Exploring the use of rootstocks from xeric areas to improve the tolerance to drought in *Castanea sativa* Mill.

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Abstract

Nut production by the European sweet chestnut (*Castanea sativa* Mill.) in grafted orchards is threatened by the increasing drought stress associated to current global warming. To explore if the tolerance to drought in *C. sativa* can be improved by the use of drought-tolerant rootstocks, trees from humid (H) and xeric (X) populations of Spain were used to establish intra-familiar (H/H and X/X) and reciprocal (X/H and H/X) grafts. The effects of the scion, the rootstock and grafting as a wounding stress on the vegetative budbreak, secondary growth and drought tolerance were studied. Drought tolerance was assessed by measuring leaf gas exchange, chlorophyll fluorescence, water status and leaf wilting two weeks after water deprivation and tree and scion mortality two months after recovery, complemented with hormones (ABA, SA, JA and JA-Ile) and proline quantification in leaves and roots. Rootstocks and scions from xeric origin induced an earlier flushing and improved drought tolerance of both scions and rootstocks from humid origin. After drought, tree mortality of H/X trees was 57 % lower than mortality of H/H trees, and scion loss due to drought was 47 % lower in H/X as compared to X/H trees. The grafting (wounding) effect had no influence on the tolerance to drought of trees, although it delayed vegetative budbreak and tended to reduce tree secondary growth. Under drought stress, differences in the hormone and proline contents of trees reflected their different dehydration levels reached. Results support using rootstocks from xeric areas to improve the drought tolerance of chestnuts and suggest that the southern Iberian *C. sativa* gene pool could be exploited as a source of drought tolerant rootstocks to be used in further chestnut breeding programs.

Key words

ABA, climate change, grafting, hormonal profiling, JA-Ile, orchard management

Introduction

Sweet chestnut (*Castanea sativa* Mill.) is a multipurpose tree species widely distributed throughout the Mediterranean Basin. It occurs in forests and it is cultivated by grafting in orchards for nut production. At present, orchards undergo increasing drought stress associated to climate change (Conedera et al. 2010; Carnicer et al. 2011; Buras and Menzel 2019). This situation is aggravated by the replacement of native *C. sativa* rootstocks with inter-specific hybrid rootstock clones (*C. sativa* x *C. crenata*) which are resistant to *Phytophthora cinnamomi* Rands. but have low tolerance to drought (López-Villamor et al. 2018). Drought-tolerant rootstocks may be used to mitigate the impacts of climate change on chestnut cultivation (Soylu and Serdan 2000), similarly to other woody crops (Serra et al. 2013; Zhang et al. 2016; Tworkoski et al. 2016; Han et al. 2019). However, breeding programs on chestnut are based on increasing rootstock resistance to *P. cinnamomi* and on enhancing rootstock compatibility with traditional *C. sativa* varieties (Pereira-Lorenzo and Fernández-López 1997; Pereira-Lorenzo and Ramos-Cabrer 2004; Grauke and Thompson 2010; Warschefsky et al. 2016). The influence of the scion and the rootstock on the budbreak phenology, growth and drought tolerance of chestnut is largely unknown, since research is mainly focused on the compatibility between the scion and the rootstock (e.g. Huang et al. 1994; Pereira-Lorenzo and Fernandez-Lopez 1997; Serdar and Soyla 2005; Bueno et al. 2009; Serdar et al. 2010; Warmund et al. 2012; Ada and Ertan 2013; Iliev et al. 2013). Grafting a tree implies a wounding stress during the early stages of graft union healing, which 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 be reduced due to incomplete vascular reconnection at the graft union (Torii et al. 1992;

66 Serra et al. 2014) while changes in the production of hormones and other metabolites
67 during the regeneration of tissues (Mo et al. 2017; Melnyk et al. 2018; Nanda and Melnyk
68 2018) might affect tree phenology, growth and drought tolerance.
69
70 *Castanea sativa* inhabits regions with marked water availability gradients (e.g. in the
71 Iberian Peninsula and Turkey) leading to a genetically-based differentiation in traits
72 related to drought adaptation (Pigliucci et al. 1990; Lauteri et al. 1999; Fernández-López
73 et al. 2005; Ciordia et al. 2012; Míguez-Soto and Fernández-López 2015; Míguez-Soto
74 et al. 2019). This evolutionary pressure has permitted to obtain rootstock genotypes
75 contrasting in drought tolerance. In the Iberian Peninsula there are two *C. sativa* ecotypes
76 adapted to different climatic conditions, the first located in wet and mild northern areas
77 and the second in xeric central and southern regions (Ciordia et al. 2012; Míguez-Soto
78 and Fernández-López 2015; Míguez-Soto et al. 2018; Alcaide et al. 2019). Xeric *C. sativa*
79 populations show early phenology, low plant growth and higher root development in
80 comparison to mesic populations, because of adaption to summer drought conditions
81 (Lauteri et al. 1999; Fernández-López et al. 2005; Ciordia et al. 2012; Míguez-Soto and
82 Fernández-López 2015; Míguez-Soto et al. 2018).
83
84 Phytohormones are stress signaling molecules that help plants adapt to adverse
85 environmental conditions including drought through a complex crosstalk that implies
86 changes in primary and secondary metabolism. They also play an important role in the
87 scion/rootstock communication (Aloni et al. 2010; Albacete et al. 2015) what makes them
88 ideal candidates for studying the mechanisms by which rootstocks enhance drought
89 tolerance (Allario et al. 2013; Tworkoski et al. 2016; Silva et al. 2018). However, it is
90 unknown if biochemical responses related to stress signaling may 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) and recent studies have shown that rootstock-induced changes in the content of ABA play an important role in defining the tolerance to drought of grafted plants (Allario et al. 2013; Liu et al. 2016; Santana-Viera et al., 2016; Tworkoski et al. 2016; Silva et al. 2018). Salicylic acid (SA) and jasmonates (JAs) are phytohormones well-known for regulating plant defense against pests and pathogens but their involvement in responses of plants to drought is increasingly recognized (De Diego et al. 2012; Jesús et al. 2015; Shenxie et al. 2015; 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; Matos 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) like 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 performs also stress signaling functions and is commonly used as a drought stress marker, its content being 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 analyzing the constitutive and drought-induced hormonal profiles of two families contrasting in tolerance to drought. The following hypotheses were tested in chestnut: (i) vegetative budbreak, tree growth and drought tolerance

responses depend on the rootstock and are influenced by a 'grafting' effect and (ii) there are constitutive and/or drought-induced differences in the hormone and proline content of leaves and roots of trees from humid and xeric origins.

Materials and methods

Plant material, grafting and growth conditions

Four *C. sativa* families (H_1, H_2, X_1 and X_2; half-sibling trees) were used. H_1 and H_2 came from a mild, humid coastal location in north western Spain (Bergondo, Galicia region, 43º18'32"N 8º13'57"W, mean annual temperature 13 ºC, annual rainfall 1,105 mm), and X_1 and X_2 came from a xeric location in southern Spain (Constantina, Andalusia region, 37º53'16"N 5º36'13"W, mean annual temperature 15.5 ºC, 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 that were at least 100 m apart from each other were randomly selected in each population and their nuts were massively collected. Seeds were immersed in water and those which floated were discarded as non-viable. Viable seeds were sterilized 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 obtained seedlings 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-familiar grafts) and grafted trees using scions from a different location as the rootstock (inter-familiar grafts).

This grafting design resulted into reciprocal grafts between each pair of families with contrasted 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, 40º02´N, 6º05´W; 374 m asl, western Spain), fertilized with Osmocote Pro 3-4M (Osmocote® Pro) at 4 g L^{-1} and grown under optimal watering conditions (soil volumetric water content around 30 %).

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. Potted plant material was arranged in a complete randomized block design of six blocks, each block containing at least one observation per scion/rootstock combination and non-grafted control. All plant material was merged into six groups of trees considering whether trees were grafted or not and the origin of the scion and the rootstock family. This resulted into H and X (non-grafted controls of the H_1 and H_2 and the X_1 and X_2 families, respectively), H/H and X/X (intra-familiar grafts of the H_1 and H_2 and the X_1 and X_2 families, respectively), and X/H and H/X (reciprocal inter-familiar grafts between the H_1 and H_2 and the X_1 and X_2 families) groups of trees (see Table SM1). To test the hypotheses that vegetative budbreak, tree growth and drought tolerance are influenced by the rootstock, the H/H, X/X, X/H and H/X tree-groups were assessed. This way, the relative contribution of the scion and the rootstock were taken into account. 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. Because of the genetic proximity of the scion and the rootstock in intra-familiar grafts, differences relative to non-grafted controls are expected to be mainly due to the effect of the graft union rather than to the interaction between two genetically distinct individuals.

Assessment of budbreak phenology and tree growth under optimal watering conditions

Vegetative budbreak was assessed in all trees in April 2017. 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 of stem diameter in April 2017 and July 2017 (before the application of the drought treatment) and expressed as percentage. Stem diameters were calculated by the average of two measurements made orthogonally ca. 5 cm from the ground level, where a white stripe in April was painted. In July, diameters were measured at the stripes. Tree height was measured in all plants before the application of the drought treatment.

Drought treatment

The drought treatment was imposed over all plants during July 2017 and consisted of watering pots to field capacity (day 0) and withdrawing watering for two weeks. At day 14, to assess 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, the plants were

191 rewatered to field capacity for recovery and maintained under optimum watering
192 conditions (30 % SVWC) until the end of the experiment (September 2017).
193
194 *Morpho- Physiological assessment of tree drought tolerance*
195 The degree of drought tolerance was assessed 14 days after the drought treatment started
196 by two approaches: (i) evaluation of external symptoms due to damage caused by drought
197 in all trees and (ii) a physiological assessment of gas exchange parameters, chlorophyll
198 fluorescence and water status in leaves of a subsample of trees. Leaf wilting was visually
199 estimated as the percentage of plant foliage showing turgor loss while tree mortality and
200 scion mortality (if any) were assessed two months after the drought treatment finished.
201 Assessment after two months was done because some trees died after rewatering due to
202 drought-induced damage. Leaf gas exchange related parameters, net carbon assimilation
203 (A) and stomatal conductance (g_s), were measured with a portable differential infrared
204 gas analyzer (IRGA) (Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf
205 chamber (Alcaide et al., 2019). Measurements were performed between 10.00-12.00 h
206 with photosynthetically active radiation (PAR) ranging from 300 to 500 µmol photons
207 m^{-2} s^{-1}. For chlorophyll fluorescence (*Fv/Fm* the maximum quantum yield of
208 photosystem II (PSII)), readings were obtained from 8.00 to 10.00 h with a Multimode
209 Chlorophyll Fluorometer OS5p device (Opti-Science Inc., USA) in dark-adapted leaves
210 (30 min). Leaf relative water content (RWC) was evaluated at noon, following:

211 $$\mathrm{RWC}\ (\%) = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{HW} - \mathrm{DW})} \cdot 100$$

212 Where FW is the fresh weight of leaves at the time of sampling, HW is the hydrated
213 weight of leaves after soaking in distilled water for 24 h at 4 ºC in darkness, and DW is
214 the dry weight of leaves after complete oven dehydration (48 h, 60 ºC). Two apical fully
215 expanded leaves per tree were used.

Hormone and proline quantification in leaves and roots of trees

On day 0 (optimum watering conditions) and on day 14 after the drought treatment started, hormone and proline content in leaves and roots of a subsample of trees were assessed. For both sampling points, non-grafted controls, intra-familiar grafts and reciprocal grafts of the families H_1 and X_1 were used, and selection of these two families was done by random. Around 15 plants from each of the six groups selected were sampled. Leaves were sampled by collecting the apex of one fully-developed top-stemmed 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 together (n=5) to get a sample size of three biological replicates per group of trees. Samples were kept at -80 ºC until freeze drying with a FreeZone 6 Liter Benchtop (Labconco, Kansas City, USA). Samples were further 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. (2019). Shortly, fifty milligrams of lyophilized powdered plant tissue were wetted with a 10% methanol aqueous solution containing hormonal internal standards, vortexed and incubated. Then, samples were mixed, centrifuged and the supernatant was recovered for a 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,

241 Mildford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex)
242 connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK).
243 Further quantification was done using external calibration curves. The chromatographic
244 and mass spectrometry conditions were the same as in Gamir et al. (2012).
245
246 Proline was determined by slight modifications to the classical protocol by Bates et al.
247 (1973). 20 mg of dry powdered tissue was homogenized with 1.5 ml of sulphosalicylic
248 acid (3%, w/v) and centrifuged (10 min, 4 °C, 10,000g). 1 ml of supernatant was mixed
249 with 1 ml of ninhydrin acid and 1 ml of glacial acetic acid, and the mix was incubated (30
250 min, 100 °C). After cooling down on ice, 2 ml of toluene were added and absorbance was
251 read at 520 nm. A free proline standard curve was used for quantification, using three
252 technical replicates per biological replicate.
253
254
255 *Statistical analysis*
256 The effect of the origin of the scion and rootstock and the effect of grafting on vegetative
257 budbreak, secondary growth, leaf physiology parameters and leaf wilting were analysed
258 by Linear Mixed Models (LMM) and Generalized Linear Mixed Models (GLMM),
259 depending on whether errors were normally distributed or not. Data were first checked
260 for normality and homoscedasticity by Shapiro–Wilk and Levene's tests. When assessing
261 the effect of the origin of the scion and rootstock, intra- and inter-familiar grafts (i.e.,
262 H/H, X/X, H/X and X/H scion/rootstock combinations) were used and the 'scion origin',
263 the 'rootstock origin' and their interaction were considered as fixed effects. Tree mortality
264 was analyzed with a cumulative link mixed model (CLMM) in which the outcome
265 variable consisted of three ordered categories: 0 (dead plant), 1 (basal or epicormic

resprouting with scion loss) and 2 (scion alive). CLMM are similar to logistic regression but they can handle ordered categorical outcomes with more than two categories. When assessing the effect of grafting, non-grafted controls and their respective intra-familiar grafts (i.e., H, X, H/H and X/X trees) were used and 'grafting' (two levels: 'grafted' and 'non-grafted'), the 'rootstock origin' and their interaction were specified as fixed effects in models. The effect of grafting on tree mortality was analysed with a logistic mixed model where the dependent variable was coded as 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. The hormone and proline content in leaves and roots was analysed with GLMM using the 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. The relations between hormones 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).

Results

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

Vegetative budbreak of grafted trees was influenced by the origin of the rootstock and its interaction with the origin of the scion (Table 1). Whenever X material was used either as scion or rootstock, budbreak occurred earlier. The 'grafting effect' was highly

290 significant (Table 2), inducing a late vegetative budbreak in chestnut, especially in trees
291 from H areas (Fig. 1a).
292
293 Secondary growth within grafted chestnuts was not influenced by the origin of the scion
294 and the rootstock (Table 1). Secondary growth tended to be lower in H/H and X/X trees
295 relative to their non-grafted controls (significant 'grafting' effect; Table 2), although
296 differences were not significant in both cases.
297
298 *Effect of the scion, the rootstock and grafting on drought tolerance in C. sativa*
299 Under drought conditions, trees with X rootstocks (X/X and H/X) showed higher net
300 photosynthesis and stomatal conductance (g_s) values in comparison to trees with H
301 rootstocks (H/H and X/H) (Fig. 2a, b). Grafts with X material either as scion or as
302 rootstock showed higher g_s values (significant 'scion origin' × 'rootstock origin'
303 interaction, Table 1, Fig. 2b). *Fv/Fm* and leaf RWC mean values followed similar patterns
304 to each other, being maximum for X/X and H/X trees and minimum in H/H and X/H trees
305 (Fig. 2c, d).
306
307 Regardless of the scion, grafts with H rootstocks wilted more in comparison to grafts with
308 X rootstocks (Table 1, Fig. 2e). Tree mortality induced by drought was mainly influenced
309 by the 'rootstock origin' (Table 1), being highest in H/H (81%) and X/H (50%) grafts and
310 lowest in X/X (19%) and H/X (35%) grafts (Fig. 2f), and to a lesser degree also by the
311 'scion origin' (Table 1). Mortality of X rootstocks increased if a H scion instead of a X
312 scion was used while mortality of H rootstocks decreased if a X scion instead of a H scion
313 was used (Fig. 2f). The capacity of trees to maintain the scion alive after drought was
314 lowest in grafts with H rootstocks (0 and 22% for H/H and X/H trees, respectively) in

315 comparison to grafts with X rootstocks (67 and 49% for X/X and H/X trees, respectively)
316 (Fig. 2b). Tree height was significant in all models (Table 1) and positively associated to
317 leaf wilting and tree mortality.
318
319 Grafting itself had no effect on the tolerance of trees to drought stress (Table 2).
320 Differences in gas exchange parameters, leaf wilting and tree mortality were exclusively
321 attributed to the 'rootstock origin' and 'tree height' effects (Table 2, Fig. 2). Only in plant
322 material from H origin (significant 'grafting' × 'rootstock origin' interaction, Table 2),
323 the effect of grafting diminished values of leaf RWC and *Fv/Fm* in trees (Fig. 2c, d).
324
325 *Constitutive and drought-induced hormone and proline content in leaves and roots*
326 Under optimal watering (day 0), no significant differences in the content of ABA, SA,
327 JA, JA-Ile and proline between non-grafted grafted H and X plant material were observed
328 (Fig. 3). However, when pooling non-grafted and grafted trees together, leaf ABA and
329 proline content were significantly higher in X than in H trees (250 *vs* 187 ng/g DW, and
330 146 µg/g *vs* 94 µg/g DW, respectively; $P < 0.05$; *t*-test).
331
332 Two weeks after water deprivation, ABA and proline content significantly increased in
333 leaves and roots of all groups of trees (Fig. 3). SA content in leaves increased relatively
334 more in H, X/H and H/H trees in comparison to X, H/X and X/X trees. While JA-Ile
335 content in leaves increased with drought, JA-Ile and JA content in roots decreased in
336 almost all trees (Fig. 3e-f and 3g-h). H/H trees showed the highest levels of ABA in roots
337 and the highest levels of JA-Ile and proline in leaves (Fig. 3b, 3g and 3i). The lowest
338 concentrations of JA-Ile in roots were observed in H and H/H trees (Fig. 3h).
339

Relations between hormone content and parameters related to drought stress

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

Discussion

C. sativa families from xeric origin advance vegetative budbreak when used as rootstock and scion

The results obtained in this work 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. 2013; Tworkoski et al. 2016; Han et al. 2019) and show that rootstocks from xeric origins could be used to induce early flushing in scions from humid origins. However, the fact that X scions grafted onto H rootstocks also advanced tree budbreak indicates that the origin of the scion partly influences vegetative budbreak too. Grafting-induced shifts in budbreak phenology have been attributed to changes in endogenous factors of the scion including hormones (e.g. auxins, Tworkoski and Miller 2007), which could explain why budbreak of X scions was not delayed by H rootstocks. The 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 would enhance

365 tree growth and flowering. Although the species is highly sensitive to late frosts
366 (Fernández-López et al. 2005; Míguez-Soto et al. 2019) we cannot assume, contrarily to
367 X rootstocks, that H rootstocks could be used in areas with continental climates to reduce
368 the exposure of chestnut trees to late frost events.
369
370 *Grafting induces stress in terms of budbreak phenology and growth but does not*
371 *predispose* C. sativa *trees to drought*
372 The finding that grafting delayed budbreak and tended to reduce stem secondary growth
373 of trees in relation to non-grafted controls is in agreement with studies in other woody
374 species indicating that grafting is perceived as a wounding stress by the plant, at least
375 during the graft union healing (Cookson et al. 2014). Other abiotic stresses including
376 drought (Kuster et al. 2014; Čehulić et al. 2019), heat (Luedeling et al. 2013) or salinity
377 (Van Zandt and Mopper 2004) alter plant phenology. In our two-year-old grafts, the graft
378 union was not perfectly sealed in most of the cases (see Fig. SM1b) which supports the
379 existence of a wounding effect during the study. This result suggests that commercial
380 chestnut rootstocks of known phenology under non-grafted conditions may flush later and
381 grow less after being grafted, at least during the graft union healing. The delay in
382 budbreak phenology induced by grafting may partially explain why grafts had a lower
383 stem secondary growth, as a more delayed flushing determines a shorter vegetative
384 period. This is supported by the positive correlation between the vegetative budbreak
385 scores and stem secondary growth ($r = 0.37$; $P < 0.001$; results not shown). Growth–stress
386 defense tradeoffs are thought to occur in plants due to resource restrictions, which demand
387 prioritization towards either growth or defense with impacts on plant fitness (Huot et al.
388 2014). Thus, a trade-off in the investment of resources between wound healing and stem
389 secondary growth in grafted chestnuts is also plausible.

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*, as such effect could be ephemeral. These studies may 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 may turn the design of rootstocks for specific environments in a feasible target (Gregory et al. 2013).

As a wounding stress, no evidence that grafting predisposes *C. sativa* trees to drought stress was found (Table 2). If any, the effect of grafting was overcome by the effect of the origin of the rootstock. In fact, mechanical wounding may have a positive outcome by leading to the activation of stress defense responses improving plant performance, yet jeopardizing growth, by triggering signaling compounds such as jasmonates (Koo et al. 2009; Wasternack and Feussner 2018). From an applied perspective, such result encourages the implementation of grafting as an adaptive tool to mitigate the impacts of climate change and optimize site- specific production of chestnuts.

Rootstocks from xeric areas increase the tolerance to drought in C. sativa

Drought tolerance was mainly determined by the rootstock origin in *C. sativa* grafts, and rootstocks from xeric areas increased the tolerance to drought of the more drought-sensitive trees from humid origin. Under drought, X rootstocks improved the plant fitness of H scions (as indicated by leaf gas exchange rates, the maximum quantum yield of PSII and the leaf RWC), which resulted into 50% lower leaf wilting and 57% lower tree mortality. The major role of the rootstock in controlling drought tolerance in grafted woody plants has been reported elsewhere, as rootstocks regulate the water extraction capacity and control scion transpiration (Serra et al. 2013; Tworkoski et al. 2016; Han et

al. 2019). From an agronomical point of view, the high capacity of X rootstocks to maintain the scion alive after drought has important implications for the maintenance of chestnut orchards productivity and profitability. However, the scion also had an influence on the drought tolerance of trees (expressed as tree mortality, Table 1), suggesting that the drought response of the scion also needs to be considered to improve drought tolerance in *C. sativa*. Feedback loops between the scion and the rootstock exist that affect drought tolerance of trees (Tworkoski et al. 2016) in an intricate bidirectional signalling network (Gregory et al. 2013; Albacete et al. 2015).

Hormone and proline contents in C. sativa *trees from humid and xeric origins*

The *C. sativa* trees sampled for hormone analysis had a contrasted tolerance to drought in terms of leaf physiology and mortality, but the biochemical changes induced by water deprivation in H and X trees were not so different. Possibly, sampling was 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), in some of them occurring near to tree death. In consequence, hormone levels in our study reflected the different stress levels undergone by trees, likely as a consequence of differential drought adaptive mechanisms between H and X trees. As an instance, the highest values of ABA in roots (and of proline in leaves) of H/H trees indicated their extremely stressful situation prior to death. Variation in xylem sap ABA as a function of variable levels of drought stress were reported by Soar et al. (2006) in *Vitis* rootstocks. Under drought, differences in the leaf ABA *vs.* leaf RWC relationship between the X and H trees (Fig. 4b) could be due to the different stress levels in trees, although they could also suggest a stricter control of plant dehydration through ABA-induced stomatal closure in the X trees. Intra-specific variability in the ABA metabolism

of plants affecting adaptation to drought exists (Mahajan and Tuteja 2005; Nguyen et al. 2017).

Constitutively, some biochemical features observed in X trees may partially explain the delay in plant dehydration induced by X rootstocks. These include their higher content of ABA in leaves and proline in roots as compared to H rootstocks. High constitutive leaf ABA levels can induce stomatal closure under well-watered conditions, thus reducing water loss and delaying tree dehydration after drought begins (Allario et al. 2013; Tworkoski and Fazio 2016). Stomatal sensitivity to ABA in *C. sativa* was reported by Maurel et al. (2004). Elevated levels of the osmolytic amino-acid proline found in roots of X rootstocks may have enabled a more effective osmotic adjustment in these trees during initial stages of drought, thus contributing to delay dehydration.

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

Conclusions

This study highlights the potential of grafting to shape phenotypical variation in *C. sativa* trees and shows that drought tolerant (xeric origin) *C. sativa* rootstocks (and scions) could be used to improve tolerance of sensitive chestnuts. Results may imply changes in the management of *Castanea* spp. orchards and suggest that the southern *C. sativa* gene pool could be exploited as a source of drought tolerant rootstocks to be used in further chestnut breeding programs in the face of ongoing global warming. Under drought stress, differences in the hormone and proline content of leaves and roots between trees from humid and xeric origins were mainly related to the different stress levels reached as a consequence of different adaptive strategies between H and X trees.

Acknowledgements

This work was funded by grant AGL2014-53822-C2-1-R from the Spanish Ministry of Economy and Competitiveness, and the European Regional Development Fund (FEDER) from the European Union. VR (TA18022) is grateful to the Regional Government of Extremadura (Spain) for financial support ('Atracción de Talento Investigador' Programme). M. Vivas was supported by a 'Juan de la Cierva program' funded by the Ministry of Economy, Industry and Competitiveness of the Spanish Government. Thanks are due to FCT/MCTES for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020), through Portuguese national funds. Thanks to Fernando Silla and Sonia Mediavilla (University of Salamanca) for allowing the use of the ball mill and to the Serveis Centrals of the Universitat Jaume I for the technical support.

References

Ada S, Ertan E (2013) Cytological study of the graft union of the Chestnut (*Castanea sativa* Mill)/Oak (*Quercus vulcanica* Boiss). Agriculture, Forestry and Fisheries 2:110-115.

Albacete A, Martínez-Andújar C, Martínez-Pérez A, Thompson AJ, Dodd IC, Pérez-Alfocea F (2015) Unravelling rootstock×scion interactions to improve food security. J Exp Bot 66: 2211–2226.

Alcaide F, Solla A, Mattioni C, Castellana S, Martín MA (2019) Adaptive diversity and drought tolerance in *Castanea sativa* assessed through EST-SSR genic markers. Forestry 92:287–296.

Allario T, Brumos J, Colmenero-Flores JM, Iglesias DJ, Pina JA, Navarro L, Talon M, Ollitrault P, Morillon R (2013) Tetraploid Rangpur lime rootstock increases drought tolerance via enhanced constitutive root abscisic acid production. Plant Cell Environ 36:856–868.

Aloni B, Cohen R, Karni L, Aktas H, Edelstein M (2010) Hormonal signaling in rootstock–scion interactions. Sci Hortic 127:119–126.

Arbona V, De Ollas C, Argamasilla R, López-Climent MF, Gómez-Cadenas A (2015). Hormone and metabolite traits related to abiotic stress tolerance in citrus Acta Hortic 1065:1275-1281.

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207.

Bueno SCS, Tokunaga T, Berti AJ, Coutinho EL, Maia AH, Yamanishi OK (2009). Grafting compatibility among eleven chestnut cultivars and hybrids. Acta Hortic 844:127–132.

Buras A, Menzel A (2019) Projecting Tree Species Composition Changes of European Forests for 2061–2090 Under RCP 4.5 and RCP 8.5 Scenarios. Front Plant Sci 9:1986.

Camisón Á, Martín MA, Sánchez-Bel P, Flors V, Alcaide F, Morcuende D, Pinto G, Solla A (2019) Hormone and secondary metabolite profiling in chestnut during susceptible and resistant interactions with *Phytophthora cinnamomi*. J Plant Physiol 241:153030.

Carnicer J, Coll M, Ninyerola M, Pons X, Sánchez G, Peñuelas J (2011) Widespread crown condition decline, food web disruption, and amplified tree mortality with increased climate change-type drought. Proc Natl Acad Sci USA 108:1474-1478.

Čehulić I, Sever K, Katičić Bogdan I, Jazbec A, Škvorc Ž, Bogdan S (2019) Drought Impact on Leaf Phenology and Spring Frost Susceptibility in a *Quercus robur* L. Provenance Trial. Forests 10:50.

Ciordia M, Feito I, Pereira-Lorenzo S, Fernández A, Majada J (2012) Adaptive diversity in *Castanea sativa* Mill. half-sib progenies in response to drought stress. Environ Exp Bot 78:56–63.

Conedera, M., Barthold, F., Torriani, D. and Pezzatti, G.B. (2010). Drought sensitivity of *Castanea sativa*: case study of summer 2003 in the southern Alps. Acta Hortic 866:297-302.

Cookson SJ, Clemente Moreno MJ, Hevin C, et al (2014) Heterografting with nonself rootstocks induces

genes involved in stress responses at the graft interface when compared with autografted controls. J Exp Bot 65:2473–2481.

Cuenca B, Lario FJ, Luquero L, Ocaña L, Mandujano M (2018). Early grafting of chestnut by green grafting. Acta Hortic 1220:141-148.

De Diego N, Saiz-Fernandez I, Rodriguez J L, Perez-Alfoce P, Sampedro M C, Barrio R J, Lacuesta M, Moncalean P (2015) Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. J Plant Physiol 188:64–71.

De Diego N, Pérez-Alfocea F, Cantero E, Lacuesta M, Moncaleán P (2012) Physiological response to drought in radiata pine: phytohormones implication at leaf level Tree Physiol 32:435–449.

De Ollas C, Dodd IC (2016). Physiological impacts of ABA-JA interactions under water-limitation. Plant Mol Biol 91:641–650.

De Ollas C, Arbona V, Gómez-Cadenas A, Dodd IC (2018) Attenuated accumulation of jasmonates modifies stomatal responses to water deficit. J Exp Bot 69:2103-2116.

De Ollas C, Hernando B, Arbona V, Gómez-Cadenas A (2012) Jasmonic acid transient accumulation is needed for abscisic acid increase in citrus roots under drought stress conditions. Physiol Plant 147:296-306.

Dutra de Souza J, de Andrade Silva EM, Coelho Filho MA, Morillon R, Bonatto D, Micheli F et al. (2017) Different adaptation strategies of two citrus scion/rootstock combinations in response to drought stress. PLoS One 12:e0177993.

Fernández-López J, Zas R, Blanco-Silva R, Díaz R (2005) Geographic differentiation in adaptive traits of wild chestnut Spanish populations (*Castanea sativa* Miller) Invest Agrar Sist Recur For 14:13-26.

Gamir J, Pastor V, Sanchez-Bel P, Agut B, Mateu D, Garcia-Andrade J, Flors V (2018) Starch degradation, abscisic acid and vesicular trafficking are important elements in callose priming by indole-3-carboxylic acid in response to *Plectosphaerella cucumerina* infection. Plant J 96:518–531.

Grauke LJ, Thompson TE (2010) Rootstock Development in Temperate Nut Crops. Acta Hortic 622:553–566.

Gregory PJ, Atkinson CJ, Bengough AG, Else MA, Fernández-Fernández F, Harrison RJ, Schmidt S (2013) Contributions of roots and rootstocks to sustainable, intensified crop production. J Exp Bot 64:1209–1222.

Han Q, Guo Q, Korpelainen H, Niinemets Ü, Li C (2019) Rootstock determines the drought resistance of

poplar grafting combinations. Tree Physiol 39:1855–1866.

Huang H, Norton JD, Boyhan GE, Abrahams BR (1994) Graft Compatibility among Chestnut *(Castanea)* Species. J Amer Soc Hort Sci 119:1127–1132.

Huot B, Yao J, Montgomery BL, He SY (2014) Growth-defense tradeoffs in plants: a balancing act to optimize fitness. Mol Plant 7:1267-1287.Iliev N, Varbeva L, Milev M (2013) The effects of different methods and rootstocks phenological stages on Sweet chestnut (*Castanea sativa* Mill.) spring grafting. For Ideas 19:41–48.

Jesus C, Meijón M, Monteiro P, Correia B, Amaral J, Escandón M, Cañal MJ, Pinto G (2015) Salicylic acid application modulates physiological and hormonal changes in *Eucalyptus globulus* under water deficit. Environ Exp Bot 118:56–66.

Jogaiah S, Oulkar DP, Banerjee K, Sharma J, Patil AG, Maske SR, Somkuwar RG (2013) Biochemically Induced Variations During Some Phenological Stages in Thompson Seedless Grapevines Grafted on Different Rootstocks. S Afr J Enol Vitic 34:36–45.

Kabbadj A, Makoudi B, Mouradi M, Pauly N, Frendo P, Ghoulam C (2017) Physiological and biochemical responses involved in water deficit tolerance of nitrogen-fixing *Vicia faba*. PLoS One 12:e0190284.

Koo AJ, Gao X, Daniel Jones A, Howe GA (2009) A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J 59:974-986.

Kumari A, Kumar J, Kumar A, Chaudhury A, Singh SP (2015) Grafting Triggers Differential Responses between Scion and Rootstock. PLoS ONE 10:e0124438.

Kuster TM, Dobbertin M, Günthardt-Goerg MS, Schaub M, Arend M (2014) A Phenological Timetable of Oak Growth under Experimental Drought and Air Warming. PLoS One 9:e89724.

Liu S, Li H, Lv X. et al (2016) Grafting cucumber onto luffa improves drought tolerance by increasing ABA biosynthesis and sensitivity. Sci Rep 6:20212.

Lauteri M, Monteverdi MC, Sansotta A, Cherubini M, Spaccino L, Villani F, Küçük M (1999) Adaptation to drought in european cherstnut. Evidences from a hybrid zone and from controlled crosses between drought and wet adapted populations. Acta Hortic 494:345–354.

López-Villamor A, Fernández-López J, Míguez-Soto B, Sánchez ME (2018) Resistance to *Phytophthora cinnamomi* in *Castanea* spp. is under moderately high genetic control mainly because of additive genetic variance. Euphytica 214:230.

Luedeling E, Guo L, Dai J, Leslie CA, Blanke M (2013) Differential responses of trees to temperature

variation during the chilling and forcing phases. Agr Forest Meteorol 181:33–42.

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: An overview. Arch Biochem Biophys 444:139–158.

Neves DM, Almeida LAdH, Santana-Vieira DDS et al (2017) Recurrent water deficit causes epigenetic and hormonal changes in citrus plants. Sci Rep **7**:13684

Maurel M, Robin C, Simonneau T et al (2004) Stomatal conductance and root-to-shoot signaling in chestnut saplings exposed to *Phytophthora cinnamomi* or partial soil drying. Funct Plant Biol 31:41–51.

Melnyk CW, Gabel A, Hardcastle TJ, Robinson S, Miyashima S, Grosse I, Meyerowitz EM (2018) Transcriptome dynamics at *Arabidopsis* graft junctions reveal an intertissue recognition mechanism that activates vascular regeneration. Proc Natl Acad Sci USA 115:2447–2456.

Míguez-Soto B, Fernández-Cruz J, Fernández-López J (2019) Mediterranean and Northern Iberian gene pools of wild *Castanea sativa* Mill. are two differentiated ecotypes originated under natural divergent selection. PLoS One 14:e0211315.

Míguez-Soto B, Fernández-López J (2015) Variation in adaptive traits among and within Spanish and European populations of *Castanea sativa*: selection of trees for timber production. New Forest 46: 23–50.

Mo Z, He H, Su W, Peng F (2017) Analysis of differentially accumulated proteins associated with graft union formation in pecan (*Carya illinoensis*). Sci Hortic 224:126–134.

Nanda AK, Melnyk CW (2018) The role of plant hormones during grafting. Int J Plant Res 131:49–58.

Naser L, Kouroshi V, Bahman K, Reza A (2010) Soluble sugars and proline accumulation play a role as effective indices for drought tolerance screening in Persian walnut (*Juglans regia* L.) during germination. Fruits 65:97–112.

Nguyen QN, Polle A, Pena R (2017) Intraspecific variations in drought response and fitness traits of beech (*Fagus sylvatica* L.) seedlings from three provenances differing in annual precipitation. Trees 31:1215–1225.

Pereira-Lorenzo S, Fernandez-Lopez J (1997) Propagation of chestnut cultivars by grafting: Methods, rootstocks and plant quality. Int J Hortic Sci 72:731–739.

Pigliucci M, Villani F, Benedettelli S (1990) Geographic and climactic factors associated with the spatial structure of gene frequencies in *Castanea sativa* Mill. from Turkey. J Genet 69:141–149.

Santana-Vieira D, Freschi L, Almeida L et al (2016) Survival strategies of citrus rootstocks subjected to

610 drought. Sci Rep 6:38775.
611 Serdar U, Demirsoy H, Macit I, Ertürk U (2010) Graft compatibility in some Turkish chestnut genotypes
612 (*C. sativa* Mill.). Acta Hortic 866:285–290.
613 Serdar U, Soyla A (2005) Investigation of anatomical structure of graft union for T and inverted T buddings
614 and whip grafting in chestnut. Acta Hortic 693:165-170.
615 Serra I, Strever A, Myburgh PA, Deloire A (2014) Review: the interaction between rootstocks and cultivars
616 (*Vitis vinifera* L.) to enhance drought tolerance in grapevine. Aust J Grape Wine R 20:1–14.
617 Sharma A, Shahzad B, Kumar V, Kohli SK, Sidhu GPS, Bali AS, Handa N, Kapoor D, Bhardwaj R, Zheng
618 B (2019) Phytohormones Regulate Accumulation of Osmolytes Under Abiotic
619 Stress. Biomolecules 9:285.
620 Shenxie X, Xiaopeng L, Qun N, Xiaoli Z (2015). The effect of water stress on abscisic acid, jasmonic acid
621 and physiological characteristics of citrus. Acta Hortic 1065:1385–1391.
622 Silva VA, Prado FM, Antunes WC, Paiva RMC, Ferrão MAG, Andrade AC, Mascio PD, Loureiro ME,
623 DaMatta FM, Almeida AM (2018) Reciprocal grafting between clones with contrasting drought
624 tolerance suggests a key role of abscisic acid in coffee acclimation to drought stress. Plant Growth
625 Regul 85:221–229.
626 Soar CJ, Dry PR, Loveys B (2006) Scion photosynthesis and leaf gas exchange in *Vitis vinifera* L. cv.
627 Shiraz: Mediation of rootsock effects via xylem sap ABA. Aust J Grape Wine R 12:82–96.
628 Solla A, López-Almansa JC, Martín JA, Gil L (2014). Genetic variation and heritability estimates of *Ulmus*
629 *minor* and *Ulmus pumila* hybrids for budburst, growth and tolerance to *Ophiostoma novo-ulmi*. iForest
630 8:422–430.
631 Soylu A, Serdar U (2000) Rootstock selection on chestnut (*Castanea sativa* Mill.) in the middle of Black
632 sea region in Turkey. Acta Hortic 538:483–487.
633 Taïbi K, Del Campo AD, Vilagrosa A, et al (2017) Drought Tolerance in *Pinus halepensis* Seed Sources
634 As Identified by Distinctive Physiological and Molecular Markers. Front Plant Sci 8:1202.
635 Torii T, Kasiwazaki M, Okamoto T, Kitani O (1992) Evaluation of graft-take using a thermal camera. Acta
636 Hortic 319:631-634.
637 Tworkoski T, Fazio G, Glenn DM (2016) Apple rootstock resistance to drought. Sci Hortic 204:70–78.
638 Tworkoski T, Fazio G (2016) Hormone and growth interactions of scions and size-controlling rootstocks
639 of young apple trees. Plant Growth Regul 78:105–119.

640 Tworkoski T, Miller S (2007) Endogenous hormone concentrations and bud-break response to exogenous
641 benzyl adenine in shoots of apple trees with two growth habits grown on three rootstocks. J Hortic Sci
642 Biotech 82:960–966.
643 Van Rensburg L, Kruger GHJ, Kruger H (1993) Proline Accumulation as Drought-tolerance Selection
644 Criterion: its Relationship to Membrane Integrity and Chloroplast Ultrastructure in *Nicotiana tabacum*
645 L. J Plant Physiol 141:188–194.
646 Van Zandt PA, Mopper S (2004). The effects of maternal salinity and seed environment on germination
647 and growth in *Iris hexagona*. Evol Ecol Res 6:813–832.
648 Wasternack C, Feussner I (2018) The Oxylipin Pathways: Biochemistry and Function Annu Rev Plant
649 Biol 69:363–386.
650 Warmund MR, Cumbie BG, Coggeshall MV (2012) Stem Anatomy and Grafting Success of Chinese
651 Chestnut Scions on 'AU-Cropper' and 'Qing' Seedling Rootstocks. HortScience 47:893–895.
652 Warschefsky EJ, Klein LL, Frank MH, Chitwood DH, Londo JP, von Wettberg EJB, Miller AJ (2016)
653 Rootstocks: Diversity, Domestication, and Impacts on Shoot Phenotypes. Trends Plant Sci 21:418-
654 437.
655 Zhang L, Marguerit E, Rossdeutsch L, Ollat N, Gambetta GA (2016) The influence of grapevine rootstocks
656 on scion growth and drought resistance Theor Exp Plant Physiol 28:143–157.
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Tables and figures

Table 1. Results of the mixed models used to analyze the main effects of the 'scion origin', the 'rootstock origin' and their interaction on the indicated variables in *Castanea sativa* grafted trees. The 'tree height' was used as a covariate for those variables measured under drought stress (see Fig. SM2).

		Budbreak phenology		**Secondary growth**		***g_s***		***A***		***Fv/Fm***		**Leaf RWC**		**Leaf wilting**		**Tree mortality**	
Fixed factors	**df**	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***
Scion origin (S)	1	0.91	0.337	3.19	0.076	0.78	0.374	1.42	0.23	2.82	0.092	3.67	0.055	1.96	0.16	4.69	**<0.05**
Rootstock origin (R)	1	10.34	**<0.01**	0.02	0.883	0.10	0.741	0.86	0.350	8.48	**<0.01**	4.58	**<0.05**	22.51	**<0.001**	7.40	**<0.01**
S x R	1	4.32	**<0.05**	0.58	0.441	6.71	**<0.01**	1.34	0.240	1.00	0.31	2.26	0.13	1.35	0.24	0.66	0.415
Covariate																	
Tree height	1	-	-	-	-	28.09	**<0.001**	15.84	**<0.001**	4.44	**<0.05**	30.96	**<0.001**	15.45	**<0.001**	30.77	**<0.001**

Degrees of freedom (df) and χ^2 statistics for the fixed factors are shown. Significant *P*-values are indicated in bold. 'block' and 'rootstock family' were used as random factors in the models. g_s: stomatal conductance; *A*: net photosynthesis.

Table 2. Results of the mixed models used to analyze the main effects of 'grafting', the 'rootstock origin' and their interaction on the indicated variables in *Castanea sativa* trees. The 'tree height' was used as a covariate for those variables measured under drought stress (see Fig. SM2).

		Budbreak phenology		**Secondary growth**		***A***		***g_s***		***Fv/Fm***		**Leaf RWC**		**Leaf wilting**		**Tree mortality**	
Fixed factors	**df**	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***
Grafting (G)	1	22.04	**<0.001**	7.01	**<0.01**	0.00	0.993	0.97	0.322	0.00	0.961	0.56	0.450	2.96	0.085	0.068	0.803
Rootstock origin (R)	1	3.95	**<0.05**	0.06	0.791	6.31	**<0.05**	4.24	**<0.05**	1.56	0.210	2.15	0.141	8.24	**<0.01**	11.27	**<0.001**
G x R	1	4.63	**<0.05**	0.58	0.440	0.01	0.920	0.00	0.99	10.10	**<0.01**	13.10	**<0.001**	3.45	0.063	0.342	0.553
Covariate																	
Tree height	1	-	-	-	-	25.89	**<0.001**	38.72	**<0.001**	6.05	**<0.05**	21.92	**<0.001**	13.86	**<0.001**	18.36	**<0.001**

Degrees of freedom (df) and χ^2 statistics for the fixed factors are shown. Significant *P*-values are indicated in bold. 'block' and 'rootstock family' were used as random factors in the models. g_s: stomatal conductance; *A*: net photosynthesis.

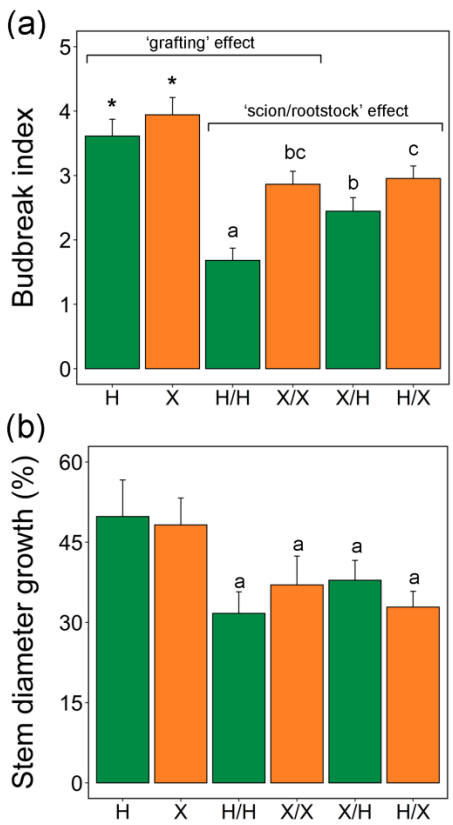

Figure 1. Mean values of (a) budbreak index and (b) stem secondary growth in non-grafted controls (H and X), intra-familiar grafts (H/H and X/X) and reciprocal grafts (X/H and H/X) established using *Castanea sativa* families from humid and xeric areas. Error bars indicate one standard error of the mean. '*' indicates differences in means between non-grafted controls and their respective intra-familiar grafts ('grafting' effect) while different letters indicate differences in means among grafted trees ('scion/rootstock' effect) (P<0.05; Tukey's HSD).

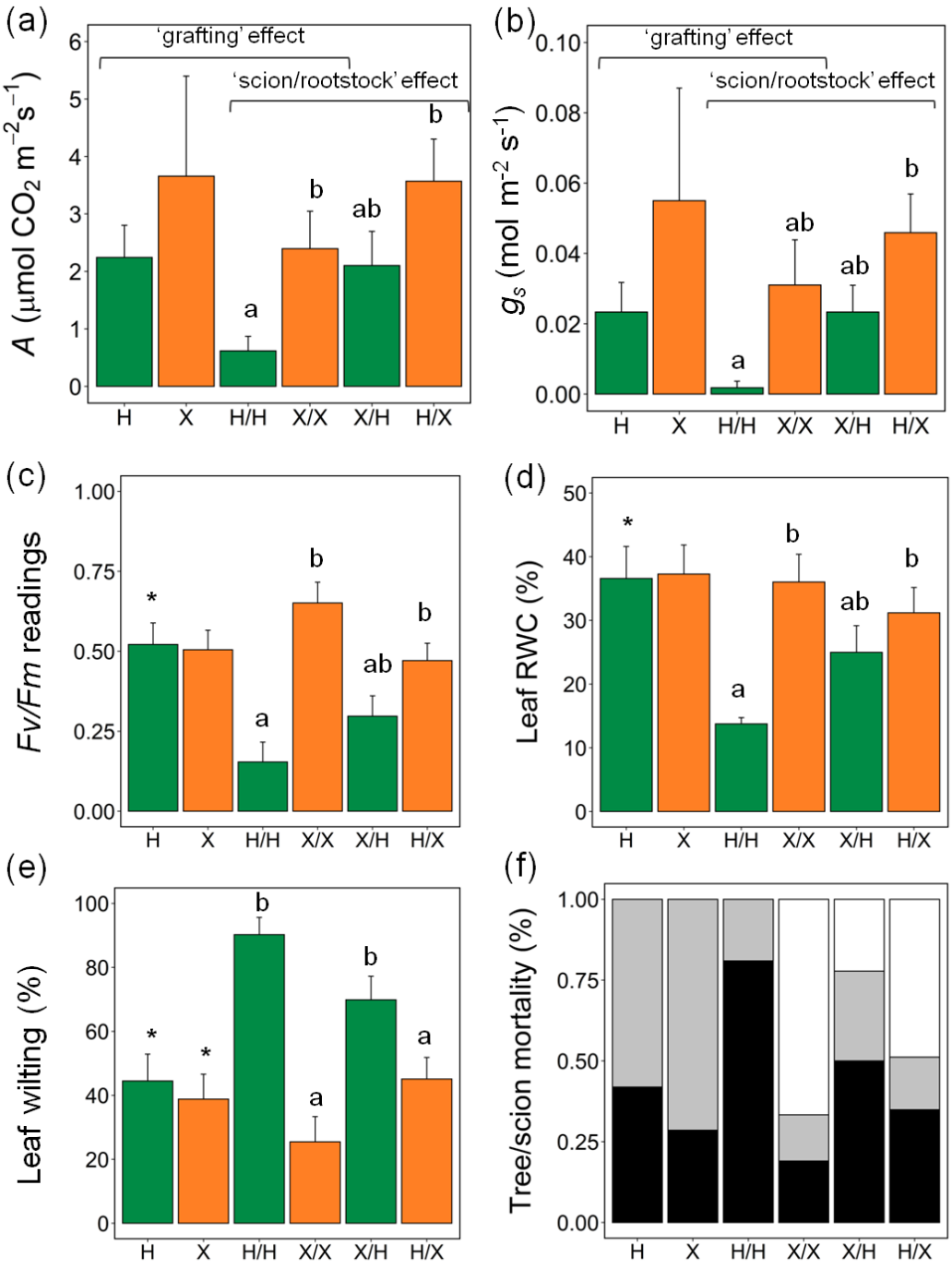

Figure 2. Mean values of (a) leaf net photosynthesis (*A*), (b) stomatal conductance (g_s), (c) *Fv/Fm* readings, (d) leaf RWC, (e) leaf wilting and (f) tree/scion mortality in non-grafted controls (H and X), intra-familiar grafts (H/H and X/X) and reciprocal grafts (X/H and H/X) established using *Castanea sativa* families from humid and xeric areas during drought. In (f), black, grey and white areas within bars of grafted trees represent dead trees, resprouting trees and trees with the scion alive after drought, while only dead (black) and alive (grey) categories are represented for non-grafted controls. '*' indicates differences in means between non-grafted controls and their respective intra-familiar grafts ('grafting' effect) while different letters indicate differences in means among grafted trees ('scion/rootstock' effect) (P<0.05; Tukey's HSD).

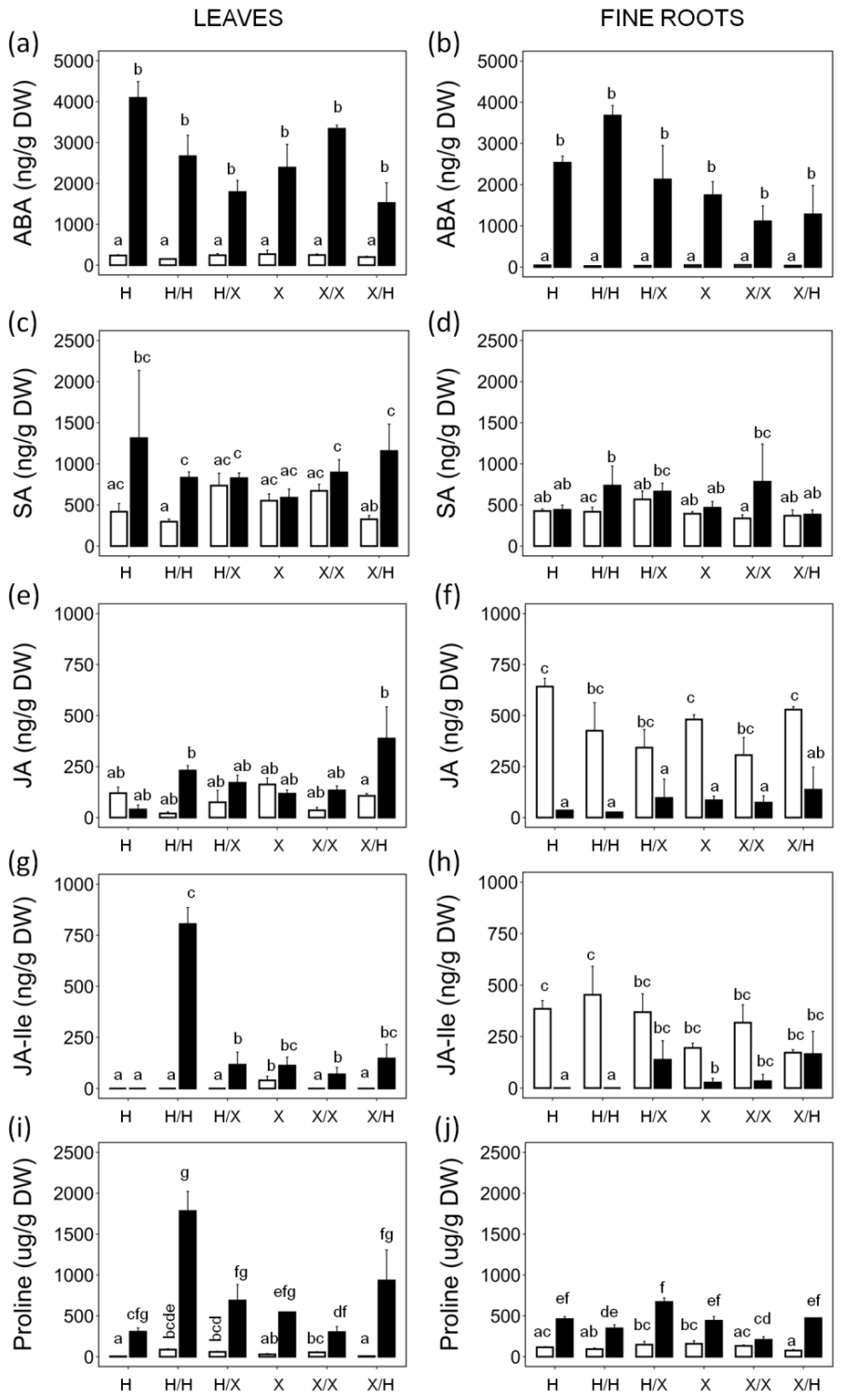

Figure 3. Content of abscisic acid (ABA) (a, b), salicylic acid (SA) (c, d), jasmonic acid (JA) (e, f), jasmonic acid-isoleucine (JA-Ile) (g, h) and proline (i, j) before (white bars) and during (black bars) drought in leaves and fine roots of non-grafted controls (H and X), intra- (H/H and X/X) and inter-familiar (H/X and X/H) grafts of *Castanea sativa* material from humid (H) and xeric (X) origin. Error bars indicate one standard error of the mean (n=3) and different letters indicate significant differences between means (P < 0.05; Tukey's HSD).

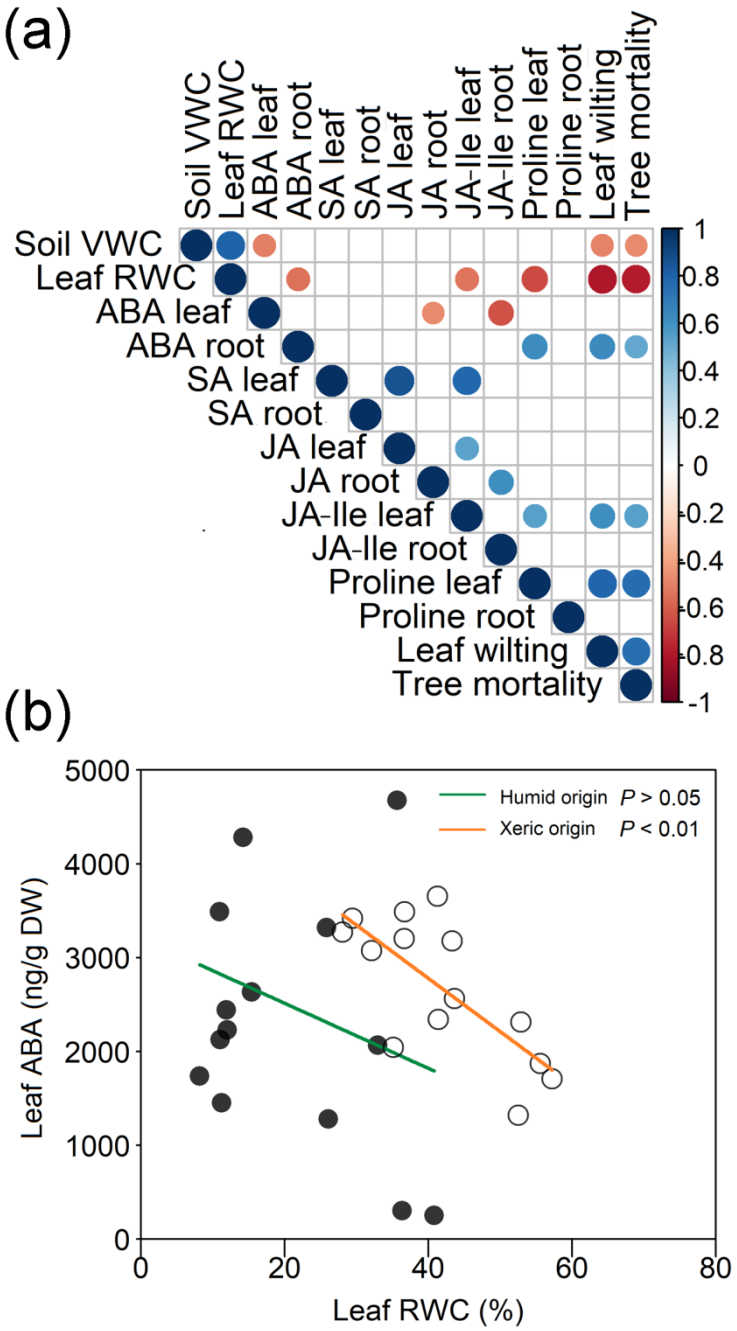

Figure 4. Matrix of significant ($P < 0.05$) Pearson correlation coefficients (a) among the water content in soil and leaves (soil VWC and leaf RWC), the contents of hormones and proline in leaves and roots, and external symptoms induced by drought (leaf wilting-and tree mortality) obtained during drought stress. The relationship between leaf ABA content and leaf RWC during drought in the X (open circles; fit in orange) and H families (closed circles; fit in green) is shown in (b). Significance of linear fits is shown (*P*).

Supplementary materials

Table SM1. Scion/rootstock combinations used in the study resulting from grafting scions of *Castanea sativa* families onto rootstocks of the same family as the scion (intra-familiar grafts, codes H_i/ H_i and X_i/ X_i) and onto rootstock families with contrasted origin (inter-familiar grafts, codes H_i/ X_i and X_i/ H_i).

		Scion family			
		H_1	**H_2**	**X_1**	**X_2**
Rootstock family	**H_1**	H_1/ H_1* (H/H)	–	X_1/ H_1* (X/H)	X_2/ H_1 (X/H)
	H_2	–	H_2/ H_2 (H/H)	X_1/ H_2 (X/H)	X_2/ H_2 (X/H)
	X_1	H_1/ X_1* (H/X)	H_2/ X_1 (H/X)	X_1/ X_1* (X/X)	–
	X_2	H_1/ X_2 (H/X)	H_2/ X_2 (H/X)	–	X_2/ X_2 (X/X)

H_1, H_2: *C. sativa* families from humid origin. X_1, X_2: *C. sativa* families from xeric origin. Scion/rootstock combinations with '–' were not used and those combinations selected for hormone and proline analysis are denoted with '*'. The codes in parhenthesis indicate the resulting scion/rootstock combinations according to the humid or xeric origin of the scion and rootstock used.

Figure SM1. (a) *Castanea sativa* trees one year after grafting by the 'green grafting' technique (note the V-shaped graft union in the detail) and (b) graft union at the time when the experiment was performed, not totally fused.