loss after drought was 47 % lower in H/X than in X/H trees. The 'grafting' (i.e., wounding) effect delayed vegetative budbreak and reduced secondary growth of trees but did not predispose trees to drought. We report for the first time differences in hormone and proline content in leaves and fine roots of chestnut trees of humid and xeric origins exposed to drought. The results suggest the use of scions and rootstocks from xeric areas to improve drought tolerance in chestnut.

1. Introduction

Sweet chestnut (*Castanea sativa* Mill.) is a multipurpose tree species widely distributed throughout the Mediterranean Basin. It occurs in forests and is cultivated by grafting in orchards for nut production. Orchards and forest plantations are undergoing increasing drought stress associated with climate change (Conedera et al., 2010; Carnicer et al., 2011; Bussotti et al., 2015; Buras and Menzel, 2019; Camisón et al., 2020). This situation is aggravated by the replacement of native *C. sativa* rootstocks with inter-specific hybrid rootstock clones (*C. sativa* × *C. crenata*) that are resistant to *Phytophthora cinnamomi* Rands. but have low drought tolerance (López-Villamor et al., 2018). Drought-tolerant rootstocks could be used to mitigate the impacts of climate change on chestnut cultivation, as in other woody crops (Serra et al., 2014; Zhang et al., 2016; Tworowski et al., 2016; Han et al., 2019).

However, chestnut breeding programmes are based on increasing rootstock resistance to *P. cinnamomi* and enhancing rootstock compatibility with traditional *C. sativa* varieties (Pereira-Lorenzo and Fernández-López, 1997; Grauke and Thompson, 2010; Warschefsky et al., 2016). The influence of the scion and the rootstock on chestnut budbreak phenology, growth and drought tolerance is largely unknown, because research has primarily addressed scion and rootstock compatibility (e.g., Pereira-Lorenzo and Fernández-López, 1997; Serdar and Soyula, 2005; Bueno et al., 2009; Serdar et al., 2010; Warmund et al., 2012; Ada and Ertan, 2013; Iliev et al., 2013). Grafting a tree involves a wounding stress during the early stages of graft union healing, which also interacts with the effects of the scion and the rootstock (Albacete et al., 2015). However, little is known about the effect of grafting as a wounding stress in chestnut. Root-to-leaf water flow can decrease due to incomplete vascular reconnection at the graft union (Torii et al., 1992;

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Serra et al., 2014), while changes in the production of hormones and other metabolites during tissue regeneration (Mo et al., 2017; Melnyk et al., 2018; Nanda and Melnyk, 2018) may affect the phenology, growth and drought tolerance of the grafted tree.

Castanea sativa inhabits regions with marked water availability gradients (e.g., the Iberian Peninsula and Turkey), leading to genetically-based differentiation in traits related to drought adaptation (Pigliucci et al., 1990; Lauteri et al., 1999, 2004; Fernández-López et al., 2005; Ciordia et al., 2012; Míguez-Soto and Fernández-López, 2015; Míguez-Soto et al., 2019; Castellana et al., 2021). This evolutionary pressure has made it possible to obtain rootstock genotypes with contrasting drought tolerance. The Iberian Peninsula has two *C. sativa* ecotypes adapted to different climate conditions, the first located in wet and mild northern areas and the second in xeric central and southern regions (Ciordia et al., 2012; Míguez-Soto and Fernández-López, 2015; Míguez-Soto et al., 2019; Alcaide et al., 2019). Xeric *C. sativa* populations show early phenology, low plant growth and higher root development than mesic populations because of adaptation to summer drought conditions (Lauteri et al., 1999, 2004; Fernández-López et al., 2005; Ciordia et al., 2012; Míguez-Soto and Fernández-López, 2015; Míguez-Soto et al., 2019).

Phytohormones are stress signalling molecules that help plants adapt to adverse environmental conditions, including drought, through a complex crosstalk involving changes in primary and secondary metabolism. They also play an important role in scion/rootstock communication (Aloni et al., 2010; Albacete et al., 2015), making them ideal candidates for studying the mechanisms by which rootstocks enhance drought tolerance (Allario et al., 2013; Tworowski et al., 2016; Silva et al., 2018). However, it is not known whether biochemical responses related to stress signalling contribute to differences in drought tolerance in *C. sativa*. The hormone abscisic acid (ABA) is the principal mediator of plant responses to drought because it regulates stomatal closure and water loss (de Ollas and Dodd, 2016). Recent studies have shown that rootstock-induced changes in ABA content play an important role in defining the drought tolerance of grafted plants (Allario et al., 2013; Liu et al., 2016; Santana-Vieira et al., 2016; Tworowski et al., 2016; Silva et al., 2018). Salicylic acid (SA) and jasmonates (JAs) are phytohormones that are well known for regulating plant defence against pests and pathogens (Martín et al., 2010; Vivas et al., 2012; López-Villamor et al., 2021), but their involvement in plant drought responses is increasingly recognised (De Diego et al., 2012; Jesus et al., 2015; Shenxie et al., 2015; De Ollas and Dodd, 2016). In citrus trees under severe drought, SA was reported to increase along with ABA, presumably promoting stomatal closure jointly (Santana-Vieira et al., 2016; Neves et al., 2017). In roots of a commercial citrus rootstock, a transient burst of jasmonic acid was required to trigger ABA accumulation (De Ollas et al., 2012). Accumulation of compatible solutes (osmoprotectants) such as the free amino acid L-Proline is crucial to bind plant water during plant dehydration, a process that is largely mediated by phytohormones (reviewed in Sharma et al., 2019). Proline also performs stress signalling functions and is commonly used as a drought stress marker because its content is often positively correlated to drought tolerance (van Rensburg et al., 1993; Naser et al., 2010; De Diego et al., 2015; Kabbadj et al., 2017; Taïbi et al., 2017).

In this work, we used reciprocal grafts between Iberian *C. sativa* families from humid and xeric provenances to explore the capacity of xeric rootstocks to improve drought tolerance in chestnut. Additionally, we analysed the constitutive and drought-induced hormonal profiles of two families with contrasting drought tolerance. The following hypotheses were tested in chestnut: (i) vegetative budbreak, tree growth and drought tolerance responses are influenced by the rootstock and (ii) the 'grafting' injury itself, and (iii) there are constitutive and/or drought-induced differences in the hormone and proline content of leaves and roots of trees of humid and xeric origins.

2. Materials and methods

2.1. Plant material, grafting and growth conditions

Four *C. sativa* families (H₁, H₂, X₁ and X₂; half-sibling trees) were used. H₁ and H₂ came from a mild, humid coastal location in northwest Spain (Bergondo, Galicia region, 43°18'32"N 8°13'57"W, mean annual temperature 13 °C, annual rainfall 1105 mm), and X₁ and X₂ came from a xeric location in southern Spain (Constantina, Andalusia region, 37°53'16"N 5°36'13"W, mean annual temperature 15.5 °C, annual rainfall 628 mm). Previous research showed significant differences in drought tolerance between trees from these two populations (Alcaide et al., 2019). In October 2015, two mature, healthy-looking mother trees at least 100 m apart were randomly selected in each population and all their nuts were collected. Seeds were immersed in water and any that floated were discarded as non-viable. Viable seeds were sterilised in a fungicide solution (2 g l⁻¹ Thiram 80GD, ADAMA Inc., Spain) for 10 min, rinsed, and stratified for 2 months at 4 °C in moistened blond peat (Pindstrup Mosebrug Inc., Spain). After stratification, nuts were sown in 100-cell rigid plastic root trainers (300 ml volume; 18 cm high, 5.3 × 5.3 cm upper surface). The seedlings obtained were transplanted into 2-l pots containing a mixture of peat, vermiculite and perlite (1:1:1).

In July 2016, seedlings of each family were divided into three groups: non-grafted controls, grafted trees using scions from the same family (intra-family grafts), and grafted trees using scions from a different location as rootstock (inter-location grafts). This grafting design resulted in reciprocal grafts between each pair of families of contrasting origin and included 12 scion/rootstock combinations (three per family according to Table SM1). Trees were grafted using the 'green grafting' technique (Cuenca et al., 2018, Fig. SM1a). In January 2017, the plant material was placed in the greenhouse at the Faculty of Forestry of the University of Extremadura (Plasencia, 40°02'N, 6°05'W; 374 m asl, western Spain), in natural light under a mesh that reduced solar radiation by 50 %, fertilised with Osmocote Pro 3–4 M (Osmocote® Pro) at 4 g l⁻¹ and grown under optimal watering conditions (soil volumetric water content around 30 %).

2.2. Experimental design

The experiment was performed from April to September 2017, when trees were two years old, at the greenhouse of the Faculty of Forestry of Plasencia, under natural conditions of light and temperature. The experiment included 188 trees with a sample size of 7–18 plants (11.75 ± 3.47; mean ± SD) for non-grafted controls and scion/rootstock combinations. The plant material was divided into six groups of trees based on whether or not the trees had been grafted and the origin of the scion and the rootstock family. This resulted in H and X (non-grafted controls of the H₁ and H₂ and the X₁ and X₂ families, respectively), H/H and X/X (intra-family grafts of the H₁ and H₂ and the X₁ and X₂ families, respectively), and X/H and H/X (reciprocal inter-location grafts between the H₁ and H₂ and the X₁ and X₂ families) groups of trees (see Table SM1). To test the hypothesis that vegetative budbreak, tree growth and drought tolerance are influenced by rootstock, the H/H, X/X, X/H and H/X tree-groups were assessed, thus taking into account the relative contribution of the scion and the rootstock. To test the hypothesis that vegetative budbreak, tree growth and drought tolerance are influenced by the wounding effect of grafting, H, X, H/H and X/X tree-groups were assessed. In H/H and X/X trees, scions and rootstocks from the same individual were used, to avoid grafting effects between two genetically distinct individuals. Potted plant material was arranged in a complete randomised block design with six blocks, each containing 1–3 trees per scion/rootstock combination and non-grafted controls. All plants were subjected to a single drought treatment (see below).

2.3. Assessment of budbreak phenology and tree growth under optimal watering conditions

Vegetative budbreak was assessed in April 2017 in all trees. Bud development was assessed as follows (Solla et al., 2014): 1= dormant buds; 2= swollen buds, but scales closed; 3= bud scales open and extremities of the first leaf visible at the apex of the buds; 4= extremities of all leaves out; and 5= two or more leaves completely expanded. Secondary growth of all plants was obtained by the difference in stem diameter in April 2017 and July 2017 (before drought treatment) and expressed as a percentage. Stem diameters were calculated by the average of two measurements made orthogonally ca. 5 cm from ground level, where a white stripe was painted in April. Tree height was measured in all plants before drought treatment.

2.4. Drought treatment

In July 2017, all plants were subjected to a drought treatment consisting of watering pots to field capacity (day 0) then withdrawing watering for two weeks. At day 14, to determine the effect of drought, trees were assessed for morpho-physiological parameters and samples were taken for further hormone and proline quantification in leaves and roots. Immediately after morpho-physiological assessment, plants were rewatered to field capacity for recovery and maintained under optimal watering conditions (30 % SVWC) until the end of the experiment (September 2017).

2.5. Morpho-physiological assessment of tree drought tolerance

Tree drought tolerance was assessed 14 days after the start of drought treatment using two approaches: (i) evaluation of external symptoms due to drought damage in all trees and (ii) physiological assessment of gas exchange parameters, chlorophyll fluorescence and water status in leaves from a subsample of trees. Leaf wilting was visually estimated as the percentage of plant foliage showing turgor loss (Camisón et al., 2019a), while tree mortality and scion mortality (if any) were assessed two months after the drought treatment had finished. Assessment was performed after two months because some trees died after rewatering due to drought-induced damage. Leaf gas exchange related parameters, net carbon assimilation (A) and stomatal conductance (g_s) were measured with a portable differential infrared gas analyser (IRGA) (Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf chamber (Alcaide et al., 2019). Measurements were made from 10.00–12.00 h with photosynthetically active radiation (PAR) ranging from 300 to 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For chlorophyll fluorescence (F_v/F_m the maximum quantum yield of photosystem II (PSII)), readings were obtained from 8.00–10.00 h with a Multimode Chlorophyll Fluorometer OS5p device (Opti-Science Inc., USA) in dark-adapted leaves (30 min). Leaf relative water content (RWC) was evaluated at noon, following:

$$\text{RWC}(\%) = \frac{(\text{FW} - \text{DW})}{(\text{HW} - \text{DW})} \cdot 100,$$

where FW is the fresh weight of leaves at the time of sampling, HW is the hydrated weight of leaves after soaking in distilled water for 24 h at 4 °C in darkness, and DW is the dry weight of leaves after complete oven dehydration (48 h, 60 °C) (Camisón et al., 2020). Two apical fully expanded leaves per tree were used.

2.6. Hormone and proline quantification in leaves and roots

On day 0 (optimal watering conditions) and day 14 after the start of drought treatment, hormone and proline content in leaves and roots from a subsample of trees were assessed. For both sampling points, non-grafted controls, intra-family grafts and reciprocal grafts of the families

H₁ and X₁ were used, after random selection of the two families. Around 15 plants from each of the six groups selected were sampled. Leaves were sampled by collecting the apex of one fully developed top-stem leaf from the scion (and non-grafted trees). Roots were sampled by carefully excising and collecting five outermost fine root segments from the root ball of rootstock (and non-grafted trees). After collection, samples were immediately frozen in liquid N and pooled (n = 5) to obtain a sample size of three biological replicates per group of trees. Samples were kept at -80 °C until freeze drying with a FreeZone 6 l Benchtop Dryer (Lab-conco, Kansas City, USA). Samples were ground in a ball mill (Mixer Mill MM 400, Retsch, Germany) and passed through a 0.42 mm screen.

The acidic plant hormones abscisic acid (ABA), salicylic acid (SA) and the jasmonates jasmonic acid (JA) and its conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) were determined in leaves and roots as described in more detail in Camisón et al. (2019b). Briefly, 50 mg of lyophilised powdered plant tissue was wetted with a 10 % methanol aqueous solution containing hormonal internal standards, vortexed and incubated. Samples were then mixed and centrifuged and the supernatant was recovered for double partitioning against diethyl ether and drying in a centrifuge evaporator. Samples were suspended in a 10 % methanol aqueous solution for chromatographic separation with an Acquity Ultra Performance Liquid Chromatography system (UPLC) (Waters, Milford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex) connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). Further quantification was performed using external calibration curves. The chromatographic and mass spectrometry conditions were the same as in Gamir et al. (2018).

Proline was determined by slight modifications to the traditional protocol of Bates et al. (1973). Twenty mg dry powdered tissue was homogenised with 1.5 mL sulphosalicylic acid (3%, w/v) and centrifuged (10 min, 4 °C, 10,000 g). One ml supernatant was mixed with 1 mL ninhydrin acid and 1 mL glacial acetic acid, and the mix was incubated (30 min, 100 °C). After cooling on ice, 2 mL toluene was added and absorbance was read at 520 nm. A free proline standard curve was used for quantification, using three technical replicates per biological replicate.

2.7. Statistical analysis

The effect of scion and rootstock origin and grafting on vegetative budbreak, secondary growth, leaf physiology parameters and leaf wilting were analysed by Linear Mixed Models (LMM) and Generalized Linear Mixed Models (GLMM), depending on whether errors were normally distributed or not. Data were first checked for normality and homoscedasticity by Shapiro–Wilk and Levene’s tests. When assessing the effect of the scion and rootstock origin, intra- and inter-location grafts (i. e., H/H, X/X, H/X and X/H scion/rootstock combinations) were used and ‘scion origin’, ‘rootstock origin’ and their interaction were considered as fixed effects. Tree mortality was analysed with a cumulative link mixed model (CLMM) in which the outcome variable consisted of three ordered categories: 0 (dead plant), 1 (basal or epicormic resprouting with scion loss) and 2 (live scion). CLMM are similar to logistic regression but can handle ordered categorical outcomes with more than two categories. When assessing the effect of grafting, non-grafted controls and their respective intra-family grafts (i. e., H, X, H/H and X/X trees) were used, and ‘grafting’ (two levels: ‘grafted’ and ‘non-grafted’), ‘rootstock origin’ and their interaction were specified as fixed effects in the models. The effect of grafting on tree mortality was analysed with a logistic mixed model in which the dependent variable was coded 0 or 1 if the tree survived or not, respectively. All models considered ‘block’ and ‘rootstock family’ as random factors. The covariate ‘tree height’ was included in models that analysed variables measured under drought stress. Hormone and proline content in leaves and roots were analysed with GLMM using tree identity as random factor to account for non-independence of observations. Differences between means ($p < 0.05$) for all variables were tested with Tukey’s HSD test with the Bonferroni

correction. Relations between hormone and proline content in leaves and roots, leaf wilting, and plant mortality were assessed by correlation and regression analysis. Statistical analyses were carried out in R software environment version 3.4.2 (R Foundation for Statistical Computing, <http://www.R-project.org>).

3. Results

3.1. Effect of scion, rootstock and grafting on budbreak phenology and growth in *C. sativa*

Vegetative budbreak of grafted trees was influenced by ‘rootstock origin’ and the ‘scion origin’ × ‘rootstock origin’ interaction (Table 1). Whenever X material was used either as scion or rootstock, budbreak occurred earlier. The ‘grafting’ effect was highly significant (Table 2), inducing a late vegetative budbreak in chestnut, especially in trees from H areas (Fig. 1a).

Secondary growth within grafted chestnuts was not influenced by ‘scion origin’ or ‘rootstock origin’ (Table 1). Secondary growth was lower in H/H and X/X trees than in their non-grafted controls (significant ‘grafting’ effect in Table 2; results not shown).

3.2. Effect of scion, rootstock and grafting on drought tolerance in *C. sativa*

Under drought conditions, trees with X rootstocks (X/X and H/X) showed higher mean net photosynthesis and stomatal conductance (g_s) values than trees with H rootstocks (H/H and X/H) (Fig. 2a, b). Grafts with X material either as scion or rootstock showed higher g_s values than H/H trees (significant ‘scion origin’ × ‘rootstock origin’ interaction, Table 1, Fig. 2b). *Fv/Fm* and leaf RWC mean values followed a similar pattern and were maximum in X/X and H/X trees and minimum in H/H and X/H trees (Fig. 2c, d).

Regardless of the scion, grafts with H rootstocks wilted more than grafts with X rootstocks (Table 1, Fig. 2e). Tree mortality induced by drought was mainly influenced by ‘rootstock origin’ (Table 1), being highest in H/H (81 %) and X/H (50 %) grafts and lowest in X/X (19 %) and H/X (35 %) grafts (Fig. 2f), and to a lesser extent also by ‘scion origin’ (Table 1). Mortality of X rootstocks increased if an H scion instead of an X scion was used, whereas mortality of H rootstocks decreased if an X scion instead of an H scion was used (Fig. 2f). The capacity of trees to keep the scion alive after drought was lower in grafts with H rootstocks (0 and 22 % for H/H and X/H trees, respectively) than in grafts with X rootstocks (67 and 49 % for X/X and H/X trees, respectively) (Fig. 2b). Tree height was significant in all models (Table 1) and positively associated with leaf wilting and tree mortality.

Grafting had no effect on tree tolerance to drought stress (Table 2). Differences in gas exchange parameters, leaf wilting and tree mortality

were attributed exclusively to the ‘rootstock origin’ and ‘tree height’ effects (Table 2, Fig. 2). The effect of grafting decreased leaf RWC and *Fv/Fm* values in trees (Fig. 2c, d) only in plant material of H origin (significant ‘grafting’ × ‘rootstock origin’ interaction, Table 2).

3.3. Constitutive and drought-induced hormone and proline content in leaves and roots

Under optimal watering (day 0), no significant differences were observed in ABA, SA, JA, JA-Ile or proline content between non-grafted and grafted H and X plant material (Fig. 3). However, when non-grafted and grafted trees were pooled, leaf ABA and proline content were significantly higher in X than in H trees (250 vs 187 ng/g DW, and 146 μg/g vs 94 μg/g DW, respectively; $p < 0.05$; *t*-test).

Two weeks after water deprivation, ABA and proline content significantly increased in leaves and fine roots of all tree groups (Fig. 3a, b, i, j). SA content in leaves increased relatively more in H, X/H and H/H trees than in X, H/X and X/X trees. While JA-Ile content in leaves increased with drought (Fig. 3g), JA-Ile and JA content in fine roots decreased in most tree combinations (Fig. 3d, f). H/H trees showed the highest levels of ABA in roots (Fig. 3b) and the highest levels of JA-Ile and proline in leaves (Fig. 3g, i). The lowest concentrations of JA-Ile in roots were observed in H and H/H trees (Fig. 3h).

3.4. Relations between hormone content and parameters related to drought stress

Under drought stress, root ABA content and leaf JA-Ile content were good predictors of leaf RWC, leaf wilting and tree mortality (Fig. 4a). Leaf proline content was also a good indicator of leaf wilting and tree mortality (Fig. 4a). The relationship between leaf ABA content and leaf RWC during drought differed in *C. sativa* depending on the rootstock origin (significant ‘leaf RWC’ × ‘origin’ interaction, Fig. 4b). In X rootstocks, leaf ABA content increased as leaf RWC decreased, while no linear relationship ($p > 0.05$) was found for H rootstocks (Fig. 4b).

4. Discussion

4.1. *C. sativa* families of xeric origin advance vegetative budbreak when used as rootstock and scion

The results are in accordance with other studies reporting that phenology in grafted woody plants is mainly influenced by the rootstock (Jogaiah et al., 2013; Serra et al., 2014; Tworowski et al., 2016; Han et al., 2019) and demonstrate in chestnut that rootstocks of xeric origin could be used to induce early flushing in scions of humid origin. X scions grafted onto H rootstocks also advanced tree budbreak, indicating that scion origin also partly influences vegetative budbreak.

Table 1

Results of the mixed models used to analyse the main effects of ‘scion origin’, ‘rootstock origin’ and their interaction on the indicated variables in *Castanea sativa* grafted trees. ‘Tree height’ was used as a covariate for variables measured under drought stress. Significant *p*-values are indicated in bold.

		Budbreak phenology		Secondary growth		A ^a		g _s ^b		Fv/Fm		Leaf RWC		Leaf wilting		Tree mortality	
Fixed factors ^c	df ^d	χ ^{2e}	<i>p</i>	χ ²	<i>p</i>	χ ²	<i>p</i>	χ ²	<i>p</i>	χ ²	<i>p</i>	χ ²	<i>p</i>	χ ²	<i>p</i>	χ ²	<i>p</i>
Scion origin (S)	1	0.91	0.337	3.1	0.076	1.4	0.23	0.7	0.374	2.8	0.092	3.6	0.055	1.9	0.16	4.6	<0.05
Rootstock origin (R)	1	10.3	<0.01	0.0	0.883	0.8	0.350	0.1	0.741	8.4	<0.01	4.5	<0.05	22.5	<0.001	7.4	<0.01
S × R	1	4.3	<0.05	0.5	0.441	1.3	0.240	6.7	<0.01	1.0	0.31	2.2	0.13	1.3	0.24	0.6	0.415
Covariate																	
Tree height	1	–	–	–	–	15.8	<0.001	28.1	<0.001	4.4	<0.05	30.9	<0.001	15.4	<0.001	30.7	<0.001

^a Net photosynthesis.

^b Stomatal conductance.

^c ‘Block’ and ‘rootstock family’ were used as random factors in the models (results not shown).

^d Degrees of freedom.

^e Chi-square statistic.

Table 2

Results of the mixed models used to analyse the main effects of ‘grafting’, ‘rootstock origin’ and their interaction on the indicated variables in *Castanea sativa* trees. ‘Tree height’ was used as a covariate for variables measured under drought stress. Significant *p*-values are indicated in bold.

Fixed factors ^c	df ^d	Budbreak phenology			Secondary growth		A ^a		g _s ^b		Fv/Fm		Leaf RWC		Leaf wilting		Tree mortality	
		χ^2 ^e	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	χ^2	<i>p</i>	
Grafting (G)	1	22.0	<0.001	7.0	<0.01	0.0	0.993	0.9	0.322	0.0	0.961	0.5	0.450	2.9	0.085	0.06	0.803	
Rootstock origin (R)	1	3.9	<0.05	0.0	0.791	6.3	<0.05	4.2	<0.05	1.5	0.210	2.1	0.141	8.2	<0.01	11.2	<0.001	
G x R	1	4.6	<0.05	0.5	0.440	0.0	0.920	0.0	0.99	10.1	<0.01	13.1	<0.001	3.4	0.063	0.34	0.553	
Covariate																		
Tree height	1	–	–	–	–	25.8	<0.001	38.7	<0.001	6.0	<0.05	21.9	<0.001	13.8	<0.001	18.3	<0.001	

^a Net photosynthesis.

^b Stomatal conductance.

^c ‘Block’ and ‘rootstock family’ were used as random factors in the models (results not shown).

^d Degrees of freedom.

^e Chi-square statistic.

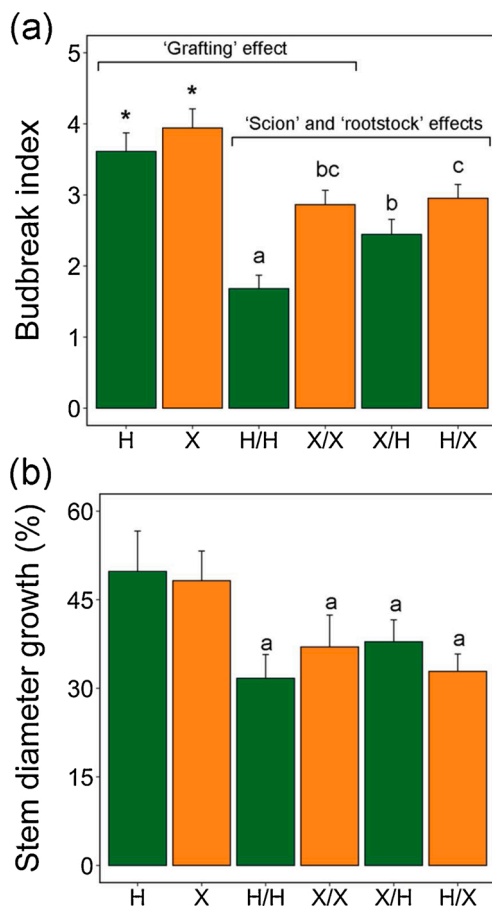


Fig. 1. Mean values of (a) budbreak index and (b) stem secondary growth in non-grafted H and X controls, intra-family grafts (H/H and X/X) and reciprocal grafts (X/H and H/X) of *Castanea sativa* of humid (H) and xeric (X) origin. Vertical bars indicate standard errors, asterisks indicate differences between mean values of non-grafted controls and their respective intra-family grafts (‘grafting’ effect), and different letters indicate differences between mean values of grafted trees (‘scion’ and ‘rootstock’ effects) ($p < 0.05$; Tukey’s HSD).

Grafting-induced shifts in budbreak phenology have been attributed to changes in endogenous factors of the scion, including hormones (e.g., auxins, Tworowski and Miller, 2007), which could explain why budbreak of X scions was not delayed by H rootstocks. Use of rootstocks to modulate budbreak phenology has received little attention in the management of *C. sativa* orchards. Chestnut growers could benefit from X rootstocks that advance budbreak in areas with mild climates, especially

if early budbreak enhanced tree growth, flowering, and presumably fruit production. Although this species is highly sensitive to late frost (Fernández-López et al., 2005; Míguez-Soto et al., 2019), we cannot assume, contrary to X rootstocks, that H rootstocks could be used in areas with a continental climate to reduce exposure of chestnut trees to late frost events.

4.2. Grafting influences budbreak phenology and growth but does not predispose *C. sativa* trees to drought

The finding that grafting delayed budbreak and reduced stem secondary growth of trees compared to non-grafted controls is in agreement with studies in other woody species reporting that plants perceive grafting as a wounding stress, at least during graft union healing (Cookson et al., 2014). Other abiotic stresses including heat (Luedeling et al., 2013), salinity (Van Zandt and Mopper, 2004) and drought (Čehulić et al., 2019) alter plant phenology. In our two-year-old grafts, the graft union was not perfectly sealed in most cases (see Fig. SM1b), supporting the existence of a wounding effect during the study. This result suggests that commercial chestnut rootstocks of known phenology under non-grafted conditions may flush later and grow less if grafted, at least during graft union healing. It is ignored if the delay in budbreak phenology induced by grafting may partly explain why grafts had lower stem secondary growth, as more delayed flushing determines a shorter vegetative period. However, a positive correlation between the vegetative budbreak scores and stem secondary growth was obtained ($r = 0.37$; $p < 0.001$; results not shown). Growth–stress defence tradeoffs in plants can occur due to resource restrictions, and prioritisation towards either growth or defence can impact plant fitness (Huot et al., 2014). A trade-off in resource investment between wound healing and stem secondary growth may have occurred in our grafted trees. Long-term studies are needed to evaluate the persistence of the effect of the graft union on budbreak phenology and tree growth in *C. sativa*, because this effect may not last. Such studies could provide new insights into the multiple types of mobile signals that confer a wide range of effects on scion development (Kumari et al., 2015) and make the design of rootstocks for specific environments a feasible target (Gregory et al., 2013).

There was no evidence that the grafting itself (*sensu* injury) predisposed *C. sativa* trees to drought stress (Table 2). If any, the effect of grafting was overcome by the effect of the rootstock origin. Mechanical wounding can have a positive outcome by leading to activation of stress defence responses that would improve plant performance but jeopardise growth by triggering signalling compounds such as jasmonates (Koo et al., 2009; Wasternack and Feussner, 2018). From a practical point of view, because grafting has little influence in predisposing trees to drought, it could be implemented as an adaptive tool to mitigate the impacts of climate change and optimise site-specific nut production.

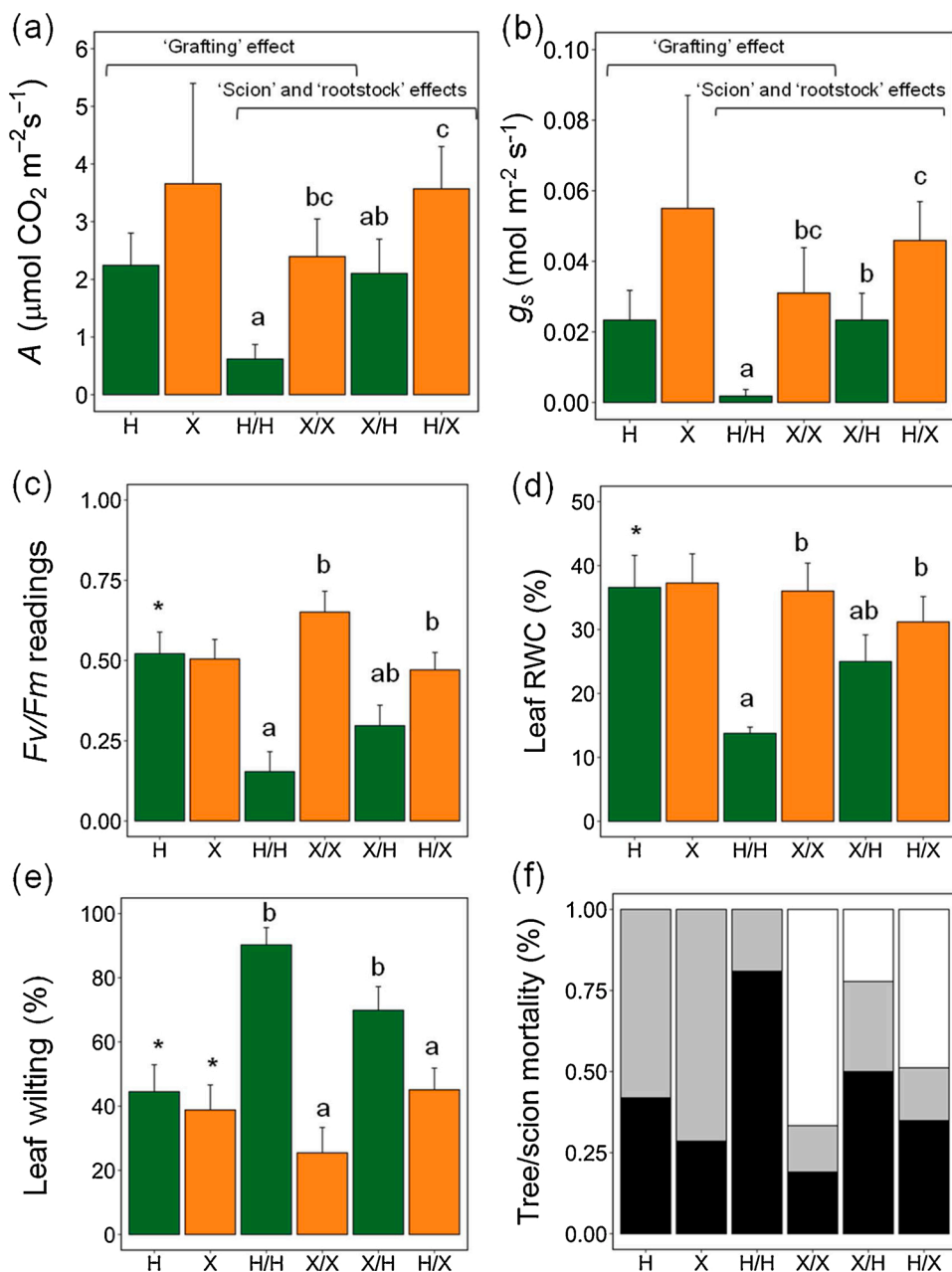


Fig. 2. Mean values during drought treatment of (a) leaf net photosynthesis (A), (b) stomatal conductance (g_s), (c) Fv/Fm readings, (d) leaf RWC, (e) leaf wilting and (f) tree/scion mortality in non-grafted H and X controls, intra-family grafts (H/H and X/X) and reciprocal grafts (X/H and H/X) of *Castanea sativa* of humid (H) and xeric (X) origin. Vertical bars indicate standard errors, asterisks indicate differences between mean values of non-grafted controls and their respective intra-family grafts ('grafting' effect), and different letters indicate differences between mean values of grafted trees ('scion' and 'rootstock' effects). In (f) bars, black, grey and white indicate dead trees, sprouted trees and trees with a live scion after drought, respectively ('scion/rootstock' effect) ($p < 0.05$; Tukey's HSD).

4.3. Rootstocks from xeric areas increase drought tolerance in *C. sativa*

Rootstocks from xeric areas increased the drought tolerance of the more drought-sensitive trees of humid origin. Under drought, X rootstocks improved the plant fitness of H scions (as indicated by leaf gas exchange rates, maximum quantum PSII yield and leaf RWC), resulting in 50 % lower leaf wilting and 57 % lower tree mortality. The role of rootstock in controlling drought tolerance in grafted woody plants has been reported previously, as rootstocks regulate water extraction capacity and control scion transpiration (Serra et al., 2014; Tworkoski et al., 2016; Han et al., 2019). From an agronomical point of view, the high capacity of X rootstocks to extend the life expectancy of trees subjected to drought has significant implications in maintaining chestnut orchard productivity and profitability. Moreover, the scion also influenced the drought tolerance of chestnuts (expressed as tree mortality, Table 1), suggesting that the scion also needs to be taken into account for improving drought tolerance in *C. sativa*. There may be feedback loops between scion and rootstock that affect tree drought

tolerance (Tworkoski et al., 2016), operating in an intricate bidirectional signalling network (Gregory et al., 2013; Albacete et al., 2015).

4.4. Hormone and proline content in *C. sativa* trees of humid and xeric origin

The *C. sativa* trees sampled for hormone analysis had contrasting drought tolerance in terms of leaf physiology and mortality, but the biochemical changes induced by water deprivation in H and X trees did not differ much. Sampling may have been performed at a very advanced stage of water stress for H trees (Soil Volumetric Water Content at sampling of 4.7 % for grafts with H rootstocks vs. 7.2 % for grafts with X rootstocks, data not shown), given that some H trees wilted severely. In consequence, hormone levels in our study reflected the different stress levels experienced by trees, likely as a consequence of differential drought adaptive mechanisms between H and X trees. As an example, the highest values of ABA in roots (and of proline in leaves) in H/H trees indicated their extremely stressful situation before death. Variation in

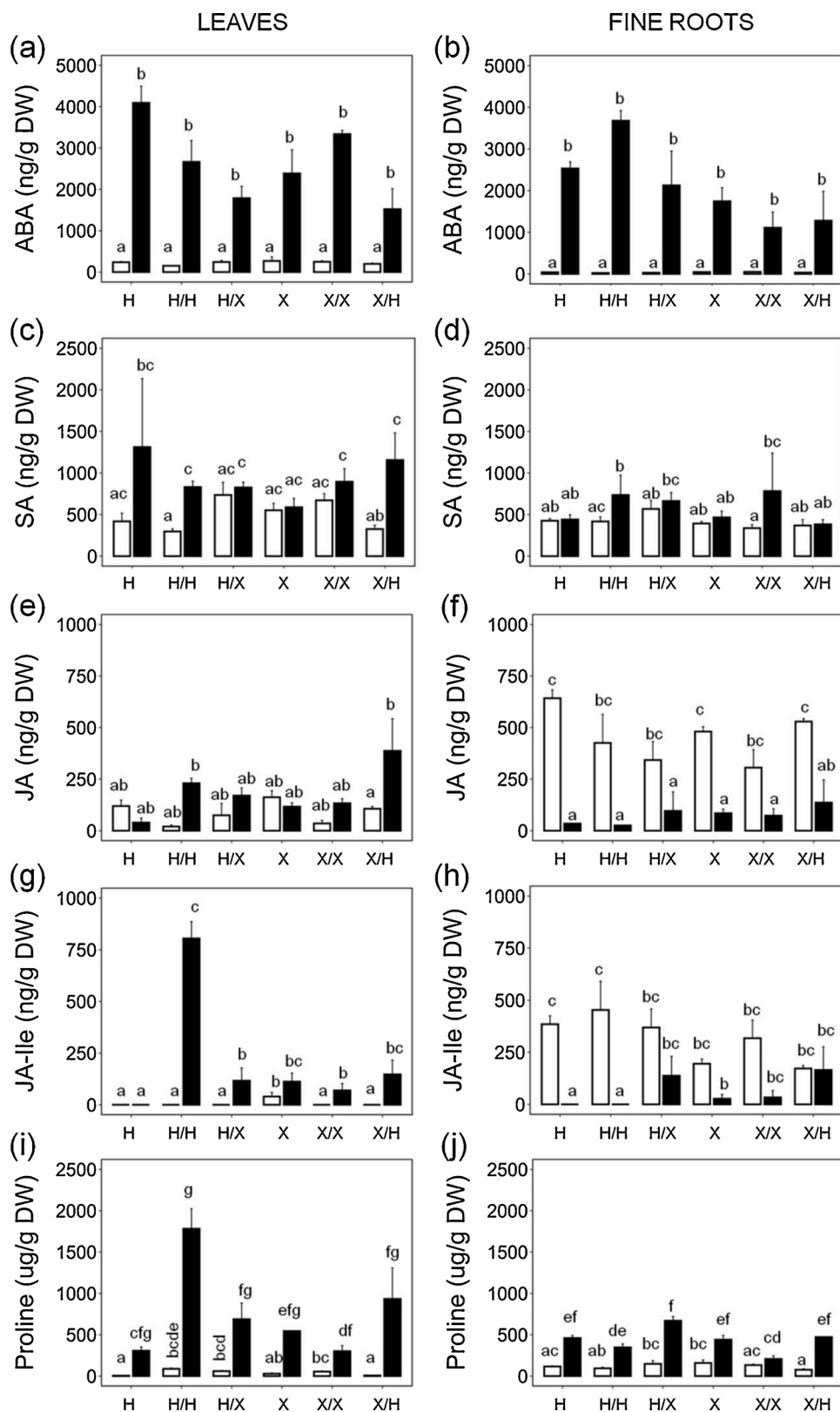


Fig. 3. Content of (a, b) abscisic acid (ABA), (c, d) salicylic acid (SA), (e, f) jasmonic acid (JA), (g, h) jasmonic acid-isoleucine (JA-Ile) (i, j) and proline before (white bars) and during (black bars) drought treatment in leaves and fine roots of non-grafted H and X controls, intra- (H/H and X/X) and inter-location (H/X and X/H) grafts of *Castanea sativa* of humid (H) and xeric (X) origin. Vertical bars indicate standard errors and different letters indicate significant differences between means ($p < 0.05$; Tukey's HSD).

xylem sap ABA depending on variable levels of drought stress was reported by Soar et al. (2006) in *Vitis* rootstocks. Under drought, differences in leaf ABA vs. leaf RWC relationship between X and H trees (Fig. 4b) could be due to the different stress level of trees, and may also suggest a stricter control of plant dehydration through ABA-induced stomatal closure in X trees. This is sustained by intra-specific

variability in the ABA metabolism affecting adaptation to drought in *Fagus sylvatica* (Nguyen et al., 2017).

Some constitutive chemical compounds observed in X trees may partly explain the delay in plant dehydration induced by X rootstocks. These include their higher leaf ABA content and root proline control than in H rootstocks. High constitutive leaf ABA levels can induce

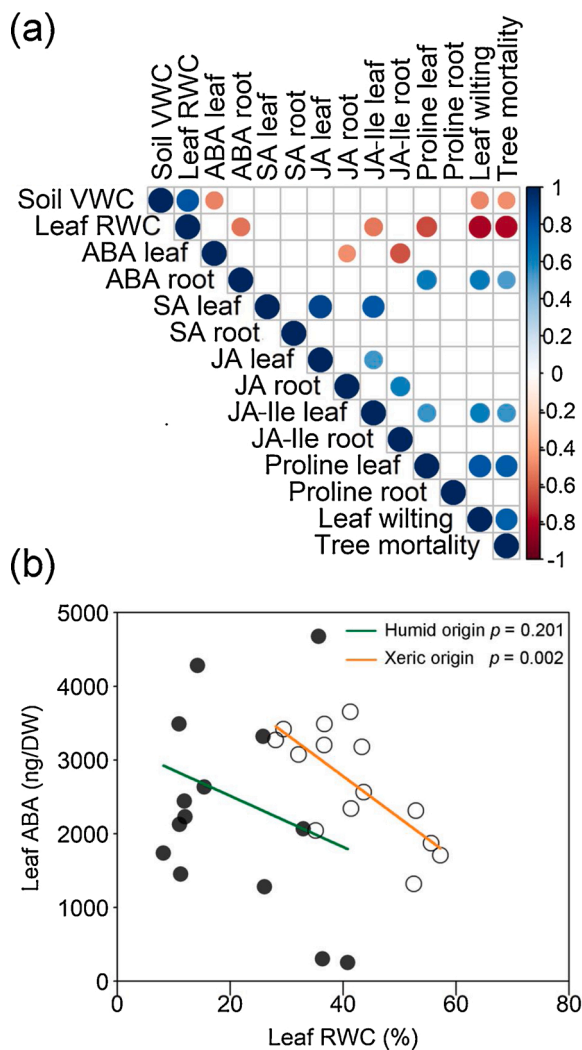


Fig. 4. (a) Matrix of significant ($p < 0.05$) Pearson correlation coefficients among water content in soil and leaves (soil VWC and leaf RWC), hormone and proline content in leaves and roots, and external symptoms (leaf wilting and tree mortality) induced in *Castanea sativa* by drought treatment, and (b) relation between leaf ABA content and leaf RWC during drought treatment in *C. sativa* trees of humid (closed circles; green line) and xeric (open circles; orange line) origin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

stomatal closure under well-watered conditions, thus reducing water loss and delaying tree dehydration after drought begins (Allario et al., 2013; Tworowski and Fazio, 2016). Stomatal sensitivity to ABA in *C. sativa* was reported by Maurel et al. (2004). High levels of the osmolytic amino-acid proline found in roots of X rootstocks may have enabled a more effective osmotic adjustment in trees during initial stages of drought, thus contributing to delay dehydration.

Our study reports for the first time the involvement of jasmonates in the response of *C. sativa* to drought. Involvement of ABA in the response of *C. sativa* to drought was previously reported (Maurel et al., 2004). Under drought conditions, JA-Ile in leaves can regulate biosynthesis, accumulation and signalling of ABA (De Ollas and Dodd, 2016; De Ollas et al., 2018), and both hormones can in turn modulate stomatal closure (De Ollas et al., 2018). The increased leaf JA-Ile and down-regulation of jasmonates (JA and JA-Ile) in roots coinciding with ABA accumulation in roots and leaves was a hallmark of *C. sativa* response to drought. This result suggests a significant role of belowground jasmonates in the drought response of chestnut trees.

5. Conclusions

This study reveals the potential use of grafting to shape the phenology of *C. sativa* trees and improve the drought tolerance of sensitive chestnuts using scions and rootstocks of xeric origin. The results may allow changes in the management of *Castanea* spp. orchards, and suggest that the southern *C. sativa* gene pool in Spain could be used as a source of drought tolerant scions and rootstocks in the face of ongoing climate change. We report for the first time differences in hormone and proline content in leaves and fine roots of chestnut trees from humid and xeric provenances exposed to drought, probably occurring as a consequence of different adaptive strategies of trees depending on their origin.

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.

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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.envexpbot.2021.104467>.

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