UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Castanea sativa Mill. ante *Phytophthora cinnamomi* Rands, estrés hídrico y otros factores del cambio global

Álvaro Camisón Caballero

Programa de Doctorado en Biología Molecular y Celular, Biomedicina y Biotecnología

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2020

La conformidad de los directores de la tesis consta en el original en papel de esta Tesis Doctoral

D. Alejandro Solla Hach, Catedrático de Universidad del Departamento de Ingeniería del Medio Agronómico y Forestal de la Universidad de Extremadura y Dª Mª Ángela Martín Cuevas, Profesora Titular de Universidad del Departamento de Genética de la Universidad de Córdoba, informan:

Que la presente Tesis Doctoral titulada '***Castanea sativa* Mill. ante *Phytophthora cinnamomi* Rands, estrés hídrico y otros factores del cambio global**' presentada por Álvaro Camisón Caballero para la obtención del título de Doctor ha sido realizada bajo nuestra supervisión en el Departamento de Ingeniería del Medio Agronómico y Forestal de la Universidad de Extremadura, y entendiendo que se halla finalizada y cumple los requisitos necesarios, autorizan su presentación para ser juzgada por el correspondiente tribunal.

Y para que conste y surta los efectos oportunos, firman la presente en Plasencia, a 24 de Julio de 2020.

La conformidad de los directores de la tesis consta en el original en papel de esta Tesis Doctoral

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Landscape integration between natural forests of *Quercus* spp. (background uphill) and grafted orchards of *Castanea sativa* for nut production (foreground) in Las Villuercas (region of Extremadura, Spain). Photo by F. Javier Dorado.

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RESUMEN

Factores relacionados con el cambio global incluyendo los patógenos forestales invasores y el cambio climático amenazan la productividad y persistencia de los ecosistemas forestales y los cultivos arbóreos en todo el mundo. Por tanto, desentrañar las complejas respuestas fisiológicas de las especies forestales frente a los factores del cambio global es fundamental para comprender y predecir mejor su dinámica futura en un mundo cambiante. *Castanea sativa* Mill. (castaño europeo) es una especie forestal multipropósito y la única especie de su género nativa de Europa, donde es importante por sus valores ecológicos y se cultiva para la producción de madera y castañas en sotos injertados. Sin embargo, la especie sufre el impacto de 'la tinta', una enfermedad consistente en una grave pudrición radicular causada por el oomiceto invasor *Phytophthora cinnamomi* Rands. (*Pc*), y está amenazada por la ocurrencia cada vez más frecuente de eventos climáticos extremos como la sequía y el anegamiento inducidos por el cambio climático actual.

Los avances científicos recientes han permitido mejorar la comprensión de las respuestas del castaño a *Pc* y al estrés por sequía al nivel genético, molecular y fisiológico. Sin embargo, las bases de la resistencia a *Pc* y la tolerancia a la sequía en la especie están aún lejos de ser bien entendidas. Además, se sabe mucho menos acerca de las respuestas de *C. sativa* al estrés por anegamiento. A nivel bioquímico, lagunas importantes de conocimiento en *C. sativa* incluyen el efecto de los fenómenos climáticos extremos sobre los carbohidratos no estructurales (NSC), y también el papel que las hormonas vegetales desempeñan en la modulación de la resistencia a *Pc* y la tolerancia a la sequía. Los NSC están involucrados en los procesos de mortalidad inducida por sequía en árboles, mientras que las hormonas son compuestos de señalización del estrés que ayudan a las plantas a adaptarse a condiciones adversas. Los efectos maternos intergeneracionales se refieren a los cambios en el fenotipo y el fitness de la descendencia que se deben al entorno parental, pero se desconoce si el entorno parental puede afectar a la susceptibilidad de *C. sativa* frente a la sequía y *Pc*. El injertado es también responsable de cambios en el fenotipo de los árboles incluyendo modificaciones en la

susceptibilidad al estrés biótico y abiótico. Sin embargo, cómo el injertado cambia la resistencia a *Pc* y la tolerancia a la sequía, así como la importancia relativa de la púa y el patrón en el control de estos cambios, son desconocidos en *C. sativa*. Esto limita el potencial del injertado para la gestión adaptativa de los sotos de castaño frente al cambio global. Con un enfoque multidisciplinar, esta tesis tuvo por objeto aportar conocimiento sobre la interacción entre *C. sativa* y factores relacionados con el cambio global mediante la realización de experimentos de invernadero complementados con análisis bioquímicos en el laboratorio.

En el primer capítulo se exploraron y compararon los efectos de la sequía y el anegamiento sobre la fisiología, el crecimiento y el contenido de N y carbohidratos no estructurales de *C. sativa*. Plántulas de dos años de edad fueron sometidas durante dos meses a tratamientos regulares de riego, sequía y anegamiento para identificar diferencias en la dinámica de los azúcares solubles, el almidón y los NSC totales. La sequía y el anegamiento tuvieron un impacto perjudicial similar sobre la conductancia estomática, la fotosíntesis neta y el crecimiento de las plántulas, pero efectos diferentes en la dinámica de los azúcares solubles y el almidón en hojas, tallos y raíces, evidenciando estrategias diferentes de uso del carbono para hacer frente a los desafíos del cambio climático. Bajo sequía, el almidón se agotó rápidamente para producir azúcares solubles y no se observó inanición de carbono, probablemente permitiendo a las plantas revertir los embolismos del xilema inducidos por la sequía. Bajo anegamiento, los NSC se acumularon en los tallos y raíces de las plantas a lo largo del tiempo, probablemente debido a la asignación activa de carbono para la formación de reservas y/o a una inhibición en el uso de los NSC. La dinámica de los NSC en las plantas de *C. sativa* explica por qué la especie se adapta bien a las condiciones de sequía pero no a las de anegamiento.

En el segundo capítulo se investigó la existencia de efectos maternos intergeneracionales en *C. sativa* explorando si la susceptibilidad al estrés por sequía y a *Pc* depende del estado de salud de los árboles madre. Se obtuvieron plántulas a partir de semillas recogidas de castaños sanos y enfermos de tinta, evaluándose el marchitamiento foliar tras la exposición a la sequía y la mortalidad de las plantas tras la inoculación con el patógeno. La

descendencia de los árboles enfermos de tinta tuvo una menor altura de planta y biomasa radicular que la descendencia de los árboles sanos, con el tamaño de las semillas mediando este efecto. El marchitamiento foliar debido al estrés hídrico fue similar en la descendencia de árboles sanos e infectados por *Pc*, pero se observó una mayor tolerancia a *Pc* en las plántulas de pequeño tamaño originadas a partir de árboles enfermos, sugiriendo que la tolerancia a *Pc* puede implicar costes de crecimiento. Los resultados indican que un patógeno invasor puede modificar las características morfológicas de la descendencia y potenciar una respuesta defensiva en la próxima generación de una especie de árbol forestal, generando presiones selectivas conflictivas relacionadas con el tamaño de la planta.

En el tercer capítulo se estudió la relación entre hormonas, metabolitos y varios parámetros de intercambio de gases con la resistencia a *Pc*. Antes y después de la inoculación con *Pc* y bajo condiciones controladas, se tomaron muestras de hojas y raíces finas de clones de 2 años de edad susceptibles y resistentes al patógeno (clon de *C. sativa* Cs14 y clon de *C. sativa* × *C. crenata* 111-1, respectivamente) para su análisis bioquímico. Tras la infección, el clon resistente mostró una respuesta más dinámica en relación a los parámetros de intercambio de gases, hormonas y metabolitos asociada a una sinergia entre las hormonas ABA y JA-Ile en las raíces, mientras que en el clon susceptible se observaron pocos cambios hormonales. Dado que la hormona JA-Ile fue detectable tanto de forma constitutiva como inducida por *Pc* tan sólo en el clon resistente, esta hormona podría ser un biomarcador de resistencia a *Pc* en castaño.

En el cuarto capítulo se estudió la influencia del injertado y la púa sobre el perfil hormonal, la brotación, el crecimiento y la susceptibilidad de *Castanea* spp. a *Pc*. En un experimento de invernadero, se utilizaron controles no injertados de dos años de edad, autoinjertos e injertos recíprocos entre dos clones de *C. crenata* x *C. sativa* resistentes a *Pc* y dos clones de *C. sativa* susceptibles. Se tomaron muestras de hojas y raíces de los clones Cs14 y 111-1 para el análisis de las hormonas relacionadas con la defensa antes y durante la infección con *Pc*. El injerto influyó significativamente en el perfil hormonal, la brotación e incrementó la susceptibilidad a *Pc* de los patrones tanto susceptibles como resistentes. Las alteraciones

hormonales debidas al injertado estuvieron mediadas por un efecto de herida, lo que probablemente influyó en el aumento en la susceptibilidad a *Pc* observado en los árboles injertados. En éstos, el patrón y no la púa controló el perfil hormonal y la resistencia a *Pc*. Por último, la presencia constitutiva de la hormona JA-Ile en las hojas del clon 111-1, la cual es sugerida en el tercer capítulo como un posible biomarcador de resistencia a *Pc*, es debida a un mecanismo derivado de las raíces.

En el quinto capítulo se exploró el potencial de los patrones tolerantes a la sequía para mejorar la tolerancia a la sequía en *C. sativa*. Se utilizaron árboles de regiones húmedas (H) y xéricas (X) de España para establecer injertos intrafamiliares (H/H y X/X) y recíprocos (X/H y H/X). Se estudiaron los efectos de la púa, el patrón y el injertado como estrés por herida sobre la brotación, el crecimiento secundario y la tolerancia a la sequía. Dos semanas después de la suspensión del riego, la tolerancia a la sequía fue evaluada midiendo el intercambio de gases foliar, el marchitamiento foliar y la mortalidad de los árboles junto con el contenido de hormonas (ABA, SA, JA y JA-Ile) y prolina en hojas y raíces. Las púas y los patrones de origen xérico indujeron una brotación más temprana y mejoraron la tolerancia a la sequía de los patrones y las púas de origen húmedo. Al final del período vegetativo, la mortalidad de los árboles y la pérdida de la púa debido a la sequía en los árboles H/X fue menor que en los árboles H/H. El efecto del injertado como estrés por herida no influyó en la tolerancia de los árboles a la sequía, pero sí indujo un retraso en la brotación y tendió a reducir el crecimiento secundario de los árboles. Bajo sequía, las diferencias en el contenido de hormonas y prolina de los árboles reflejaron los diferentes niveles de estrés alcanzados. Los resultados sugieren el uso de patrones de zonas xéricas para mejorar la tolerancia a la sequía en castaño.

ABSTRACT

ABSTRACT

Global change-related factors including invasive forest pathogens and climate change challenge the productivity and persistence of forest ecosystems and tree crops worldwide. Unraveling the complex physiological responses of tree species to global change factors is therefore crucial to better understand and predict their future dynamic in a changing world. *Castanea sativa* Mill. (European sweet chestnut) is a multipurpose tree forest species and the only species within its genus native to Europe, where it is important for its ecological values and is cultivated for the production of timber and edible nuts in grafted orchards. However, the species undergoes the impact of 'ink disease', a serious root rot caused by the invasive oomycete *Phytophthora cinnamomi* Rands. (*Pc*), and is threatened by the more frequent occurrence of extreme climatic events like drought and waterlogging induced by the current climate change.

Recent scientific advances have allowed for an improved understanding of chestnut responses to *Pc* and drought stress at the genetic, molecular and physiological levels, but the basis of resistance to *Pc* and tolerance to drought in the species are not well understood yet. In addition, much less is known about the *C. sativa* responses to waterlogging stress. At the biochemical level, important knowledge gaps in *C. sativa* include the effect of extreme climate events on the tree non-structural carbohydrates (NSC) but also the role that plant hormones play in modulating resistance to *Pc* and tolerance to drought. NSC are involved in drought-induced tree mortality while hormones are stress signalling compounds that help plants adapt to stressful conditions. Intergenerational maternal effects refer to changes in the phenotype and fitness of the offspring which are due to the parental environment and it is unknown whether the parental environment can affect susceptibility of *C. sativa* trees to drought stress and *Pc*. Grafting is also responsible for changes in the phenotype of trees including modifications in the susceptibility to biotic and abiotic stress. However, changes in the resistance to *Pc* and tolerance to drought by grafting and the relative importance of the scion and the rootstock in controlling these changes are unknown in *C. sativa*. This limits the potential of grafting for the adaptive management of chestnut orchards under global change. With a multidisciplinary approach, this thesis aimed to

fill in the above knowledge gaps by performing greenhouse experiments complemented with biochemical analysis at the laboratory.

In the first chapter, the effects of drought and waterlogging on physiology, growth and N and non-structural carbohydrates content of *C. sativa* were explored and compared. Two-year-old seedlings were subjected for 2 months to regular watering, drought and waterlogging treatments to identify differences in the dynamics of soluble sugars, starch and total NSC. Drought and waterlogging had a similar detrimental impact on the stomatal conductance, net photosynthesis and growth of *C. sativa* seedlings but different effects on the dynamic of soluble sugars and starch in leaves, stems and roots, evidencing different strategies of carbon use to cope with the challenges of climate change. Under drought, starch was rapidly depleted to yield soluble sugars with no evidence of carbon starvation, probably allowing plants to reverse drought-induced xylem embolisms. Under waterlogging, NSC accumulated over time in plant stems and roots, probably as consequence of active allocation of carbon to reserve formation and/or inhibited utilisation of NSC. NSC dynamics in *C. sativa* plants provided evidence of why this species adapts well to dry but not to waterlogging conditions.

In the second chapter, the existence of intergenerational maternal effects in *C. sativa* was explored by investigating if the susceptibility to drought stress and *Pc* depends on the health status of mother trees. Plants were grown from seeds collected from healthy and ink-diseased chestnut trees and leaf wilting after drought exposure and plant mortality after pathogen inoculation were assessed. Offspring of ink-diseased trees had poorer performance in plant height and root biomass than offspring of healthy trees, with allocation of biomass to seeds mediating this effect. Leaf wilting due to water stress was similar in offspring of healthy and *Pc*-infected trees while increased tolerance to *Pc* was observed in small-sized seedlings, suggesting that tolerance to *Pc* may involve growth costs. The results suggest that an invasive pathogen can regulate the performance and prime a defence response of a forest tree species in the subsequent generation, and generate conflicting selection pressures related to plant size.

Castanea sativa Mill. facing global change factors. Abstract

In the third chapter, the role of hormones, metabolites and gas exchange parameters determining tree resistance to *Pc* was studied. Under controlled conditions, before and after inoculation with *Pc*, leaves and fine roots from susceptible *C. sativa* and resistant *C. sativa* × *C. crenata* 2-year-old clones (Cs14 and 111-1, respectively) were sampled for biochemical analysis. Upon infection, a more dynamic response of gas exchange-related parameters, hormones and metabolites linked to a synergistic crosstalk between the hormones ABA and JA-Ile in roots was observed in the resistant clone while few hormonal changes in the susceptible clone were found. Because constitutive and *Pc*-induced levels of JA-Ile were only detectable in the resistant clone, this hormone could be a potential biomarker for *Pc* resistance.

In the fourth chapter, the influence of the scion and grafting on the hormone profile, vegetative budburst, growth and susceptibility of *Castanea spp.* to *Pc* were studied. In a greenhouse experiment, two-year old non-grafted controls, autografts and reciprocal grafts between two *Pc* resistant *C. crenata* x *C. sativa* clones and two *Pc* susceptible *C. sativa* clones were used. Leaves and roots of clones Cs14 and 111-1 were sampled for analysis of defense-related hormones before and during *Pc* infection. Grafting significantly influenced the hormone profile, vegetative budburst, and increased susceptibility to *Pc* in susceptible and resistant rootstocks. Hormonal alterations by grafting were mediated by a wounding effect, which probably influenced the increased susceptibility of grafted trees to *Pc*. The rootstock and not the scion controlled the hormone profile and resistance to *Pc* in grafted trees. Finally, the constitutive presence of JA-Ile in leaves of the 111-1 clone, which was suggested in the third chapter as a potential biomarker for *Pc* resistance, appears to be triggered by roots.

In the fifth chapter, the potential of drought-tolerant rootstocks to improve drought tolerance in *C. sativa* was explored. Trees from humid (H) and xeric (X) regions 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. Two weeks after water deprivation, drought tolerance was assessed by measuring leaf gas exchange, leaf wilting and tree mortality

together with hormones (ABA, SA, JA and JA-Ile) and proline 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. At the end of the vegetative period, tree mortality and scion loss due to drought in H/X trees was lower than in H/H trees. The grafting (wounding) effect had no influence on the tolerance to drought of trees, although it induced delayed vegetative budbreak and tended to reduce secondary growth of trees. Under drought stress, differences in the hormone and proline contents of trees reflected their different dehydration levels. Results suggest using rootstocks from xeric areas to improve the tolerance to drought of chestnuts.

GENERAL INTRODUCTION

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Invasive forest pathogens and climate change as factors of global change

Global change refers to a broad suite of planetary-scale biophysical and socioeconomic changes affecting the functioning of the Earth system as a whole. It highlights the large change in the relationship between human being and environment occurring over the last few centuries as well as the increasing capacity of humans to influence the functioning of the Earth system. In this sense, there is evidence that planetary-scale changes (e.g. climate change, biodiversity loss and changes in the distribution patterns of species and ecosystems) are occurring rapidly over the past few decades linked to human activities (IPCC 2013).

The increasing emergence of novel forest diseases and pests as a consequence of human activities is one of the best documented processes associated to ongoing global change (Dukes et al. 2009; Stenlid et al. 2011; Santini et al. 2013; Desprez-Loustau et al. 2016; Ghelardini et al. 2017). Emergent forest diseases and pests are generally caused by exotic, invasive microorganisms and insects that are introduced into new areas and establish new associations with naïve tree hosts that lack a co-evolutionary story with. Invasive forest pathogens can be important drivers of forest ecosystem change and succession through their direct impact on the demography of host tree populations (Brunet et al. 2014). Moreover, invasive forest pathogens have profound economic impacts because of reductions in plantations' yield, value loss of forestry products as well as the cost of removing dead trees and control operations to reduce disease/pest expansion, among others (Stenlid et al. 2011). Human global mobility (globalization) and current climate change are main drivers for the introduction, establishment and spread of non-native pathogens and pests into new areas (Stenlid and Oliva 2016; Ghelardini et al. 2017). Changes in climate have the potential to shift the distribution of current forest pathogens and insects that serve as vectors of pathogens (Dukes et al. 2009; Sturrock et al. 2011; Redondo et al. 2015; Stenlid and Oliva 2016).

In Europe, the number of invasive forest pathogens introduced has increased exponentially over the last two centuries (Santini et al. 2013; Ghelardini et al. 2017) and the relative importance of pathogenic records in forests associated to exotic pathogens has largely increased relative to the total number of records (Desprez-Loustau et al. 2016). For instance, invasive oomycetes within the genus *Phytophthora* including *P. cinnamommi*, *P. quercina*, *P. ramorum* and *P. alni* s.l. are involved in the decline of Central European and Mediterranean oaks and also alder trees (*Alnus* spp.) (Jung et al. 2018).

Climate change is another major factor within the context of global change because climate determines the distribution of forest ecosystems and tree species, the patterns of biodiversity and primary productivity, and the viability of crops (Davis and Shaw 2001). On average, mean global temperature is rising coupled to the increasing release of greenhouse gases into the atmosphere, a phenomenon that is referred to as 'global warming'. Also, it is expected a higher frequency and lasting of extreme climatic events including hotter droughts, heat waves and heavy rains (Perkins-Kirkpatrick and Gibson 2017; Giorgi et al. 2019). The Mediterranean Basin is especially susceptible to global warming (Perkins-Kirkpatrick and Gibson 2017; Peñuelas et al. 2017; Lionello and Scarascia 2018). With increasing mean global temperature, in the Mediterranean region it is expected that precipitation will be reduced by around 4 %/ºC whilst temperature may rise 20 % over the global average (Lionello and Scarascia 2018), and the area affected by drought in Europe is expected to increase by 40 % ± 24 % with a 3 ºC increase in mean temperature (the current forecasted temperature increase) (Samaniego et al. 2018).

Widespread tree mortality and forest die-off increasingly occur worldwide as a consequence of anthropogenic global warming, particularly because of the more frequent occurrence of hotter droughts and is expected to continue rising along the XXI century (Allen et al. 2010, 2015; Choat et al. 2018; Anderegg et al. 2019; Taccoen et al. 2019). Drought events alter forest structure and function (Anderegg et al. 2019) and dry or semi-arid ecosystems like the Mediterranean Basin are especially susceptible to drought-induced mortality. In these

already drought-prone areas, small increases in water stress can overcome compensation and tolerance mechanisms at the physiological and ecosystem levels (Carnicer et al. 2011; Peñuelas et al. 2017; Sánchez-Salguero et al. 2017a, 2017b; Anderegg et al. 2019).

Study species

Brief overview of the genus *Castanea* Miller

The genus *Castanea* (chestnut trees) within the *Fagaceae* family distributes throughout temperate regions of the northern hemisphere in Asia, Europe and eastern North America and encompasses three sections with 7-12 species of shrubs and trees (Lang et al. 2007; Pereira-Lorenzo et al. 2016). Eastern Asia (China and Japan) is the center of origin of the genus (Lang et al. 2007; Corredoira et al. 2017) and is home to five *Castanea* species including *C. mollissima* Blume (Chinese chestnut), *C. crenata* Sieb. and Zucc. (Japanese chestnut) and *C. henryi* (Skan) Rehd. & E.H. Wils. (Henry chestnut). *Castanea sativa* Mill. (European sweet chestnut) inhabits Europe while eastern North America is home to *C. dentata* (Marsh.) Borkh. (American chestnut), *C. pumila* (Benth.) Wedd. (Allegheny chinquapin) and *C. ozarkensis* Ashe (Ozark chinquapin), among others (Pereira-Lorenzo et al. 2016). *Castanea* species within the Section Eucastanon including *C. sativa*, *C. dentata*, *C. mollissima* and *C. crenata* are especially relevant for the human being because they are exploited as tree crops for their edible nuts (Corredoira et al. 2017).

Castanea sativa in Europe: habitat and its ecological and socio-economical importance

European sweet chestnut is a majestic forest tree species up to 20-40 m tall and the only species within the genus *Castanea* native to the Mediterranean Basin and Central European regions (Kosňovská et al. 2013; Conedera et al. 2016). It is a mesophilous, thermophilous species with an altitudinal range between 200-1800 masl that requires a vegetation season lasting between

160–180 days a year, a minimum annual rainfall between 600-800 mm and a mean annual temperature between 8 - 15 °C (Kosňovská et al. 2013; Conedera et al. 2016). *Castanea sativa* grows on well-drained, acidic to neutral soils and is highly sensitive to late frosts while being a vigorous re-sprouter after fire-disturbances (Conedera et al. 2016). It has a large, disjunct distribution range covering 2.53 million hectares in Europe and is present in 25 countries ranging from Southern Europe (e.g. Iberian Peninsula, Italy and Balkans) and North Africa (Morocco) to North-Western Europe (England) and Western Asia (Turkey, Armenia, Georgia, Syria) (Conedera et al. 2004a; Conedera and Krebs 2008; Corredoira et al. 2017). *Castanea sativa* commonly shares habitat with other thermophilous broadleaved tree forest species such as *Quercus petraea*, *Q. robur*, *Q. pyrenaica*, *Q. faginea*, *Q. pubescens*, *Alnus glutinosa*, *Prunus avium, Fraxinus* spp. and *Tilia* spp. (Camisón et al. 2015; Conedera et al. 2016; Conedera et al. 2017; Silla et al. 2018). Its current distribution range is due to the range dynamics of the species during the Last Glacial Maximum (around 18,000-22,000 yr B.P.) coupled with the human influence that further favoured the expansion of the species for its edible nuts (Jarman et al. 2019; Krebs et al. 2019). This makes it difficult to establish the naturalness of the sweet chestnut in many areas where it currently thrives (Conedera and Krebs 2008). Cultivation of *C. sativa* started early and extended its range into areas beyond its ecological optimum where the species has nowadays become naturalized (Conedera et al. 2004a, 2004b).

The European sweet chestnut is currently a paradigm of multipurpose tree species for both the socio-economical and ecological values it owns, being considered a good model of integration between natural and human-driven distribution of biodiversity (Martín et al. 2012). Most of the chestnut forested area concentrates in a few countries with a long tradition of chestnut cultivation where the species is currently economically important in mountainous and hilly rural areas for the production of timber and especially nuts. France and Italy account for 79.3 % of the chestnut forest area and Spain, Portugal and Switzerland account for another 9.7 %, the remaining 11.0 % being dispersed in other countries (Conedera and Krebs 2008). 1.75 million ha of the chestnut-growing area (79.0%) is intended for timber and wood production as

coppice system (1.48 million ha) and high forests (0.29 million ha) while the area devoted to nut production in grafted orchards amounts to 0.43 million hectares (19.3%) (Conedera and Krebs 2008). Socio-economical changes occurring since the end of the Second World War and the presence of introduced pests and diseases have negatively impacted *C. sativa* orchards, despite which nut production has continued to be relevant in Europe. In the period 2010-2018, Europe accounted for the 6.4 % (1,230,044 tonnes) of the total nut production globally, whit Asia and the Americas accounting for the remaining 90 % and 3.6 %, respectively (FAOSTAT 2020, faostat.fao.org). Within Europe, the main nut-producer countries from higher to lower importance are Turkey, Italy, Greece, Portugal and Spain (FAOSTAT 2020, faostat.fao.org). Interest in sweet chestnut for nut production has increased along the last decades in Europe motivated by a renewed attention to sustainable agroforestry, leading to the establishment of new plantations and the recovery of old, abandoned orchards in rural areas (Díaz-Varela et al. 2018). *Castanea sativa* stands also offer complementary secondary productions including tannins for the industry, edible mushrooms, honey and pasture for cattle when stands are managed in agroforestry systems (Conedera et al. 2016).

From an ecological point of view, *C. sativa* stands supply important ecosystem services and externalities some of which include regulation (water quality, erosion control, disturbance prevention, gene pool, climate regulation...) and cultural services (aesthetic landscape, traditional knowledge...) (Roces-Diaz et al. 2018; Pérez-Girón et al. 2020). Within externalities, *C. sativa* orchards may constitute biodiversity spots in area-based biodiversity conservation strategies (Diaz-Varela et al. 2018). Consequently, the need to protect *C. sativa* stands is recognized at the European level and they are listed as 'habitat of Community interest' (code 9260) in the Habitat Directive 92/43/EEC. This habitat is described as 'supra-Mediterranean and sub-Mediterranean *Castanea sativa*-dominated forests and old established plantations with semi-natural undergrowth', and includes coppices, orchards and natural and semi-natural *C. sativa* forests.

C. sativa facing global change factors: *Phytophthora cinnamomi* and climate change

Impact of *P. cinnamomi* on *C. sativa*

Castanea sativa is a paradigmatic example of a tree species subjected to the negative effects of multiple diseases and pests caused by exotic organisms with consequences for the persistence of forests, orchards and coppices. In terms of their ecological and economical impact, the main organisms affecting *C. sativa* in Europe are the insect pest *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera, Cynipidae), the fungus *Cryphonectria parasitica* (Murr.) Barr. (Ascomycota, Cryphonectriaceae) and especially the soilborne pathogen *Phytophthora cinnamomi* Rands. (*Pc*) (Oomycota, Peronosporaceae).

Pc is the causal agent of the root rot disease known as 'ink disease' and the most dangerous exotic organism affecting *C. sativa* in Europe. The pathogen was first described in Sumatra by Rands as the causal agent of stripe canker of cinnamon (*Cinnamomum burmannii*) (Rands 1922) and is thought to be native to Taiwan or Papua New Guinea (Sena et al. 2018). In Europe, ink disease was recorded for the first time in Portugal in 1838, although it is thought that the pathogen was already established in Spain since 1726 (Crandall 1950). Asian chestnut species including *C. mollissima* and *C. crenata* are considered fully resistant due to a co-evolutionary history with *Pc* (Corredoira et al. 2017). Ink disease can be also caused by *P. cambivora* (Petri) Buisman, a species with different ecological requirements which is responsible for ink disease in Italy and Greece, while *Pc* prevails in the Iberian Peninsula, France and England (Vannini and Vettraino 2001; Vettraino et al. 2001, 2005; Robin et al. 2006). However, *Pc* is globally much more dangerous to chestnut trees than *P. cambivora* due to its higher virulence and invasiveness. *Pc* is listed in the Global Invasive Species Database (http://www.issg.org) as one of the 100 worst invasive exotic species and has become widespread in North America, Europe, Australia, and South Africa causing severe economic losses in agriculture and forestry (Hardham and Blackman 2018; Sena et al. 2018). Economically important woody species impacted by *Pc* include avocado, peach, walnut and pineapple (Sena et al. 2018) although the pathogen has also a huge impact on natural

ecosystems (Hardham and Blackman 2018; Sena et al. 2018). *Pc* is involved in the decline of holm and cork oaks (*Quercus ilex* and *Q. suber*) across the Mediterranean Basin and has a tremendous ecological impact on the Australian flora (Jung et al. 2018; Sena et al. 2018).

Infection by *Pc* occurs through fine roots by motile asexual zoospores when soil moisture is high. Zoospores are produced in sporangia and are capable of swimming in water because of the presence of a flagellum, localizing suitable infection sites through chemical cues (chemotaxis). *Pc* is hemibiotrophic and after encystment and germination of zoospores, hyphae penetrate in root epidermal and cortical cells and develop haustoria, consistent with a biotrophic lifestyle (Redondo et al. 2015; Ruiz Gómez et al. 2015). The pathogen switches to a necrotrophic lifestyle when hyphae enter phloem cells and elongate, resulting in cell degradation and phloem transport blockage. In *C. sativa*, the main symptoms of ink disease include necrosis of fine and main roots which can spread to the collar and stem, leading to the appearance of characteristic black exudates that give the name to the disease (Vettraino et al. 2005). As a consequence of root destruction, aboveground symptoms appear including microphylly, leaf chlorosis, wilting and dieback of shoots. Mature *C. sativa* trees can be killed more or less suddenly within 1-3 years after initial infection or slowly through progressive decline, depending on environmental factors (Vannini and Vettraino 2001; Vettraino et al. 2005).

Spreading of ink disease into *C. sativa* orchards, coppices and forests has caused devastating epidemics along the 19th and 20th Centuries with dramatic economic and ecological consequences (Vettraino et al. 2005; Robin et al. 2006). For instance, the distribution area of chestnut in Portugal decreased by 27.3 % between 2002 and 2004 owing to *Pc* infections (Martins et al. 2007). Eradication of *Pc* from infected soils is not feasible in most circumstances because of legal and environmental reasons (Jung et al. 2018; Zhebentyayeva et al. 2019) and, unlike *D. kuriphilus* and *C. parasitica*, the identification of biological control agents to protect trees from *Pc* remains so far very limited (Rigling and Prospero 2017; Avtzis et al. 2019; Ferracini et al. 2019; Hardham and Blackman 2018). Tree breeding for resistance to *Pc* has proven the most effective long-term strategy to overcome the negative impacts of ink disease in

C. sativa (Pereira-Lorenzo et al. 2016; López-Villamor et al. 2018). Breeding programs based on the hybridization between *C. sativa* and the Asian chestnuts *C. crenata* and *C. mollissima* has been undertaken in numerous chestnut-growing countries including Portugal, France, and Spain since 1940 and continue nowadays (Urquijo-Landaluce 1957; Costa et al. 2011; Pereira-Lorenzo et al. 2016; Míguez-Soto et al. 2016). As a result, a number of *Pc*-resistant hybrid clones are available in the market and are used for timber production in coppices and as rootstocks of *C. sativa* traditional varieties for nut production (López-Villamor et al. 2018; Pereira-Lorenzo et al. 2016).

Breeding chestnuts to mitigate the impacts of ink disease requires a comprehensive understanding of the genetic, molecular and physiological basis of resistance to *Pc* within the genus *Castanea* (Costa et al. 2011; Zhebentyayeva et al. 2019). Currently, the knowledge about resistance to *Pc* comes from a few studies performed in economically important tree species namely eucalyptus, avocado, oaks and chestnuts (Zhebentyayeva et al. 2019). Within the genus *Castanea* spp., studies based on linkage mapping suggest that resistance to *Pc* in chestnut trees is a complex polygenic trait (Santos et al. 2017b; Zhebentyayeva et al. 2019). Other studies using simple sequence repeat (SSR) markers from expressed sequence tags (ESTs) to characterize the adaptive genetic diversity of chestnuts for resistance to *Pc* further improved our understanding about the genetic basis of resistance (Santos et al. 2015, Alcaide et al. 2020). Nevertheless, more research is needed to link genotype and phenotype that can assist future breeding programs (Santos et al. 2015; López-Villamor et al. 2018). Likewise, numerous knowledge gaps regarding the defence mechanisms to *Pc* still limit our capacity to understand and predict resistance in chestnut. In particular, the influence of the hormonal signalling before and during the interaction between *Castanea* spp. and *Pc* has never been studied. Plant hormones are signalling molecules known to be central regulators of plant defence responses to pathogens (de Torres Zabala et al. 2009; Perez-Clemente et al. 2019). In trees, the importance of sources of phenotypic variability unrelated to genetics for the modulation of the resistance to biotic and abiotic stress is increasingly recognized (Vivas et al. 2020). These sources of variability include ‘maternal effects’, i.e., changes in the phenotype and fitness of the offspring

which are due to the parental environment rather than to modifications in the DNA sequence (Vivas et al. 2020). There are examples illustrating how the maternal environment (e.g. favorable *vs* unfavorable) affects the performance of the offspring upon challenged with pathogens and abiotic stress (Vivas et al. 2020). However, it is unknown if the maternal environment can affect the resistance to *Pc* and other stresses in the offspring of *C. sativa* trees.

Impact of climate change on *C. sativa*

In the Mediterranean range, *C. sativa* populations are subjected to increasing water stress as a result of progressively drier and warmer conditions (Conedera et al. 2010; Carnicer et al. 2011; Alcaide et al. 2019; Perez-Girón et al. 2020). The species is especially sensitive to water deficit during the growing season (spring and summer) which threatens its persistence in Europe (Conedera et al. 2010; Menéndez-Miguélez et al. 2015; Buras et al. 2019; Lionello and Scarascia 2018; Perez-Girón et al. 2020). *Castanea sativa* is also highly sensitive to prolonged flooding and waterlogging conditions which lead to root hypoxia (Glenz et al. 2006), events that are expected to increase their magnitude along the 21th Century across most of the distribution range of the species in Europe (e.g. northern Spain) (Roudier et al. 2016).

Due to habitat suitability loss, *C. sativa* is expected to retreat from Mediterranean areas while increasing its relative abundance in Central Europe under both optimistic (RCP 4.5) and pessimistic (RCP 8.5) climate change scenarios (Buras et al. 2019). Variability in responses to drought is high within the species due to its wide distribution range and populations growing at the dry rear edges are more drought tolerant than those populations adapted to wet and temperate climates (Míguez-Soto and Fernández-López 2015; Míguez-Soto et al. 2019; Alcaide et al. 2019). Nonetheless, even in these drought-adapted populations, increasing climate stress may exceed the adaptive compensating mechanisms of the species (Anderegg et al. 2019), negatively affecting the provision of ecosystem services and the economic viability of coppices and orchards in the Mediterranean region (Conedera et al. 2010; Pérez-Girón et al. 2020).

Understanding the genetic basis and the molecular and physiological mechanisms underlying tolerance to drought (and also waterlogging) in *C. sativa* is desirable to better predict the future evolution of the species, and to develop appropriate mitigation measures (Ciordia et al. 2012). As compared to resistance to *Pc*, less is known about the genetic basis for adaptation to drought stress in the genus *Castanea* (but see Alcaide et al. 2019). In *C. sativa*, differences in traits with adaptive significance to drought tolerance including growth, morphology, phenology, water use efficiency and carbon isotope discrimination have been reported among populations from contrasted climates (Lauteri et al. 1997, 1999, 2004; Ciordia et al. 2012; Míguez-Soto and Fernández-López 2015; Míguez-Soto et al. 2019). Tree non-structural carbohydrates (NSC) including soluble sugars and starch are important for plant adaptation to stress and are involved in drought-induced tree mortality processes through the mechanisms of 'carbon starvation' and 'hydraulic failure' (Dietze et al. 2014). Likewise, plant hormones are key signalling compounds for stress adaptation and regulate tree drought responses. However, both the effects of drought on the NSC and the role of plant hormones for tolerance to drought stress have never been studied in the species. On the other hand, and even though *C. sativa* is listed as intolerant to waterlogging conditions (Glenz et al. 2006), much less is known about the responses its to waterlogging stress. From both a physiological and biochemical point of view, no study so far has addressed the responses of the species to waterlogging stress.

The importance of grafting in *C. sativa* and its interaction with *P. cinnamomi* and drought stress

Grafting is practiced by humans since ancient times and numerous techniques has been developed for agronomical applications (Wang et al. 2017). Grafting of tree crop species is widely used for the propagation of cultivars but also for the modification of agronomical characteristics of interest like phenology, growth, fruit quality or resistance to biotic and abiotic stress. This is because grafting normally induces phenotypic changes in the grafted plants

(Warschefsky et al. 2015; Wang et al. 2017). Sources of phenotypic variation in grafted plants are more complex than in non-grafted plants because they usually combine two different genotypes (the scion and the rootstock) that interact with each other and with the environment, causing scion × rootstock × environment interactions (Albacete et al. 2015; Wang et al. 2017). In addition, the effect of the graft union itself partially mediates the scion × rootstock communication. Since wounding caused by grafting disrupts the plant vasculature, a proper healing and development of the graft union is the primary requisite for a successful graft (Wang et al. 2017). The outcome of all these complex interactions ultimately determines the influence of the rootstock and the scion on the plant's phenotype (Albacete et al. 2015). Nonetheless, there is consensus that the rootstock is the primary source of phenotypic variation in grafted trees (Warschefsky et al. 2015). In recent decades, grafting has also emerged as an important scientific tool to study diverse aspects of the root-to-shoot signalling and systemic processes in plants (Wang et al. 2017).

Since ancient times, the cultivation of *C. sativa* in orchards for nut production relies on grafting (Grauke and Thompson 2010; Pereira-Lorenzo et al. 2016; Warschefsky et al. 2015). Traditionally, *C. sativa* cultivars selected for their high quality nuts are propagated by grafting onto *C. sativa* rootstocks originated from seed. While this practice continues nowadays, the replacement of *C. sativa* rootstocks from seed with *P. cinnamomi*-resistant hybrid rootstocks is increasingly frequent in order to avoid the negative impact of ink disease in orchards (Grauke and Thompson 2010; Pereira-Lorenzo et al. 2016; López-Villamor et al. 2018). By contrast to other widely grafted tree crops such as apple, peach, cherry or citrus trees (Sorce et al. 2002; Allario et al. 2013; Olmstead et al. 2010; Warschefsky et al. 2015; Zhou et al. 2020), in chestnut very little is known about how the phenotype is changed by grafting and the relative contribution of the scion, the rootstock and the graft union to these changes. Within the genus *Castanea*, all the studies performed dealing with grafting has been focused on compatibility issues between cultivars of interest and commercial hybrid rootstocks (Grauke and Thompson 2010; Warschefsky et al. 2015). In consequence, the lack of studies addressing the grafting-

induced changes in the phenotype of chestnuts limits the application of grafting for the adaptive management of *C. sativa* orchards under global change. For instance, *C. sativa* rootstock could be used to improve tree drought tolerance in areas under subjected to increasing drought stress.

GENERAL AIM AND OBJECTIVES

GENERAL AIM AND OBJECTIVES

The overall aim of this thesis was to contribute, through a multidisciplinary approach, to the knowledge of *Castanea sativa* responses to global change-related factors including drought, waterlogging and infection by *Phytophthora cinnamomi* (*Pc*). The specific objectives were:

I- To evaluate the effects of drought and waterlogging on the dynamics of soluble sugars, starch and total non-structural carbohydrates in leaves, stems and roots of *C. sativa*, complemented with measurements of plant physiological parameters, growth and nitrogen content.

II- To test the presence of maternal effects in *C. sativa* by evaluating if the susceptibility to drought stress and *P. cinnamomi* depends on the health status of mother trees, complemented with expression analysis of candidate genes for resistance to *Pc*.

III- To explore the role that constitutive and *Pc*-induced stress-related signals (hormones and metabolites) play for *Pc* resistance, complemented with the evaluation of changes in leaf physiological parameters.

IV- To explore the influence of the scion, the rootstock and grafting on the hormone profile, vegetative budburst, growth and susceptibility of *Castanea spp.* to *Pc*.

V- To assess the influence of the scion, the rootstock and grafting for drought tolerance in *C. sativa*, and the potential of rootstocks from xeric areas to improve tree drought tolerance.

Greenhouse experiments combined with different biochemical analysis at the laboratory were performed. The results are compiled and discussed in five chapters, which are referred to with Roman numerals (I-V).

MATERIALS AND METHODS

MATERIALS AND METHODS

A short explanation about the materials and methods is provided. Detailed information can be found within the five specific chapters, which are referred to with Roman numerals (I-V).

Materials

Plant material

Young chestnut material up to two years old was used in all experiments. *Castanea sativa* seedlings were used to evaluate the effects of drought and waterlogging on the dynamic of non-structural carbohydrates of the species and to evaluate maternal effects in the species (chapters I and II, respectively). The *Pc*-susceptible *C. sativa* clones 'Cs12' and 'Cs14' and the *Pc*-resistant *C. sativa* x *C. crenata* clones 'PO11' and '111-1' were used to evaluate the relation of constitutive and *Pc*-induced hormones and metabolites to *Pc* resistance (chapter III) and to explore the influence of the scion and grafting on the hormone profile, vegetative budburst and susceptibility to *Pc* (chapter IV). In chapter IV, non-grafted controls, autografts (self-grafted plants) and reciprocal grafts between the clones contrasting in susceptibility to the pathogen were used. In chapter V, non-grafted controls and intra- and inter- familiar grafts between two mesic and two xeric Iberian open-pollinated *C. sativa* families were used to evaluate the modulation of drought tolerance by grafting. Plant material was always acquired from a specialized tree nursery except for chapter I.

P. cinnamomi isolates

Two aggressive *P. cinnamomi* A2 strains were indistinctly used in the experiments involving inoculation (chapters II, III and IV): Ps-1683 strain, isolated from a *C. sativa* tree in northern Spain, and UEx1 strain, isolated from a *Quercus ilex* tree in Valverde de Mérida in SW Spain.

Methods

Experimental design

Four experiments using potted chestnut material were carried out under natural conditions of light and temperature at the greenhouse of the Faculty of Forestry of Plasencia (40°02´N, 6°05´W; western Spain). The results exposed in chapters III and IV were obtained from the same experiment. Experiments were based on subjecting the plant material under study to waterlogging (chapter I), drought stress (chapters I, II and V) and inoculations with *P. cinamommi* (chapters II, III and IV). The experimental design varied according to the experiment. In particular, a randomised complete blocks design (chapters I, IV and V), a split-plot random design replicated in blocks (chapter II), and a complete randomized bi-factorial design (chapter III) were used.

Application of waterlogging, drought stress and inoculations with P. cinnamomi.

Waterlogging was applied in chapter I while drought stress was applied in chapters I, II and V. Inoculations with *P. cinnamomi* were performed in chapters II, III and IV. In chapter I, waterlogging was applied by maintaining potted plants in containers filled with tap water to the root collar of plants; drought was applied by daily measurements of the soil volumetric water content using a soil moisture meter (Field Scout™ TDR Soil Moisture Meter, Spectrum Technologies, Inc.) so as the soil volumetric water content was around 5 %. In chapter II, drought stress was applied by watering plants to field capacity every 10 days. In chapter V, a soil drying – rewetting cycle was imposed by watering pots to field capacity and withdrawing watering for two weeks afterwards. *P. cinnamomi* inoculum was always prepared according to Jung et al. (1996) and inoculations were conducted in roots by soil infestation.

Tree assessment

Tree height and stem diameter at the base were evaluated in all experiments and height and diameter relative growth rates were calculated in chapter I, IV and V. Plant dry biomass was

measured in chapters I and II. *Castanea sativa* seeds (nuts) used in chapter II were individually assessed for their weight before sowing, together with radicle length, germination rate, time to emerge after sowing and leaf mass per area (g cm^{-2}) of seedlings. The length of the scion was measured in grafts used in chapters IV and V. Vegetative budbreak was evaluated in April in chestnut plants used in chapters IV and V. Damage induced by drought stress to plants was recorded in chapters I, II and V through several parameters including the percentage of leaf wilting and tree mortality. Development of foliar symptoms and plant mortality after inoculation with *P. cinnamomi* were assessed in chapters II, III and IV.

In vivo leaf physiological measurements

In vivo and immediate leaf physiological measurements were performed depending on the specific research objectives of this thesis, including measurements of the leaf and stem relative water content (RWC), leaf gas exchange parameters and chlorophyll fluorescence parameters (maximum quantum yield of photosystem (PS) II and leaf chlorophyll content). Leaf and/or stem RWC was measured in chapters I and V to assess the plant's water status following:

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

where FW is the fresh weight at the time of sampling, HW is the hydrated weight of tissues after soaking in distilled water for 24 h at 4 °C in darkness, and DW is the dry weight of tissues after complete oven dehydration (48 h, 60 °C). In chapters I, III and V, gas exchange parameters such as stomatal conductance (g_s), net leaf photosynthesis (A) and leaf transpiration (E) were determined early in the morning under natural conditions of photosynthetically active radiation (PAR) with a portable differential infrared gas analyser (IRGA) (Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf chamber. Chlorophyll fluorescence *Fv*/*Fm* readings (the maximum quantum yield of PSII) were obtained in chapters III and V with a Multimode Chlorophyll Fluorometer OS5p device (Opti-Science Inc., USA) in dark-adapted leaves, and leaf chlorophyll content was evaluated through SPAD readings in chapters I, II and III using a

chlorophyll fluorescence meter (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, Inc., USA).

Analysis of total nitrogen and carbon content

Total nitrogen (N) and carbon content in tissues was determined in chapter I with a DUMATHERM® CN Analyser (C-Gerhardt Analytical Systems) following the Dumas method. About 50 mg dry powdered tissue were combusted at 990 °C in the presence of catalysts and concentrations were expressed as a percentage of dry weight. Total N and carbon concentrations were used to calculate the total carbon-to-total N ratio (C/N) for each plant tissue.

Analysis of non-structural carbohydrates

The content of non-structural carbohydrates (NSC) in tissues including soluble sugars (chapters I and III) and starch (chapter I) was determined on a dry weight basis (%) following modified protocols by Haissig and Dickson (1979) and Hansen and Møller (1975). In chapter I, the concentrations of soluble sugars and starch in tissues were used to calculate (i) the total NSC content for each tissue (the sum of soluble sugars and starch concentrations), (ii) the sugar:starch ratios and (iii) the NSC concentrations at the whole-plant level.

Gene expression analysis

Gene expression analysis of candidate genes for *P. cinnamomi* resistance in chestnut (*Cast_Gnk2-like*, *Cast_SAP11* and *Cast_MYB44*) standardized relative to the expression of the housekeeping gene *Actin-7* was performed in chapter II by Real-time PCR. Fine roots were collected and submerged in RNAlater® (R0901 – Sigma- Aldrich®) to prevent RNA degradation and total RNA isolation was done with slight modifications to the protocol developed by Chang et al. (1993). Total RNA integrity and concentration were assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) and cDNA synthesis was performed with an iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR reactions were performed by mixing 1 µL non-diluted template cDNA, 0.25 pM of each primer and 1X SSoFast EvaGreen reaction mixture

(Bio-Rad) and melting curve analysis was carried out for each gene to check gene-specific amplification. Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001).

Analysis of plant hormones

Plant hormones in leaves and roots were determined in chapters III, IV and V. Hormones were extracted from 50 mg dried powdered tissue in one milliliter of 10% methanol aqueous solution containing a pool of hormonal internal standards. Hormones were chromatographically separated in an Acquity Ultra Performance Liquid Chromatography system (UPLC) (Waters, Mildford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex) connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). Quantification was done using external calibration curves with each pure chemical standard.

Analysis of proline, total polyphenolics, condensed tannins and flavonoids

The concentrations of the free amino acid proline (chapters III and V) and phenolic compounds (total phenolics, condensed tannins and flavonoids, chapter III) were determined by colorimetric methods. Proline was analysed by slight modifications to the classic protocol by Bates et al. (1973). Total polyphenolics and condensed tannins were determined by the Folin-Ciocalteu and Porter methods, respectively. Flavonoids were analysed in the same chapter with a slight modification to the AlCl3-NaNO2-NaOH protocol described in Pękal and Pyrzynska (2014). Concentrations were expressed on a dry weight basis.

Statistical analysis

To analyse the sources of variation of variables and significant differences between groups, Linear Mixed Models (LMM) and Generalized Linear Mixed Models (GLMM) were used for normal and non-normal variables, respectively. *Post-hoc* comparison of means was based on Tukey's HSD test with the Bonferroni correction ($P = 0.05$) excepting for chapter II, where Fisher's LSD test was used. Mixed logistic models and Survival Time analysis techniques

including Cox models and Kaplan-Meier survival curves were used to further analyse plant mortality and time to death in those experiments involving inoculation with *P. cinnamomi*. Tree mortality after drought stress was analysed in chapter V with a cumulative link mixed model. Regression and correlation analysis were performed between the measured variables in chapters I, II and V. Principal Component Analysis (PCA) was used in chapters III and IV to reduce the dimensionality of data.

CHAPTER I. Changes in carbohydrates induced by drought and waterlogging in *Castanea sativa*

Castanea sativa Mill. facing global change factors. Chapter I.

Abstract

Drought and flooding events, which cause water and oxygen deprivation in tree roots, are expected to occur more frequently due to climate change. The effects of drought and waterlogging on physiology, growth and N content of *Castanea sativa* Mill. were explored. Through a manipulative experiment, growth-limiting conditions in *C. sativa* seedlings were induced to identify differences in the dynamics of soluble sugars, starch and total non-structural carbohydrates (NSC) in leaves, stems and roots. Two-year-old seedlings were subjected for 2 months to regular watering, drought and waterlogging treatments. Drought and waterlogging induced similar effects on plants, including reduced stomatal conductance, net photosynthesis and growth. However, chlorophyll degradation was detected only in plants subjected to waterlogging. N content and C/N ratios differed between treatments and were highest in leaves of drought stressed plants and roots of control plants, respectively. Under drought, starch was rapidly depleted to yield soluble sugars and afterwards remained constant, and no change in total NSC was observed, probably allowing plants to reverse drought-induced xylem embolisms. Under waterlogging, a net gain of NSC over time in plant stems and roots was observed, suggesting that plants were unable to utilise them. NSC dynamics in *C. sativa* plants provide evidence of why this species adapts well to dry but not to waterlogging conditions.

Keywords: gas exchange, carbon starvation, root hypoxia, soluble sugars, starch mobilization

Introduction

Plant non-structural carbohydrates (NSC) include readily usable soluble sugars (e.g. glucose and sucrose) and stored starch, providing the 'building blocks' and energy for plant growth and survival (Dietze et al. 2014; Kono et al. 2019). Although most NSC are used for respiration (Martínez-Vilalta et al. 2016; Yang et al. 2018), NSC storage is a buffer that allows trees to recover after periods of stress (Vivas et al. 2014; Maguire et al. 2015; Tomasella et al. 2017; Smith et al. 2018; Camisón et al. 2019b). Climate-driven tree mortality is an increasingly frequent natural process worldwide due to higher occurrence of drought and flooding events associated with ongoing climate change (Niu et al. 2014; Peñuelas et al. 2017; Colangelo et al. 2018). While drought is a primary cause of tree mortality (Allen et al. 2010; Peñuelas et al. 2017; Anderegg et al. 2019), the influence of waterlogging on tree health has been less studied (Niu et al. 2014; Tei et al. 2019). Similarly, many studies have addressed the effect of drought on tree NSC (McDowell et al. 2011; Maguire and Kobe 2015; Rodríguez- Calcerrada et al. 2017), but much less is known about the effect of waterlogging on NSC content and distribution in trees (Kreuzwieser and Rennenberg 2014; Yang et al. 2019).

Hydraulic failure and carbon starvation are the main processes occurring during drought-induced mortality of trees (Savi et al. 2016; Dai et al. 2017; Tomasella et al. 2017; Trifilò et al. 2017; Trugman et al. 2018). Hydraulic failure occurs when xylem tension increases above a critical threshold that causes vessel embolism and hinders xylem hydraulic functioning (Hammond et al. 2019). Soluble sugars are involved in refilling xylem vessels after drought-induced embolism (Nardini et al. 2011), and the loss of adequate tissue carbohydrate content required for refilling may limit xylem embolism recovery (Tomasella et al. 2017; Trugman et al. 2018). Carbon starvation occurs when carbon supply by photosynthesis and mobilisation is lower than the metabolic carbon demand (negative balance). It can be observed in trees as whole-plant or organ-specific depletion in NSC concentration (McDowell 2011). Characterising NSC dynamics in trees is, therefore, important to better understand the two processes of drought-induced mortality.

The negative impact of waterlogging in trees is caused by oxygen deprivation in the roots (hypoxia). Hypoxia tolerance largely depends on the species, age and genotype of the plant and the severity, timing and duration of waterlogging (Kozlowski and Pallardy 2002; Kreuzwieser and Rennenberg 2014). Plant responses to waterlogging are mediated by hormones such as abscisic acid and ethylene, and depend on species-specific adaptations that are genetically determined (Phukan et al. 2016). Over time, root hydraulic conductivity can decrease, thus reducing xylem sap flow through the plant and impacting the plant water status (Islam and Macdonald 2004; Repo et al. 2016). Waterlogging can also reduce phloem transport capacity (Peuke et al. 2015). Moreover, waterlogging is often associated with changes in plant metabolism to manage energy production and consumption (Pedersen et al. 2017). Waterlogging tolerant and non-tolerant trees can be differentiated by their efficiency to maintain their carbon metabolism (Parent et al. 2008; Ashraf et al. 2012; Delgado et al. 2018). While flooding-tolerant trees are able to maintain a steady supply of NSCs to roots to sustain fermentative rates and growth over time, sensitive trees fail to maintain sufficient NSC supply to roots (Parelle et al. 2006; Gérard et al. 2009; Ferner et al. 2012; Martínez- Alcántara et al. 2012; Kreuzwieser and Rennenberg 2014). Rapid responses of plants to waterlogging include stomatal closure and associated decreases in net assimilation and transpiration rates (Reeksting et al. 2014; Repo et al. 2016; Silva Branco et al. 2017; Ow et al. 2019). Drought and waterlogging negatively impact the tree nitrogen (N) metabolism, usually by impeding nitrate and ammonium uptake by roots (Kreuzwieser et al. 2002; Horchani and Aschi-Smiti 2010; Martínez-Alcántara et al. 2012; He and Dijkstra 2014; Ow et al. 2019).

Natural populations of *C. sativa* have variable responses to drought stress (Ciordia et al. 2012; Alcaide et al. 2019; Míguez-Soto et al. 2019) but low tolerance to waterlogging (Glenz et al. 2006). Because of the low tolerance of *C. sativa* to waterlogging, the species thrives mainly on well-drained soils (Glenz et al. 2006). The low tolerance of *C. sativa* to waterlogging conditions is not well understood and has never been studied through physiology. Under Atlantic and continental climates, *C. sativa* is listed as a dominant species on mid-slope areas.

However, in areas where water availability is low, *C. sativa* shares its habitat with riparian species such as *Alnus glutinosa* (L.) Gaertn., *Ulmus spp.* and *Fraxinus angustifolia* Vahl., which are tolerant to waterlogging. Offspring of chestnut will probably not survive summer drought unless seedlings are able to develop a deep tap root for vertical water exploration by taking advantage of the riparian water table (Camisón et al. 2019a). Because of this, the European chestnut has sometimes been considered a riparian tree (Canhoto and Graça 1996; Pazianoto et al. 2019). In valleys of southern Portugal, Spain, Italy and Greece, *C. sativa* trees inhabiting upper slopes are exposed to drought in summer, whereas trees on river banks could be exposed to waterlogging conditions during the rest of the year. Lack of precipitation during dry years (e.g. 2012, 2017 in Portugal and Spain) and/or heavy rain (e.g. in June 2016 in France and September 2019 in southern Spain) exacerbate this situation and negatively influence the health status of *C. sativa*, especially if soils are infested by *Phytophthora cinnamomi* Rands (Dal Maso and Montecchio 2015; Martín et al. 2016). In floodplains, where chestnut is now often planted and regularly irrigated, waterlogging also occurs (Fig. 1). Although *C. sativa* responses to drought stress have been extensively characterised using numerous physiological and genetic approaches (Lauteri et al. 2004; Maurel et al. 2004; Ciordia et al. 2012; Alcaide et al. 2019), information about the effects of waterlogging on *C. sativa* physiology and performance is notably lacking. More importantly, the effects of drought and waterlogging on chestnut carbon metabolism are unknown. In this work, we explored the effects of drought and waterlogging on physiology, growth and N content of *C. sativa* seedlings and described the dynamics of soluble sugars, starch and total NSC in leaves, stems and roots. Through a manipulative experiment, we induced growth-limiting conditions in *C. sativa* to identify differences in tree functioning between drought and waterlogging treatments.

Figure 1. Failure of a *Castanea sativa* tree plantation on a floodplain near Torre de Don Miguel (Extremadura, Spain) because of waterlogging. In Extremadura there are ca. 6000 ha of *C. sativa* forests and 3000 ha of chestnut fruit orchards (4500–5000 Tm nuts yr^{-1}). Landowners sometimes try to establish plantations in suitable climate sites without appropriate soil conditions for the species. Arrows indicate dead trees

Materials and methods

Plant material

In January 2015, 60 1-year-old *C. sativa* seedlings germinated from nuts collected at the Hervás chestnut forest (40º15′N, 5º52′W; 805 m asl, western Spain) were transplanted into 2-litre pots containing a mixture of peat and sand (3:1 *v/v*). Plants were amended with Osmocote Pro 3–4 M slow release fertiliser (Osmocote® Pro) at 4 g L^{-1}, and grown for one season under optimal watering conditions (soil volumetric water content around 30%). Among several chestnut populations in Spain, Hervás has been characterized as drought-tolerant (Míguez-Soto et al. 2019). The experiment began on June 1 2016, when seedlings were 2 years old, and was

performed in a greenhouse at the Faculty of Forestry of Plasencia (40º02'N, 6º04'W; 374 m asl, Extremadura region, Spain).

Experimental design and application of watering treatments

Sixty seedlings were arranged in a randomised complete block design comprising five blocks subjected to three watering treatments: regular watering or control (C), severe drought (D) and waterlogging (W) (n = 20). Treatments were applied for 2 months. To assess the effects of treatments on plant physiology, growth and NSC dynamics, five seedlings per treatment (one per block) were destructively sampled 1 day before treatments and on days 20 and 60 of treatment. Effective application of treatments was accomplished by daily soil volumetric water content measurements using a soil moisture meter (Field Scout™ TDR Soil Moisture Meter, Spectrum Technologies, Inc.). Soil volumetric water content was around 30% and 5% for C and D treatments. Previous work by our group indicated that this volumetric water content induces severe drought stress on *C. sativa* seedlings (leaf wilting and plant mortality) (Alcaide et al. 2019). W treatment was applied by keeping the soil permanently under water saturation conditions, maintaining the pots in containers filled with tap water to the root collar of plants. The soil was drained 2 days a week throughout the experiment to prevent plant mortality (Martín et al. 2016).

Plant sampling and physiology measurements

One day before treatments and on days 20 and 60 of treatment, stomatal conductance (g_s, mol m^{-2} s^{-1}) and net photosynthesis (A, μmol CO_2 m^{-2} s^{-1}) were measured in three top-stemmed mature leaves per seedling (n = 5) under natural light conditions (PAR 1014–1350 μmol photons m^{-2} s^{-1}). Measurements were performed between 9:00 and 11:00 h with an IRGA device (Li-6400, Li-Cor Inc., Lincoln, NE, USA). Leaf chlorophyll content was assessed using a

chlorophyll meter (SPAD 502 Plus, Spectrum Technologies, Inc.) in the same leaves where A and g_s were measured. The water status of seedlings was assessed by measuring leaf and stem relative water content (RWC) at noon, following:

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

where FW is the fresh weight at the time of sampling, HW is the hydrated weight of tissues after soaking in distilled water for 24 h at 4 °C in darkness, and DW is the dry weight of tissues after complete oven dehydration (48 h, 60 °C). Stem and leaf RWC were obtained for each plant by averaging the individual RWC of three top-stemmed excised leaves and two 2 cm-long stem segments. Taking into account the diurnal oscillation of NSC content in plant tissues (Tixier et al. 2018), plants were destructively sampled between 14:00 and 15:00 h. Leaves, aboveground woody tissues (stem plus small twigs, hereafter 'stems') and taproots of each seedling were used for dry biomass determination and NSC analysis. Leaves that fell off because of treatments were not included for dry biomass determination and NSC analysis. Within 30 min of harvesting, excised organs were microwaved (80 s, 800 W) to deactivate enzymes and avoid noise in determination of actual NSC content induced by cellular respiration during the drying process (Hoch et al. 2002). Samples were then oven dried (48 h, 60 °C), weighed and ground to fine powder in a ball mill (Mixer Mill MM 400, Retsch, Germany) to pass through a 0.42-mm screen. Total plant dry biomass, comprising dry weight of all tissues used for RWC and NSC determinations, was used as a proxy for plant growth. One day before treatments and on days 20 and 60 of treatment, plant height and stem diameters at ground level were measured.

Determination of total nitrogen and carbon content

Total N in tissues was assessed with a DUMATHERM® CN Analyser (C-Gerhardt Analytical Systems) according to the Dumas method and expressed as a percentage of dry weight. About 50 mg dry powdered tissue was combusted at 990 °C in the presence of catalysts into NO_x, CO_2 and water. NO_x were then reduced to elemental N (N_2), water was separated and CO_2 was adsorpted, and the remaining N_2 was measured in a thermal conductivity detector. Finally, CO_2

was desorpted and total carbon content was measured. This value was used to obtain the total carbon-to-total N ratio (C/N) for each tissue.

Determination of non-structural carbohydrate content

Total NSC content in tissues was expressed as the sum of soluble sugars and starch concentrations on a dry matter basis (%). Soluble sugars and starch were determined following modified protocols by Haissig and Dickson (1979) and Hansen and Møller (1975). Briefly, 25 mg powdered tissue was washed three times with a 5-ml mixture of methanol, and water (12:5:3) and the extracts were combined. After adding 9 ml distilled water, the extracts were left overnight (4 °C) and the pellet was oven dried for further starch quantification. For soluble sugar analysis, 0.5 ml supernatant in the methanol, chloroform and water extract was collected and boiled for 10 min with 5 ml anthrone (Sigma A1631), and absorbance was read at 625 nm in a spectrophotometer (Helios Beta, Spectronic Unicam, England). For starch determination, the pellet was gelled by boiling it in sodium acetate acid buffer (pH 4.5) for 15 min. After cooling down on ice, amyloglucosidase (Sigma 10115) was added and the solution incubated for 24 h at 50 °C. After centrifugation, 0.5 ml of the digested solution was collected and thoroughly mixed with 5 ml peroxidase-glucose-oxidase complex solution. After incubation (30 min, 37 °C), absorbance of samples was measured at 450 nm. All determinations were performed in triplicate, and a D (+) glucose anhydrous standard curve was used to quantify soluble sugars and glucose released from digested starch. All analyses were performed at the Faculty of Forestry (Plasencia) using the same protocol. Soluble sugar:starch ratios were calculated in each plant tissue and used as a proxy for mobilisation of starch to soluble sugars (Piper 2011). NSC concentration at whole-plant level was calculated by a weighted mean, taking into account the total NSC concentration of each tissue and the relative contribution of each tissue's biomass to plant total dry biomass, using the following equation:

$$\text{Whole plant NSC (\%)} = ((NSC_l \times a_l + NSC_s \times b_s + NSC_r \times c_r) / (a_l + b_s + c_r)) \times 100,$$

where NSC_l, NSC_s and NSC_r are the total NSC concentrations in leaves, stems and roots, respectively, and a_l , b_s and c_r are the proportions by which each tissue contributes to plant dry biomass.

Statistical analysis

Statistical analyses were performed in R software environment version 3.4.2 (R Foundation for Statistical Computing, http://www.R-proje ct.org). Data were checked beforehand for normality and homoscedasticity by Shapiro– Wilk and Levene's tests. When data were normally distributed, the effect of 'watering treatments' and 'time of measurement' on leaf gas exchange parameters, total dry biomass, NSC content (soluble sugars, starch and total NSC) and soluble sugar:starch ratios was analysed by linear mixed models. 'Watering treatment' (three levels, C, D and W), 'time of measurement' (three levels, 0, 20 and 60 days) and their interaction were included as fixed effects in the model, while 'block' was used as random factor. When data were not normal, the errors were the same model specification as above. Nitrogen data were analysed in the same way, but considering the 'tissue' (three levels, leaves, stems and taproot), 'watering treatment' and their interaction as fixed effects. Individual plant identity was used as random factor to account for the correlation between the N content in different tissues within a plant. Tukey's HSD test with Bonferroni correction was used for post hoc identification of differences between means across and within treatments for all variables ($P < 0.05$). To determine how watering treatments affected the relations between selected variables, regression analysis within each treatment was performed.

Results

Effect of treatments on plant water relations and plant growth

Throughout the experiment, g_s and A rates in D and W plants were similar and close to zero (Fig. 2a, b). RWC values of C plants did not change over time, in contrast to RWC values of D plants, which significantly decreased, especially in leaves (Fig. 2c, d). At the end of the

experiment, the stems of W plants had lower RWC values than the stems of C plants (Fig. 2c, d). D and W treatments did not allow plants to increase in height (Fig. Supplementary 1a) or weight (Table 1), in contrast with plants subjected to C treatment. After 2 months of C, D and W treatments, stem diameters of plants at ground level increased 3.2, 11.0 and 15.9 %, respectively (Fig. Supplementary 1b). Leaves of control plants were green and turgid during the experiment, but leaves of plants subjected to drought wilted and some turned brown and fell off. In D plants, dieback was observed (Fig. 3). In W plants, chlorosis, necrotic borders and senescence of leaves were observed (Fig. 3). Chlorophyll degradation in the central part of leaves was detected only in plants subjected to W (Fig. 3).

Table 1. Total dry mass (g) of two-year-old *Castanea sativa* seedlings (n = 5) under regular watering (control), drought and waterlogging treatments. Values are means ± SE and different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between sampling points and treatments.

		Treatment		
		Control	Drought	Waterlogging
Days of treatment	0	4.7 ± 0.3 b	6.0 ± 0.4 ac	6.8 ± 0.8 ac
	20	9.3 ± 1.0 c	6.2 ± 0.8 ac	7.3 ± 1.2 ac
	60	8.5 ± 0.9 c	4.0 ± 0.5 a	6.0 ± 0.5 ac

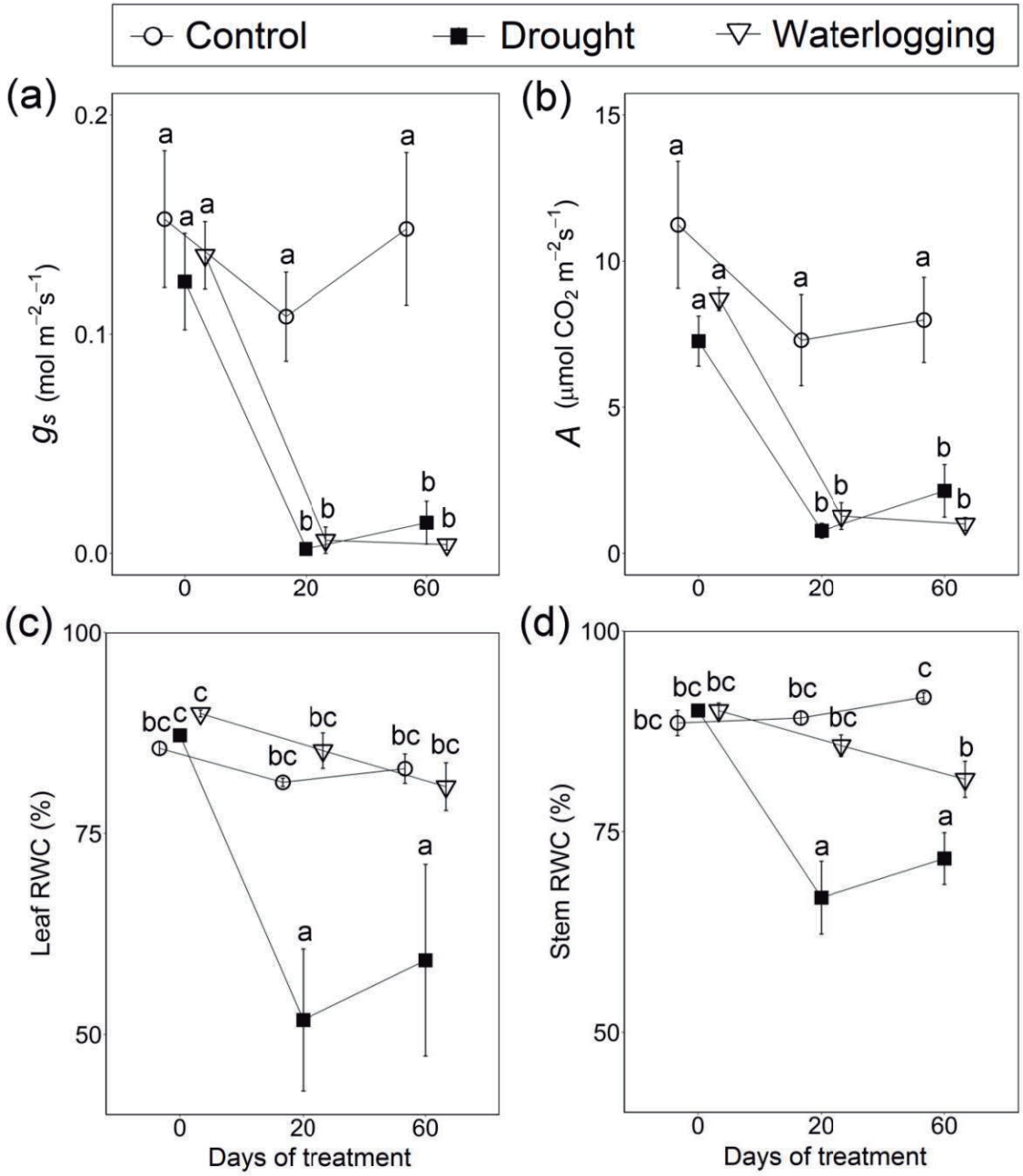


Figure 2. Variation in (a) stomatal conductance (g_s), (b**)** net photosynthesis (A), (c) leaf relative water content (RWC), and (d**)** stem RWC in two-year-old *Castanea sativa* seedlings subjected to regular watering (control), drought and waterlogging treatments. Vertical bars are standard errors of means (n = 5) and different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between sampling points and treatments.

Table 2. N content (% dry weight) and C/N ratios in leaves, stems and roots of two-year-old *Castanea sativa* seedlings (n = 5) after 60 days of regular watering (control), drought and waterlogging treatments. Values are means ± SE and different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between plant tissues and treatments.

		Treatment		
		Control	Drought	Waterlogging
N	Leaf	1.17 ± 0.11 c	2.20 ± 0.54 d	1.07 ± 0.39 bc
	Stem	0.48 ± 0.04 a	0.69 ± 0.26 ab	0.62 ± 0.26 a
	Root	0.43 ± 0.07 a	0.68 ± 0.38 ab	0.69 ± 0.42 ac
C/N	Leaf	37.2 ± 4.6 ab	21.7 ± 5.6 a	43.3 ± 13.4 bc
	Stem	90.2 ± 9.4 d	69.0 ± 19.6 bd	77.6 ± 28.0 cd
	Root	102.9 ± 16.8 d	75.0 ± 28.8 cd	75.0 ± 29.0 cd

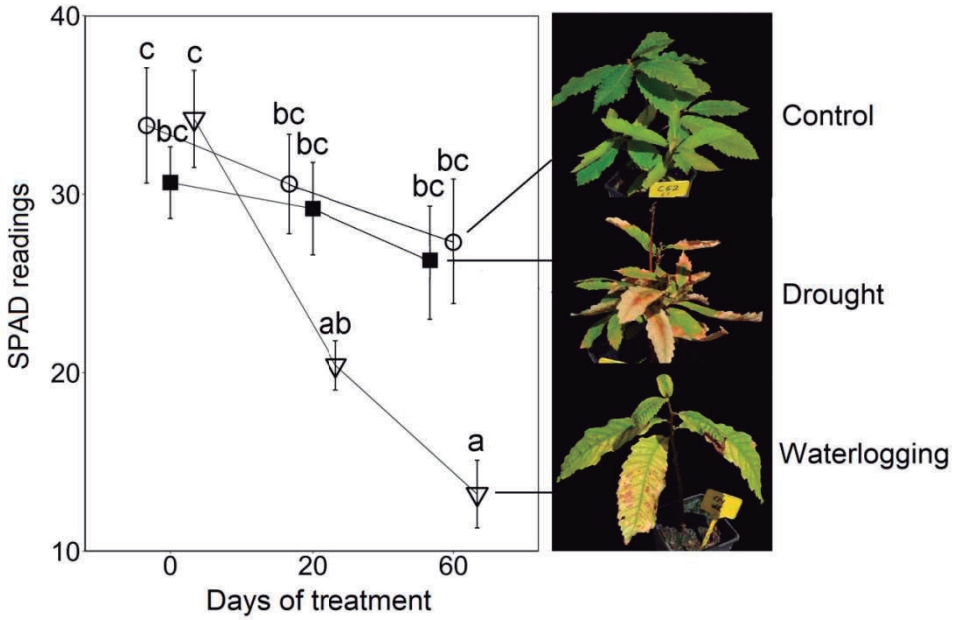


Figure 3. Variation in SPAD readings in two-year-old *Castanea sativa* seedlings subjected to regular watering (control, circles), drought (squares) and waterlogging (triangles) treatments. Representative seedlings from each treatment at day 60 are shown. Vertical bars are standard errors of means (n = 5) and different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between sampling points and treatments.

Total nitrogen content and C/N ratios

On day 60 of treatment, N content in C and D plants was higher in leaves than in stems and roots ($P < 0.05$), whereas in W plants, N content was similar in leaves and roots ($P = 0.22$; Table 2). N content and C/N ratios differed between treatments only in leaves (Table 2). N content and C/N ratios were highest in leaves of D plants and roots of C plants, respectively (Table 2).

Soluble sugars, starch and total NSC

Soluble sugar concentration in C plants barely changed during the experiment (Fig. 4a, d, g). Plants responded to D by a rapid, sharp increase in soluble sugar concentration in all tissues, followed by a slight but non-significant decrease in values at day 60. In W plants, soluble sugar concentration increased progressively over time (Fig. 4a, d, g). Leaf starch content decreased in the first 20 days, particularly in D plants, and was similar between treatments at the end of the experiment (Fig. 4b, e, h). In D plants, starch content in stems and roots was nearly depleted at days 20 and 60, in contrast with W plants, in which starch accumulated (Fig. 4b, e, h). Soluble sugar:starch ratios barely changed in C and W plants except in leaves, where values peaked at day 20 due to starch depletion (Fig. 4c, f, i). Soluble sugar:starch ratios peaked at day 20 and decreased at day 60 in all tissues of D plants (Fig. 4c, f, i). Total NSC content in leaves of C seedlings significantly decreased throughout the experiment, while total NSC in stems and roots were similar at the beginning and the end (Fig. 5a–c). In D seedlings, total NSC content of plants did not change throughout the experiment, except for a transient increase in leaves in the first 20 days. However, total NSC accumulated in the stems and roots of W seedlings over time (Fig. 5a–c). At the whole-plant level, in C and D seedlings' total NSC content did not change during the experiment, in contrast to W seedlings, in which total NSC increased interactions are shown in Table Supplementary 1.

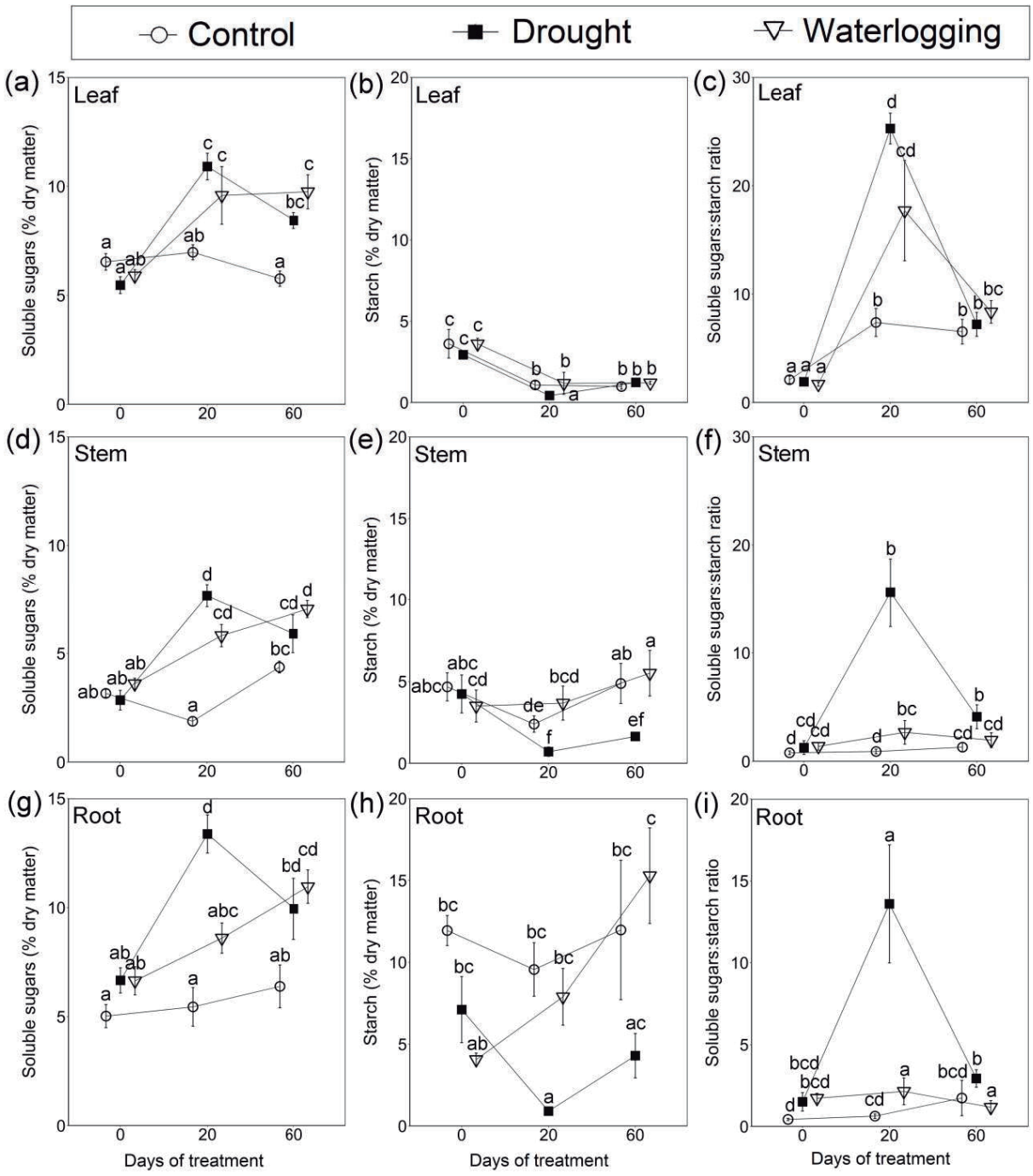


Figure 4. Concentrations of soluble sugars and starch, and soluble sugar:starch ratios in leaves (a,b,c), stems (d,e,f) and roots (g,h,i) of two-year-old *Castanea sativa* seedlings subjected to regular watering (control), drought and waterlogging treatments. Vertical bars are standard errors of means (n = 5) and different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between sampling points and treatments

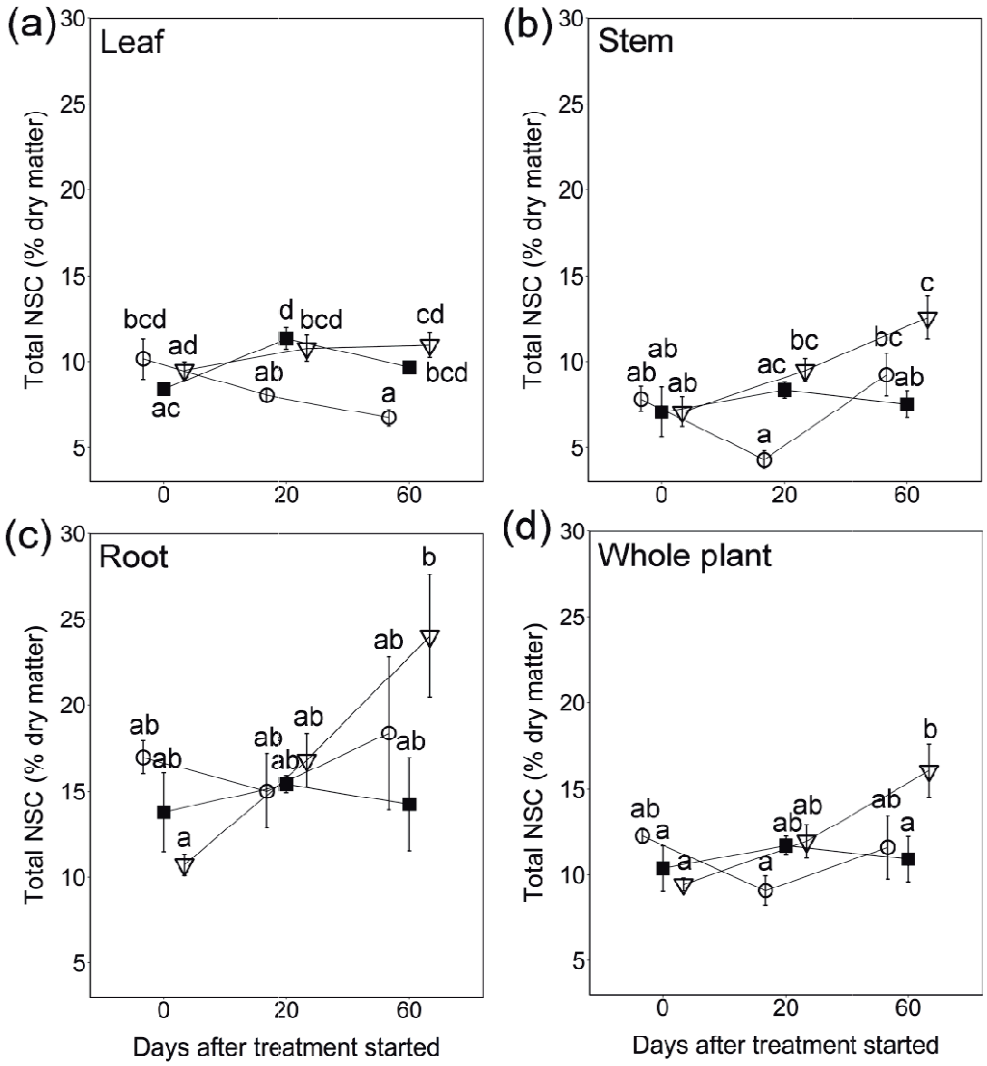


Figure 5. Total NSC concentration in (a) leaves, (b) stems, (c) roots and (d) the whole plant in two-year-old *Castanea sativa* seedlings subjected to regular watering (control, circles), drought (squares) and waterlogging (triangles) treatments. Vertical bars are standard errors of means (n = 5) and different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between sampling points and treatments.

Relations between variables

Stomatal conductance and A were tightly and positively correlated in all treatments (Fig. Supplementary 2, $R^2 > 0.6$, $P < 0.001$). Within-treatment regression analysis showed a positive association between gs and leaf RWC (and also stem RWC, data not shown) only for D plants (Fig. 6a). A positive relation between A and SPAD readings was observed only for C treatment (Fig. 6b). In W plants and marginally in D plants, A and leaf soluble sugar content were

negatively associated (Fig. 6c). There was a significant trend of increased concentration of soluble sugars in stems and roots with declining leaf RWC (and stem RWC, data not shown) during drought, while the increased concentration of root soluble sugars during waterlogging was not associated with a decrease in leaf or stem RWC (Fig. 6d).

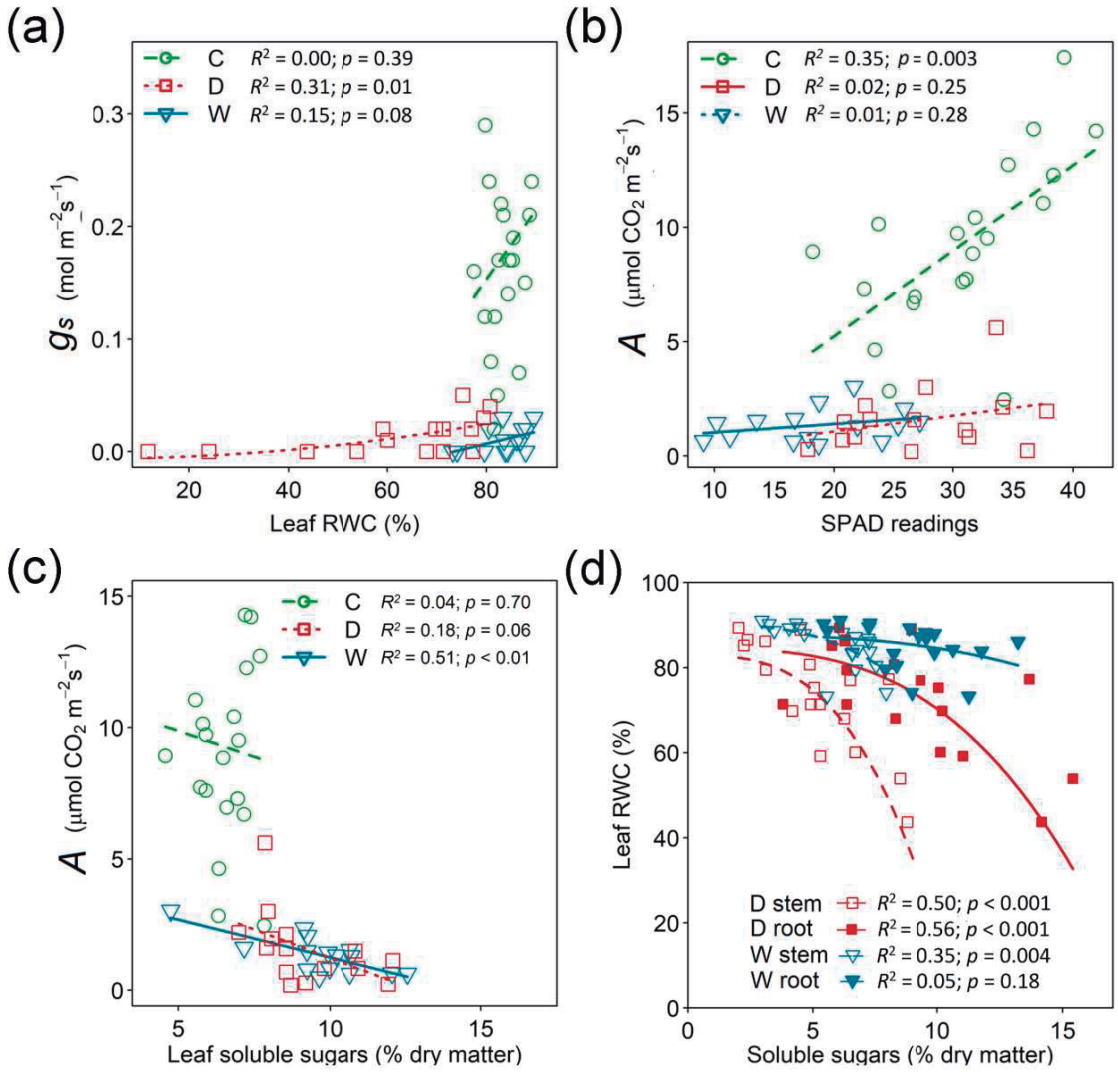


Figure 6. Within-treatment relations between (a) stomatal conductance (g_s) and leaf relative water content (RWC), (b) photosynthesis (A) and SPAD readings, (c) A and leaf soluble sugars and (d) leaf RWC and soluble sugars in stems and roots of two-year-old *Castanea sativa* seedlings subjected to regular watering (control, C, circles), drought (D, squares), and waterlogging (W, triangles) treatments. The explained variance (R^2) and significance (p) of the models are shown.

Discussion

Water relations, gas exchange and N content in C. sativa under drought vs waterlogging

Stomatal closure is a general response of plants on exposure to drought and waterlogging (Farooq et al. 2012; Reeksting et al. 2014; Silva Branco et al. 2017; Trifilò et al. 2017), but mechanisms driving the behaviour of stomata during drought and waterlogging are not fully understood (Maurel et al. 2004; Else et al. 2009; Reeksting et al. 2014; Kreuzwieser and Rennenberg 2014). In *C. sativa* exposed to drought, regulation of g_s depends on both chemical (e.g. the hormone ABA) and hydraulic signals transmitted from roots to leaves (Maurel et al. 2004). In this study, the decreased g_s due to drought was clearly associated with a decline in RWC (Figs. 2c, d and 6a), indicating hydraulic control on stomatal closure. However, the decreased g_s due to waterlogging was not associated with decreased RWC, suggesting involvement of chemical signals mediating stomatal closure in W plants. Stomatal conductance and photosynthetic uptake are interdependent, mutually regulating processes (Lawson et al. 2010), as observed here in *C. sativa* irrespective of treatments (Fig. Supplementary 2). Apart from stress-induced stomatal closure, additional factors such as chlorophyll degradation and soluble sugar accumulation in leaves could have contributed to the reduction in A (Fig. 6b, c). Chlorophyll degradation, frequently associated with photosynthesis depletion in plants (Reis et al. 2009), was particularly high in W plants. Moreover, soluble sugar accumulation occurred rapidly in D and W plants. High leaf soluble sugar concentrations have been associated with down-regulation of A (Azcón-Bieto et al. 1983, Franck et al. 2006).

N content was not affected by treatments except in leaves, the tissue with the highest N concentrations. Leaf N was highest in D seedlings, probably because of N resorption from senescent to non-senescent leaves. N concentration increased as a result of decreased leaf biomass due to drought (Mediavilla and Escudero 2003), i.e. 3.58 *vs* 1.19 g total leaf biomass at the end of the experiment for C and D plants, respectively ($P = 0.030$). In *Quercus rubra* plants subjected to drought, resorption of amino acids at rates higher than 90% were reported (Suseela et al. 2015). N concentration in leaves of W seedlings did not increase and despite some leaf shedding (Fig. 3), total leaf biomass at the end of the experiment in W plants was similar to that

of C plants (2.07 and 1.19 g, respectively; $P = 0.181$). Nitrate and ammonium uptake are often strongly impaired by waterlogging (Kreuzwieser and Rennenberg 2014), but this effect was not mirrored by decreased N concentration in our W plants, even though their leaves were significantly more chlorotic than the leaves of C plants (Fig. 3).

Carbohydrate changes in C. sativa *under drought vs waterlogging*

Starch decreased over time in leaves of C plants, confirming the existence of a seasonal trend (Martínez-Vilalta et al. 2016; Tixier et al. 2018) that was also observed in D and W plants. Moreover, treatment × time interactions were also observed (Table Supplementary 1). For example, in the first 20 days, starch in leaves of D plants decreased more than starch in leaves of C and W plants. At day 20, the highest reduction of starch content in leaves of D plants (Fig. 4b), coincident with the peak values of soluble sugars in leaves of D plants (Fig. 4a), indicates that leaf starch was mobilised in response to water scarcity. Increased leaf soluble sugars content likely contributed to plant osmoregulation. In waterlogged trees, starch was reported to accumulate in leaves, and this phenomenon is thought to be linked to impaired phloem transport (Peuke et al. 2015; Kreuzwieser and Rennenberg 2014; Silva Branco et al. 2017). We did not observe starch accumulation in leaves of W plants, but stable content of starch and soluble sugars over time.

NSC dynamics in stems and roots of *C. sativa* plants under drought were characterised by a balance between soluble sugars and starch without change in total tissue NSC content. As indicated by other authors (Martínez-Vilalta et al. 2016), starch may have behaved as an NSC reservoir, being depleted when drought started, while soluble sugars may have performed immediate functions (cell metabolism, cellular osmotic adjustment), without being depleted. The ability of plants to convert starch into soluble sugars without net change in total NSC is characteristic of drought-tolerant species (Maguire and Kobe 2015), and, therefore, the results confirm evidence of chestnuts from Hervás being drought tolerant as reported elsewhere (Míguez-Soto et al. 2019). The role of soluble sugars in cellular osmotic adjustment during plant dehydration (Irigoyen et al. 1992; Sala et al. 2012) was evidenced here by the negative

relation between leaf RWC and soluble sugars in stems and roots of D plants (Fig. 6d). Although hydraulic conductivity was not assessed here, soluble sugars may have also been involved in xylem vessel refilling after drought-induced embolism (Trifilò et al. 2017; Trugman et al. 2018; Trifilò et al. 2019). Soluble sugars released into embolised vessels from adjacent parenchyma cells generate an osmotic gradient that promotes refilling of air with water (Nardini et al. 2011; Klein et al. 2018). European chestnut is vulnerable to drought-induced embolism due to (i) its anisohydric regulation of water status and (ii) its ring-porous xylem anatomy (Hipps et al. 1997; Martínez-Sancho et al. 2017). Under drought conditions, our *C. sativa* plants contained high concentrations of soluble sugars and high soluble sugars to starch ratios in stems, indicating that this Mediterranean species may effectively reverse xylem drought-induced embolisms.

NSC dynamics in *C. sativa* plants under waterlogging were characterised by an initial increase in soluble sugar content in all tissues, followed by starch storage in stems and roots. As observed here, soluble sugars usually accumulate in the leaves of waterlogged plants (Kreuzwieser and Rennenberg 2014). Although not quantified, it is presumed that function loss of phloem did not occur in W plants, at least not totally, given that leaf soluble sugar content stabilized over time while soluble sugar and starch content (originating from leaves and transported through the phloem) increasingly accumulated in stems and roots. Accumulation of soluble sugar and starch in stems and roots probably occurred because carbon supply by photosynthesis was not synchronized with carbon demand for functions such as growth and respiration (Sala et al. 2012). As a consequence of waterlogging, a passive allocation of carbon to reserve formation could have occurred (if carbon supply had exceeded carbon demand), and/or an active allocation of carbon to reserve formation may have occurred because of stress (Sala et al. 2012; Dietze et al. 2014; Hartmann et al. 2015). Given the low *A* rates observed in W plants (Fig. 2b), an active allocation of carbon to reserve formation probably occurred. In any case, chestnuts were able to supply carbohydrates to stems (probably inducing aerenchyma formation at the root collar according to Fig. Supplementary 1b) and roots, which was unexpected for a waterlogging-intolerant species. A recent study reported that carbon storage

does not necessarily confer tolerance to waterlogging (Delgado et al. 2018). We suggest that the inability of *C. sativa* to properly utilise carbohydrates during flooding could explain its low tolerance to waterlogging. Varieties of *Sorghum bicolor* non-tolerant to flooding showed decreased activity of key enzymes for glycolysis (PFK, FBP aldolase and PK) in leaves and roots during waterlogging, in contrast to tolerant varieties, in which activity of enzymes for glycolysis increased (Singla et al. 2003). Lack of activation of glycolytic enzymes in roots could also explain why carbohydrates were not used (Kreuzwieser et al. 2004) and stored. Measuring carbohydrate use in *C. sativa* may have allowed us to better explain the carbon accumulation in stem and roots induced by waterlogging.

Lack of carbon limitation in C. sativa *under drought and waterlogging*

After 2 months of drought or waterlogging, total NSC content of *C. sativa* plants remained unchanged or increased, respectively. Treatments were not severe enough to induce carbon limitation in *C. sativa* seedlings. This was unexpected, owing to the low photosynthetic rates of plants, plant growth cessation and external symptoms related to plant stress. The non-negative whole-plant carbon balance observed is in agreement with recent studies reporting high NSC content in trees subjected to drought and waterlogging conditions (Piper et al. 2017; Hammond et al. 2019; Yang et al. 2019) and carbon limitation (if any) occurring only under extremely stressful conditions (see Martínez-Vilalta et al. 2016). The lack of carbon limitation in our plants could be explained by the sink-limitation hypothesis (Körner 2003; Palacio et al. 2014; Piper et al. 2017). Growth cessation during D and W treatments could be explained because plant cell expansion and division are more sensitive to environmentally driven limitations than to photosynthesis (Palacio et al. 2014). Decreased NSC in plants can be avoided by down-regulation of energy-consuming processes such as growth during stress (Zhang et al. 2015; Rodríguez-Calcerrada et al. 2017). Decreased total NSC in response to drought can be also buffered by decreases in whole-plant respiratory rates (Rodríguez-Calcerrada et al. 2017; Kono et al. 2019). It should be noted that to prevent plant mortality, D seedlings were irrigated only slightly and the soil of W plants was drained 2 days a week throughout the experiment. This

could have allowed plants to recover and assimilate carbon during short periods of time (not assessed). In chestnut plantations for fruit production, irrigation of trees is highly advisable (Mota et al. 2018). During years in which water availability is scarce, tree irrigation in summer is minimal, as simulated here with D plants. However, during years in which water availability is abundant, farmers irrigate their trees daily in summer, at times exceeding crop water requirements. In soils compacted by livestock or with low permeability, drip irrigation of trees may also generate transient waterlogging conditions.

Differences in plant respiratory rates (and thermoregulation) could account for the different total NSC content observed between D and W seedlings. Plant respiration increases with temperature (Atkin and Tjoelke 2003) and leaf transpiration allows thermoregulation of the plant (Gomes-Laranjo et al. 2012). Because leaves of W plants were more hydrated than leaves of D plants, a higher transpiration in W plants could have occurred for a similar stomatal opening, leading to more efficient thermoregulation and lower plant respiratory rates than in D plants. The lack of carbon limitation in D plants is in agreement with the general assumption that anisohydric tree species like chestnut are not limited by carbon availability during drought (McDowell 2011). Additional reasons explaining differences in whole-plant carbon balances between D and W may include more intense leaf-shedding in D plants and the inability of *C. sativa* to use carbohydrates during waterlogging, as suggested above.

Conclusions

This is the first study to describe NSC dynamics in *C. sativa* under the effects of drought and waterlogging. For 2 months, drought and waterlogging treatments had a similar detrimental impact on g_s, A and growth in *C. sativa* seedlings but different effects on soluble sugars and starch in leaves, stems and roots. Carbon dynamics of seedlings under drought differed from those of seedlings under waterlogging, evidencing different strategies of carbon use in *C. sativa* to cope with the challenges of climate change. The dynamic of NSC in *C. sativa* plants under drought was characterized by an initial mobilisation of stored starch to yield soluble sugars, followed by the presence of high soluble sugar concentrations in aboveground tissues. Total

NSC concentration did not decrease, indicating that 2 months of drought conditions did not induce carbon starvation in the *C. sativa* population studied. The results provide evidence of the ability of this Mediterranean species to reverse drought-induced xylem embolism and tolerate drought, as observed in trees located on xeric mid-slopes. The dynamic of NSC in *C. sativa* plants under waterlogging comprised soluble sugar and starch accumulation in stems and roots, probably as a consequence of active allocation of carbon to reserve formation and inhibited utilisation of NSC. This inability to use carbohydrates may explain the low tolerance of *C. sativa* to waterlogging conditions, which is related to the low occurrence of chestnut trees on river banks and the high mortality of trees in plantations established in soils with low permeability (Fig. 1).

Dead trees by the oomycete *Phytophthora cinnamomi* in a traditional *Castanea sativa* orchard located in La Garganta (Extremadura region, Spain)

CHAPTER II. Increased tolerance to *Phytophthora cinnamomi* in offspring of ink-diseased chestnut (*Castanea sativa* Miller) trees

Castanea sativa Mill. facing global change factors. Chapter II.

Abstract

P. cinnamomi Rands (*Pc*) is responsible for the widespread and destructive ink disease in sweet chestnut (*Castanea sativa* Miller). We investigated if the susceptibility of *C. sativa* to *Pc* and drought stress depends on the health status of mother trees. Plants were grown from seeds collected from healthy and ink-diseased chestnut trees. In a greenhouse assay, leaf wilting after drought exposure and plant mortality after pathogen inoculation were assessed, complemented with gene expression analysis of candidate genes for *Pc* resistance. Offspring of ink-diseased trees had poorer performance in plant height and root biomass than offspring of healthy trees, with allocation of biomass to seeds mediating this effect. Leaf wilting due to water stress was similar in offspring of healthy and *Pc*-infected trees. However, increased tolerance to *Pc* was observed in small-sized seedlings, suggesting that tolerance to *Pc* in *C. sativa* may involve growth costs. No differences in the defense-related gene expression patterns were found between the offspring of healthy and ink-diseased chestnut trees tough. This is the first report of increased tolerance to *Pc* in plants germinating from a diseased tree. The results suggest that an invasive pathogen can regulate the performance and prime a defence response of a forest tree species in the subsequent generation, and generate conflicting selection pressures related to plant size.

Keywords: tree regeneration, maternal effects, invasive pathogen, priming, stress memory

Introduction

Phytophthora cinnamomi Rands (*Pc*) is responsible for ink disease in *Castanea dentata* (Marsh.) Borkh. (USA) and *C. sativa* Mill. (Atlantic areas of Europe), leading to extensive mortality of chestnut trees (Vettraino et al. 2005; Gonthier and Nicolotti 2013; Jung et al. 2018). Infected chestnuts initially show small-sized chlorotic foliage, followed by defoliation and dieback, eventually resulting in whole crown dieback and tree mortality (Phillips and Burdekin 1982; Jung et al. 2018; Camisón et al. 2019a). Symptoms are caused by extensive root loss and necrosis in the inner bark of main roots and collar. In Spain, *C. sativa* is probably the most culturally valued tree, but soils of nearly all *C. sativa* forests are infested by *Pc* (Paloma Abad-Campos, unpublished results). The hybrid *P.* × *cambivora*, associated with ink disease in central and southeast Europe (Vettraino et al. 2005), has not been reported in Spain.

Long-term persistence of *C. sativa* depends on the success of tree regeneration. In areas where *Pc* is present, it is likely that this invasive pathogen will modulate current and future forest dynamics of *C. sativa* forests. Chronic presence of *Phytophthora* spp. constrains tree regeneration at different stages, reducing seed production in infected mother trees, influencing seed germination and causing plant mortality in infested soils (Martín-García et al. 2015; Jung et al. 2018). Moreover, if exposed to drought, young *C. sativa* seedlings suffering root rot caused by *Phytophthora* spp. will probably be less competitive than non-susceptible species. In drought prone areas of the Mediterranean basin such as southern and central Spain, climate change is causing temperature rise and drought stress events in *C. sativa* stands (Ciordia et al. 2012; Camisón et al. 2019a). Combined effects of infested soils and water deprivation could further limit the success of *C. sativa* regeneration in the understory. In this context, it is crucial to understand the differences in performance between offspring of weakened, infected mother trees and offspring of non-infected trees. Infections in the mother tree during seed development may alter the fitness of seedlings subjected to *Pc*, drought stress and other plant competitors.

The high genetic diversity in *C. sativa* (Martin et al. 2010; Cuestas et al. 2017; Martín et al. 2017), reflected in within- and among-population variation in traits of adaptive significance associated with resistance to *Phytophthora* sp. (Robin et al. 2006; Santos et al. 2015; Alcaide et

al. 2020) and drought stress (Pliura and Eriksson 2002; Ciordia et al. 2012; Alcaide et al. 2019), suggests that the species is highly adaptable to environmental changes. Phenotypic plasticity for adaptation can also be achieved through transmitted maternal effects (Marshall and Uller 2007; Vivas et al. 2019). Epigenetics is the study of heritable phenotypic changes that do not involve alterations in the DNA sequence. Maternal effects are a subcategory of transgenerational epigenetics and are defined as the influences of maternal environment, phenotype and/or genotype on offspring phenotypes, independently of the offspring genotypes (Ho 2014). Although the response of germination and seedling phenology to maternal temperatures has been studied in *Pinus pinaster* and *Populus nigra* (Zas et al. 2013; Dewan et al. 2018), the influence of the health status of forest trees on offspring has only been addressed in *P. pinaster* (Vivas et al. 2013, 2014a, 2014b). It was recently reported that maternal environments with low water availability produced *Eucalyptus* seedlings with more efficient water use than maternal environments with high water availability (Vivas et al. 2019). Repeated exposure of trees to stress may cause transcriptional memory in a process known as priming (D'Urso and Brickner 2017; Mauch-Mani et al. 2017).

Transcriptional memory implies that stress-responsive genes are influenced by a stress experience and show differential expression in response to stress repetition. However, evidence for transgenerational passage of acquired resistance to stress is sparse (Avramova 2019). In this work, it is hypothesised that ink disease in *C. sativa* trees influences offspring performance and susceptibility to drought stress and *Pc*. The first objective of this study was to compare, in a common garden experiment, morphological traits of seedlings obtained from healthy mother trees with those of seedlings obtained from *Pc*-infected mother trees. The second objective was to assess tolerance to drought stress and *Pc* in offspring of healthy and diseased mother trees. The third objective was to quantify expression of three genes related to ink-disease resistance (*Cast_Gnk2-like*, *Cast_SAP11* and *Cast_MYB44*) in offspring of healthy and diseased mother trees in response to drought stress and *Pc*.

Materials and methods

Plant material

Plant material came from the *C. sativa* forest in Hervás, Extremadura region, southwest Spain (40° 15' N, 5° 52' W; 805 m a.s.l.). The Hervás chestnut forest has a Mediterranean climate (MAT = 14.9 °C; P = 1004.7 mm) and is currently threatened by severe drought episodes (e.g. total precipitation in 2015 and 2017 was 410 and 550 mm, respectively) and the occurrence of several Phytophthora species, thus providing an ideal test-bed for studying global change factors affecting offspring (Pazianoto et al. 2019) . In October 2015, isolations from the rhizosphere of four healthy and four symptomatic trees (Fig. 1) suggested that symptomatic trees were infected by *Pc* whereas no *Pc* was detected in then soil under healthy trees. Occurrence of *Pc* was assessed by taking fine roots and soil 2–3 m from the trunks, at depths of 10–40 cm from 2 cardinal points. Roots were plated on Petri dishes containing NARPH selective medium, and the soil was baited using leaflets of *Quercus suber* and Granny Smith apples, following conventional methods (Jung et al. 1996; Mora-Sala et al. 2018).

Three non-infected (asymptomatic) and three *Pc*-infected (symptomatic) *C. sativa* trees were selected (Table 1). Trees were > 50 m apart to prevent healthy trees from sharing parts of their rhizosphere with infected trees. They were located close to a stream bank where soil and light conditions were favourable for growth. Trees were ~70 years old and had similar height and basal diameter (ca. 20 m and 80 cm, respectively). Infected trees showed typical symptoms of ink disease (Vettraino et al. 2005), including branch dieback, reduced shoot growth and reduced leaf and nut size (Table 1). To determine whether offspring of healthy and *Pc* -infected *C. sativa* trees have different biological performance when exposed to stress, a greenhouse experiment with 1-year-old seedlings was performed. The greenhouse is located at the Faculty of Forestry in Plasencia, University of Extremadura, Spain (40° 02' N, 6° 04' W; 374 m a.s.l.). In November 2015, nuts from selected mother trees were hand collected from the ground and stored in a cold chamber at 4 °C for 2 weeks. Nuts were immersed in water to determine their viability and those that floated were discarded as non-viable. Viable seeds were immersed in a fungicide solution (2 g L^{-1} Thiram 80GD, ADAMA Inc., Spain) for 10 min and rinsed, then

stratified in moistened blond peat (Pindstrup Mosebrug Inc., Spain) for 1 month at 4 °C (Soylu et al. 1999).

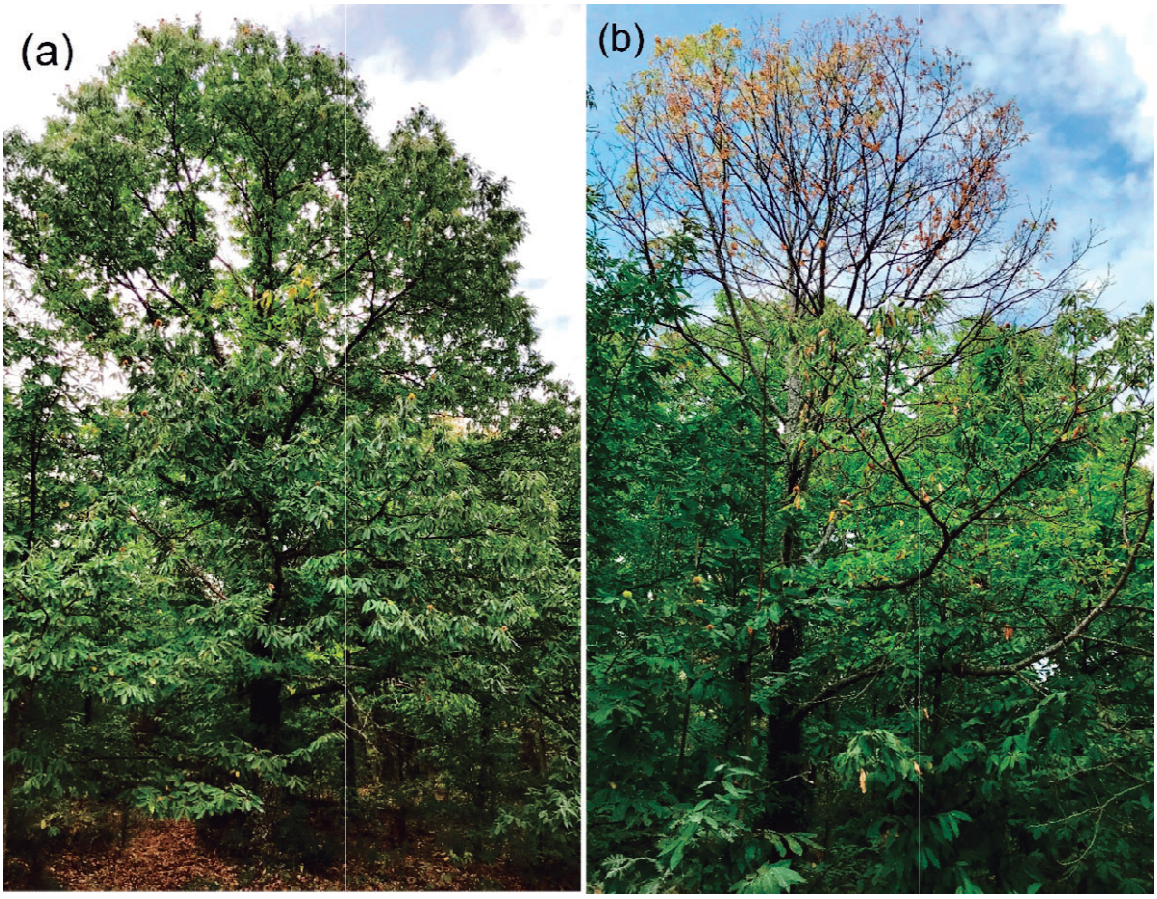


Figure 1. Healthy, non-infected (a) and symptomatic *Phytophthora cinnamomi*-infected (b) *Castanea sativa* trees used to assess biological performance of offspring. Hervás forest, Extremadura region, SW Spain.

Experimental design and treatments

In January 2016, stratification ended and nuts were individually sown in 48-cell rigid plastic root trainers (cells 330 mL in volume; 18 cm high, 5.3 × 5.3 cm upper surface) containing sand and peat (1:3, pH 6). Earlier research by Cubera et al. (2012) showed that this pot size would provide oak seedlings with unrestricted root growth during treatments. Plants were arranged following a split-plot random design replicated in five blocks, with treatments acting as the

main factor (three categories: control, drought stress and *Pc* infection; whole plots) and health status of mother trees as the split factor (two categories: asymptomatic and symptomatic, as shown in Fig. 1; split plots). In all five blocks, health status of mother trees was represented in each whole plot by eight individuals from each of the three mother trees selected. One block consisted of three root trainers, with each root trainer receiving a different treatment. Individuals were randomly positioned within each block and treatment. In total, 720 plants were distributed over 5 blocks × 3 treatments × 2 health statuses of mother trees × 3 progenies × 8 individuals. The term progeny will be further used to refer to half-sib plants sharing the same mother tree. Plants were kept in natural daylight under greenhouse shade that reduced solar radiation by 50% and hand watered to field capacity every 4 days until they were well established. When plants were 5 months old and about 10 cm in height, five root trainers were left as controls, five were subjected to drought stress and five were infested with *Pc*. Drought stress consisted of watering plants to field capacity every 10 days. The remaining plants were watered to field capacity 2 days per week. Soil moisture was checked in four cells per block and treatment using a TDR 100 soil moisture meter (Spectrum Technologies Inc., Plainfield, Illinois, USA) and 12-cm-length rods, and plant gas exchange was assessed using a portable differential infrared gas analyser (IRGA) (LCi, ADC Bio Scientific Ltd., UK) (Alcaide et al. 2019). Measurements confirmed significant differences in soil water content and stomatal conductance between control and water stressed plants (results not shown). Watering treatments lasted 1 month. A single *Pc* A2 strain isolated from roots of a *Quercus ilex* tree in Valverde de Mérida, SW Spain (38° 55′ N, 6° 11′ W; Corcobado et al. 2013), and highly virulent to seedlings of *Q. ilex* (Corcobado et al. 2017) and *C. sativa* was used. Inoculum was prepared following the procedure described by Jung et al. (1996). Briefly, a mix of 500 cm^3 fine vermiculite, 40 cm^3 oat grains and 350 ml multivitamin juice broth (200 mL/L juice, 800 mL/L distilled water with 3 g/L $CaCO_3$) was autoclaved twice in 1-L Erlenmeyer flasks. Individual plugs of *Pc* were then added to the flasks containing the medium and incubated at 20 °C for 5 weeks. Soil was infested in May 2016, using a spoon to carefully mix 12 mL inoculum into the first 3 cm soil of each plant. After inoculation, plants were watered slightly and flooded the following day with chlorine-free

water to stimulate sporangia production and zoospore release and spread. To prevent cross-contamination of plants within blocks, all root trainers were individually placed in large plastic boxes (58 cm × 38 cm × 40 cm, 88 L in volume). In September 2016, *Pc* was successfully re-isolated from root samples collected from inoculated plants only, following the method of Martín-García et al. (2015).

Plant measurements and plant performance

Nuts were individually weighed before sowing, and radicle length was individually measured in nuts starting to germinate (53.1 % of total nuts). Radicle length was interpreted as a proxy of time to germinate, and short (if any) and long radicles were assumed to indicate late and early seed germination, respectively. Time to emerge of first leaves was assessed weekly. Plant height was measured before inoculation and at the end of the experiment, on 30 September 2016. After drought stress treatment, the percentage of foliage showing wilting was visually estimated using a 5 % interval. After inoculation treatment, plant mortality was assessed weekly for 2.5 months. At the end of the vegetative period in all control plants, height, stem diameter and biomass were assessed. Plants were destructively sampled and oven dried at 60 °C for 48 h and their above- and belowground biomass were determined. Roots were separated into fine (diameter < 2 mm) and coarse (diameter ≥ 2mm) (Cubera et al. 2012), usually corresponding to secondary and tap roots, respectively. Before drying, leaves were scanned at 300 × 300 dpi resolution and the area of each leaf was determined using ImageJ 1.x software (Schneider et al. 2012). Leaf mass per area (g cm^{-2}) was calculated for each seedling by dividing leaf dry weight by foliar area.

Gene expression analysis

We selected genes *Cast_Gnk2-like*, *Cast_SAP11* and *Cast_MYB44* for expression analysis because they have been reported to be associated with expression of resistance to *Pc* in *Castanea* spp. and *Castanea* hybrids (Serrazina et al. 2015; Santos et al. 2017) (Table Supplementary 1). To compare gene expression in response to treatments, progenies from healthy mother trees (numbers 1 and 3) and from *Pc*-infected mother trees (numbers 4 and 6)

were selected at random and three seedlings per progeny and treatment were sampled. Gene expression analysis included 36 plants corresponding to 2 health status of mother trees × 2 progenies × 3 treatments (control, drought stress and *Pc* infection) × 3 seedlings. Sampling times were chosen based on the literature (Serrazina et al. 2015; Redondo et al. 2015). Samples were taken 15 days after drought stress started and 2, 4 and 7 days after inoculation. About 3–4 fine roots per plant were excised, washed with water and immediately submerged in RNAlater® (R0901 – Sigma- Aldrich®) to prevent RNA degradation. protocol developed by Chang et al. (1993): samples were incubated for 10 min instead of 15 min, and one-third instead of one-fourth of volume of LiCl 8 M was added, with a final concentration of 750 mM. RNA integrity and concentration were assessed by loading samples into an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). cDNA synthesis was performed with an iScript cDNA Synthesis kit (Bio-Rad) using 200 ng total RNA in a 20-µl total volume and incubating samples in a PCR thermocycler for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. Primers for target genes and *Actin-7*, chosen as housekeeping gene (Table Supplementary 1), were designed with Primer 3 (Untergasser et al. 2012) and the following settings: product size 80–150 bp, primer size 18–25 bp, primer Tm 57–62 °C, primer GC content 40–60% and Max 3′ self complementarity 2 bases. Three technical replicates consisting of 20 µL volume were used for each biological sample. Real-time PCR reactions were performed by mixing 1 µL non-diluted template cDNA, 0.25 pM of each primer and 1X SSoFast EvaGreen reaction mixture (Bio-Rad). For negative controls, 1 µL nuclease-free water was used instead of template cDNA. Primer efficiency was assessed by including one standard curve for each gene in the assay. The PCR conditions were 2 min at 90 °C followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C for annealing and 30 s at 72 °C. After amplification, melting curve analysis was carried out for each gene to check gene-specific amplification. Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001).

Statistical analysis

Plant tolerance to drought stress was assessed by comparing 'leaf wilting' (continuous variable indicating wilting intensity from 0 to 100 %) in offspring of healthy mother trees with 'leaf wilting'in offspring of ink-diseased mother trees. A linear mixed model was performed using 'leaf wilting' as the dependent variable, 'health status of mother tree', 'block' and 'health status of mother tree × block' as the fixed factors, 'mother tree' (nested within 'health status of mother tree') as a random factor, and 'seed weight', 'radicle length', 'time to emerge' and 'plant height' as covariates. Means of leaf wilting among progenies were compared using the linear mixed model. Plant tolerance to *Pc* was assessed by comparing 'time to death' (survival time since inoculation) in offspring of healthy mother trees with 'time to death' in offspring of ink-diseased mother trees. To analyse 'time to death' of plants and determine seedling survival probabilities, the Kaplan–Meier estimate was used (Solla et al. 2011). To test for statistical differences between survival probabilities of progenies, the log rank test was used. Survival analysis does not account for the effect of continuous covariates on survival probabilities. A first approach to take 'plant height' into account consisted of dividing seedlings into offspring 1–10 cm tall from healthy mother trees (n = 23), offspring 1–10 cm tall from *Pc* -infected mother trees (n = 43), offspring > 10 cm tall from healthy mother trees (n = 54), and offspring > 10 cm tall from *Pc*-infected mother trees (n = 41). A second approach to assess whether 'seed weight', 'radicle length', 'time to emerge' and 'plant height' covariates influenced offspring susceptibility to *Pc* consisted in using a Cox proportional hazards model which was fitted to the survival data (Zhang 2016). Cox regression estimates how plant survival is affected by several effects relative to a control group (offspring of non-infected mother trees). The measure of effect is the hazard ratio, which is the risk of failure or the probability of suffering mortality. The effects 'health status of mother tree', 'seed weight', 'radicle length', 'time to emerge', 'plant height' and the interactions of these effects were included in the model. The autocorrelation between seedlings of the same mother tree was accounted for by estimating 'robust standard errors' for the model's coefficients. Automatic stepwise variable selection based on the Akaike information criterion was applied to identify the most relevant effects. The

significance of the Cox model was evaluated by the likelihood ratio test and the Wald statistic, while the explanatory power of the model was evaluated using the C-statistic and R^2. All data were checked for normality and homoscedasticity by applying the Shapiro-Wilk and Bartlett tests, respectively. Differences between means of phenotypic traits and 'seed weight' of mother trees (Table 1) were analysed by ANOVA. Fisher's least significant difference (LSD) was used in post hoc tests. To identify differences in gene expression either between offspring of healthy and *Pc* -infected trees or between control and treated seedlings, the Mann-Whitney test was used. Relations between the early performance of seedlings and the time to death, parameters were examined using Pearson correlation coefficients within seedlings from healthy and *Pc*-infected mother trees. All statistical analyses were performed in R software environment version 3.4.2 (R Foundation for Statistical Computing, http://www.R-project.org). Functions in the 'survival' version 2.41-3 (Therneau 2015) and 'survminer' version 0.4.0.999 (Kassambara and Kosinski 2017) packages were used for survival time analysis and optimal cut point calculations, respectively, and the 'agricolae' package was used for comparison of means between groups.

Results

Plant performance in offspring of healthy and Pc -infected trees

Severe infection of adult *C. sativa* trees by *Pc* did not delay or reduce seed germination rate (Table 2). However, offspring of healthy trees were significantly taller than offspring of *Pc*-infected trees ($P < 0.001$; Table 2). The tallest and smallest progenies belonged to the mother trees with the heaviest and lightest seeds (Tables 1 and 2). At the end of the vegetative period, seedling performance was strongly influenced by health status of mother trees (Table 2). Correlation analysis after merging data of plants from mother trees with the same health status showed that 'seed weight' was correlated to 'plant height', 'stem diameter' and woody biomass of *C. sativa* seedlings ($P < 0.05$; Table 3). Contrary to expectation, negative correlations between 'plant height' and 'radicle length' ($P < 0.05$) and positive correlations between 'plant height' and 'time to emerge' ($P < 0.001$) were observed, indicating that taller plants were

obtained from seeds germinating and emerging late (Table 3). Most correlations were substantially conditioned by health status of mother tree (Table 3).

Offspring response to drought stress

Unstressed control plants did not wilt. In response to water stress treatment, offspring of *Pc*-infected trees wilted similar to offspring of healthy trees (16.7 *vs* 18.9 %; $P > 0.05$; Table 2). According to the linear mixed model used, the only factors explaining variability in 'leaf wilting' were 'seed weight' ($F = 9.1$, $P = 0.002$) and 'plant heigh' ($F = 51.0$, $P < 0.001$) (Table Supplementary 2). Pearson correlation analysis with values of all seedlings showed that 'leaf wilting' did not correlate to 'seed weight' but did correlate to 'time to emerge' ($r = -0.14$, $P = 0.011$), 'plant height' ($r = -0.30$; $P < 0.001$), 'biomass of fine roots' ($r = -0.25$, $P < 0.01$) and 'biomass of tap roots' ($r = -0.15$; $P < 0.05$).

Offspring response to Pc

Plant mortality due to *Pc* was similar in offspring of healthy and *Pc* -infected trees (Fig. 2a, Table 2). However, the group of plants 1–10 cm tall from *Pc* infected trees died less and more slowly than the other groups of plants (Fig. 2b). Cox proportional hazards model ($P < 0.001$, $R^2 = 0.13$, concordance = 63.6%) showed that the risk of plants dying on inoculation was significantly influenced by 'health status of mother tree' (if infected by *Pc*), 'radicle length' and the 'health status of mother tree × plant height' interaction (Table 4). Offspring had on average 85% lower risk of dying if germinated from *Pc*-infected instead of from healthy mother trees. A 1 mm increase in radicle length increased the daily probabilities of death in offspring of both healthy and *Pc* -infected trees by 3%. The Cox model predicted survival probabilities of 52% for short and 22% for tall offspring from *Pc* -infected trees, and 22% for short and 28% for tall offspring from healthy trees. Higher variability in response to *Pc* was observed in offspring of *Pc*-infected trees than in offspring of healthy trees. Pearson correlation analysis confirmed the negative relation between 'time to death' and 'radicle length' in both groups of offspring ($P <$

0.05). In offspring of *Pc*-infected trees only (Fig. 3), 'time to death' was related to 'plant height' (r = − 0.19, $P = 0.002$).

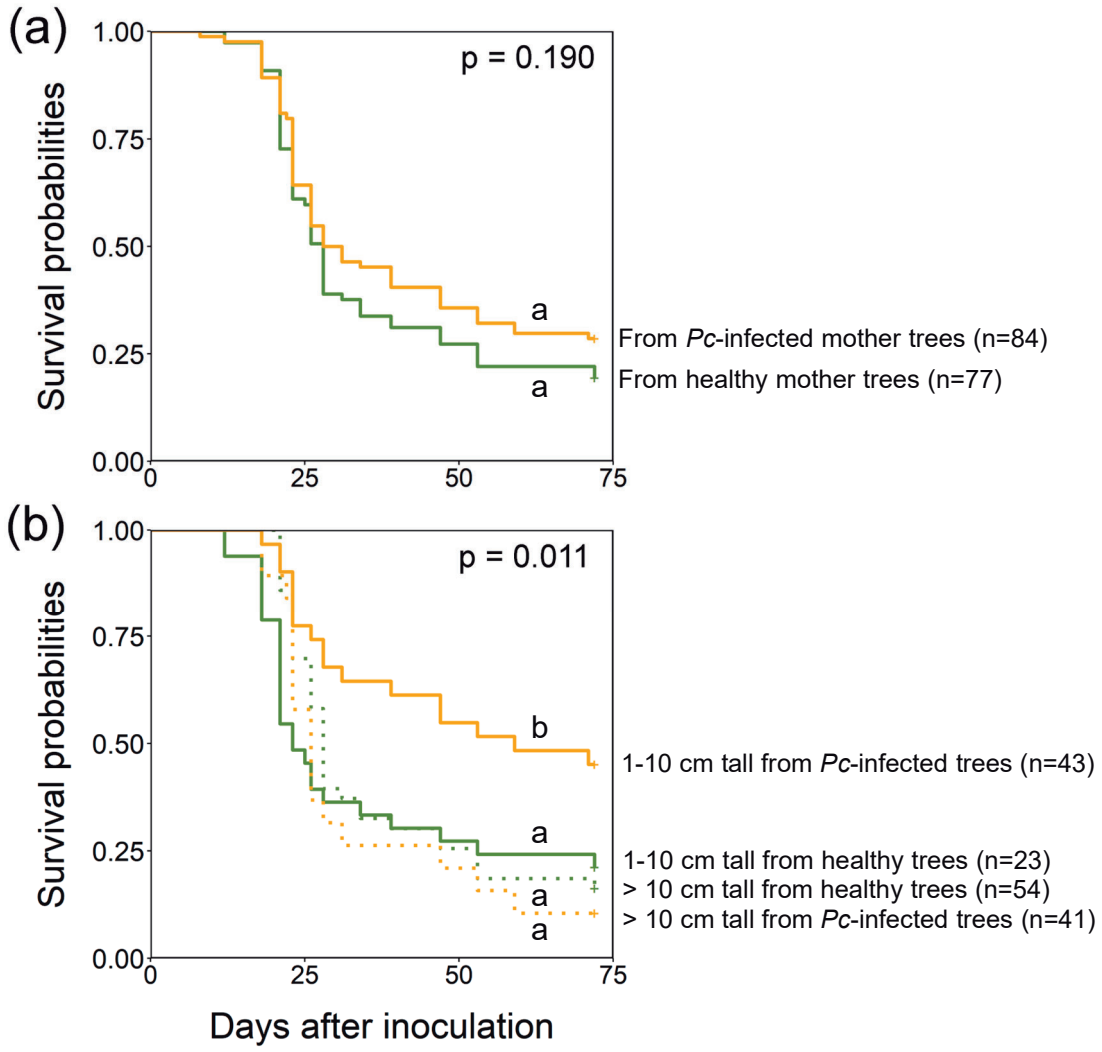


Figure 2. Estimated survival probabilities of *Castanea sativa* seedlings after *Phytophthora cinnamomi* inoculations considering health status of mother trees (a) and both health status of mother trees and seedling height (b) as grouping variables. Seedlings germinated from healthy and *Phytophthora cinnamomi* (*Pc*)-infected mother trees. A p value is provided for the global log rank test, with different letters indicating significant differences between survival curves ($P < 0.05$) according to the log rank test.

Gene expression

In response to drought stress treatment, no significant differences of gene expression between offspring of healthy and *Pc* -infected mother trees were observed (results not shown). At day 15 after drought stress had started, only gene *Cast_SAP11* was significantly more expressed in drought-stressed than in control plants (5.2- *vs* 0.3-fold, respectively, $P = 0.0021$, *t*-test). Gene expression was constant over time in non-inoculated control plants (Fig. 4). Two days after inoculation (dai), gene expression did not differ between control and inoculated plants (Fig. 4). At four and seven dai, the *Cast_Gnk2-like* gene was more expressed in inoculated plants than in controls (Fig. 5a). Expression levels of *Cast_SAP11* peaked at four dai in inoculated plants (Fig 5b) and *Cast_MYB44* was not regulated in response to *Pc* (Fig. 5c). No significant differences were observed in gene expression between offspring of healthy and *Pc* -infected mother trees (Fig. 5).

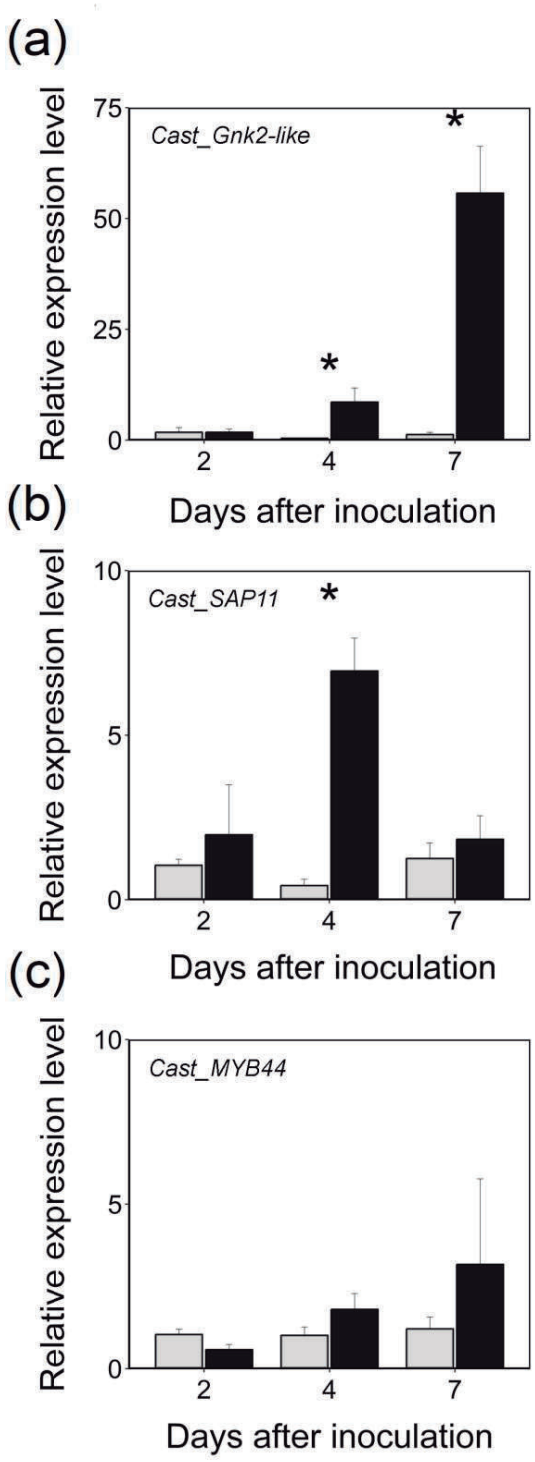


Figure 3. Relative expression of *Cast_Gnk2-like* (a), *Cast_SAP11* (b) and *Cast_MYB44* (c) genes in offspring of *Castanea sativa* not inoculated (white bars) and inoculated (black bars) with *Phytophthora cinnamomi* (n=3 plants). Observe differences in scale. Asterisks indicate significant differences ($P < 0.05$) between mean values on the same date.

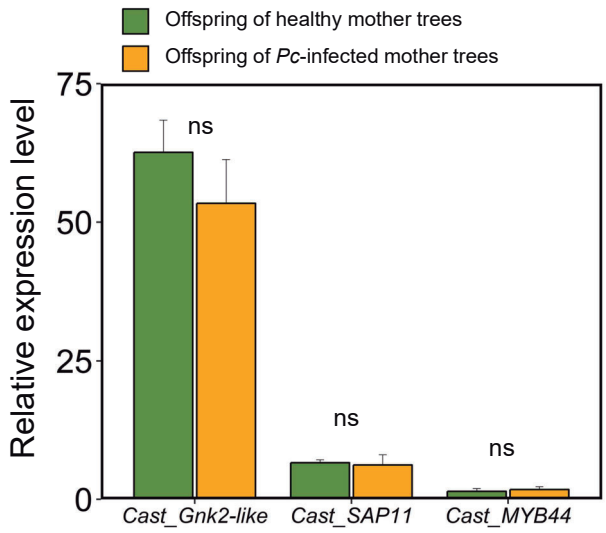


Figure 4. Relative expression of *Cast_Gnk2-like*, *Cast_SAP11* and *Cast_MYB44* genes in offspring of healthy and *Phytophthora cinnamomi* (*Pc*)-infected *Castanea sativa* trees (n=3 plants). Ns indicates non-significant differences between means.

Discussion

Seed weight and offspring of ink-diseased chestnuts

The performance of young trees is genetically determined, but also can be altered by hardening practices (Villar-Salvador et al. 2012) and unfavourable scenarios experienced by the mother trees (Vivas et al. 2013, 2014a, 2014b). The present study shows that *Pc* infection of chestnuts inhibits early growth of offspring. It also confirms that plant growth inhibition is mediated by seed size–dependent mechanisms, probably involving allocation of biomass to seeds (i.e. seed provisioning) (Shi et al. 2019). The morphological traits most affected were plant height and root biomass. In several studies, including this one, plant height and root biomass were directly dependent on seed mass (Çiçek and Tilki 2007; Ramírez- Valiente et al. 2009) and seed mass was directly influenced by environmental factors experienced by mother trees (Vivas et al. 2013). Large seedlings are better able to acquire and retain resources than their smaller counterparts, therefore enhancing survival chances, growth, and reproductive opportunities and success (Ramírez-Valiente et al. 2009; Younginger et al. 2017). Seed size variability, found to be higher in *Phytophthora*-infected than in healthy mother trees, was also higher in mother trees of *P. pinaster* growing under unfavourable than under favourable conditions (Zas et al. 2013).

The high variation in traits in the offspring of *Pc* -infected mother trees may be a consequence of the high and significant variation in traits in infected mother trees. Increased seed size variability could be considered an adaptive response because a wide range of phenotypes is produced in a given unfavourable environment. However, the low variability in seed size and other traits in offspring of the most symptomatic chestnut (tree 4, smallest seeds) suggests inability to adapt (Kleunen and Fischer 2005) and few chances for competition and establishment. This is in agreement with the existence of seed resource-dependent thresholds which, once exceeded, strongly limit phenotypic plasticity within a progeny (Kleunen and Fischer 2005).

Similar tolerance to drought stress in offspring of chestnuts irrespective of the health status of mother trees

Seedlings of ink-diseased trees wilted similar to seedlings of healthy trees, and gene expression analysis was consistent with this result. In consequence, *Pc* infection in *C. sativa* does not contribute to the inheritance of dehydration stress memory. Stress memory may increase a plant's survival chances by improving its tolerance abilities and may provide a mechanism for adaptation (Avramova 2019). Priming with mild drought during the flowering stage was shown to be effective in reducing the impact of subsequent severe terminal drought stress in *Oryza sativa* (Bahuguna et al. 2018). Drought priming in parental plants was recently reported to induce drought tolerance in offspring (Wang et al. 2018; Vivas et al. 2019). In our study, the reduced root water uptake capacity often associated to ink disease (Maurel et al. 2001; Dinis et al. 2011) combined with the low precipitation in 2015 at the seed collection site were not enough to induce drought-priming in offspring of *C. sativa*. The role of 'seed weight' and 'plant height' mediating tolerance to drought stress can be explained by the higher root biomass and thus higher water uptake ability in taller plants germinating from the heavier seeds (Ramírez-Valiente et al. 2009; Cubera et al. 2012). Interestingly, leaf mass per area was lower in offspring of *Pc* -infected than of healthy trees (Table 2). Low leaf mass per area (or high specific leaf

area) is usually associated to species with low tolerance to drought, which produce 'cheap' leaves as long as the season is favorable (Lopez-Iglesias et al. 2014).

Increased tolerance to Pc in small seedlings from ink-diseased chestnuts

Candidate gene expression patterns in response to *Pc* were similar in offspring of healthy and *Pc*-infected trees. However, small plants from *Pc*-infected mother trees showed increased tolerance to further infection by *Pc*, irrespective of seed weight. A recent study reported reduced and delayed mortality of *Q. ilex* seedlings if less virulent *Phytophthora* species were inoculated before more virulent *Phytophthora* species (Corcobado et al. 2017). Small chestnuts plants could have been primed for resistance (Ashraf et al. 2018), probably by elicitins and effector molecules released by *Pc* during root infection (Osswald et al. 2014), but this needs further investigation. Active adaptive plasticity of plants in response to the environment requires a specific signal perception-transduction system that moulds plant development and influences next generations (Kleunen and Fischer 2005; Mauch-Mani et al. 2017). Infection by *Pc* in *C. sativa* is perceived via pathogen recognition proteins that are coded by several defence genes (Santos et al. 2017). After recognition, transcriptional reprogramming in the host may directly provide the tree with pathogen-specific cues that may be transmitted to seeds, resulting in transgenerational phenotypic plasticity in offspring. Chestnut seedlings that invested fewer resources in growth and attained less biomass died later than their taller counterparts. This was consistently observed through Pearson's correlation, Cox proportional hazards model and survival analyses, and could suggest a growth-defence trade-off that occurred only in offspring of *Pc*-infected mother trees. This trade-off is in agreement with ecological theories on plant defence, e.g. the resource availability hypothesis (RAH) and the growth development balance hypothesis (GDBH), which indicate that plant defences involve growth costs, especially in resource-limited environments (Ferrenberg et al. 2015; Moreira et al. 2015). We did not assess fitness in offspring but morphological traits related to competitive abilities of plants (Younginger et al. 2017) and 'survival' of plants to two different stresses. Studies that use close proxies to fitness (e.g. survival) are expected to show stronger effect sizes than studies using

simplistic single traits (Uller et al. 2013). Several circumstances suggest that the three *Pc*-infected mother trees used were not tolerant to ink disease: (i) there is no evidence of *C. mollissima* and *C. crenata* germplasm in Hervás forest, in contrast to other forests in northern Spain (Alcaide et al. 2020) in which genes from Asiatic germplasm confer resistance to *Pc* in offspring; (ii) inoculation with *Pc* of progenies from 16 additional mother trees, selected in Hervás at random, resulted in circa 100% of seedling mortality (Alcaide et al. 2020); and (iii) in spring 2019, tree 4 was almost dead and tree 6 was dead. Table 2 shows relevant heterogeneity in mean height of the six progenies used, and when assessing adaptive plasticity in offspring, it may occur that seedling height could be confounded with parent effects. However, 'plant height' was included as a covariate in the linear mixed and Cox models. When addressing differences in survivorship between short and tall seedlings, only offspring of trees 5 and 6 contributed to these differences. Offspring of tree 4 did not significantly contribute to differences in survivorship between short and tall seedlings probably because plants were homogeneous in height (standard error was 0.2 *vs* 0.5 and 0.6 in offspring of trees 5 and 6, respectively), and probably because tree 4 was severely damaged by *Pc.* Tree 4 had the highest branch dieback and the lowest leaf mass per area values of all *Pc*-infected trees, and their nuts were stunted probably as a consequence of severe ink disease damage (Phillips and Burdekin 1982). A hypothetical schematic diagram of what may have occurred in offspring of *C. sativa* trees is represented in Fig. Supplementary 1. Nuts from trees 1, 2 and 3 were not primed, small-size offspring of trees 5 and 6 showed increased tolerance to *Pc* at a cost of plant growth, and offspring of tree 4 exceed the threshold for a beneficial effect of priming as a consequence of intense damage. More mother trees and more tree species should be used to confirm hypothetical Fig. Supplementary 1. Moreover, in this study, the use of more mother trees or clonally replicated material would have properly allowed distinguishing heritable genetic variation from maternal environmental effects. Unfortunately, seedlings used for gene expression analysis were tall in height, which could explain the lack of differences of gene expression between offspring of healthy and *Pc*-infected trees. Further studies on gene expression patterns would require consideration of plant size. The use of a single and highly

virulent isolate of *Pc* also limits the conclusions that can be derived from our work. Further studies on maternal effects would require the use of several isolates including some of low virulence in order to capture greater sensitivity from the offspring.

Ecological consequences

How can previous findings be transferred to *C. sativa* forests? In forests where *Pc* is absent, *C. sativa* is able to regenerate and reach the main canopy under closed overstories (Camisón et al. 2015; Silla et al. 2018). The findings suggest that in *Pc*-infested sites, *C. sativa* trees will generate small seeds whose viability is not affected by the health status of mother trees. Small seeds are usually associated with early emergence (Solla et al. 2011; *Pc*-infected mother tree 1), giving the added advantage of seeds being less exposed to soil-borne pathogens like *Phytophthora* spp. (Martín-García et al. 2015). In *Phytophthora*-infested sites, sexual regeneration may be dominated by small-sized primed seedlings, which respond better than larger conspecifics to *Pc* in the short term. However, small-sized *C. sativa* seedlings will not maintain their competitive ability over other forest species, because of reduced access to light, water and nutrients. Small-sized *C. sativa* seedlings with fine roots partially killed by *Pc* will probably not survive summer drought unless they are able to develop a deep tap root for vertical water exploration. Previous assumptions should be taken with caution for *C. sativa* and validated by long-term assessment of seedlings (from healthy and ink-diseased mother trees) grown in the field.

Conclusions

Offspring of ink-diseased chestnut trees had poorer performance in plant height and root biomass than offspring of healthy chestnut trees. This effect was probably mediated by seed size–dependent mechanisms involving seed provisioning. *Pc* infection in chestnut did not contribute to the inheritance of dehydration stress memory. However, small plants of offspring of ink-diseased mother trees showed increased tolerance to *Pc*. Increased tolerance was not mediated by seed size and was probably a consequence of seed priming during fruit

development. The results suggest that the impact of *Pc* on *C. sativa* forests may generate conflicting selection pressures related to plant size, constraining regeneration success at the seedling stage.

Table 2. Early performance traits (±se) in *Castanea sativa* seedlings from healthy and *Phytophthora cinnamomi* (*Pc*)-infected mother trees, and leaf wilting and mortality values after water stress and *Pc* treatments, respectively. Seedlings from the untreated control treatment did not wilt or die. Different lowercase letters indicate significant differences between values within progenies, while different uppercase letters indicate significant differences between mean values within groups of progenies according to the health status of their mother trees (linear mixed model, $P < 0.05$).

Mother tree		Offspring (n ≈ 120)	Control treatment (n≈40)					Drought stress treatment (n ≈ 40)	*Pc* treatment (n≈40)
Status		Germination rate (%)	Time to emerge (days)	Plant height[a] (cm)	Woody biomass[b] (g)	Fine root biomass[b] (g)	Leaf mass per area[b] (g m^{-2})	Leaf wilting (%)	Plant mortality (%)
Healthy	1	83	41±2.3b	10.1±0.4b	1.9±0.17b	0.32±0.21b	8.02±0.52b	13.7±4.0a	80
	2	58	36±3.3b	8.7±0.6b	2.0±0.23b	0.24±0.15ab	7.41±0.42ab	34.4±8.7b	75
	3	82	40±3.6b	12.5±0.5c	2.5±0.21b	0.30±0.18b	7.50±0.35ab	17.3±3.9ab	78
	Mean	75A	39 A	10.1 B	2.1 B	0.29 B	7.6 B	18.9 A	78 A
Pc-infected	4	93	24±1.3a	5.9±0.2a	1.2±0.09a	0.17±0.09a	6.27±0.19a	16.5±3.8ab	76
	5	83	39±2.4b	10.1±0.5b	2.0±0.22b	0.26±0.19ab	7.38±0.43ab	20.2±4.8ab	75
	6	50	50±5.0b	8.7±0.6b	1.2±0.21a	0.21±0.12ab	7.53±0.45ab	10.7±4.9a	60
	Mean	75A	35 A	7.9 A	1.4 A	0.21 A	6.7 A	16.7 A	71 A

[a]Measured before inoculation. Significantly related to stem diameter (r=0.68, $P < 0.001$; data not shown); [b]Assessed in untreated plants at the end of the vegetative period;

[c]Assessed one month after water stress treatment started; [d]Assessed 2.5 months after inoculation.

Table 3. Pearson values from individual correlations among early performance variables in *Castanea sativa* seedlings from healthy (above the diagonal) and *Phytophthora cinnamomi*-infected (below the diagonal) mother trees. Asterisks indicate levels of significance at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; ns = not significant.

	Seed weight	Radicle length	Time to emerge	Plant height	Woody biomass	Taproot biomass	Fine root biomass	Leaf mass per area
Seed weight	X	ns	ns	0.42***	0.53***	0.46***	ns	ns
Radicle length	-0.28**	X	-0.23**	-0.26**	ns	ns	ns	ns
Time to emerge	0.21**	-0.56***	X	0.29***	ns	ns	ns	ns
Plant height	0.50***	-0.35***	0.28***	X	0.38**	ns	ns	ns
Woody biomass	0.60***	ns	ns	0.69***	X	0.90***	0.24*	ns
Taproot biomass	0.60***	ns	ns	0.59***	0.94***	X	0.25*	ns
Fine root biomass	0.35**	ns	ns	0.49***	0.62***	0.59***	X	ns
Leaf mass per area	0.47*	-0.38*	ns	ns	0.46*	0.47*	0.40*	X

Table 4. Parameters of the Cox regression model used to analyse survival data in the offspring of *Castanea sativa* trees after artificial inoculations with *Phytophthora cinnamomi*. The reference group was the offspring of healthy mother trees. Likelihood ratio test (7 df) = 21.79; $P = 0.0013$.

Effect	Estimate	Hazard ratio[b] (± robust SE)	95% Confidence interval	Z score	p value
Health status of mother tree (*Pc*-infected)	-1.78	0.15 ±0.53	0.05, 0.44	-3.47	<0.001
Seed weight	-0.00	0.99 ± 0.01	0.93, 1.03	-0.24	0.816
Radicle length	0.03	1.03 ± 0.00	1.02, 1.04	4.93	<0.001
Time to emerge	0.00	1.00 ± 0.00	0.99, 1.01	0.92	0.352
Plant height	-0.02	0.97 ± 0.03	0.91, 1.03	-0.84	0.390
Health status of mother tree (*Pc*-infected) × plant height[a]	0.11	1.12 ± 0.02	1.06, 1.17	4.58	<0.001
Health status of mother tree (healthy) × plant height	-0.02	0.97 ± 0.02	0.92,1.02	-1.00	0.311

[a]The only significant interaction.

[b]Non-significant values or ratios = 1 indicate no effect on plant survival; significant ratios < 1 indicate plant survival increases due to the effect; significant ratios > 1 indicate plant survival decreases due to the effect. The effect of a variable on plant survival (%) in relation to the reference group can be obtained as (1 – hazard ratio) × 100

Phytophthora cinnamomi - resistant hybrid rootstocks are used to protect susceptible *Castanea sativa* scions. In *P. cinnamomi*-infected soils, trees with traditional *C. sativa* rootstocks die (dying trees in the foreground). Photo by F. Javier Dorado. Jaraíz de la Vera (Extremadura region, Spain)

CHAPTER III. Hormone and secondary metabolite profiling in chestnut during susceptible and resistant interactions with *Phytophthora cinnamomi*

Castanea sativa Mill. facing global change factors. Chapter III.

Abstract

Phytophthora cinnamomi (*Pc*) is a dangerous pathogen that causes root rot (ink disease) and threatens the production of chestnuts worldwide. Despite all the advances recently reported at molecular and physiological level, there are still gaps of knowledge that would help to unveil the defence mechanisms behind plant-*Pc* interactions. Bearing this in mind the constitutive and *Pc*-induced stress-related signals (hormones and metabolites) were quantified complemented with changes in photosynthetic related parameters by exploring susceptible and resistant *Castanea* spp.-*Pc* interactions. In a greenhouse experiment, five days before and nine days after inoculation with *Pc*, leaves and fine roots from susceptible *C. sativa* and resistant *C. sativa* × *C. crenata* clonal 2-year-old plantlets were sampled (clones Cs14 and 111-1, respectively). In the resistant clone, stomatal conductance (g_s) and net photosynthesis (A) decreased significantly and soluble sugars in leaves increased, while in the susceptible clone *gs* and *A* remained unchanged and proline levels in leaves increased. In the resistant clone, higher constitutive content of root SA and foliar ABA, JA and JA-Ile as compared to the susceptible clone were observed. Total phenolics and condensed tannins were highest in roots of the susceptible clone. In response to infection, a dynamic hormonal response in the resistant clone was observed, consisting of accumulation of JA, JA-Ile and ABA in roots and depletion of total phenolics in leaves. However, in the susceptible clone only JA diminished in leaves and increased in roots. Constitutive and *Pc*-induced levels of JA-Ile were only detectable in the resistant clone. From the hormonal profiles obtained in leaves and roots before and after infection, it is concluded that the lack of effective hormonal changes in *C. sativa* explains the lack of defence responses to *Pc* of this susceptible species.

Keywords: hormonal profiling, jasmonates, stress signaling, oomycetes, induced defence, crosstalk

Introduction

Chestnuts (*Castanea* spp.) are highly valuable trees of the temperate zone for their nutritious nuts, timber and ecosystem services. Ink disease caused by the invasive soil oomycete *Phytophthora cinnamomi* (*Pc*) is the most destructive disease affecting susceptible *Castanea* spp. globally (Jung et al., 2018), and has contributed to drastic reduction of chestnut distribution areas around the world (Martins et al. 2007; Sena et al. 2018). The root rot pathogen *Pc* is hemibiotrophic and able to infect around 5000 plant species worldwide (Hardham and Blackman 2018). Sweet chestnut (*C. sativa* Mill.) is the most susceptible European tree to *Pc* in contrast to the Korean chestnut (*C. crenata* Sieb. & Zucc.), native to Japan and South Korea, which is considered a fully resistant species (Crandall et al. 1945). *C. crenata* germplasm has been used in chestnut breeding programs in several European countries as a source of resistance to *Pc* (Lopez- Villamor et al. 2018) and several *Pc* resistant *C. crenata* × *C. sativa* hybrid clones are currently available in the market, used as rootstocks of traditional varieties of sweet chestnut, and cultivated in orchards for nut production (Miranda-Fontaina et al. 2007; Fernandez-Lopez and Fernandez-Cruz 2015). Considerable effort has been devoted to characterize the changes induced by *Pc* in susceptible and/or resistant chestnuts through studies involving root histology, plant water relations, root-to-shoot signalling, mineral nutrition and biochemical parameters (Maurel et al. 2001a, 2001b; Gomes-Laranjo et al. 2004; Maurel et al. 2004; Dinis et al. 2011; Medeira et al. 2012; Serrazina et al. 2015). Despite the substantial advances achieved recently at molecular level (Santos et al. 2017a, 2017b), there are still important knowledge gaps, which are crucial to link genotype to phenotype traits involved in plant defence and allow validation of resistant genotypes. More data and experiments are needed in relation to signalling occurring belowground, at the front of pathogen recognition, and more effort is needed to integrate this information with aboveground responses during *Pc* infection in chestnut trees.

Phytohormones are small signalling molecules known to be central regulators of plant responses to a wide range of biotic and abiotic stresses (de Torres Zabala et al. 2009; Martin et al. 2012; van den Berg et al. 2018; Perez-Clemente et al. 2019). In general terms, salicylic acid

(SA)-mediated signalling is important for plant defence against biotrophic pathogens (Spoel and Dong 2008). The non-bioactive derivatives 2-O-β-d-glucoside (SAG) and salicylate glucose ester (SGE) contribute to the regulation and homeostais of the bioactive SA, and are worth to be quantified together with SA *in planta* in response to pathogen attack (Allasia et al. 2018). Jasmonates (JAs) are fatty acid-derived hormones which regulate defence against necrotrophic pathogens (Spoel and Dong 2008). JAs include jasmonic acid (JA) and its amino acid conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile). Conjugation of phytohormones to amino acids is commonly associated with storage and inactivation, but JA-Ile is the bioactive form of JAs perceived by cells (Piotrowska and Bajguz 2011). Other plant hormones such as abscisic acid (ABA) and auxins including indole-3-acetic acid (IAA), that have been thoroughly described to regulate plant development and growth, have recently emerged as key regulators of plant immunity (Denance et al. 2013). So far, only two studies quantified hormones in trees after infection by *Pc*, reporting decreased cytokinins and ABA content in xylem sap of susceptible *Eucalyptus marginata* (Cahill et al. 1986) and *C. sativa* (Maurel at al. 2004) seedlings, respectively. No study so far has determined which hormones change and in which tissues they do upon *Pc* infection. An urgent call to investigate differences in hormonal responses between *Pc* susceptible and resistant *Castanea* spp. is derived from the work by Santos et al. (2017b), who found one QTLs for *Pc* resistance (designated *Pc_E*) associated to hormonal signalling processes.

Hormones work in a complex signalling network with other known stress-related metabolites. Soluble sugars are relevant in primary metabolism, providing plants with energy and structural material, and interacting as signal molecules with hormones (Ljung et al. 2015). Sugars enhance oxidative burst at early stages of infection by stimulating the synthesis of antioxidant phenolic compounds, which are also involved in plant signalling (*e.g.* flavonoids and SA) and defence (*e.g.*tannins) (Bolouri Moghaddam and Van den Ende 2012). Plants accumulate the amino acid proline in response to a multitude of environmental stresses. Proline acts as a beneficial solute allowing plants to increase cellular osmolarity during water limitation. Moreover, proline metabolism has roles in redox buffering and energy transfer and is involved

in plant-pathogen interactions and programmed cell death (Verslues and Sharma 2010). Proline metabolism is regulated through a fine-tuned coupling with 1-pyrroline-5-carboxylic acid (P5C) (Cecchini et al. 2011; Qamar et al. 2015). With such an intricate toolbox, plants activate appropriate and effective defence responses against pathogens, and balance defence with growth (Bolouri Moghaddam and Van den Ende 2012).

The main objectives of this study were to evaluate the impact of *Pc* infection on the main plant leaf physiological function, photosynthesis, and to quantify hormones and stress-related metabolites in leaves and roots of two *Castanea* spp. clonal genotypes of contrasted susceptibility to *Pc*. The resistant '111-1' clone, an F2 *C. sativa* x *C. crenata* hybrid containing 67% of exclusive Asian alleles (Gonzalez et al., 2011) was selected because is one of the most commonly planted *Pc*-resistant commercial rootstocks in Spain and Portugal (Miranda-Fontaina et al. 2007). A pure *C. sativa* clone termed 'Cs14' native to the north-western coast of Spain was selected as susceptible material. This clone was previously used as 'susceptible control' when screening chestnuts for *Pc* resistance in Spain (Cuenca et al. 2009). It is hypothesised that in these two clones there are both constitutive and *Pc*-induced differences in the content of hormones and other signalling metabolites in leaves and roots.

Material and methods

Plant material, growth conditions and experimental design

The plant material was obtained by *in vitro* micropropagation (Vidal et al. 2015) and acquired from a specialized chestnut supplier company (Grupo TRAGSA-SEPI, Maceda, Spain). In October 2016, one-year-old plantlets of each clone were planted in 2-liter pots containing a mixture of peat, vermiculite and perlite (1:1:1) and placed at the greenhouse of the Faculty of Forestry of Plasencia (40°02´N, 6°05´W; 374 m asl, western Spain). In January 2017, they were fertilized with Osmocote Pro 3–4M (Osmocote® Pro) at 4 g L^{-1} and arranged in a complete randomized bi-factorial design considering 'susceptibility to *Pc*' (two categories: susceptible (Cs14) and resistant (111-1)) and 'inoculation with *Pc*' (two categories: yes and no) as factors. In total, there were 60 plantlets distributed over two susceptibilities × two treatments × 15

replicates. Plantlets were inoculated with *Pc* in July 2017, at the age of two years. Clones 111-1 and Cs14 were 107.7 ± 9.6 and 91.5 ± 10.3 cm in height ($P > 0.05$, *t*-test), and 1.1 ± 0.4 and 0.8 ± 0.4 cm in diameter ($P > 0.05$, *t*-test), respectively. Five days before inoculation and nine days after inoculation the same individuals were sampled aboveground and belowground. Sampling at day nine after inoculation was done because at this stage of infection the first external symptoms (leaf wilting and yellowing) occurred in half of the plants within each chestnut clone. *In vivo* and immediate measurements of gas exchange-related parameters and chlorophyll fluorescence parameters (including maximum quantum yield of photosystem (PS) II and leaf chlorophyll content) were performed at each sampling date. Leaves and roots were collected, frozen in liquid nitrogen and kept at −80 °C for further quantification of hormones and metabolites.

Pc inoculation and symptom assessment

An aggressive single A2 strain (Ps-1683) isolated from a declining *C. sativa* tree in northern Spain was used. The inoculum was prepared following Jung et al. (1996) and was incubated during 5 weeks inside Erlenmeyer flasks. Soil infestation was conducted by mixing 12 ml of the inoculum with the first 3 cm of soil of each plant. After inoculation, plants received a slight watering and were flooded for two days in chlorine-free water to encourage production of sporangia and the release and spread of zoospores. External symptom assessment and plant mortality was recorded daily during four months. Root rot was not assessed. In October 2017, to confirm Koch's postulates, fine roots of inoculated plants were sampled, plated in PARPH selective medium, and incubated for 7 days at 25 °C (Martin-Garcia et al. 2015). *Pc* was successfully re-isolated from root samples collected in inoculated plants.

In vivo leaf physiological measurements

Gas exchange parameters such as stomatal conductance (g_s) and net leaf photosynthesis (A) were determined using 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 performed from 10.00 to 12.00 h at a photosynthetically active radiation (PAR) ranging from 300 to 500 μmol photons m^{-2} s^{-1}. Chlorophyll fluorescence *Fv*/*Fm* readings (the maximum quantum yield of PSII) were obtained from 8.00 to 10.00 h with a Multimode Chlorophyll Fluorometer OS5p device (Opti-Science Inc., USA) after adapting leaves to the dark for 30 min. Leaf chlorophyll content was evaluated through SPAD readings that were obtained at noon with a chlorophyll fluorescence meter (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, Inc., USA). All parameters were assessed in the same leaves of plant. Two apical fully expanded leaves in about 12 plants per clone and treatment were used.

Leaf and root sampling

Before and after inoculation, one leaf and five fine roots per tree were sampled, frozen and used for hormone and metabolite determination. Fully-developed leaves close to the shoot tip were used. The outermost fine roots were excised after carefully lifting the root ball from the pot. After collection, samples from five trees were pooled together to get a sample size of three replicates per clone and treatment. Samples were immediately frozen in liquid nitrogen and kept at -80 °C until further freeze drying with a FreeZone 6 L Benchtop (Labconco, Kansas City, USA). Subsequently, samples were ground in a ball mill (Mixer Mill MM 400, Retsch, Germany) to pass through a 0.42 mm screen, and used for further biochemical analysis.

Hormone extraction and quantification

The plant hormones salicylic acid (SA), jasmonic acid (JA) and its conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), abscisic acid (ABA), indolacetic acid (IAA) and the non-active derivatives of SA, 2-O-β-d-glucoside (SAG) and salicylate glucose ester (SGE) were determined. Hormone extraction was performed from dried powdered plant tissue following Sanchez-Bel et al. (2016). One milliliter of 10 % methanol aqueous solution containing a pool of deuterated and dehydrogenated hormonal internal standards was added to 50 mg of plant tissue. The mixture was vortexed and incubated (30 min, 4 °C) to allow for samples to rehydrate. After mixing in a mixer mill with glass beds (3 min, 30 Hz), samples were

centrifuged (30 min, 4 °C, 13.000 rpm) and the supernatant was recovered. The pH of the supernatant was adjusted to 2.5–2.7 with acetic acid and partitioned twice against diethyl ether. The two organic fractions were joined and concentrated in a centrifuge evaporator at room temperature until dryness. Samples were suspended in 1 ml of 10% methanol aqueous solution with 0.01% of HCOOH leading to a final concentration of internal standards of 100 ng ml−1. Quantification was performed using external calibration curves with each pure chemical standard. Hormones were chromatographically separated in an Acquity Ultra Performance Liquid Chromatography system (UPLC) (Waters, Mildford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex) connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The chromatographic and mass spectrometry conditions were those used by Gamir et al. (2012).

Metabolite quantification

Soluble sugars, proline, total polyphenols, condensed tannins and flavonoids were quantified by colorimetric methods. Soluble sugars were analyzed following modified protocols by Haissig and Dickson (1979) and Hansen and Moller (1975). For this purpose, 25 mg of powdered tissue were washed three times with a 5-ml mixture of methanol, chloroform and water (12:5:3) and extracts were combined. Then, 0.5 ml of supernatant was collected, incubated with 5 ml anthrone (10 min, 100 °C), and the absorbance was read at 625 nm using a spectrophotometer (Helios Beta, Spectronic Unicam, England). A D (+) glucose anhydrous standard curve was used for quantification. Proline was analyzed by slight modifications to the protocol explained in Bates et al. (1973). First, 20 mg of powdered tissue was homogenized with 1.5 ml of sulphosalicylic acid (3 %, w/v) and centrifuged (10 min, 4 °C, 10,000*g*). Then, 1 ml of supernatant was mixed with 1 ml of ninhydrin acid and 1 ml of glacial acetic acid, and the mix was incubated (30 min, 100 °C). After cooling down on ice, 2 ml of toluene were added and absorbance was read at 520 nm. A free proline standard curve was used for quantification. Total polyphenolics and condensed tannins were analyzed by the Folin-Ciocalteu and Porter methods, respectively. 50 mg of powdered tissue were extracted in 1 ml of 70 % aqueous methanol by

applying a sonic bath for 15 min followed by orbital shaking for one hour. After centrifugation (5 min, 4 °C, 10,000*g*), the supernatant was collected and used to determine total polyphenolics and condensed tannins. For the former, 0.2 ml of the 20-fold diluted extract was reacted with 1 ml Folin reagent and 0.8 ml sodium carbonate, and absorbance was read at 725 nm after 45 min in darkness. For condensed tannins, 45 µl of non-diluted extract were mixed with 1.5 ml Porter reagent, incubated (45 min, 70 °C) and cooled down on ice. Absorbance was measured at 550 nm and procyanidin B2 (Extrasynthese, GenayCedex, France) was used as standard. Flavonoids were analyzed with slight modification to the $AlCl_3$-$NaNO_2$-NaOH protocol described in Pękal and Pyrzynska (2014). Shortly, 20 mg of powdered tissue were washed 4 times with 1.25 ml of 70 % aqueous methanol, supernatants were combined and then brought to 10 ml volume with 70 % methanol, and frozen overnight (-80 °C). An aliquot was mixed with 5 % $NaNO_2$ and left in dark for 6 min. Then 10 % $AlCl_3$ was added and incubated for 6 min in dark, and 4 % NaOH was added. The solution was shaken and absorbance read at 510 nm with a plate reader (Synergy HT, BioTek Instruments, USA). For quantification, a standard curve of catechin was used. Three technical replicates per pooled sample were analysed and then averaged. Concentrations were expressed on a dry weight (DW) basis.

Statistical analysis

To analyse 'time-to-death' of plantlets and determine survival time probabilities after inoculation with *Pc*, the Kaplan–Meier estimate was used (Solla et al., 2011). Statistical differences between survival curves were tested by the log rank test. Metabolite and leaf physiological parameters were analyzed with generalized linear mixed models (GLMM), in which the factors 'susceptibility to *Pc*', 'inoculation with *Pc*' and their interaction were considered 'fixed' effects. To account for non independence of observations, the individual plant identity was included as 'random' factor in the models. For each organ, differences between susceptible and resistant interactions were tested through Tukey's HSD test with the Bonferroni correction. Principal component analysis (PCA) was applied to detect patterns of hormone and metabolite variation due to 'susceptibility to *Pc*' and 'inoculation with *Pc*'. Only

data corresponding to the hormones showing significant differences between susceptible and resistant interactions in either leaves or roots were included in the analysis. Data were checked for normality and homocedasticity with Shapiro-Wilk and Levene tests, respectively, and statistical analyses were performed in R software environment version 3.4.2 (R Foundation for Statistical Computing).

Results

Symptom development, plant mortality and changes in leaf physiology

Disease progression and changes in leaf physiology were markedly different between the clones (Figs. 1-3). Both clones developed aerial symptoms (leaf wilting and shedding) indicating *Pc*-induced damage (Fig. 1a and b) but, while in plants of the susceptible Cs14 clone the symptoms preceded sudden or progressive plant death, most of plants of the resistant 111-1 clone were able to recover and survive. Accordingly, strong differences in plant mortality were observed between the susceptible and resistant clones: twelve and two out of fifteen plants died along the experiment, respectively (Fig. 2). In the susceptible clone, values of A and g_s 9 days after inoculation were similar as before inoculation despite that half of plants turned yellow (Fig. 1a). By contrast, in plants of the resistant clone, values of A and g_s significantly decreased (Fig. 3a and b). *Fv*/*Fm* readings significantly decreased upon inoculation regardless of clone (Fig. 3c), and SPAD values were unaltered (Fig. 3d).

Figure 1. Leaf symptoms of 2-year-old chestnut clones 9 days after soil infestation with *Phytophthora cinnamomi*. In the susceptible clone Cs14 (a), foliage turned yellow, wrinkled and buds dried out, the leaves remaining attached to twigs after plant death. In the resistant clone 111-1 (b), foliage turned brown in an acropetal progression and most plants underwent subsequent defoliation, but buds were viable and new foliage rapidly developed.

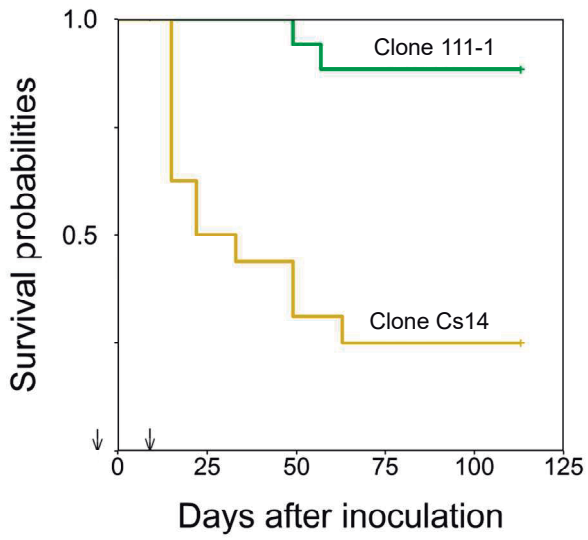


Figure 2. Plot of survival probabilities showing differences in tree mortality of chestnut clones Cs14 (susceptible) and 111-1 (resistant) after inoculation with *Phytophthora cinnamomi* ($P < 0.001$; log rank test, n=15 plants per clone). Arrows indicate time of plant measurements and tissue sampling (5 days before inoculation and 9 days after inoculation).

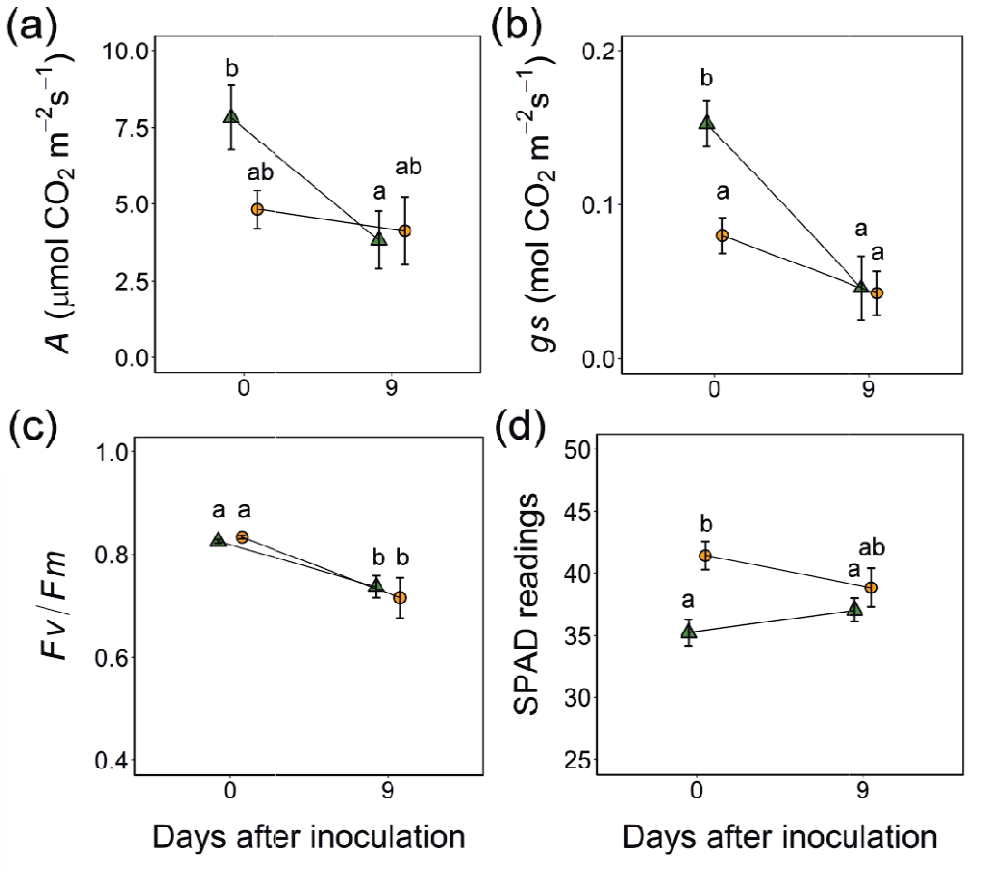


Figure 3. Effect of experimental inoculation of 2-year-old chestnut clones with *Phytophthora cinnamomi* on *A* (a), g_s (b), *Fv/Fm* (c), and SPAD readings (d). Measurements were done 5 and 9 days before and after inoculation with *Pc*, respectively. Error bars indicate one standard error of the mean (n = 10-15), while different letters indicate significant differences between clones and sampling points (Tukey's HSD test, $P < 0.05$).

Hormonal profile of leaves and roots

In the absence of infection, leaves of both clones displayed higher basal levels of hormones as compared with the roots (Figs. 4 and Supplementary 1). The resistant clone showed higher basal SA levels in roots than the susceptible clone while levels of JA, JA-Ile and ABA were similar. However, the resistant clone showed higher basal levels of JA, JA-Ile and ABA in leaves than the susceptible clone (Fig. 4). Constitutive levels of SAG plus SGE and IAA were similar in roots and leaves of both clones (Fig. Supplementary 1). At day 9 after inoculation both clones responded by decreasing JA levels in leaves and increasing them in roots. Moreover, the resistant clone displayed a dramatic increase of ABA and JA-Ile levels in roots, suggesting an enhanced response to the infection. No other hormonal change in leaves was detected in the

susceptible clone after infection and JA-Ile in leaves and roots was close to the detection limit. In the resistant clone, the levels of ABA, JA and JA-Ile in leaves were significantly reduced by infection (Fig. 4b-d). *Pc* had no significant impact on the accumulation of SA, SAG plus SGE and IAA neither in the susceptible nor in the resistant interaction (Fig. 4a and Supplementary 1a and b). Principal Component Analysis (PCA) based on content of SA, ABA, JA and JA-Ile in leaves and roots revealed segregation between the susceptible and resistant chestnut clones before *Pc* inoculation (Fig. 5a). PCA also showed a different hormonal profile between clones after infection. A strong impact in the roots of the resistant clone was observed according to the variation of the position of samples before and after infection (Fig. 5a).

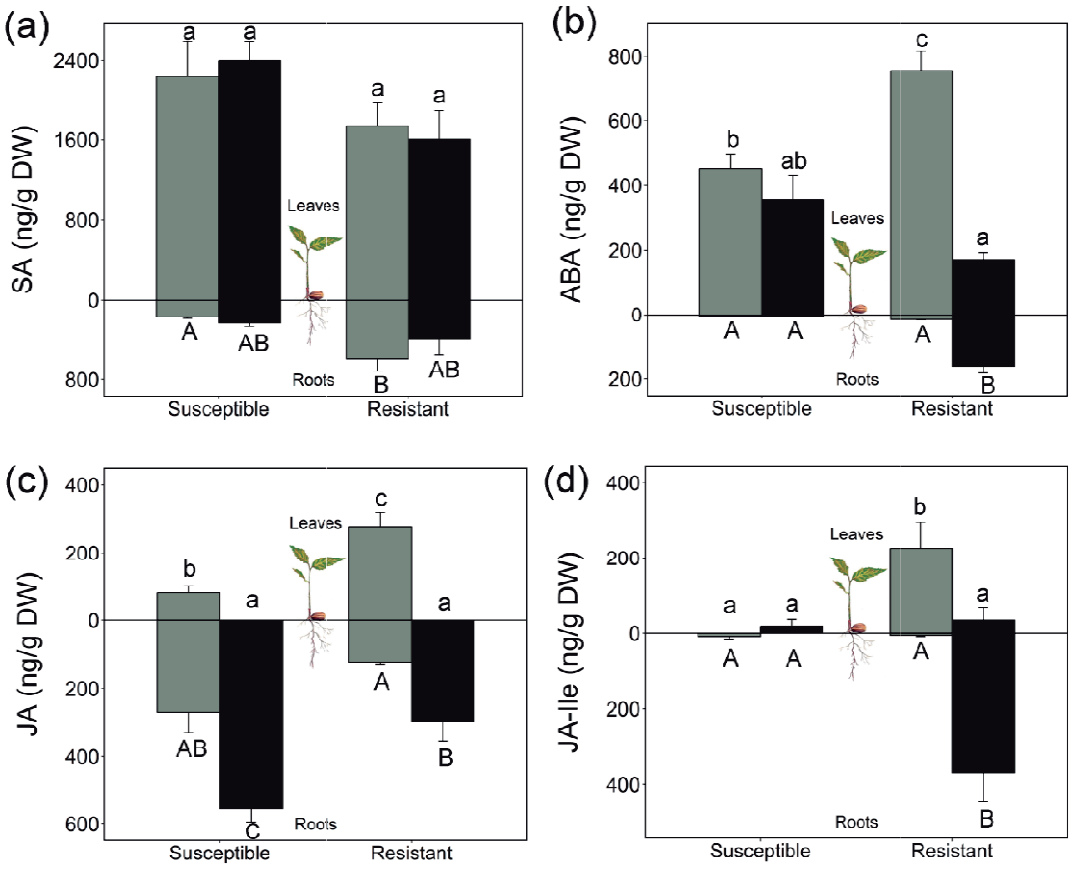


Figure 4. Hormonal content in leaves (above the zero-line) and roots (below the zero-line) of susceptible and resistant chestnut clones 5 days before (grey bars) and 9 days after (black bars) inoculation with *Phytophthora cinnamomi* for SA (a), ABA (b), JA (c) and JA-Ile (d). Note the distinct scales. Error bars indicate one standard error of the mean (n = 3), while different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between clones and sampling points within leaves (lower case letters) and roots (upper case letters).

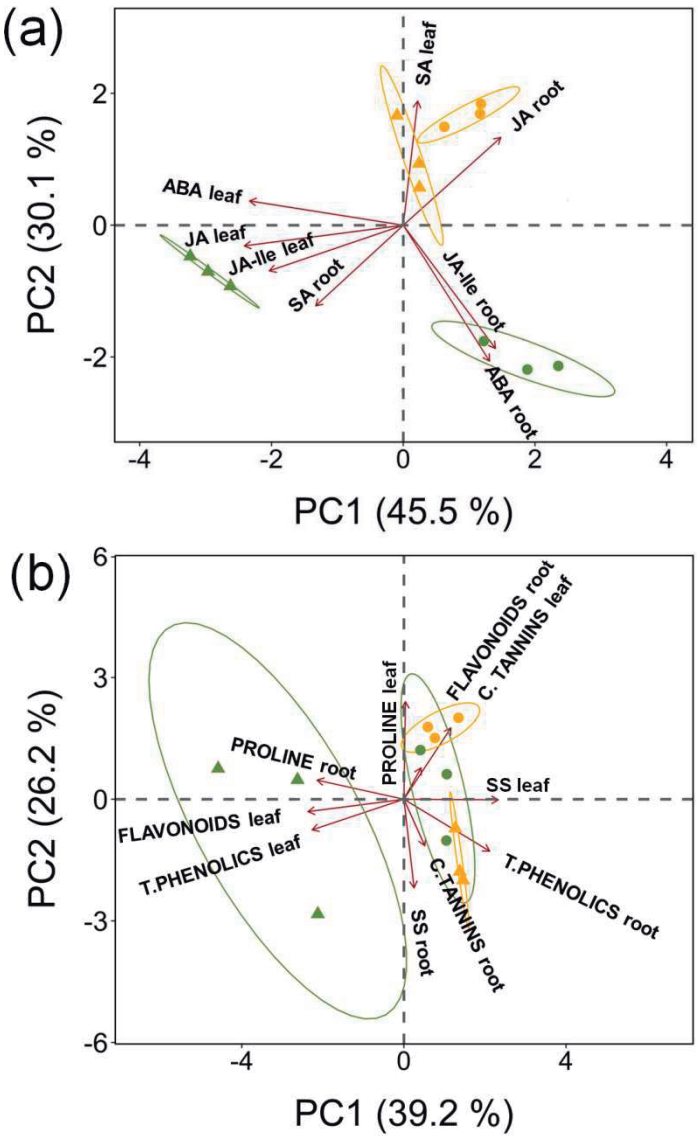


Figure 5. PCA biplots showing the ordination of non-inoculated (triangles) and inoculated (circles) plantlets of the susceptible (yellow) and resistant (green) clones along the two first principal components defined by (a) the content of SA, JA, JA-Ile and ABA in leaves and roots and (b) all the studied stress-related metabolites in leaves and roots. Names of variables are indicated along with their vectors, and the variance explained by each axis is shown in parenthesis. SS: soluble sugars.

Metabolites in leaves and roots

Before inoculation, the concentration of soluble sugars was higher in leaves of the susceptible clone than in leaves of the resistant clone (Fig. 6a), while proline was similar in both clones (Fig. 6b). Content of total polyphenolics and flavonoids were higher in leaves of the resistant clone (Fig. 6c and d). Roots had similar levels of constitutive metabolites between clones,

except for total polyphenolics and condensed tannins, which were higher in the susceptible clone (Fig. 6). After infection, leaf soluble sugars significantly increased in the resistant clone, whereas no change in root soluble sugars content was detected in none of the clones (Fig. 6a). The susceptible clone responded to *Pc* by increasing the levels of proline and condensed tannins in leaves, and the latter were reduced in roots (Fig. 6b and c). The resistant clone responded to the infection by reducing phenolics and by increasing condensed tannins in leaves (Fig. 6c), whereas in roots the opposite pattern was observed, as kind of compensation. The infection produced a reduction of flavonoids in the leaves of the resistant clone and in the roots of the susceptible clone (Fig. 6d). Similar to hormones, the PCA based on all the secondary metabolites studied showed a clear separation between non-inoculated susceptible and resistant plantlets (Fig. 5b), indicating a different preformed metabolite profile. This separation seemed to be mainly due to a different partitioning of total polyphenolics and flavonoids between leaves and roots in each clone (Fig. 5b). *Pc* had a strong impact in the behaviour of the secondary metabolites taken together since both the susceptible and the resistant clones positioned closer following infection (Fig. 5b). Changes in leaf proline and root total polyphenolics were responsible for the segregation between susceptible and resistant interactions after infection (Fig. 5b), confirming what was observed in Fig. 6.

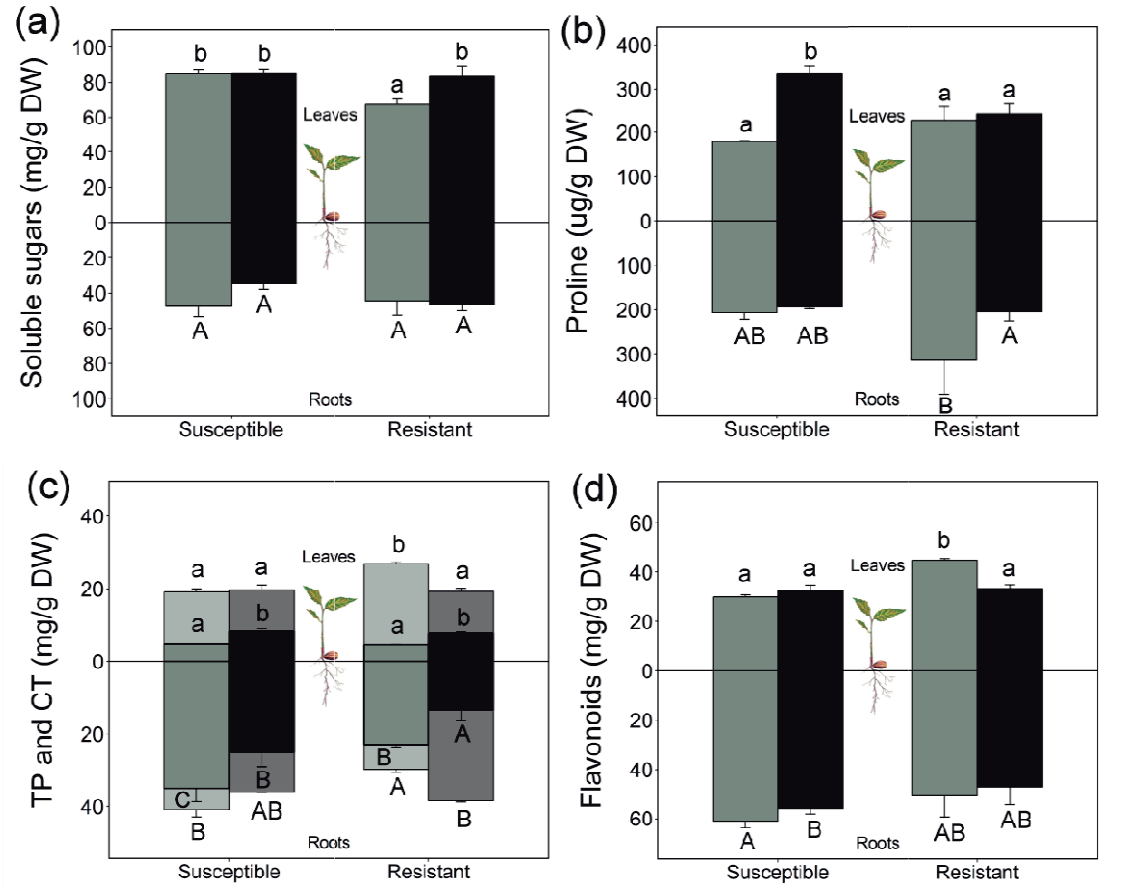


Figure 6. Secondary metabolite content in leaves (above the zero-line) and roots (below the zero-line) of susceptible and resistant chestnut clones 5 days before (grey bars) and 9 days after (black bars) inoculation with *Phytophthora cinnamomi* for soluble sugars (a), proline (b), total phenolics (TP, lighter bars) including condensed tannins (CT, darker bars) (c) and flavonoids (d). Note the distinct scales. Error bars indicate one standard error of the mean (n = 3), while different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between clones and sampling points within leaves (lower case letters) and roots (upper case letters).

Discussion

The two studied genotypes showed a contrasted defense-related hormone and metabolite profile, confirming the hypothesis that there are constitutive and *Pc*-induced differences in the hormone and metabolite contents of leaves and roots in chestnut.

Leaf physiology and general overview of metabolic responses of chestnuts during early interactions with Pc

In the present study, both chestnut clones showed foliar symptoms nine days after infection but leaf physiology and mortality patterns between clones were drastically different. Gas exchange parameters (g_s and A) were not significantly altered by *Pc* infection in the susceptible clone but in the resistant one, where *gs* and A decreased. This is in contrast to the decreased g_s and A reported by Dinis et al. (2011) 9 days after *Pc* infection in a susceptible chestnut cultivar while no change was observed in a *Pc* resistant chestnut hybrid. Weeks after infection, Maurel et al. (2001a) showed that g_s was substantially reduced in *C. sativa* seedlings under severe and very severe *Pc* root rot. Differences could have been due to the different plant material, inoculation method (inoculation in stem wound *vs* soil infestation) and the *Pc* strain used. However, the strong reduction in g_s and A in the resistant clone is in agreement with the strengthened metabolic response to infection observed in *Pc* resistant chestnuts (Dinis et al. 2011; Serrazina et al. 2015; Santos et al. 2017a). Decreased *Fv/Fm* during both susceptible and resistant interactions reflected changes in the efficiency of PSII of infected chestnuts, which could be attributed to increased photoinhibitory damage in response to *Pc* (Corcobado et al. 2015; Camilo-Alves et al. 2017). The general overview of the metabolic responses to *Pc* obtained by PCA analysis revealed a different constitutive metabolite and hormone fingerprinting between genotypes, and indicated a highly dynamic response of the resistant clone. This is in agreement with observations in leaf physiology. Results suggest that preformed accumulation of stress-related metabolites (ABA, JA, JA-Ile, flavonoids and total phenolics in leaves, and SA and proline in roots), and a dynamic accumulation of stress-related metabolites (JA-Ile, ABA and total phenolics in roots) are relevant for chestnuts to resist *Pc*. Differences in the hormone

profile between the chestnut clones suggests that more research has to be done using more genotypes.

Constitutive hormones and stress-related metabolites in leaves and roots of susceptible and resistant chestnut clones

In roots, 111-1 resistant trees displayed enhanced SA levels as compared with the susceptible clone before infection, which may have a positive influence during the very early stages of pathogenic penetration and disease establishment. SA has been widely described to trigger and coordinate defence against biotrophic pathogens (de Torres-Zabala et al. 2009; Denance et al. 2013), and is a component of the signal transduction pathway leading to defence reactions against pathogens *via* endochitinase production and H_2O_2 oxidative burst in *C. sativa* (Harfouche et al. 2008). After exogenous SA application, enhanced resistance to *Pc* due to SA accumulation in roots has been reported for *P. americana* and *Lupinus angustifolius* (Garcia-Pineda et al. 2010; Groves et al. 2015). In our study, JA-Ile was only constitutively detectable in leaves of the resistant clone. JAs-mediated signalling is involved in the production of antifungal metabolites in many plant species including *Castanea* spp. (Antico et al. 2012; Serrazina et al. 2015; Di et al. 2017). Whether the constitutive signalling by JA-Ile played a role in the defensive status of resistant plants is unknown, but this result suggests the use of hormones as potential stress biomarkers, which has been pointed out before but rarely applied (Kosakivska 2008). The constitutive JA-Ile should be explored across more chestnut taxa and genotypes. Phenolic compounds including simple phenolic acids and flavonoids are a large class of plant secondary metabolites involved in plant defence against herbivores and pathogens (Martin et al. 2008; Conrad et al. 2017; Gallardo et al. 2019), being also precursors of phytoalexins (Treutter 2006). The constitutive total phenolics and condensed tannins contents in roots of the susceptible clone, higher than in the resistant one, were not enough to hinder *Pc* at the site of infection. This is in line with root preformed phenol assessments made in *Eucalyptus* spp. before *Pc* inoculation (Cahill and McComb 1992; Cahill et al. 1993).

Pc-induced hormones and stress-related metabolites in leaves and roots of susceptible and resistant chestnut clones

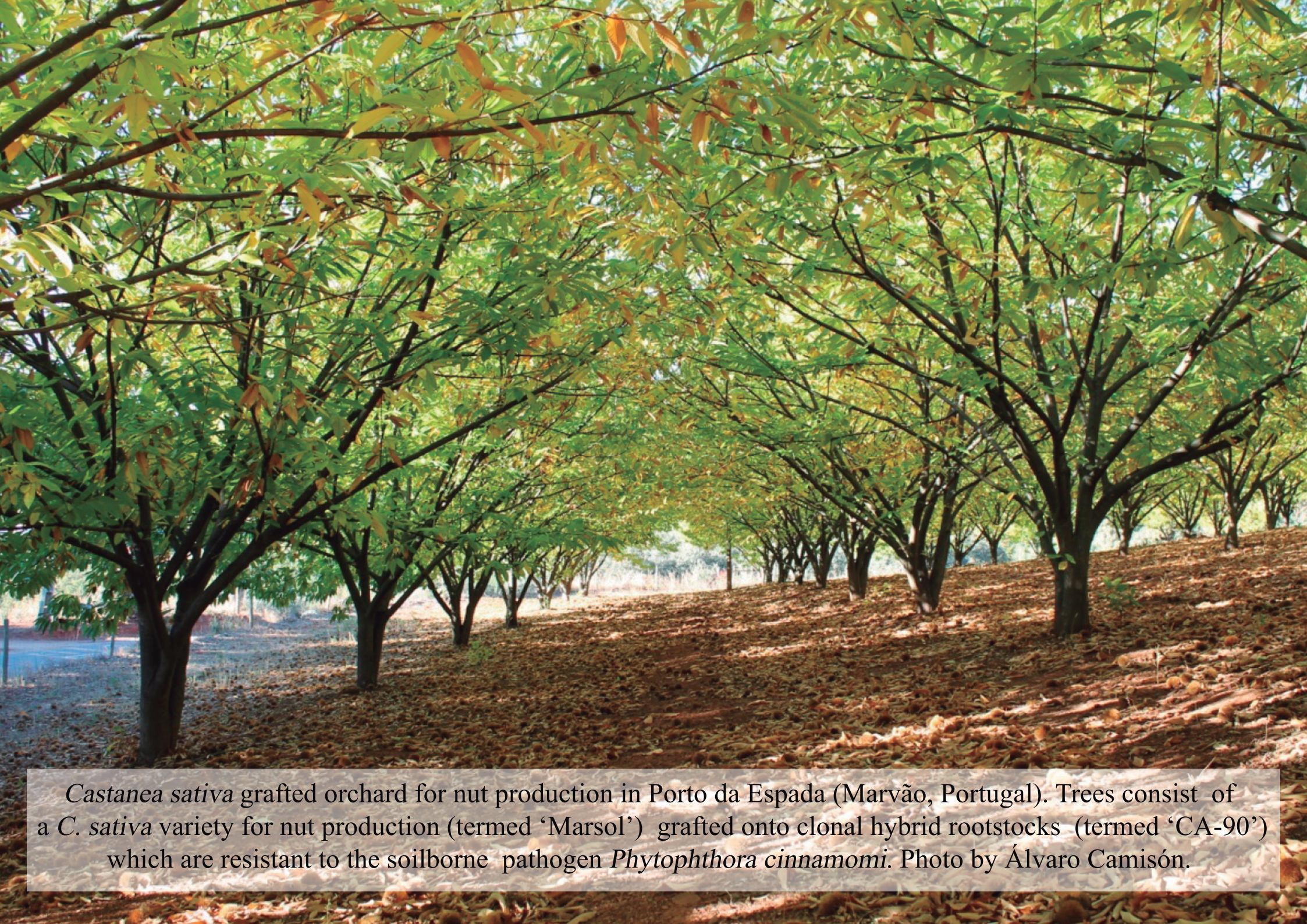
Unlike the susceptible clone, the resistant one showed a highly dynamic hormonal response by increasing the root JA-Ile and ABA levels following infection. ABA has recently emerged as a key player in callose deposition against necrotrophs and was linked to starch degradation and sugar mobilization in infected tissues of plants (Ton et al. 2009; Mohr and Cahill 2007; Liu et al. 2016a; Gamir et al. 2018). Because ABA modulates JAs-induced defences and acts synergistically with JAs on the expression of the MYC genes from the JAs pathway (Proietti et al. 2018), a positive synergistic effect of ABA and JA-Ile on the defence response of plants to *Pc* could be expected. This may explain why plants from the resistant clone recovered while plants from the susceptible clone started to die. Phytohormones regulate plant functioning and defence by regulating the expression of many hormone-responsive genes (Chapman and Estelle 2009). Therefore, differences in hormonal responses between susceptible and resistant chestnut clones in our study are in line with the more abundant and higher expressed defence genes involved in response to *Pc* of the resistant *C. crenata* as compared with *C. sativa* (Serrazina et al. 2015). Several studies have shown that defence strategies of plants are trophic dependent (van den Berg et al. 2018 and references therein). For example, a biphasic defence response has been reported to occur in *P. americana* when facing *Pc*: an initial biotrophic plant-based response followed by the enrichment of JA-mediated defence during the necrotrophic phase (van den Berg et al. 2018). Because of the enrichment of JA and JA-Ile in roots after infection, our results suggest a necrotrophic plant-based response occurring during the expression of aerial symptoms. This in turn provides evidence that at 9 days after infection *Pc* has a necrotrophic lifestyle, which is in agreement with histological observations in the *Pc*-susceptible *Quercus ilex* tree (Redondo et al. 2015). The susceptible genotype was not able to convert increased JA levels into the active form of the hormone (JA-Ile). On the contrary, this step was strongly induced in the resistant 111-1 plants. We ignore if these differences are related to pathogen hijacking of the JA-dependent responses in the susceptible clone, but they should be the objective of further studies. JA-dependent signalling has been described to be a target for

pathogen effectors that interact with JAZ repressors in order to avoid effective plant defences (Kazan and Lyons 2014; Shen et al. 2018). *Pc* (as other *Phytophthora* species) is able to release a plethora of effectors highly efficient in manipulating and hijacking plant host defences (Hardham and Blackman 2018). Increases in the concentration of the amino acid proline were reported in plants both under water deficit and pathogen attack (Kaur and Asthir 2015; Cerqueira et al. 2017). In this study, proline showed a clone- and organ-specific dynamic in response to infection, increasing in leaves in the susceptible clone and decreasing in roots in the resistant clone. In the susceptible clone, proline could have increased due to *Pc*-induced water stress, which is consistent with the wrinkling of leaves observed. Sixteen days after inoculation, larger reductions in leaf water potential in susceptible *C. sativa* (93 %) plantlets than in resistant hybrid plantlets (36%) were reported (Dinis et al. 2011). In the resistant clone, decrease of proline in roots could have been occurred by stimulation of the proline-P5C cycle, although this is just a hypothesis. In *A. thaliana*, proline catabolism has been associated to pathogen defence (Cecchini et al. 2011). The increased concentration of leaf soluble sugars in the 111-1 clone after infection was also reported in a *Pc* resistant hybrid chestnut (Dinis et al. 2011), in agreement to the often reported inducible immunity mechanism in plants known as 'high-sugar resistance' (Reimer-Michalski and Conrath 2016). The increased concentration of root total phenolics in the 111-1 clone is in agreement with the often observed accumulation of phenolics in roots after *Pc* infection in resistant interactions with *Pc* (Osswald et al. 2014). Phenols can be polymerized into a matrix of lignin, reinforcing cell walls, or act as antimicrobial compounds (Martin et al. 2008; Osswald et al. 2014). The role of condensed tannins in the susceptible and resistant chestnut-*Pc* interactions is difficult to clarify, as they behaved similarly in the two genotypes. However, because of the negative crosstalk with total phenolics observed in leaves and roots of the resistant genotype after infection (Fig. 6c), the involvement of condensed tannins in a dynamic response of total phenolics to resist *Pc* cannot be ruled out. Results suggest that the switch of total phenolics between the roots and leaves during infection in resistant plants may be responsible for a better defensive status of trees. Considering that both families of compounds utilize the same phenolic acid precursors, the degradation of condensed tannins may

constitute a dynamic supply of phenolics contributing to this response. Reductions of root total flavonoids observed here are in agreement with reductions of the flavonoid epicatechin in roots of susceptible *P. americana* seedlings upon *Pc* infection (Garcia-Pineda et al. 2010). Given the antioxidant functions that flavonoids perform, our results could indicate decreased antioxidant capacity in roots of the susceptible clone, as shown in susceptible *Eucalyptus* spp. after *Pc* infection (Dempsey et al. 2012).

Conclusions

This study explored the relevance of stress-related signals like hormones to better understand *Pc* resistance mechanisms in chestnut by describing and comparing alterations of hormone content in leaves and roots during early stages of susceptible and resistant interactions with *Pc.* A more dynamic response of hormones and metabolites across organs in the resistant clone, linked to a synergistic crosstalk between ABA and JA-Ile in roots was observed. The lack of effective hormonal changes in the susceptible clone agrees with the weak defence responses of *C. sativa* to *Pc*. Because constitutive and *Pc*-induced levels of JA-Ile were only detectable in the resistant clone, quantification of this hormone in additional genotypes should be done. The use of only two clones of contrasting resistance limits the generalization of findings.



Castanea sativa grafted orchard for nut production in Porto da Espada (Marvão, Portugal). Trees consist of a *C. sativa* variety for nut production (termed 'Marsol') grafted onto clonal hybrid rootstocks (termed 'CA-90') which are resistant to the soilborne pathogen *Phytophthora cinnamomi*. Photo by Álvaro Camisón.

CHAPTER IV. Assessing scion and grating effects on the hormone profile, vegetative budburst, growth and susceptibility to ink disease of *Castanea sativa*

Abstract

Ink disease caused by the root-rot pathogen *P. cinnamomi* (*Pc*) threatens European sweet chestnut (*Castanea sativa* Mill.) orchards so *C. sativa* traditional varieties are increasingly grafted onto *Pc*-resistant hybrid rootstocks. The influence of the scion and grafting on the hormone profile, vegetative budburst, growth and susceptibility of *Castanea spp.* rootstocks to *Pc* are unknown. In a greenhouse experiment, two-year old non-grafted controls, autografts and reciprocal grafts between two *Pc* resistant (R) *C. crenata* x *C. sativa* clones and two *Pc* susceptible (S) *C. sativa* clones were used to evaluate budburst phenology, plant growth and mortality after inoculation with *Pc*. Leaves and roots of non-grafted controls, autografts and reciprocal grafts between the R clone 111-1 and the S clone Cs14 were sampled for analysis of defense-related hormones before and during *Pc* infection. The scion had no effect on the hormone profile and resistance to *Pc* of grafted chestnut trees, and they both depended on the rootstock. Grafting significantly influenced the hormone profile, vegetative budburst, and increased susceptibility to *Pc* in S and R rootstocks. Hormonal alterations by grafting were likely mediated by a wounding effect, which could drive the increased susceptibility of grafted trees to *Pc*. In roots of the R clone, grafting decreased SA while increasing JA-Ile content leading to significantly reduced SA-to-jasmonates (JA and JA-Ile) ratios, which may have negative consequences for *Pc* resistance. Grafting also modified the hormonal response of both clones to *Pc* infection and grafted plants accumulated SA in leaves in response to *Pc*, which could be related to the induction of SAR. The study reveals the importance of grafting in shaping phenotypical variation in chestnut and calls to more research to be done in grafted chestnut trees.

Keywords: hormonal profiling, orchard, SA-to-jasmonates ratio, systemic signaling, wounding

Introduction

Grafting is an horticultural technique in which two plant tissues fuse together, establish a vascular continuity and raise a new composite organism that functions as a whole (Mudge et al. 2009). After grafting, the upper part or scion of one plant grows on the root system or rootstock of another plant. Self-grafted plants (autografts) use to be fully compatible while the success of grafting different plants (heterografts) diminishes if their phylogenetic distance increase (Mudge et al. 2009; Goldschmidt 2014). Applications of grafting include vegetative propagation of cultivars, control of plant size, and resistance enhancement of plants to abiotic and biotic stress (Mudge et al. 2009).

One of the most intriguing aspects of grafting is that the physiology of the scion can be modulated by the rootstock (Warschefsky et al. 2015). Phytohormones coordinate locally and systemically the development and defence of plants to abiotic and biotic stresses, regulate the gene expression and their effect is graft-transmissible (Kalantidis 2004; Guan et al. 2012; Wang et al. 2017). Phytohormones move across the graft junction and take part in the rootstock-scion communication (Aloni et al. 2010; Wang et al. 2017). Changes in hormone content in grafted plants may explain why the rootstock controls the growth and the size of a scion (Sorce et al. 2002; Hooijdonk et al. 2011; Tworkoski and Fazio 2016), its vegetative bud burst (Tworkoski and Fazio 2016), and its resistance, for example by inducing systemic defence to bacterial and fungal diseases in leaves (Jensen et al. 2003; Russo et al. 2007; Jensen et al. 2012; Chitarra et al. 2017). Grafting susceptible cultivars into resistant rootstocks is a very effective way to protect crops against soil-borne plant pathogens (Guan et al. 2012). Most research on grafting-induced physiological changes in trees has been addressed from the point of view of the rootstock influencing the scion (Warschefsky et al. 2015; Wang et al. 2017). Very little is known about the effect of scions on the physiology and resistance of rootstocks (Tandonnet et al. 2010; Warschefsky et al. 2015; Huang et al. 2019).

Grafting implies wounding a plant. In the same way as pathogens alter the hormonal profile of infected trees during attack (de Torres Zabala et al. 2009; Pozo et al. 2014; Camisón et al. 2019b), damage caused by grafting could also be expected to alter the hormonal profile of

trees (Gainza et al. 2015; Wang et al. 2017). In apple trees, the root growth rate of rootstock was repressed significantly by grafting and mainly influenced by hormone signalling pathways (Li et al. 2016). In pepper, incidence of the soil-borne pathogen *Ralstonia solanacearum* increased after grafting (Abebe et al. 2016). The effect of grafting in the susceptibility of trees to soil-borne pathogens has been rarely investigated (Guan et al. 2012). Moreover, it is ignored how grafting influences the content of defense-related hormones in trees, and if the impact of tree-grafting on the hormone content is highest in scion or in rootstock.

Castanea sativa Mill. (Sweet chestnut, *Fagaceae*) is an important tree species in Europe for its edible nuts and valuable wood. Grafting is currently used to grow profitable *C. sativa* cultivars onto hybrid rootstocks resistant to ink disease (Fernández-Lorenzo and Crecente-Campo 2010). Ink disease in chestnuts is caused by *Phytophthora cinnamoni* Rands. (*Pc*), an invasive soil-borne pathogen widespread worldwide (Scott et al. 2019). Since the 18th century in southern Europe *Pc* has led to large economical losses for chestnut farmers (Alcaide et al. 2020). The main strategy to control ink disease in Europe consists of using hybrid rootstocks, resistant to *Pc*, obtained after crossing *C. sativa* with Asiatic *C. crenata* and *C. mollissima* species.

ºTwo hypotheses justified the need of this work: (i) the scion has an effect on the hormone profile and resistance to *Pc* of the rootstock, and (ii) grafting influences the hormone profile, vegetative budburst, growth and resistance to *Pc* of trees. The main objectives were to test previous hypothesis in chestnut. A reciprocal grafting experiment including *Castanea* spp. clones with contrasted susceptibility to *Pc* was performed.

Materials and methods

Plant material

Four *Castanea* spp. clones of known and contrasted susceptibility to *Pc* were used. The two clones 'Cs-12' and 'Cs-14' are *C. sativa* and susceptible to *Pc*. Originally from the North-Atlantic coast of Spain (Galicia), they were selected because grafted well and were characterized in previous studies (Camisón et al. 2019b; Alcaide et al. 2020). The '111-1' and

'PO11' clones are *C. sativa* × *C. crenata* hybrids. Because they are resistant to *Pc* (Camisón et al. 2019b; Alcaide et al. 2020) and show a high degree of compatibility with Iberian traditional varieties of *C. sativa* (Cuenca et al. 2018), these clones are used as rootstocks and widely planted in Spain. The hybrid clones used in this study are early flushers and highly vigorous in comparison to the *C. sativa* clones used. In August 2015, clones were micropropagated *in vitro* according to Vidal et al. (2015) and grown in a greenhouse in 2-L pots with a mixture of peat, vermiculite and perlite (1:1:1). In August 2016, some plantlets from each clone were grafted by 'green grafting' (Cuenca et al., 2018) by a specialized tree nursery (Grupo TRAGSA-SEPI, Maceda, Spain), and some others were not grafted. Grafts were performed in the main stem. In October 2016, the plant material was placed at the greenhouse of the Faculty of Forestry of Plasencia, western Spain (40º02´N, 6º05´W; 374 m asl,), fertilized with Osmocote Pro 3-4M (Osmocote® Pro) at 4 g L^{-1}, and grown under optimal watering conditions.

Experimental design

To test if the scion has an effect in the hormone profile and resistance to *Pc* of the rootstock (first hypothesis, see Fig. 1a), reciprocal grafting between clones of different susceptibly to *Pc* was performed. In consequence, scions from the resistant clones were grafted onto the susceptible clones, which acted as rootstocks (R/S heterografts), and scions from the susceptible clones were grafted into the resistant clones (S/R heterografts). Susceptible and resistant clones were also self-grated and were included as controls (S/S and R/R autografts, respectively).

To test if grafting influences the hormone profile, vegetative budburst, growth and resistance to *Pc* of chestnut plants (second hypothesis, see Fig. 1b), the above grafted plant material (S/S, R/R, R/S and S/R) was compared with non-grafted plant material (S and R controls). In this way, the 'effect of grafting', if any, could take into account the susceptibility to *Pc* of the rootstock used.

The experiment included 16 plant combinations, of which four were non-grafted controls (one per clone), four were autografts (one per clone), and eight were reciprocal grafts between scions and rootstocks of different susceptibility to *Pc* (two per clone). Sample size for

each plant combination ranged from 9 to 18 plants (mean = 13.5). To avoid confusion with the codes, S_{Cs12} and S_{Cs14} plants were considered S plants, $R_{111\text{-}1}/S_{Cs12}$, $R_{111\text{-}1}/S_{Cs14}$, R_{PO11}/S_{Cs12} and R_{PO11}/S_{Cs14} were considered R/S plants, and so on. In consequence, the different clones were used as replicates, and the plant material was merged into eight groups of plants (Table Supplementary 1). The grafting heights were 23.7 and 39.9 cm in susceptible and resistant rootstocks, respectively, because of differences in twig diameter between plants. Stem diameters at the ground level of S plants were narrower than those of R plants (6.2 ± 0.1 and 7.4 ± 0.1 cm, respectively; $P < 0.001$, *t*-test). Plants were arranged at the greenhouse of the Faculty of Forestry of Plasencia using a complete randomized block design of four blocks.

Vegetative budburst and plant growth assessment

Vegetative budburst, primary growth and secondary growth were assessed in all 212 trees of the experiment. Bud development was assessed in April 2017 as follows (Solla et al. 2015): 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. Primary growth was obtained before inoculation by the difference of plant height in January 2017 and July 2017. Secondary growth was obtained before inoculation by the difference of stem diameter in January 2017 and July 2017. Stem diameters were calculated by the average of two measurements made orthogonally ca. 5 cm from the ground level, where a white stripe in January was painted. Diameters in July were measured at the stripes.

Plant resistance to Pc *inoculations*

In July 6 2017, when the trees were two years old and 108.9 ± 35.8 cm in height (Table Supplementary 1), all the plant material was inoculated with *Pc* through the soil infestation method. An aggressive single A2 strain (coded Ps-1683) isolated from a declining *C. sativa* tree in Galicia (northern Spain) was used. The inoculum was prepared according to Jung et al. (1996) and incubated for 5 weeks in 2-L Erlenmeyer flasks. Soil infestation was done by mixing

12 ml of the inoculum with the first 3 cm of soil for each plant. After inoculation, the substrate was moistened by slight watering and the day after plants were flooded for 48 h in chlorine-free water to encourage the production and releasing of zoospores. Plant mortality was assessment weekly for four months. At the end of the experiment, in October 2017, *Pc* was successfully re-isolated from inoculated roots.

Hormone profile assessment

Fifty trees from each of the six groups of plants described in Table Supplementary 1 were sampled for hormone determination. Sampling was performed only in trees including 111-1 and Cs14 clones whose hormone and secondary metabolite profiling in response to *Pc* is described in the previous chapter of this thesis. Sampling was performed aboveground and belowground, 5 days before inoculation (July 1 2017) and 9 days after inoculation (July 15 2017). Aboveground sampling consisted of collecting the apex from one fully-developed top-stemmed leaf. Belowground sampling consisted of carefully excising five outermost fine root segments from the root ball of each plant. After collection, samples were immediately frozen in liquid N and samples from five different plants within each group were pooled together to get a sample size of three biological replicates per group of plants. 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.

Four plant hormones related to signaling of plant defense against pathogens, salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), and its conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) were determined. Hormones were extracted and quantified exactly as described in chapter III of this thesis, and therefore the reader is referred to chapter III for details on the protocol.

Data analysis

To assess the effect of the scion (or grafting) in the phenology and growth of plants, 'vegetative budburst' values and 'primary growth' and 'secondary growth' measurements were considered as dependent variables. 'Scion resistance to *Pc*' (or 'grafting'), 'rootstock resistance to *Pc*' and their interactions were considered as fixed effects. To assess the influence of the scion (or grafting) in the susceptibility of the rootstock, a logistic model was constructed using 'mortality' as the dependent variable (parametrized as 0 or 1 if the plant survived or not, respectively), and the same fixed effects described above. Previous models considered 'block' and 'clone' (nested within the 'rootstock resistance to *Pc*') as random factors, and 'plant height' as a covariate. The 'grafting height' (Table Supplementary 1) was not included in the models because of lack of significance. To assess life expectancies of different groups of plants after *Pc* inoculation, Kaplan-Meier curves representing survival probabilities through time were constructed (Solla et al. 2011). Differences between curves were tested with the log-rank test.

To assess the effect of the scion in the hormone profile of the rootstock, and *vice versa*, before and after challenging plants with *Pc*, linear models were used. In these models, 'SA', 'ABA', 'JA' and 'JA-Ile' hormones were the dependent variables, and 'scion resistance to *Pc*' (two levels, *S* or *R*), 'rootstock resistance to *Pc*' (*S* or *R*), 'inoculation with *Pc*' (two levels, *no* or *yes*), 'organ' (two levels, *leaf* or *fine root*) and their interactions were considered as fixed effects. To assess the influence of grafting in the hormone profile of plants, similar models were performed, this time considering 'grafting' (two levels, *no* or *yes*), 'rootstock resistance to *Pc*', 'inoculation with *Pc*', 'organ' and their interactions as fixed effects. Differences between means were tested with Tukey's HSD tests. To identify variation patterns in the hormone profile of groups of plants, Principal Component Analysis (PCA) of hormones in leaves and fine roots, before and after *Pc* inoculation were used. Data were checked for normality and homocedasticity with Shapiro-Wilk and Levene tests, respectively, and properly transformed when necessary prior to analysis. Statistical analyses were performed in R software environment version 3.4.2 (R Foundation for Statistical Computing).

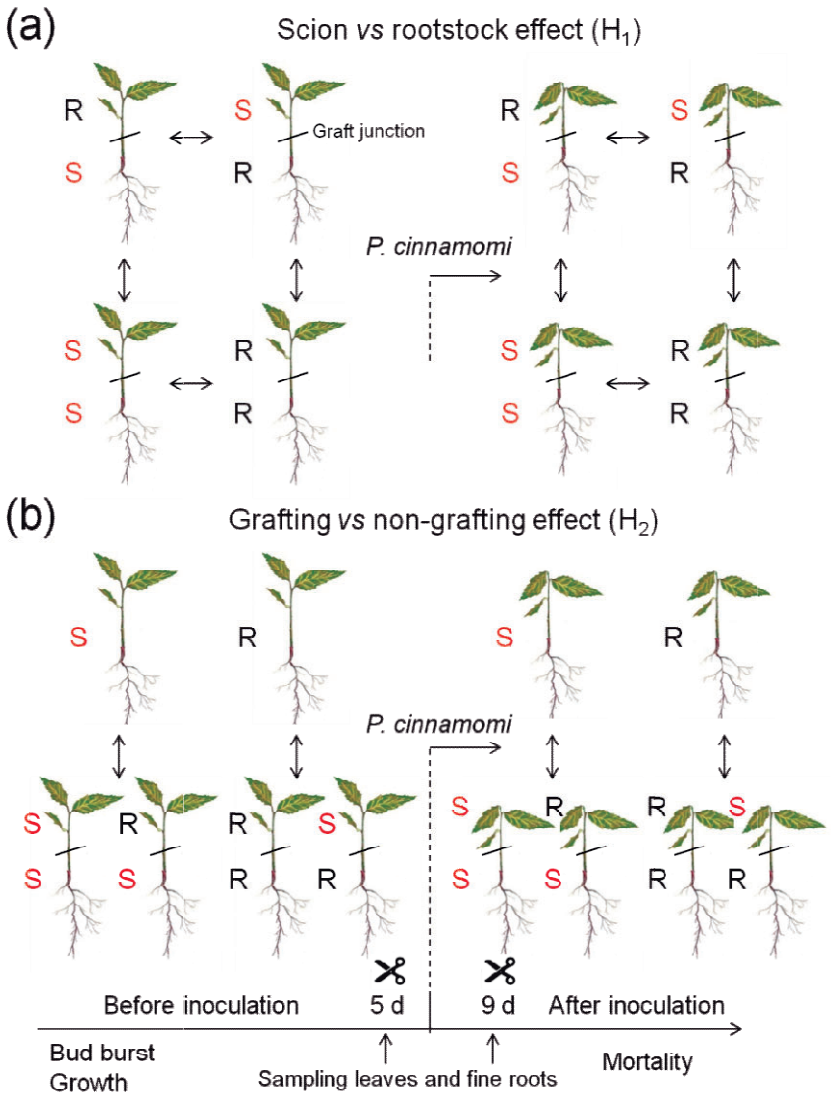


Figure 1. Experimental design to test if scion *vs* rootstock (a) and grafting (b) have an effect in the hormone profile, budburst, growth and resistance to *Phytophthora cinnamomi* (*Pc*) of *Castanea* spp. grafted trees. Two clones resistant (R) to *Pc* and two clones susceptible (S) to *Pc* were used. Sampling dates are indicated with scissors.

Results

Budburst, growth and mortality in response to Phytophthora cinnamomi *of chestnut grafts*

Vegetative budburst and primary growth were mainly determined by the scion and its interaction with the rootstock (Table 1; Fig. Supplementary 2a). R scions flushed earlier and grew more in height than S scions (4.2 *vs* 3.0 budburst values and 72 *vs* 66 cm, respectively), but differences were exacerbated when R scions were grafted onto a susceptible rootstock instead of onto a resistant rootstock (4.7 *vs* 3.1 and 3.2 *vs* 2.9 values for budburst, and 82 *vs* 65

and 68 *vs* 67 cm for height growth, respectively) (significant S × R interaction, Table 1; Fig. Supplementary 2a). Secondary growth and mortality due to *Pc* of rootstocks were not influenced by the scion (Table 1). Mortality and life expectancy of chestnut were determined by the susceptibility of the rootstock (Fig. 2a), being 92 *vs* 38% and 38 *vs* 85 days if susceptible *vs* resistant rootstocks were inoculated, respectively. Tall plants grew more and survived longer than short plants (results not shown).

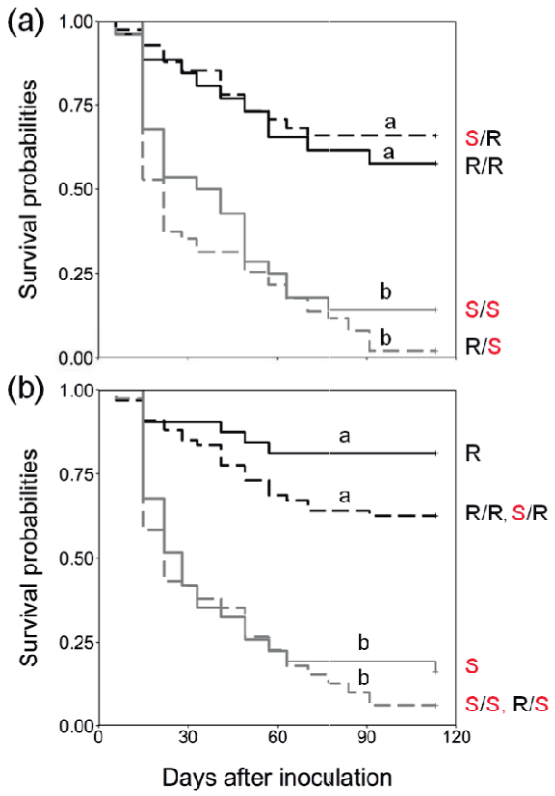


Figure 2. Kaplan-Meier survival curves representing survival probabilities after inoculation with *Pc* of susceptible (S) and resistant (R) non-grafted, self-grafted (S/S and R/R) and heterografts (R/S and S/R) of *Castanea* spp. trees. In (a), the effect of the scion resistance to *Pc* on plant survival depending on the resistance to *Pc* of the rootstock is shown, and the effect of grafting on plant survival depending on the resistance to *Pc* of the rootstock is shown in (b). Different letters indicate significant differences between survival curves ($P < 0.05$; log rank test).

Grafting significantly accelerated vegetative budburst of plants and allowed them to grow more in height and in stem diameter (Table 2; results not shown). Mortality of chestnut due to *Pc* was significantly determined by grafting irrespective of the rootstock resistance to *Pc* (non significant grafting x rootstock resistance to *Pc*, Table 2), being 67 and 51% if grafted and non-grafted chestnuts were inoculated. Survival analysis revealed that differences between life expectancies of grafted and non-grafted chestnuts (59 vs 71 days) were marginally significant ($P = 0.085$) (Fig. 2b). Considering average values, grafting increased 10 and 102% the mortality to *Pc* of susceptible and resistant un-grafted rootstocks (Table Supplementary 1, Fig. 2b).

Hormone profiling of chestnut grafts in response to Phytophthora cinnamomi

The most significant effects when assessing the reciprocal influence of scion *vs* rootstock in the hormone content of *Castanea* spp. grafted trees were the 'organ' and the 'organ' × 'inoculation with *Pc*' interaction (Table 3). SA and ABA contents were higher in leaves than in fine roots while for JA and JA-Ile it was the opposite (Fig. 3). When challenged with *Pc*, SA significantly increased only in leaves and JA significantly decreased only in leaves (Fig. 3). Scions did not significantly influence the hormone profile of grafted chestnut trees (Table 3). However, the rootstocks significantly determined SA and JA-Ile content of grafted trees (Table 3), being higher in trees grafted into R rootstocks than in trees grafted into S rootstocks (1902 ± 416 *vs* 1133 ± 212 ng/g DW, and 236 ± 24 *vs* 35 ± 25 ng/g DW for SA and JA-Ile, respectively). Previous differences were especially relevant for SA in leaves and for JA-Ile in roots, as indicated by the significant R × O interactions of the models used (Table 3). When challenged with *Pc*, SA content increased ca. 100% more in leaves of trees grafted onto R rootstocks than in leaves of trees grafted onto S rootstocks; JA-Ile decreased only in leaves of trees grafted onto R rootstocks; and JA-Ile increased only in roots of trees grafted onto R rootstocks (Fig. 3) (significant R × O × I interactions, Table 3).

Grafting influenced the SA content of chestnut plants (Table 4). Constitutive SA in leaves of the S rootstocks and in roots of the R rootstocks were diminished by grafting although not significantly in roots of R rootstocks (Fig. 3a-b). However, the non-significant decrease in root SA translated into significantly lower root SA-to-JA ratios in grafted R plants as compared to non-grafted controls (5.01 ± 2.26 *vs.* 1.15 ± 0.55 for R (non-grafted) *vs* R/R and S/R (grafted) plants, respectively (P = 0.010)). A similar trend was obtained regarding SA-to-JA-Ile ratios (data not shown). After inoculation with *Pc*, SA increase by grafting occurred more in leaves than in roots, especially in leaves of plant material including a resistant rootstock, which explains the significant interactions indicated in Table 4. The effect of grafting on ABA content depended on the organ, the rootstock resistance to *Pc* and the inoculation with *Pc* (significant G × R × O × I interaction, Table 4). Although not significantly, grafting increased the constitutive leaf (and not root) ABA content only in grafts with a susceptible rootstock while only grafts

with a resistant rootstock responded to *Pc* infection by increasing root ABA content. Grafting did not significantly alter JA content in any organ. JA-Ile content in roots of grafted plants was higher than JA-Ile content in roots of non-grafted plants (Fig. 3h), especially when using an R rootstock (significant effect of 'rootstock resistance to *Pc*', Table 4), and was similar in leaves of non-grafted and grafted plants (significant G × O interaction, Table 4, Fig. 3g).

PCA overview of the constitutive and Phytophthora cinnamomi*-induced hormonal profiles of chestnuts*

PCA based on the constitutive hormonal profile of leaves and fine roots revealed a clear segregation between R/S and S/R groups of trees (Fig. 3a-b). After *Pc* infection, segregation between R/S and S/R groups of trees was only observed for fine roots (Fig. 3c-d). Differences between R/S and S/R groups of trees were mainly associated to JA-Ile content in fine roots (Fig. 3b and d). PCA segregation was more attributable to resistance of rootstocks to *Pc* than to the resistance of scions and the effect of grafting (Fig. 3). Contents of JA and JA-Ile in leaves and SA and ABA in roots appeared to be responsible for segregation between S and R rootstocks before infection (Fig. 3a-b). Discrimination between S and S/S plants was due to differences in the constitutive SA and ABA contents in leaves (Fig. 3a), whereas discrimination between R and R/R plants was due to differences in the constitutive JA and JA-Ile in fine roots (Fig. 3b).

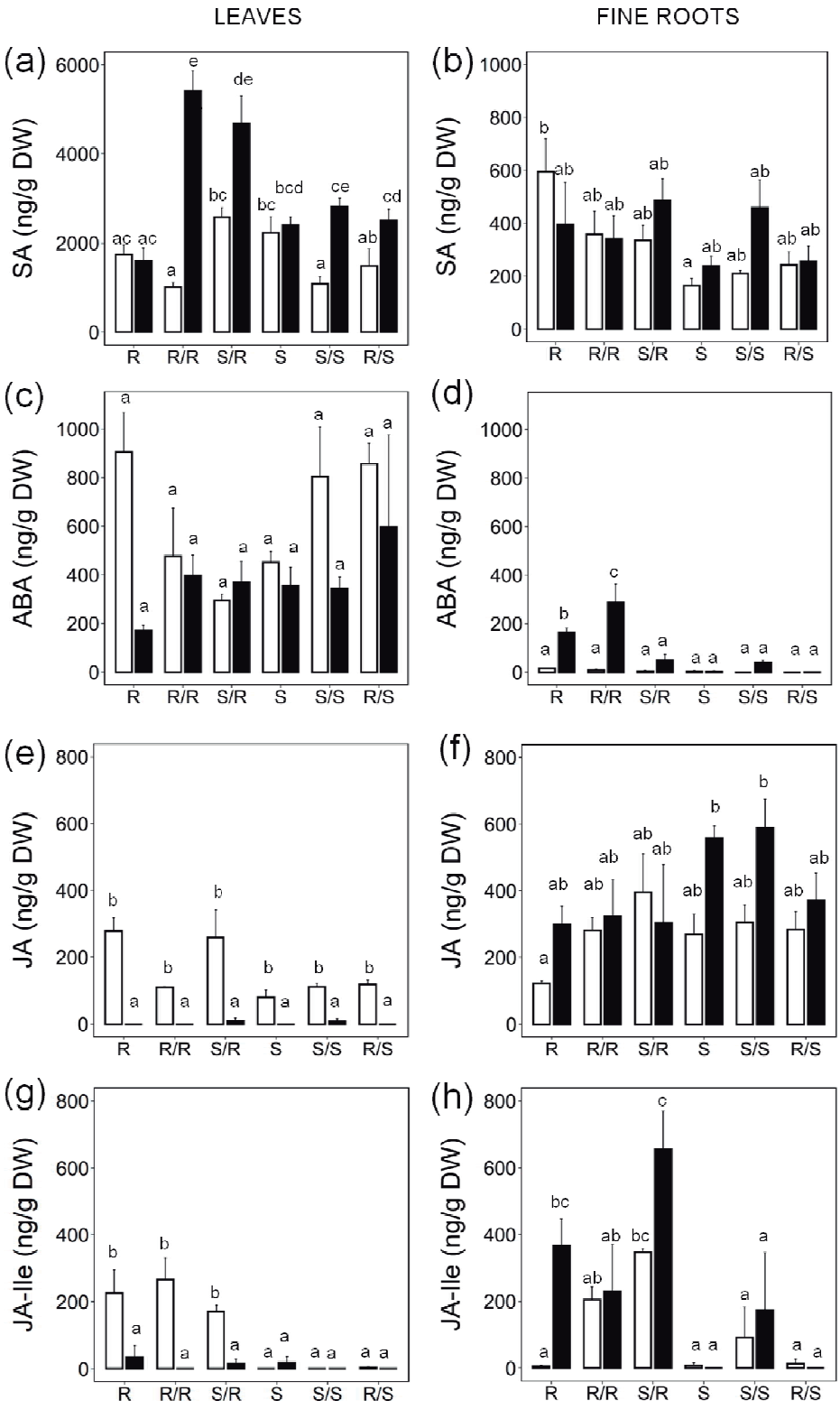


Figure 3. Salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) contents in leaves and fine roots of *Castanea* spp. trees before (white bars) and after inoculation with *Phytophthora cinnamomi* (*Pc*) (black bars). Trees included resistant (R) and susceptible (S) clones, resistant (R/R) and susceptible (S/S) autografts and heterografts (S/R, R/S). Bars indicate standard error of the mean (n = 3) while different letters indicate significant differences between means ($P < 0.05$; Tukey's HSD).

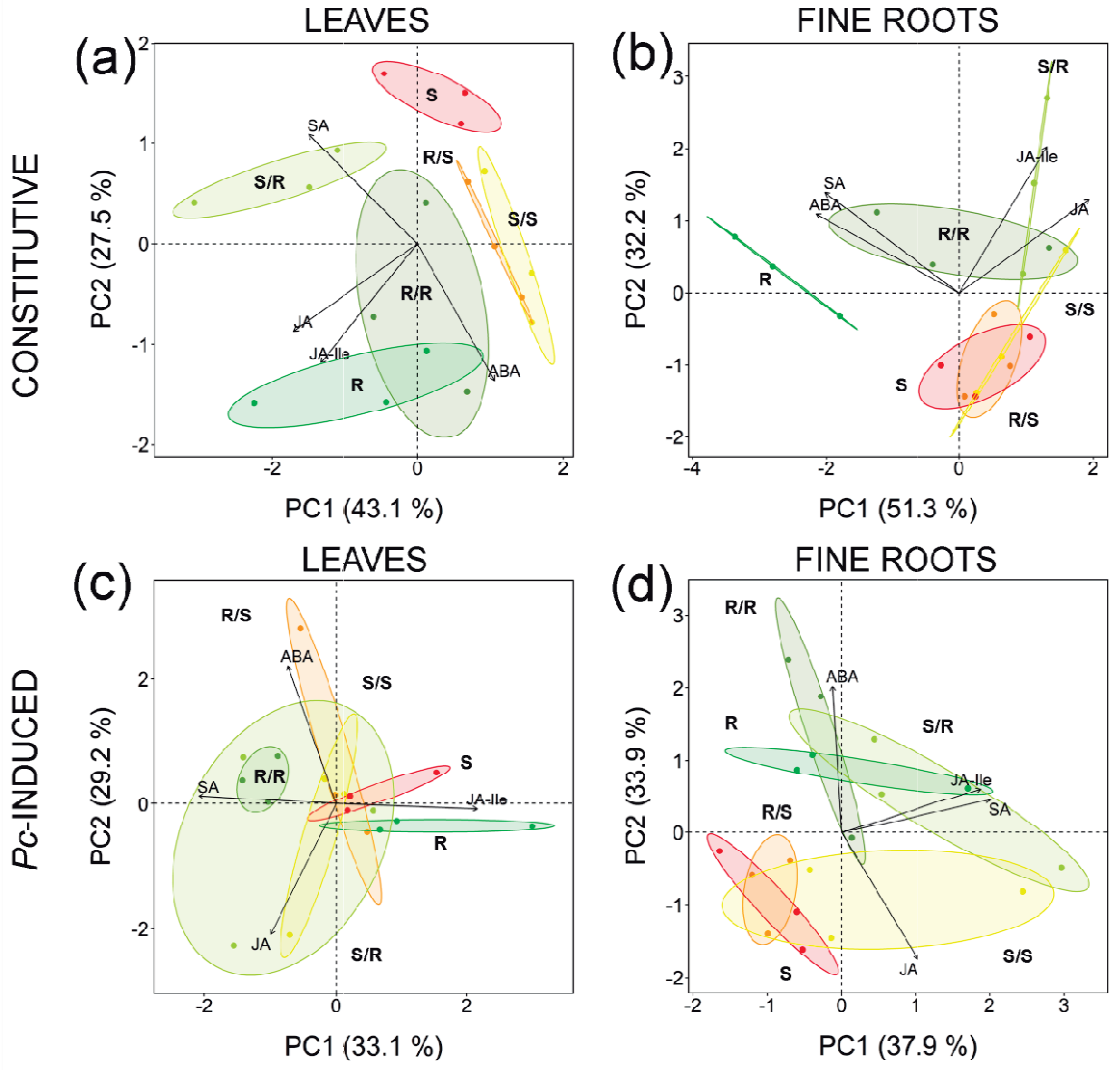


Figure 4. PCA biplots showing the ordination of susceptible (S) and resistant (R) non-grafted, self-grafted (S/S and R/R) and heterografts (R/S and S/R) of *Castanea* spp. trees the two first principal components defined by the (a) constitutive hormonal content in leaves, (b) constitutive hormonal content in fine roots, (c) *Phytophthora cinnamomi*-induced hormonal content in leaves, and (d) *Phytophthora cinnamomi*-induced hormonal content in fine roots. Explained variance by each axis is shown in parenthesis. SA: salicylic acid; ABA: abscisic acid; JA: jasmonic acid; JA-Ile: jasmonic acid-isoleucine.

Discussion

The scion determines vegetative budburst and primary growth, but does not alter the hormone profile and susceptibility to Phytophthora cinnamomi *of grafted chestnut*

Vegetative budburst and height growth of chestnuts were dependent on the scion and its interaction with the rootstock, and R scions grafted onto S rootstocks flushed earliest and had the highest primary growth (significant S × R interactions in Table 4). This is in contrast to other studies reporting that tree growth and vegetative budbreak are controlled by the rootstock rather than by the scion (Sorce et al. 2002; Jogaiah et al. 2013; Serra et al. 2013;Warschefsky et al. 2015; Tworkoski and Fazio 2016; Han et al. 2019). Assessment of other hormones like auxins and cytokinins in shoots, which controlled bud break in grafted apple trees (Tworkoski and Miller 2007), would had been necessary to explain why R/S flushed earliest. The ability of grafting to influence the timing of vegetative budburst is relevant for chestnut orchards, especially in areas under continental climate because of the high sensitivity of chestnuts to late frosts. Differences in size and grafting height of plants and the limited volume of pots (2 L) could also have influenced the growth of plants.

Grafting resistant scions onto susceptible rootstocks did not increase the resistance of plants to *Pc*. Moreover, grafting susceptible scions onto resistant rootstocks did not increase the susceptibility of plants to *Pc*. In consequence, during the interaction of trees with the pathogen, the genetic background of the rootstock prevailed over possible above-ground effects derived from the scion. The findings demonstrate that resistance to ink disease in grafted chestnuts requires resistance only in the rootstock, reasonably because *Pc* recognition and defense reactions occur belowground (Redondo et al. 2015). The scion was not able to alter the hormone profile of grafted rootstocks. According to PCA, there were no differences in the hormone profile of fine roots within S/S *vs* R/S and R/R *vs* S/R groups, before or after infection. By contrast, the R rootstock influenced the hormone profile of the scion, particularly by promoting JA-Ile accumulation in leaves. The use of reciprocal grafts allowed observing that the overexpression of JA-Ile in leaves of grafted resistant chestnut has a belowground origin. This is

important because JA-Ile has been pointed out as a potential biomarker for *Pc* resistance in chestnut (Camisón et al. 2019b).

Irrespective of the scion, the hormone profile of a resistant plant was characterized by (i) high constitutive levels of JA-Ile in leaves and SA in fine roots and (ii) high induced levels of JA-Ile and ABA in fine roots. Hormonal differences during *Pc* infection between susceptible and resistant rootstocks occurred mainly in roots as revealed here by the highly significant 'organ' effect in the models used. After infection, the depletion of JA and its derivative JA-Ile in leaves of all sampled plants irrespective of the scion, rootstock and grafting seems to be a general response of chestnuts to *Pc*. Because leaf senescence processes are regulated by jasmonates (Hu et al. 2017), the depletion of JA and JA-Ile in leaves could be explained by the occurrence of leaf wilting and browning in some plants at the time of sampling.

Grafting alters the hormone profile and susceptibility to Phytophthora cinnamomi *of chestnut*

The capacity of grafting to modify the plant's hormonal profile was highlighted by PCA overview and the lineal models used. There are probably several reasons behind the slightly higher susceptibility to *Pc* of grafted in comparison to non-grafted chestnuts, including the SA and JA-Ile changes experienced by the plants when grafted. Grafting has been reported to induce changes in the microbial community structure of the rhizosphere of plants benefiting plants against soil-borne pathogens (Guan et al. 2012; Yin et al. 2018). Microbial changes have been reported to influence the mortality of trees if soils are infested with *Pc* (Branzanti et al. 1999; Corcobado et al. 2014; Ruiz Gómez et al. 2019). Although not assessed here, it is improbable that this effect alone explained the increased susceptibility of plants. The higher susceptibility of grafted chestnuts to *Pc* can be better explained by the stress induced by grafting (Gainza et al. 2015). Biochemical alterations during stem decapitation or during the healing process, such as increased oxidative stress and changes in phenolic contents (Irisarri et al. 2015; Gainza et al. 2015) probably occurred in chestnuts after grafting. Scion-rootstock incompatibilities, which cause physiological dysfunctions in plants (Gainza et al. 2015) were not observed here. However, complete wound healing and vascular continuity did not occur in

all plants (Fig. Supplementary 1). This would hamper transport of water, nutrients, metabolites and signals throughout the graft (Sorce et al. 2002; Kalantidis 2004; Martínez-Ballesta et al. 2010), likely affecting the defense responses of chestnuts to *Pc*. Tissue healing at the graft junction is expected to be high soon after grafting (Koepke and Dhingra 2013). In consequence, the grafting effect observed in this study is probably ephemeral and differences in susceptibility of grafted and non-grafted trees are likely to disappear.

Alterations of the constitutive hormonal profile of chestnut by grafting were highest in R plants, which is in line with the more dynamic hormonal response of R chestnuts to *Pc* as compared with S chestnuts (Camisón et al. 2019b). For example, in fine roots of R plants, JA-Ile content varied drastically between non-grafted and grafted plants (in Fig. 4h compare white bars of R *vs* R/R and S/R plants). Jasmonates (JA and JA-Ile) are induced in plants in response to wounding (Pieterse et al. 2012; Wasternack and Hause 2013), which is consistent with the assumption that grafting induces a wounding effect. Jasmonates are also reported to antagonize SA-mediated signalling (Pieterse et al. 2012; Wasternack and Hause 2013). Differences were higher than those for S (0.64±0.25) *vs* S/S and R/S (0.81±0.24; P=0.383) plants which could explain why the effect of grafting was more severe in R *vs* S plants in terms of mortality (10 and 102% of mortality increase, respectively). It is ignored if accumulation of jasmonates in roots by grafting antagonized SA and in turn reduced the SA-to-jasmonates ratio with negative consequences for plant defense during the early biotrophic stages of infection. More research is needed to clarify this knowledge gap.

In the present study, soil infestation triggered aboveground accumulation of SA, particularly in leaves of grafted chestnuts. Systemic acquired resistance (SAR) is a 'whole plant' resistance response that occurs following an earlier localized exposure to a pathogen (Reimer-Michalski and Conrath 2016). The identity of the signals that move from the site of primary infection to remote, non-inoculated tissues is unclear, but there is agreement that accumulation of SA in remote tissues characterizes SAR (Reimer-Michalski and Conrath 2016). Here, it is reported for the first time in chestnut a possible occurrence of SAR, evidenced by the induction of SA by grafting and by *Pc* inoculation. After grafting or after infection, SA accumulation was

more relevant in trees grafted into R rootstocks than in trees grafted into S rootstocks. Occurrence of SAR often relates to the up-regulation of pathogenesis-related (PR) proteins (Fu and Dong 2013), not quantified here. The possible occurrence of SAR in chestnut is in agreement with the upregulation of genes related to SAR reported in roots of *C. sativa* and *C. crenata* within seven days after inoculation with *Pc* (Serrazina et al. 2015).

Conclusions

The scion has no effect on the hormone profile and resistance to *Pc* of grafted chestnut trees. Thus, it can be assumed that resistance to *Pc* is fully dependant on the rootstock. Grafting significantly influences the hormone profile, vegetative budburst, and resistance to *Pc* in chestnut. Hormone alterations by grafting are probably mediated by a wounding effect, which seems to be responsible for the increased susceptibility of grafted trees to *Pc*. The main hormonal change associated to grafting was a reduction in the SA-to-jasmonates (JA and JA-Ile) ratios in roots, which may have negative consequences for trees to resist *Pc* infection. Moreover, grafting induced SA accumulation in leaves in response to *Pc*. The study reveals the importance of grafting in modulating phenotypical variation in chestnut and calls to more research to be done on grafted chestnut trees.

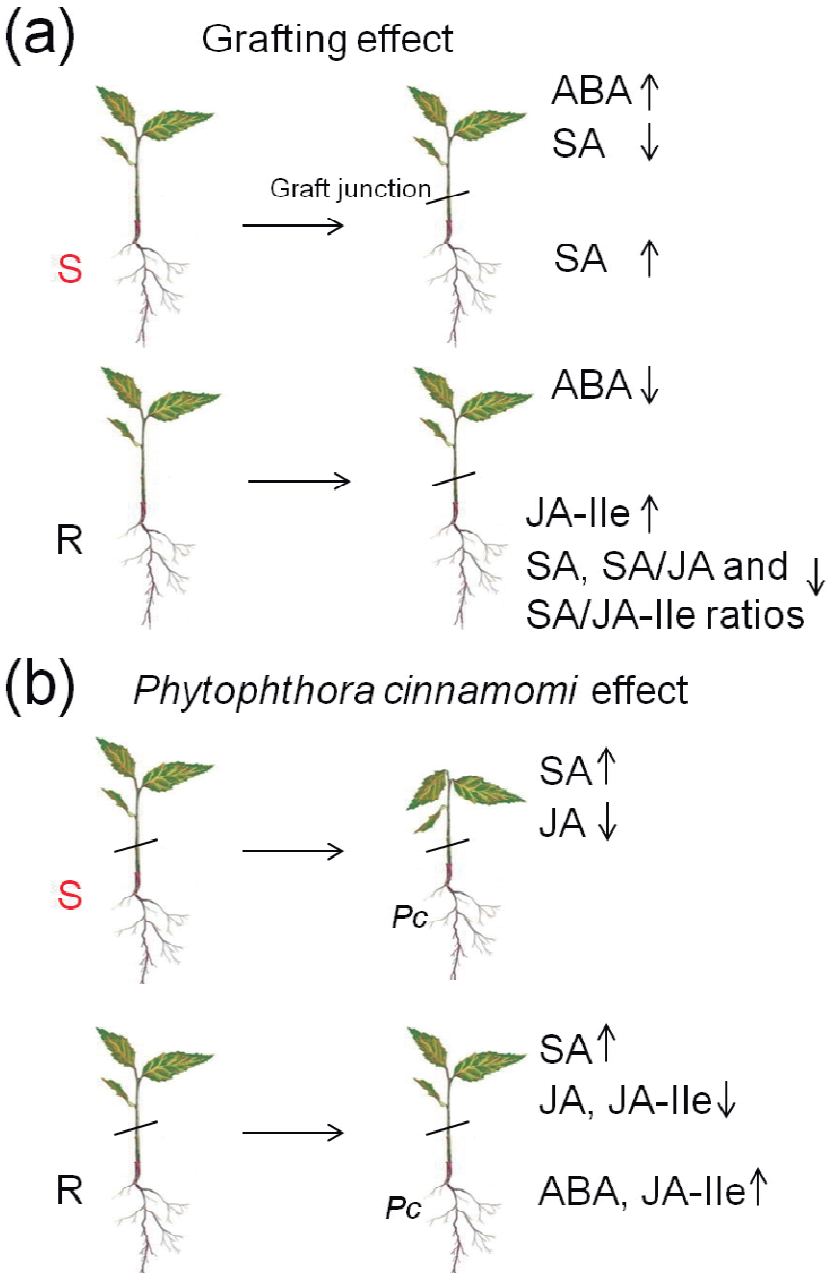


Figure 5. Main hormonal trends in response to grafting and infection by *Phytophthora cinnamomi* (*Pc*) of susceptible (S) and resistant (R) chestnut trees. The effect of grafting was evaluated ten months after grafting, and the effect of *Pc* in grafted trees was evaluated nine days after inoculation. Increased or decreased hormone contents of leaves and fine roots are indicated by arrows.

Table 1. Results of the mixed models used to analyze the reciprocal effect of scion *vs* rootstock in the vegetative budburst, growth and mortality to *Phytophthora cinnamomi* in *Castanea* spp. grafted trees.

		Vegetative budburst		Primary growth		Secondary growth		Tree mortality	
Fixed factors	DF or VarComp	F or χ^2	P	F or χ^2	P	F or χ^2	P	F or χ^2	P
Scion resistance to *Pc* (S)	1	27.3	**<0.001**	5.8	**0.017**	2.0	0.153	2.4	0.116
Rootstock resistance to *Pc* (R)	1	1.2	0.381	7.1	0.083	0.1	0.796	41.7	**0.008**
S × R	1	16.6	**<0.001**	4.1	**0.042**	3.0	0.084	0.0	0.903
Random factors									
Rootstock clone [R]	2	15.0	**<0.001**	1.3	0.260	4.7	**0.010**	1.1	0.334
Block	3	1.9	0.124	0.7	0.527	1.1	0.321	0.6	0.609
Covariate									
Plant height	1	1.8	0.173	129.6	**<0.001**	19.0	**<0.001**	9.0	**0.003**

Degrees of freedom (DF) and F-ratios for the fixed factors, and variance component (VarComp) and its associated χ^2 value for the random factors are shown. The 'rootstock clone' was nested within the 'rootstock resistance to *Pc*'. Significant P-values are indicated in bold.

Table 2. Results of the models used to analyze the effect of grafting in the vegetative budburst, growth, and mortality to *Phytophthora cinnamomi* in *Castanea* spp. grafted trees.

		Vegetative budburst		Primary growth		Secondary growth		Tree mortality	
Fixed factors	DF or VarComp	*F* or χ^2	*P*	*F* or χ^2	*P*	*F* or χ^2	*P*	*F* or χ^2	*P*
Grafting (G)	1	15.6	**<0.001**	20.9	**<0.001**	5.1	**0.024**	7.2	**0.007**
Rootstock resistance to *Pc* (R)	1	0.0	0.966	7.0	0.065	0.8	0.447	31.8	**0.019**
G × R	1	17.8	**<0.001**	3.2	0.075	1.7	0.191	0.9	0.329
Random factors									
Rootstock clone [R]	2	17.4	**<0.001**	0.8	0.411	4.2	**0.015**	3.0	0.050
Block	3	1.0	0.391	0.9	0.402	1.0	0.369	0.1	0.962
Covariate									
Plant height	1	11.5	**<0.001**	213.9	**<0.001**	21.2	**<0.001**	17.4	**<0.001**

Degrees of freedom (DF) and *F*-ratios for the fixed factors, and variance component (VarComp) and its associated χ^2 value for the random factors are shown. Significant *P*-values are indicated in bold. The 'rootstock clone' was nested within the 'rootstock resistance to *Pc*'.

Table 3. Results of models used to analyze the reciprocal effect of scion *vs* rootstock in the hormone content in leaves and roots of *Castanea* spp. grafted trees before and after inoculation with *Phytophthora cinnamomi*.

		SA		ABA		JA		JA-Ile	
Effects	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Scion resistance to *Pc* (S)	1	1.1	0.295	2.0	0.162	2.8	0.099	3.1	0.086
Rootstock resistance to *Pc* (R)	1	39.8	**<0.001**	2.2	0.143	0.1	0.726	33.3	**<0.001**
Organ (O)	1	377.2	**<0.001**	55.1	**<0.001**	58.5	**<0.001**	20.5	**<0.001**
Inoculation with *Pc* (I)	1	98.7	**<0.001**	0.5	0.476	0.7	0.379	0.0	0.932
S × R	1	0.8	0.373	0.1	0.715	0.0	0.971	0.7	0.394
S × O	1	0.2	0.646	0.3	0.535	0.3	0.550	10.5	**0.002**
R × O	1	31.3	**<0.001**	7.3	**0.010**	1.7	0.196	6.5	**0.015**
S × I	1	1.4	0.231	0.2	0.636	0.1	0.844	3.1	0.085
R × I	1	13.7	**<0.001**	3.9	0.056	3.6	0.064	0.2	0.588
O × I	1	83.3	**<0.001**	4.6	**0.037**	9.5	**0.004**	8.8	**0.005**
S × R × O	1	1.0	0.322	0.5	0.462	1.0	0.306	1.9	0.167
S × R × I	1	10.0	**0.003**	0.1	0.867	2.7	0.107	1.1	0.297
S × O × I	1	4.2	**0.048**	0.1	0.773	0.4	0.520	0.9	0.346
R × O × I	1	15.8	**<0.001**	0.7	0.393	0.9	0.337	6.0	**0.019**
S × R × O × I	1	9.1	**0.004**	1.5	0.222	0.3	0.546	0.1	0.766

Significant *P*-values are indicated in bold.

Table 4. Results of models used to analyze the effect of grafting in the hormone content in leaves and roots of *Castanea* spp. trees before and after inoculation with *Phytophthora cinnamomi*.

		SA		ABA		JA		JA-Ile	
Effects	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Grafting (G)	1	8.5	**0.005**	0.2	0.608	0.3	0.567	2.8	0.099
Rootstock resistance to *Pc* (R)	1	6.2	**0.015**	0.1	0.865	1.3	0.252	30.4	**<0.001**
Organ (O)	1	283.3	**<0.001**	90.5	**<0.001**	80.5	**<0.001**	8.2	**0.005**
Inoculation with *Pc* (I)	1	24.8	**<0.001**	5.3	**0.024**	0.0	0.913	0.4	0.512
G × R	1	15.6	**<0.001**	4.7	**0.033**	0.4	0.489	0.6	0.441
G × O	1	9.0	**0.003**	0.2	0.646	1.0	0.314	4.2	**0.043**
R × O	1	0.7	0.375	2.4	0.124	12.7	**<0.001**	3.5	0.065
G × I	1	26.7	**<0.001**	1.8	0.184	1.1	0.295	0.5	0.454
R × I	1	1.6	0.203	0.0	0.989	6.9	**0.010**	0.1	0.742
O × I	1	23.3	**<0.001**	16.4	**<0.001**	32.4	**<0.001**	13.5	**<0.001**
G × R × O	1	23.2	**<0.001**	4.3	**0.041**	3.3	0.070	0.8	0.362
G × R × I	1	6.2	**0.015**	7.0	**0.010**	0.0	0.891	0.8	0.358
G × O × I	1	20.1	**<0.001**	1.3	0.257	2.7	0.103	0.2	0.656
R × O × I	1	3.9	0.050	2.2	0.135	0.1	0.808	13.0	**<0.001**
G × R × O × I	1	4.2	**0.043**	7.1	**0.009**	1.0	0.308	0.8	0.354

Significant *P*-values are indicated in bold.

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

Castanea sativa Mill. facing global change factors. Chapter V.

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) regions 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. Two weeks after water deprivation, drought tolerance was assessed by measuring leaf gas exchange, leaf wilting and tree mortality 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. At the end of the vegetative period, 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 induced delayed vegetative budbreak and tended to reduce tree secondary growth. Under drought stress, differences in the hormone and proline contents of H and X trees reflected their different dehydration levels, with high levels of leaf JA-Ile and root ABA associated to drought-induced mortality. In the face of ongoing global warming, results support using rootstocks from xeric areas to improve the tolerance to drought of chestnuts and indicate that the southern Iberian *C. sativa* gene pool could be exploited as a source of drought tolerant rootstocks for further chestnut breeding programs.

Key words

ABA, climate change, grafting, graft union, hormonal profiling, JA-Ile, orchard

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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 in the budbreak phenology, leaf physiology 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; Lin et al. 2003; 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; Serra et al. 2014) while changes in the production of hormones, proteins and reactive oxygen species during the regeneration of tissues (Mo et al. 2017; Melnyk et al. 2018; Nanda and Melnyk 2018) might affect tree phenology, growth and drought tolerance.

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Castanea sativa inhabits regions with marked water availability gradients (e.g. in the Iberian Peninsula and Turkey) leding to a genetically-based differentiation in traits related to drought adaptation (Pigliucci et al. 1990; Lauteri et al. 1999; Fernández-López et al. 2005; Ciordia et al. 2012; Míguez-Soto and Fernández-López 2015; Míguez-Soto et al. 2019). This evolutionary pressure has made it possible to obtain rootstock genotypes contrasting in drought tolerance. In the Iberian Peninsula there are two *C. sativa* ecotypes adapted to different climatic 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. 2018; Alcaide et al. 2019). Xeric *C. sativa* populations show early phenology, low plant growth and higher root development in comparison to mesic populations, because of adaption to summer drought conditions (Lauteri et al. 1999; Fernández-López et al. 2005; Ciordia et al. 2012; Míguez-Soto and Fernández-López 2015; Míguez-Soto et al. 2018). Hormones regulate the physiological responses of plants to drought, but it is unknown if differences in hormonal responses may contribute to differences in drought tolerance in *C. sativa*.

Hormones are stress signaling molecules that help plants adapt to adverse environmental conditions through a complex crosstalk that implies changes in primary and secondary metabolism. They also play an important role in the scion/rootstock communication (Aloni et al. 2010; Albacete et al. 2015) what makes them ideal models for studying the mechanisms by which rootstocks enhance drought tolerance (Allario et al. 2013; Tworkoski et al. 2016; Silva et al. 2018). Among them, 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). In this respect, 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. 2016b; Santana-Viera et al., 2016; Tworkoski et al. 2016; Silva et al. 2018). Specifically, ABA modulates the tree drought response strategy (i.e., dehydration avoidance *vs* dehydration tolerance) and high leaf and root ABA contents before and/or during initial stages of drought are generally associated to dehydration avoidance (Allario et al. 2013; 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; Ollas and Dodd 2016). In grafted citrus trees with enhanced tolerance to drought, SA accumulated in roots and leaves in response to drought promoting stomatal closure together with ABA (Santana-Vieira et al. 2016; Matos Neves et al. 2017). JAs-mediated signaling has been also reported to regulate plant adaptation to drought stress in citrus rootstocks (de Ollas et al. 2012; Arbona et al. 2015; Shenxie et al. 2015). For example, a previous and transient burst of jasmonic acid was required to trigger a progressive accumulation of ABA in roots of a commercial citrus rootstock under severe drought (De Ollas et al. 2012). Moreover, 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 across many plant species (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, reciprocal grafts between Iberian *C. sativa* families from humid and xeric provenances were used to explore the use of rootstocks from xeric areas to improve drought tolerance in chestnut. To support the findings, the constitutive and drought-induced hormonal profiles of two families contrasting in tolerance to drought were analyzed. The following hypotheses were tested in chestnut: (i) vegetative budbreak, tree growth and drought tolerance responses depend on the rootstock, (ii) rootstocks from xeric areas may advance the phenology, reduce the growth and enhance the tolerance of trees to drought, (iii) vegetative budbreak, tree growth and tolerance to drought are influenced by a 'grafting' effect, and (iv) there are constitutive and 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 Supplementary 1). Trees were grafted using the 'green grafting' technique (Cuenca et al. 2018, Fig. Supplementary 1a). 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 %). Previously to the experiment, a characterization of the non-grafted families growing under optimum watering indicated an early vegetative budbreak of H_1 and X_1 families (Fig. Supplementary 2a) and a

conservative strategy in the X_1 family related to reduced plant size and leaf gas exchange (Fig. Supplementary 2b-d).

Experimental design and drought treatment

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. 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. 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 due to the effect of the graft union rather than to the interaction between two genetically distinct individuals.

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 non-grafted control combination. 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, soil volumetric water content (SVWC) of pots was recorded with a soil moisture meter (Field Scout™ TDR Soil Moisture Meter, Spectrum Technologies, Inc.). Immediately after

SVWC measurements, the plants were rewatered to field capacity for recovery and maintained under optimum watering conditions for two months.

Assessment of budbreak phenology and tree growth

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.

Assessment of susceptibility of trees to drought

Susceptibility to drought was assessed in all trees by two approaches: (i) assessment of gas exchange parameters in leaves, and (ii) evaluation of external symptoms due to damage caused by drought. On day 14 after the drought treatment started, net carbon assimilation (A) and stomatal conductance (g_s), were measured with a portable differential infrared gas analyzer (IRGA) (Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf chamber (Alcaide et al., 2019). Measurements were performed between 10.00-12.00 h with photosynthetically active radiation (PAR) ranging from 300 to 500 μmol photons m^{-2} s^{-1}. The day 13 after the drought treatment started, chlorophyll fluorescence (*Fv/Fm* readings, the maximum quantum yield of photosystem II (PSII)) and leaf relative water content (RWC) were assessed. *Fv/Fm* readings were obtained from 8.00 to 10.00 h with a Multimode Chlorophyll Fluorometer OS5p device (Opti-Science Inc., USA) in dark-adapted leaves (30 min). The same day, leaf 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 ºC in darkness, and DW is the dry weight of leaves after complete oven dehydration (48 h, 60 ºC). Two apical fully expanded leaves per tree were used.

Leaf wilting was evaluated two weeks after the drought treatment started. Leaf wilting was visually estimated as the percentage of plant foliage showing turgor loss. Finally, tree mortality and scion mortality (if any) were assessed two months after the drought treatment finished.

Hormone and proline quantification in leaves and roots of trees

The day before and the day 14 after the drought treatment started, hormone and proline content in leaves and roots of a subsample of trees were assessed. 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 control trees). Roots were sampled by carefully excising and collecting five outermost fine root segments from the root ball of rootstock (and control 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 plant hormones abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) and the amino acid proline were extracted and determined in leaves and roots exactly as described in chapter III, and therefore the reader is referred to chapter III for details on the protocols.

Statistical analysis

The effect of the origin of the scion and rootstock and the effect of 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 origin of the scion and rootstock, intra- and inter-familiar grafts (i.e., H/H, X/X, H/X and X/H scion/rootstock combinations) were used and the 'scion origin', the 'rootstock origin' and their interaction were considered as fixed effects. Tree mortality was analyzed 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 (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. Because of the higher water consumption of taller plants (see Fig. Supplementary 3), 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 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 the origin of the scion and the rootstock (Table 1). Secondary growth tended to be lower in H/H and X/X trees relative to their non-grafted controls (significant 'grafting' effect; Table 2), although differences were not significant in both cases.

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

Under drought conditions, trees with X rootstocks (X/X and H/X) showed higher net photosynthesis and stomatal conductance (g_s) values in comparison to trees with H rootstocks (H/H and X/H) (Fig. 2a, b). Grafts with X material either as scion or as rootstock showed higher g_s values (significant 'scion origin' × 'rootstock origin' interaction, Table 1, Fig. 2b). *Fv/Fm* and leaf RWC mean values followed similar patterns each other, being maximum for 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 in comparison to grafts with X rootstocks (Table 1, Fig. 2e). Tree mortality induced by drought was mainly influenced by the '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 degree also by the 'scion origin' (Table 1). Mortality of X rootstocks increased if a H scion instead of a X scion was used while mortality of H rootstocks decreased if a X scion instead of a H scion was used (Fig. 2f). The capacity of trees to maintain the scion alive after drought was lowest in grafts with H rootstocks (0 and 22% for H/H and X/H trees, respectively) in comparison to grafts with X rootstocks (67 and 49% for X/X and H/X trees, respectively) (Fig. 2f). Tree height was significant in all models (Table 1) and positively associated to leaf wilting and tree mortality.

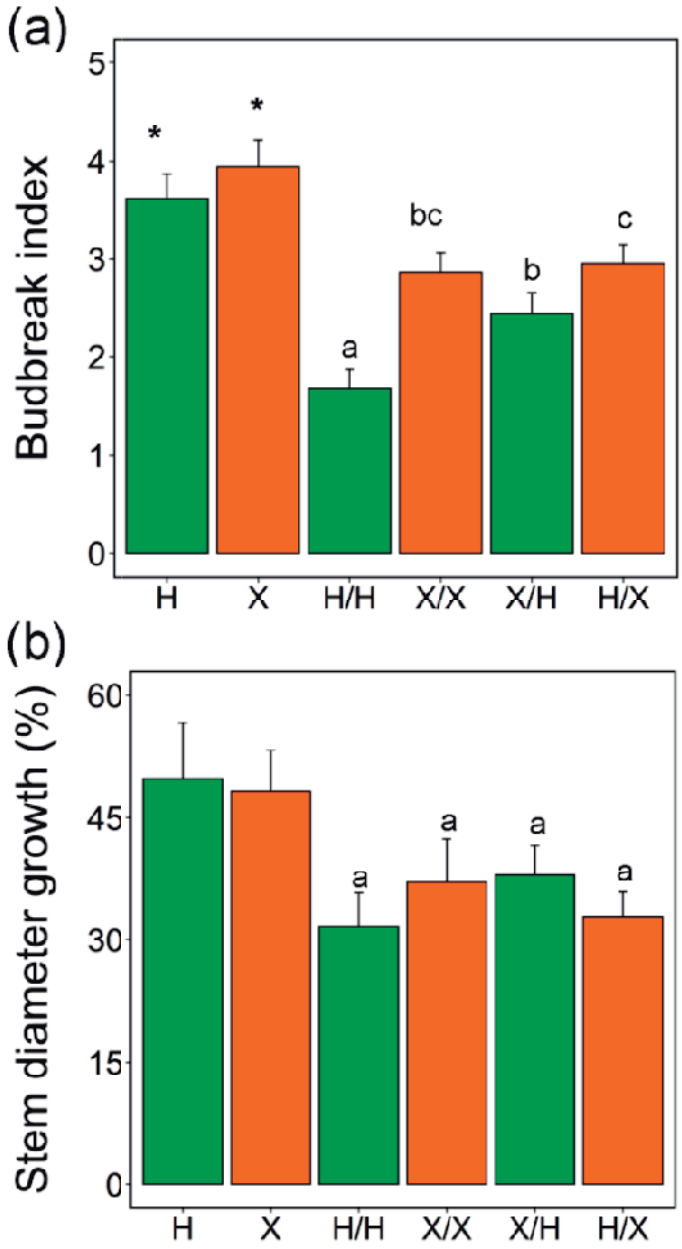

Figure 1. Mean values of (a) budbreak index and (b) stem secondary growth in non-grafted controls (H and X), intra-familiar grafts (H/H and X/X) and reciprocal grafts (X/H and H/X) established using *Castanea sativa* families from humid and xeric areas. Error bars indicate one standard error of the mean.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

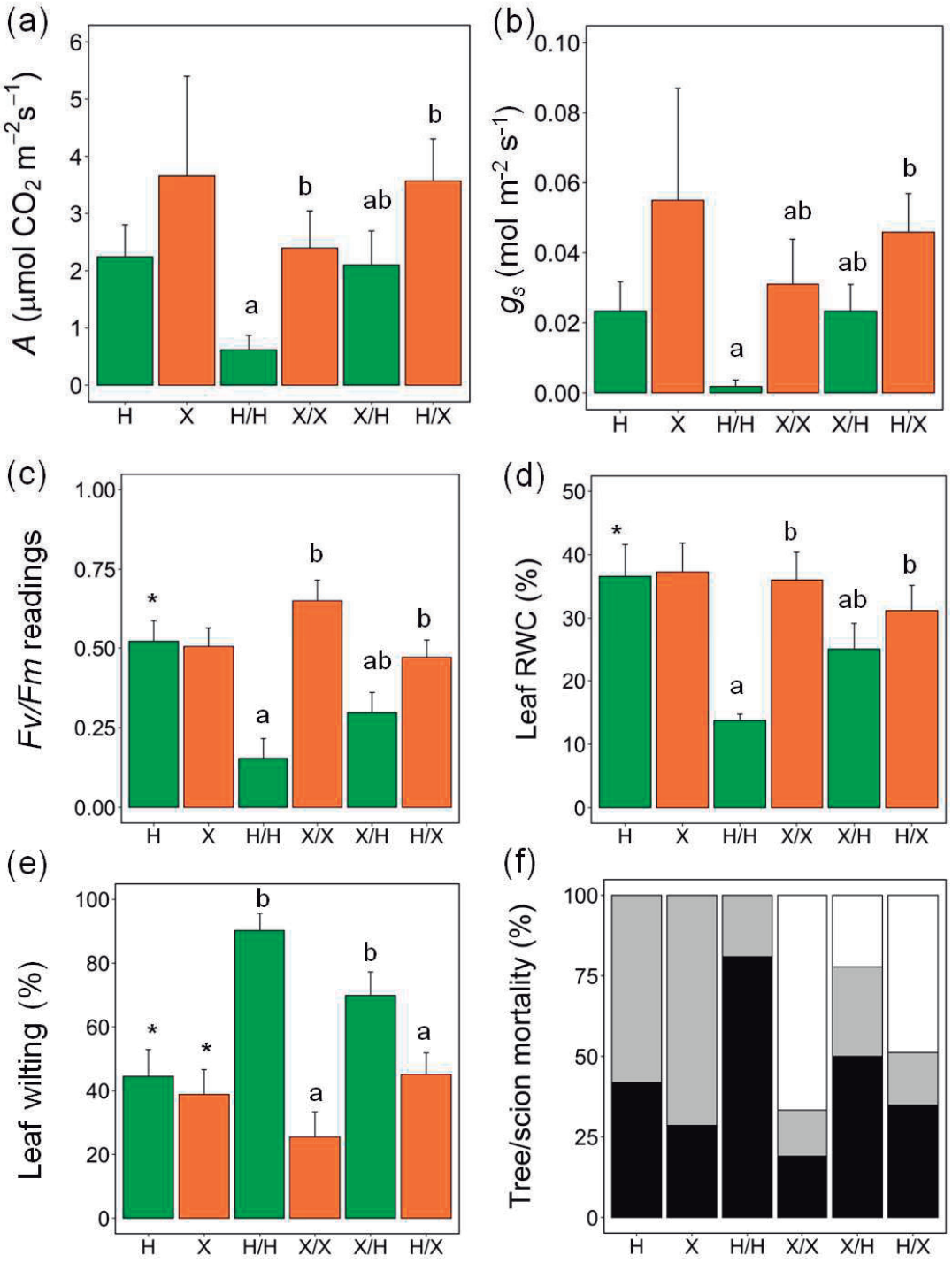

Figure 2. Mean values of (a) leaf net photosynthesis (*A*), (b) stomatal conductance (g_s), (c) *Fv/Fm* readings, (d) leaf RWC, (e) leaf wilting and (f) tree/scion mortality in non-grafted controls (H and X), intra-familiar grafts (H/H and X/X) and reciprocal grafts (X/H and H/X) established using *Castanea sativa* families from humid and xeric areas. 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. Different letters indicate significant differences between means within grafted trees, while '*' indicates whether non-grafted controls differ from their intra-familiar grafts ($P < 0.05$; Tukey's HSD).

Grafting itself had no effect on the tolerance of trees to drought stress (Table 2). Differences in gas exchange parameters, leaf wilting and tree mortality were exclusively attributed to the 'rootstock origin' and 'tree height' effects (Table 2, Fig. 2). Only in plant material from H origin (significant 'grafting' × 'rootstock origin' interaction, Table 2), the effect of grafting diminished values of leaf RWC and *Fv/Fm* in trees (Fig. 2c, d).

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

Under optimal watering, no significant differences in the content of ABA, SA, JA, JA-Ile and proline between non-grafted, and grafted H and X plant material were observed (Fig. 3). However, when pooling non-grafted and grafted trees together, 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 roots of all groups of trees (Fig. 3). SA content in leaves increased relatively more in H, X/H and H/H trees in comparison to X, H/X and X/X trees. While JA-Ile content in leaves increased with drought, JA-Ile and JA content in roots decreased in almost all trees (Fig. 3e-f and 3g-h). H/H trees showed the highest levels of ABA in roots and the highest levels of JA-Ile and proline in leaves (Fig. 3b, 3g and 3i). The lowest concentrations of JA-Ile in roots were observed in H and H/H trees (Fig. 3h).

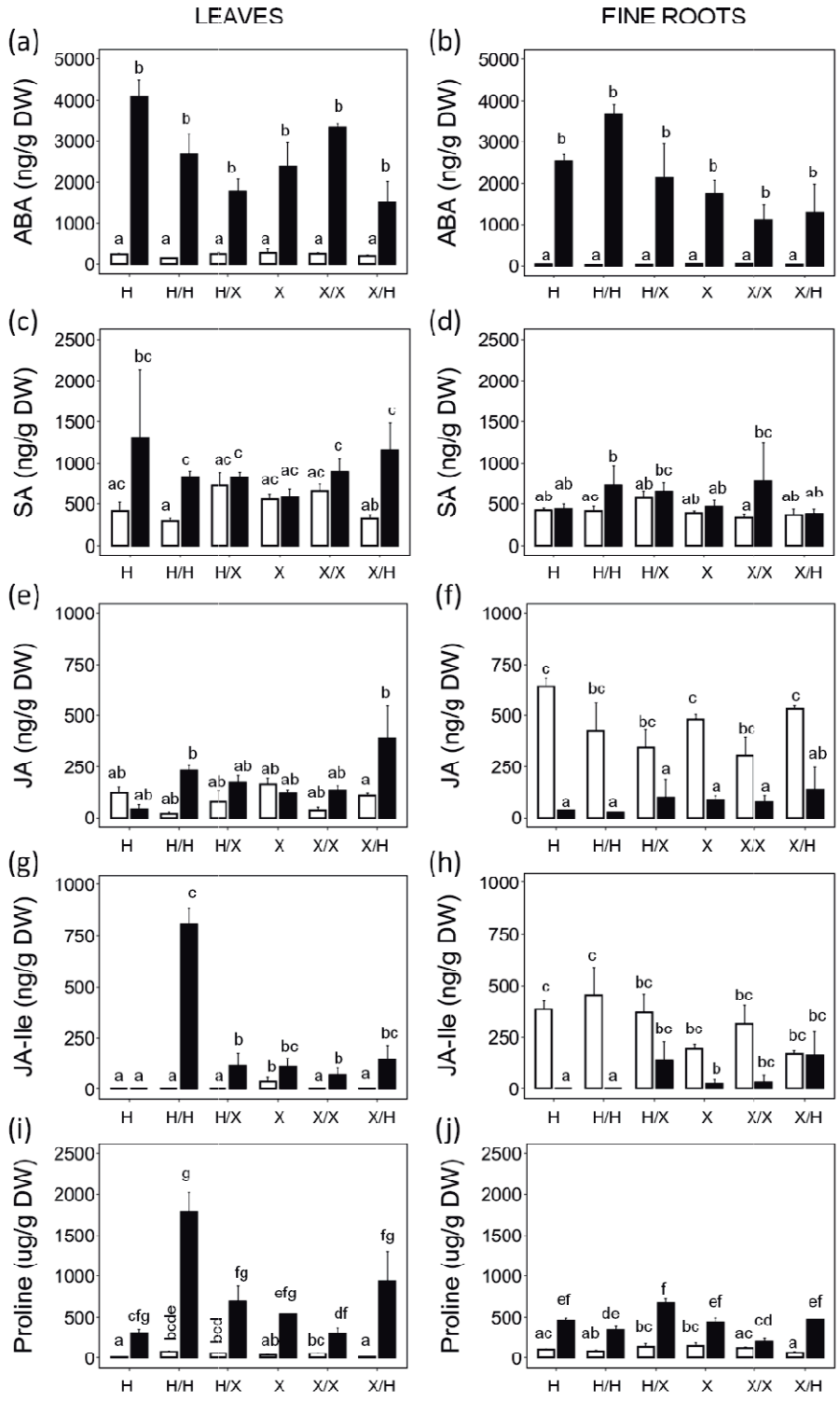

Figure 3. Content of abscisic acid (ABA) (a, b), salicylic acid (SA) (c, d), jasmonic acid (JA) (e, f), jasmonic acid-isoleucine (JA-Ile) (g, h) and proline (i, j) before (white bars) and during (black bars) drought in leaves and fine roots of non-grafted controls (H and X), intra- (H/H and X/X) and 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).

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).

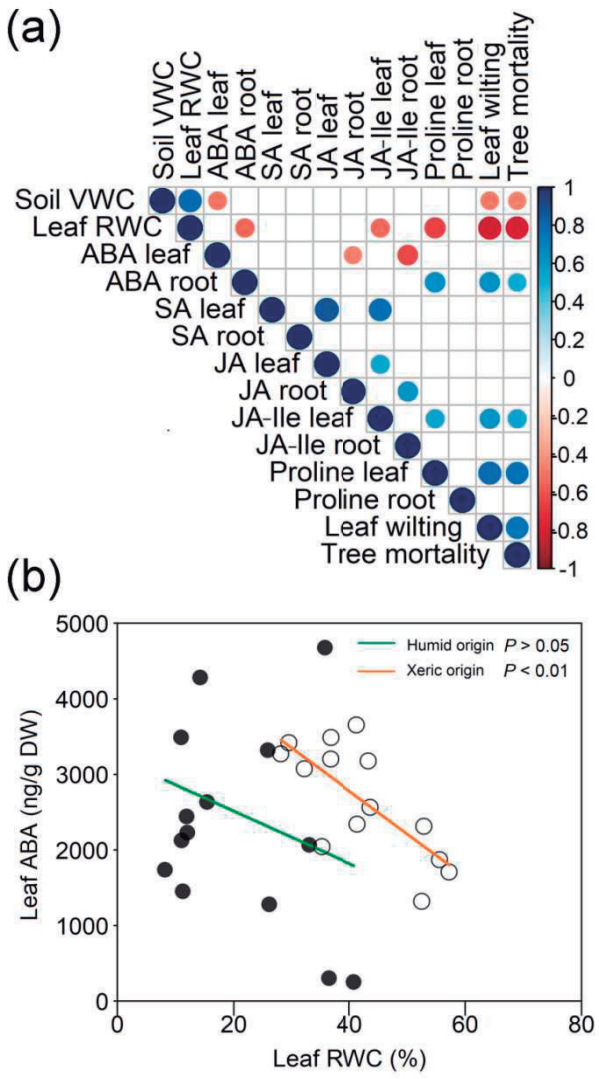

Figure 4. Matrix of significant ($P < 0.05$) Pearson correlation coefficients (a) among the water content in soil and leaves (soil VWC and leaf RWC), the contents of hormones and proline in leaves and roots, and external symptoms induced by drought (leaf wilting–and tree mortality) obtained during drought 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).

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. The fact that X scions grafted onto H rootstocks also advanced tree budbreak indicates that the origin of the scion may also partly influence vegetative budbreak. 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 tree growth and flowering. The species is highly sensitive to late frosts (Fernández-López et al. 2005; Míguez-Soto et al. 2019) although from the results obtained here it is not obvious that H rootstocks could be used in areas with continental climates to reduce the exposure of chestnut trees to late frost events.

Grafting induces stress in terms of budbreak phenology and growth but does not predispose C. sativa *trees to drought*

The finding that grafting delayed budbreak and tended to reduce stem secondary growth of trees in relation to non-grafted controls is in agreement with studies in other woody species indicating that grafting is perceived as a wounding stress by the plant, at least during the graft union healing (Cookson et al. 2014). Other abiotic stresses including drought (Kuster et al. 2014; Čehulić et al. 2019), heat (Luedeling et al. 2013) or salinity (Van Zandt and Mopper 2004) alter plant phenology. In our two-year-old grafts, the graft union was not perfectly sealed in most of the cases (see Fig. Supplementary 1b) which supports 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 after being grafted, at least during the graft union healing. The delay in budbreak phenology induced by grafting may probably explain why grafts had a lower stem secondary growth, as a more delayed flushing determines a shorter vegetative period. This is supported by the positive correlation between the vegetative budbreak scores and stem secondary growth ($r = 0.37$; $P < 0.001$; results not shown). A trade-off in the investment of resources between wound healing and stem 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. 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. From an applied perspective, such result eases the implementation of grafting as an adaptive tool to mitigate the impacts of climate change on chestnuts.

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

Drought tolerance was mainly determined by the rootstock in *C. sativa* grafts, and rootstocks from xeric areas increased the tolerance to drought of the more drought-sensitive northern trees. Under drought, X rootstocks improved the water status of H trees (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 tolerance 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).

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

Explanations for the delay in plant dehydration induced by X rootstocks may include their higher constitutive 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) and it is also supported here by the negative relationship between the leaf ABA content and values of A and g_s measured before the application of drought stress (Fig. Supplementary 4a-b). Elevated levels of the osmolytic amino-acid proline found in roots of X rootstocks may have enabled these trees to more efficiently bind water during initial stages of drought, thus contributing to delay dehydration.

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. A probable reason was that sampling was performed at a very advanced stage of water stress, in some H trees occurring near to tree death. In consequence, hormone levels in our study likely reflected the different stress levels undergone by trees rather than the different drought adaptive mechanisms between H and X trees. For 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. Differences in the leaf ABA *vs* leaf RWC relationship between the X and H trees (Fig. 4b) may 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 has been reported (Mahajan and Tuteja 2005).

While the involvement of ABA in the response of *C. sativa* to drought was previously reported (Maurel et al. 2004), this study for the first time reports 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 down-regulation of jasmonates (JA and JA-Ile) in roots coinciding with ABA accumulation in leaves was a hallmark of the *C. sativa* response to drought. This may suggest that the crosstalk between belowground jasmonates and aboveground ABA may modulate chestnut responses to drought. Interestingly, the most severely stressed chestnuts (i.e., H/H trees) showed a hormonal profile opposite to that observed in chestnuts under well-watered conditions (Fig. 3). Strongly decreased root jasmonates along with increased leaf SA, JA-Ile and root ABA associated to tree mortality, suggesting that a root-to-shoot hormonal imbalance may precede drought-induced mortality in *C. sativa*. More research is necessary to prove previous suggestions in chestnut trees.

Conclusions

This study highlights the potential of grafting to shape phenotypical variation in *C. sativa* trees and shows that drought tolerant *C. sativa* rootstocks (and to a lesser degree also 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 their different stress levels. A root-to-shoot hormonal imbalance preceded drought-induced mortality in *C. sativa*.

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.

GENERAL DISCUSSION

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This thesis contributes to a better understanding of the interaction between *C. sativa* and components of global change. Specifically, *C. sativa* was studied because of its ecological and economical importance in Europe and the Mediterranean Basin. In the context of current climate change and the spreading of *Pc*, knowledge on the physiological responses of this tree species to these stresses is desirable to anticipate its performance under varying environmental conditions (Buras et al. 2019), and to be used in tree breeding programs (Santos et al. 2017a). In this work, different approaches were used to study the responses of *C. sativa* to drought, waterlogging and *Pc* by performing greenhouse experiments which combined tree physiological assessments with molecular analysis at the laboratory. The obtained results constitute novel insights and open new research questions in the species. Nonetheless, the results could be also useful for research in other woody species impacted by *Pc* and climatic stress.

Non-structural carbohydrates dynamics in *C. sativa* under drought and waterlogging

By combining physiological assessments with measurements of non-structural carbohydrates (NSC) and nitrogen concentrations in seedlings, the results exposed in chapter I constitute a preliminary portrait of the responses of *C. sativa* to drought and waterlogging stress over time. For the first time, the effects of such stress factors on the NSC of *C. sativa* were reported. After two months of sustained drought and waterlogging, no evidence of carbon starvation (i.e. depletion of NSC in tissues) in *C. sativa* was found in spite of the reduced net photosynthesis and growth of seedlings. The dynamic of NSC under drought must be interpreted taken into account the coupling between the plant carbon status and the process of drought-induced hydraulic failure (Adams et al. 2017; Tomasella et al. 2017; Trifiló et al. 2017, 2019). A recent multi-species analysis of drought-induced mortality mechanisms totalling 26 tree species suggests that trees under drought die from either hydraulic failure alone or from a combination of hydraulic failure and reduced NSC content (Adams et al. 2017). Therefore, the results suggest that carbon starvation, at least alone, is not a major cause of drought- and waterlogging-induced mortality in *C. sativa*. More experiments measuring NSC in *C. sativa* at mortality are

necessary to confirm such a suggestion, since the experimental treatments applied in this thesis did not produce seedling mortality but severe stress. To better understand the relation of NSC dynamics to drought tolerance in *C. sativa*, further research should investigate how *C. sativa* populations contrasting in drought tolerance (e.g. northern *vs*. southern Iberian populations) differ regarding their strategies of carbohydrates use and xylem vessels refilling during drought. The observed accumulation of NSC in stems and roots of seedlings under waterlogging might be related to an inhibition to utilize carbohydrates, which explains the high susceptibility of chestnut trees to waterlogging.

The role of maternal effects for resistance to *Phytophthora cinnamomi* and tolerance to drought in *C. sativa*

Phenotypic variation for resistance to *Pc* and tolerance to drought due to maternal effects have never been studied in *C. sativa*. The results presented in chapter II suggest that infection of *C. sativa* mature trees by *P. cinnamomi* affects negatively the fitness of offspring through seed provisioning-mediated mechanisms. Seed weight decreased by the infection. However, infection seemed to prime a defense response to *Pc* in the next generation, although such effect on the tolerance to drought stress of the offspring was not detected. This inter-generational priming could be mediated by epigenetic changes, although further studies are needed to confirm it. The role of maternal effects as a source of adaptive phenotypic variation in the offspring of tree species is increasingly recognized (reviewed in Vivas et al. 2020). Importantly, which specific environmental stimulus/stress (e.g. biotic *vs* abiotic) is undergone by the mother tree needs to be carefully considered in studies dealing with maternal effects (Vivas et al. 2020). Different stimulus in the parental generation may lead to different responses to a certain stress in the offspring. While infection by *Pc* resulted into small-sized offspring with a high resistance to the pathogen, the other kinds of stimulus may prime for drought tolerance in the offspring of *C. sativa*.

At ecological time-scales, maternal effects may be particularly important for the evolution of chestnut trees because of their potential to generate rapid phenotypic changes within a population (Räsänen and Kruuk, 2007). In the Iberian Peninsula, a recent study reported a link between the genetic structure of wild *C. sativa* populations and their adaptive responses to infection by *Pc* (Alcaide et al. 2020), suggesting that pathogen-imposed selection is driving the adaptive evolution of chestnuts. In this context, the results obtained in this thesis suggest that, besides genetics, the maternal environment might also be an important source of adaptive phenotypic plasticity in response to the impact of ink disease in *C. sativa*. *Castanea sativa* seed orchards could be established in areas subjected to particular environments with certain stressors of interest (e.g. drought, nutrient deficiency, presence of ink disease…) to provide rapid phenotypic variability in the offspring, and characterization of this variability in common garden experiments could aid when establishing chestnut afforestations under a range of environmental conditions (see Vivas et al. 2020).

What can defense-related plant hormones tell us about resistance to *Phytophthora cinnamomi* in *Castanea* spp.?

Besides regulating every single developmental aspect of plants, hormones are the main mediators of plant defense responses to pathogens through multiple signalling pathways (Verma et al. 2016). This renders hormones suitable candidates to explore the mechanisms of susceptibility and resistance to *Pc* in the genus *Castanea* and in other tree species. The results exposed in chapter III constitute the first comprehensive hormonal profiling of leaves and roots of susceptible and resistant *Castanea* spp. clones, and allowed identifying several defense-related hormones whose content could potentially be related to resistance. Constitutively, the content of root SA and leaf JA-Ile could be associated to resistance since a higher content of these hormones was found associated to the resistant clone studied. Because of the well-known involvement of these hormones in the induction of plant defenses (Verma et al. 2016), this higher content could be related to a higher expression of innate defenses, which might hinder *Pc* establishment during initial stages of the infection process. Upon infection by the pathogen, the

dynamic hormonal response observed in the resistant clone linked to the accumulation of JA-Ile and ABA in roots likely enabled an effective induction of plant defenses, while the weak hormonal response of the susceptible clone suggests inability to induce plant defences. These results are in agreement with previous studies by Serrazina et al. (2015) and Santos et al. (2017a) indicating that resistance to *Pc* in chestnut relies upon the existence of constitutive physical and chemical barriers and a rapid and intense defence response upon infection. Although the infection of roots by *P. cinnamomi* triggered systemic changes in leaves (decreased JA and JA-Ile content), the biochemical changes associated to resistance were observed only in roots (increased content of JA-Ile, ABA and total phenolics), suggesting that metabolic changes localized at the infection point mediate the outcome of the interaction (compatible or incompatible). A unique sampling point for hormone analysis (i.e., the time when half of plants were symptomatic) was used here after inoculation with *Pc*, whilst other studies have demonstrated a temporal regulation of tree hormonal responses to *Pc* infection (van den Berg et al. 2018). Thus, future research should take into account the temporal variability of the hormonal responses to *Pc* across more chestnut genotypes.

One of the most noticeable results of this thesis was the fact that JA-Ile was detected in leaves exclusively in the resistant 111-1 clone before infection. The potential of this hormone as a biomarker for resistance should therefore be further explored across a range of chestnut genotypes contrasting in resistance to *Pc*. Data from chapter V additionally support the possible relationship between the leaf JA-Ile content and resistance, since JA-Ile was again constitutively almost undetectable in leaves of the *Pc*-susceptible *C. sativa* trees used. A recent study identified one EST-SSR genetic marker which could be putatively used to predict resistance to *P. cinnamomi* in chestnut (Alcaide et al. 2020). The combination of genomic and hormonal biomarkers would provide a useful tool for marker-assisted screening of *Pc*-resistant chestnuts. Interestingly, results from chapter IV demonstrate that the innate content of JA-Ile in leaves of the resistant 111-1 clone derives from the roots and is graft-transmissible.

The interaction between grafting, *P. cinnamomi* and drought stress in *C. sativa*

The complex phenotype of a grafted tree arises from the interaction between two genetically distinct partners (the scion and the rootstock), the environment and the graft union itself (Albacete et al. 2015). This makes it difficult to separately study each of these grafting-induced sources of phenotypic variation unless properly designed grafted plant material is used. Reciprocal grafts between genotypes with a contrasting trait of interest allows studying the effect of the scion, the rootstock, and their interaction on this trait, while grafts involving genetically similar partners (e.g. self-grafts and intra-familiar grafts) are suitable for the study of the graft union effect. In chapters IV and V, the use of non-grafted controls, self-grafts, intra-familiar and reciprocal grafts allowed to study the resistance to *Pc* and tolerance to drought traits. Additionally, the measurement of hormone content in grafts is a suitable way to identify possible mechanistic links between molecular and phenotypical traits because hormones can be systemically transported and are graft-transmissible (Tsutsui and Notaguchi 2017).

Overall, results from chapters IV and V agree with previous studies highlighting the prominent role of the rootstock over the scion in shaping the phenotypic responses of grafted woody plants to stress (Allario et al. 2013; Tworkoski et al. 2016; Silva et al. 2018; Han et al. 2019). Results from chapter IV showed that resistance to *Pc* in chestnut trees depends only on the rootstock, and no systemic effect of the grafted scion was observed. By contrast, results from chapter V showed that although the effect of the rootstock was the most important, the grafted scion had an effect on the tolerance to drought of chestnuts. Particularly, drought-tolerant *C. sativa* genotypes increased tolerance of drought-sensitive genotypes when used both as scions and as rootstocks. Therefore, drought tolerance of sensitive *C. sativa* varieties for nut production could be improved by grafting onto drought-tolerant rootstocks in orchards. As a possible wounding stress, grafting had an effect on the susceptibility of chestnut trees to *Pc* but not on their susceptibility to drought stress, which eases the implementation of grafting onto drought-tolerant rootstocks as a measure to mitigate the impact of current climate change on nut production in orchards.

Conflicting results regarding the effect of grafting as a wounding stress on vegetative budbreak and tree growth were found in chapters IV and V. As compared to non-grafted controls, in chapter IV grafted chestnuts grew more in height and diameter and had a more advanced budburst, while in chapter V grafting delayed budburst and decreased diameter growth of intra-familiar grafts. Explanations for this may include the different plant material used. Also, differences in the compatibility between the scion and the rootstock could be responsible for such contrasting results. Clonal material was used in chapter IV thus ensuring maximum compatibility between the scion and the rootstock while a lower compatibility could be expected for the intra-familiar grafts used in chapter V. Differences in compatibility between the scion and the rootstock could affect the timing of the graft union sealing, thus affecting tree phenology and growth. Taken together, results of chapters IV and V provide evidence that important phenotypical traits of chestnut trees including resistance to *Pc* and tolerance to drought can be modified by grafting. To the best of our knowledge, chapters IV and V constitute the first studies addressing sources of phenotypic variability in grafted chestnuts, calling to more research to be done.

CONCLUSIONS

CONCLUSIONS

I - Seedlings of *C. sativa* subjected to drought and waterlogging reflect different strategies of carbon use to cope with stress, likely related to the contrasted tolerance of the species to drought and waterlogging. Starch mobilization into soluble sugars followed by the presence of high soluble sugar concentrations in aboveground tissues could be important to tolerate drought by allowing plants to reverse drought-induced xylem embolisms. The accumulation of soluble sugars and starch in stems and roots of *C. sativa* seedlings under waterlogging could be a consequence of active allocation of carbon to reserve formation and/or inhibition to use NSC. Inhibition to use carbohydrates may explain the low tolerance of *C. sativa* to waterlogging conditions.

II – Infection of *C. sativa* mother trees by *Pc* causes poorer performance in plant height and root biomass in offspring because of reduced seed provisioning. Infection of mother trees did not contribute to the inheritance of dehydration stress memory, but small-sized plants from ink-diseased mother trees showed increased tolerance to *Pc*. Increased tolerance was not mediated by seed size and was probably a consequence of seed priming during fruit development. The impact of *Pc* on *C. sativa* forests may generate conflicting selection pressures related to plant size, constraining regeneration success at the seedling stage.

III – Differences in the constitutive and *Pc*-induced content of defense-related hormones can explain differences in susceptibility to the pathogen in chestnut trees. A dynamic response of hormones and metabolites across organs linked to a synergistic crosstalk between ABA and JA-Ile in roots seems related to resistance. On the contrary, the lack of effective hormonal changes after infection explains the susceptibility of *C. sativa* to *Pc*. The constitutive hormone JA-Ile in leaves could be a biomarker for resistance to *Pc* derived from the roots, which needs to be confirmed by using additional *Castanea* spp. genotypes.

IV – Grafting itself influences the hormone profile of chestnuts, the vegetative budburst, and increases susceptibility to *Pc* in susceptible and resistant rootstocks, likely because of a wounding effect. The rootstock and not the scion controls the hormone profile and resistance to *P. cinnamomi* of grafted chestnut trees.

V - Rootstocks and scions from xeric origin induce early flushing and improve drought tolerance of both scions and rootstocks of *C. sativa* from humid origin. Therefore, drought-tolerant rootstocks could be used to improve drought tolerance in grafted *C. sativa* orchards. The grafting (wounding) effect had no influence on the tolerance to drought of trees, although it delayed their vegetative budbreak and tended to reduce their secondary growth. High levels of leaf JA-Ile and root ABA were associated to drought-induced mortality in *C. sativa* trees.

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SCIENTIFIC PRODUCTION

SCIENTIFIC PRODUCTION

Chapters I, II and III of this thesis have been published in peer-reviewed journals. Chapters IV and V are still manuscripts to be send soon to journals for publication.

Chapter I: **Álvaro Camisón**, M. Ángela Martín, F. Javier Dorado, Gerardo Moreno, Alejandro Solla (2020). Changes in carbohydrates induced by drought and waterlogging in *Castanea sativa*. Trees 34:579–591.

Chapter II: **Álvaro Camisón**, M. Ángela Martín, Jonás Oliva, Malin Elfstrand, Alejandro Solla (2019). Increased tolerance to *Phytophthora cinnamomi* in offspring of ink-diseased chestnut (*Castanea sativa* Miller) trees. Annals of Forest Science 76:119.

Chapter III: **Álvaro Camisón**, M. Ángela Martín, Paloma Sánchez-Bel, Victor Flors, Francisco Alcaide, David Morcuende, Gloria Pinto, Alejandro Solla (2019). Hormone and secondary metabolite profiling in chestnut during susceptible and resistant interactions with *Phytophthora cinnamomi*. Journal of Plant Physiology 241: 153030.

Additional publications resulting from the collaboration in other tasks out of the scopus of this thesis were:

Álvaro Camisón, Fernando Silla, Jesús Julio Camarero (2016) Influences of the atmospheric patterns on unstable climate-growth associations of western Mediterranean forests. Dendrochronologia 40:130–142.

Antonio Gazol, Jesús Julio Camarero, Raúl Sánchez-Salguero, Sergio M. Vicente-Serrano, Xavier Serra-Maluquer, Emilia Gutiérrez, Martín de Luis, Gabriel Sangüesa-Barreda, Klemen Novak, Vicente Rozas, Pedro A. Tíscar, Juas C. Linares, Edurne Martínez del Castillo, Montse Ribas, Ignacio García-González, Fernando Silla, **Álvaro Camisón**, Mar Génova, José M. Olano, Ana-María Hereş, Jorge Curiel Yuste, Luis A. Longares, Andrea

Hevia, Miquel Tomas-Burguera, Juan Diego Galván (2020) Drought legacies are short, prevail in dry conifer forests and depend on growth variability. Journal of Ecology 00:1–12.

Antonio Gazol, Jesús Julio Camarero, Sergio M. Vicente-Serrano, Raúl Sánchez-Salguero, Emilia Gutiérrez, Martin de Luis, Gabriel Sangüesa-Barreda, Klemen Novak, Vicente Rozas, Pedro A. Tíscar, Juan C. Linares, Natalia Martín-Hernández, Edurne Martínez Del Castillo, Montserrat Ribas, Ignacio García-González, Fernando Silla, **Álvaro Camisón**, Mar Génova, José M. Olano, Luis A. Longares, Andrea Hevia, Miquel Tomás-Burguera, J. Diego Galván (2018) Forest resilience to drought varies across biomes. Global Change Biology 24:2143–2158.

Fernando Silla, **Álvaro Camisón**, Andrea Solana, Héctor Hernández, Guillermo Ríos, Miguel Cabrera, Dámaris López, Albert Morera-Beita (2018) Does the persistence of sweet chestnut depend on cultural inputs? Regeneration, recruitment, and mortality in *Quercus*- and *Castanea*-dominated forests. Annals of Forest Science 75:95.

Francisco Alcaide, Alejandro Solla, Marcello Cherubini, Claudia Mattioni, Beatriz Cuenca, **Álvaro Camisón**, M. Ángela Martín (2020) Adaptive evolution of chestnut forests to the impact of ink disease in Spain. Journal of Systematics and Evolution 58:504–516.

Marina Peña-Gallardo, Sergio M. Vicente-Serrano, Jesús Julio Camarero, Antonio Gazol, Raúl Sánchez-Salguero, Fernando Domínguez-Castro, Ahmed El Kenawy, Santiago Beguería-Portugés, Emilia Gutiérrez, Martín De Luis, Gabriel Sangüesa-Barreda, Klemen Novak, Vicente Rozas, Pedro A. Tíscar, Juan C. Linares, Edurne Martínez del Castillo, Montserrat Ribas Matamoros, Ignacio García-González, Fernando Silla, **Álvaro Camisón**, Mar Génova, José M. Olano, Luís A. Longares, Andrea Hevia, J. Diego Galván (2018) Drought Sensitiveness on Forest Growth in Peninsular Spain and the Balearic Islands. Forests 9:524.

APPENDIX

SUPPLEMENTARY MATERIALS FOR CHAPTER I

Table Supplementary 1. Results of the general linear mixed models for analysis of soluble sugars, starch, total NSC and soluble sugars:starch ratios in different tissues of *Castanea sativa* seedlings. Significant values ($P \leq 0.05$) are highlighted in bold

Variables	Tissue	Treatment		Time		Treatment × time	
		X^2	P	X^2	P	X^2	P
Sugars	Leaf	7.77	**≤0.05**	27.81	**≤0.001**	18.62	**≤0.001**
	Stem	13.07	**≤0.01**	21.89	**≤0.001**	37.19	**≤0.001**
	Root	18.66	**≤0.001**	20.96	**≤0.001**	15.49	**≤0.01**
Starch	Leaf	1.10	ns	47.95	**≤0.001**	16.93	**≤0.01**
	Stem	3.08	ns	4.86	ns	17.02	**≤0.01**
	Root	10.86	**≤0.01**	3.04	ns	27.20	**≤0.001**
Total NSC	Leaf	10.61	**≤0.01**	2.50	ns	23.55	**<0.001**
	Stem	6.88	**≤0.05**	9.10	**≤0.05**	18.45	**≤0.001**
	Root	2.00	ns	5.98	**≤0.05**	10.48	**≤0.05**
	Whole plant	2.42	ns	5.28	0.071	16.76	**≤0.01**
Sugars:starch	Leaf	24.16	**≤0.001**	235.36	**≤0.001**	6.78	ns
	Stem	41.39	**≤0.001**	31.12	**≤0.001**	2.57	ns
	Root	39.38	**≤0.001**	22.04	**≤0.001**	12.80	**≤0.05**

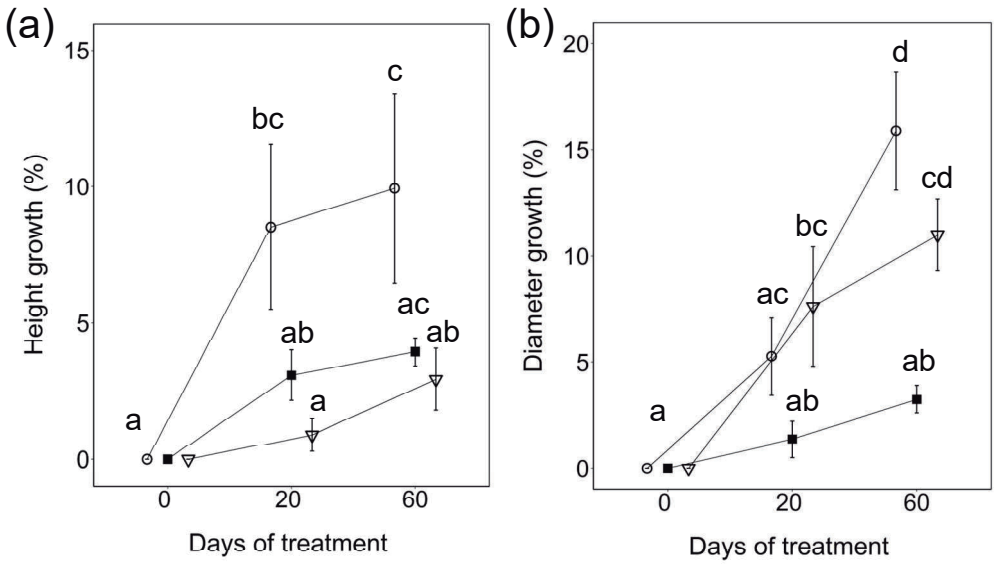

Figure Supplementary 1. Plant growth expressed as a percentage of initial height (a) and as a percentage of initial stem diameter at ground level (b) of two-year-old *Castanea sativa* seedlings subjected to regular watering (circles), drought (squares) and waterlogging (triangles) treatments. Vertical bars are standard deviations of means (n = 5) and different letters indicate significant differences (Tukey's HSD test, P < 0.05) between sampling points and treatments

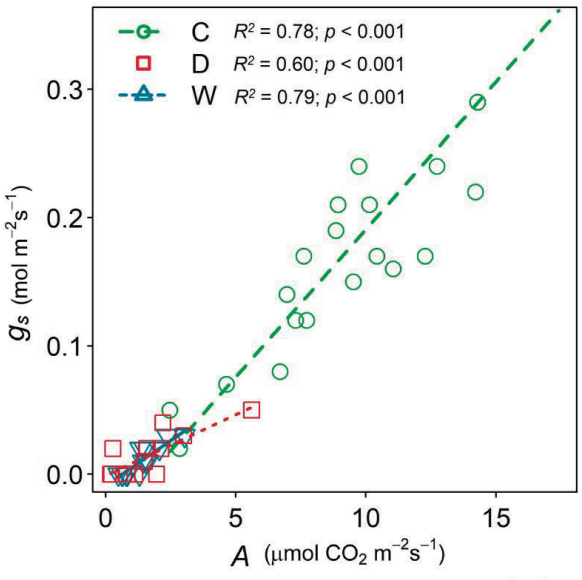

Figure Supplementary 2. Within-treatment relations between stomatal conductance (g_s) and net photosynthesis (A) in two-year-old *Castanea sativa* seedlings subjected to regular watering (control, C), drought (D) and waterlogging (W) treatments. The explained variance (R^2) and significance (p) of models are shown.

SUPPLEMENTARY MATERIALS FOR CHAPTER II

Table Supplementary 1. Genes studied in gene expression analysis by RT-qPCR. Primers sequences (forward and reverse), fragment size of resulting amplicon, melting temperature of PCR product and biological function for each gene are indicated.

Castanea sativa target gene	Primer sequence (5`- 3`)	Melting temperature (ºC)	Expected size of amplicon (bp)	Biological function
Cast_GNK2	F: GCCATCGATTTCTCCAAAGA R: CTTGGGGATCGTTGTCCTTA	76.0	99	Apoplastic antifungal protein (Wang and Ng, 2000; Miyakawa et al. 2014)
Cast_SAP11	F: ATCCGGTCAACCCTACCTTC R: GAGGGGTTCAGATTCGGATT	79.5	88	May act in protein targeting to the ubiquitin/ proteasome pathway (Giri *et al*. 2011)
Cast_MYB44	F: CTCAGGCGAGAGCTGGTTAC R: CCGAGAAACTGGTCGTTGAT	82.5	90	Modulates SA and JA signalling (Collins et al. 2003)
Actin7	F: CCAAGGCCAACAGGGAAAA R: CGGCCTGGATAGCAACATACA	79.0	100	Housekeeping gene (Serrazina *et al*. 2015)

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Table Supplementary 2. Parameters of the linear mixed model used to analyse the percentage of leaf wilting in *Castanea sativa* seedlings from mother trees with different health status (healthy *vs Phytophthora cinnamomi*-infected), exposed to drought.

Effect	df	*F*-ratio/χ2	*P*-value
Fixed factor			
Health status of mother tree	1	2.0	0.220
Block	4	0.9	0.478
Health status of mother tree × block	4	0.6	0.669
Random factor			
Mother tree (Health status of mother tree)	4	3.6	0.007
Covariates			
Seed weight	1	2.3	0.130
Radicle length	1	2.9	0.089
Time to emerge	1	1.6	0.196
Plant height	1	51.0	<0.001

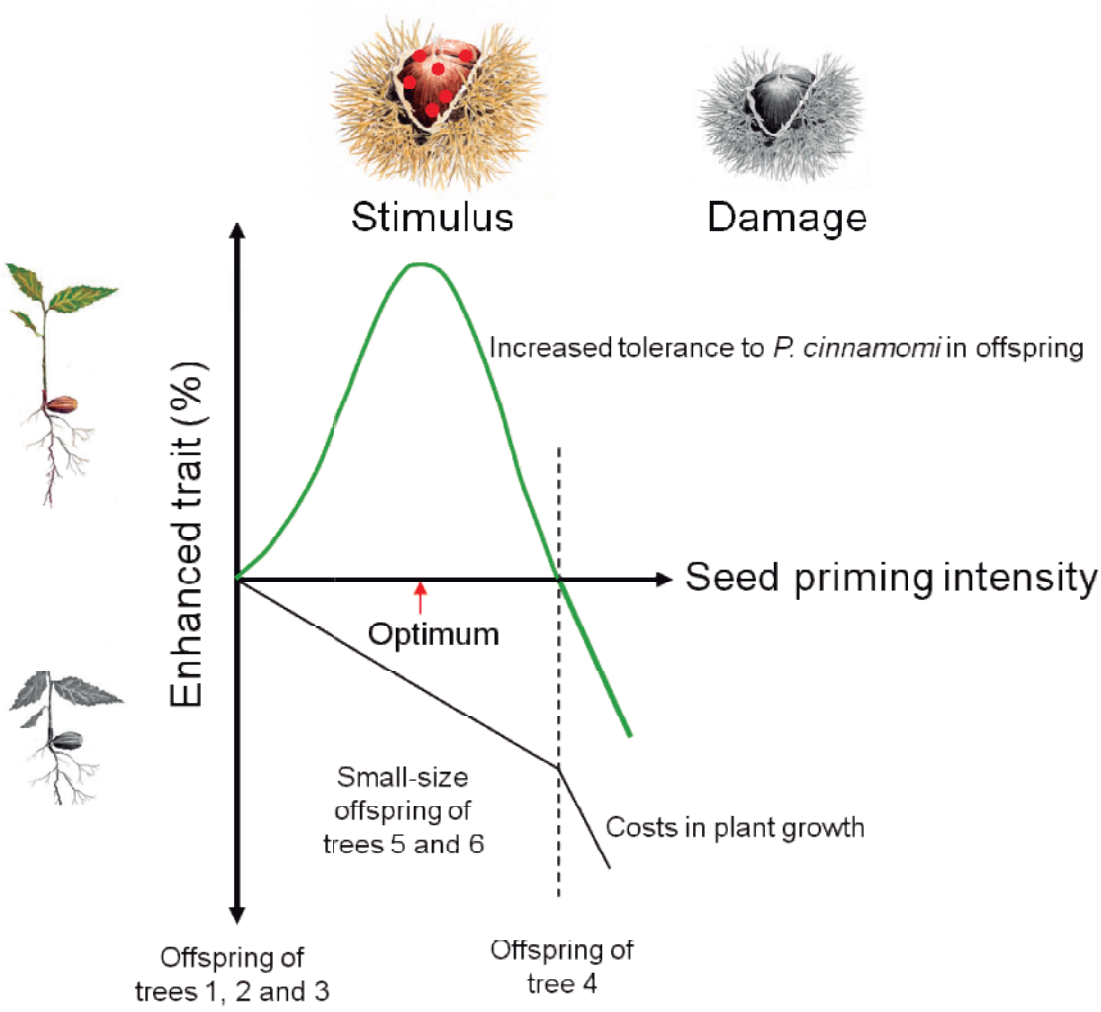

Figure Supplementary 1. Conceptual diagram of circumstances occurring in offspring of *Castanea sativa* trees after maternal priming (stimulus) or damage caused in nuts by *Phytophthora cinnamomi*.

SUPPLEMENTARY MATERIALS FOR CHAPTER III

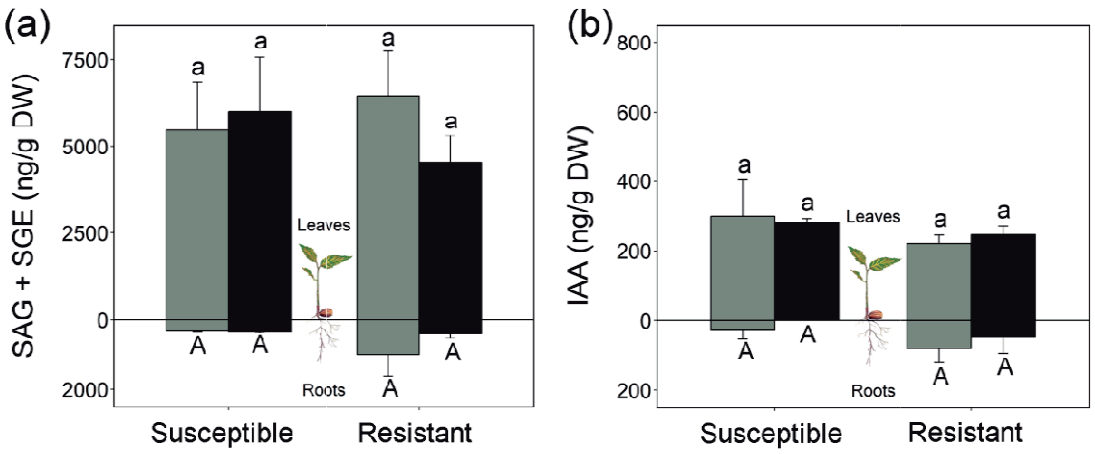

Figure Supplementary 1. Hormonal content in leaves (above the zero-line) and roots (below the zero-line) of susceptible and resistant chestnut clones 5 days before (grey bars) and 9 days after (black bars) inoculation with *Phytophthora cinnamomi* for SAG plus SGE (A) and IAA (B). Note the distinct scales. Error bars indicate one standard error of the mean (n = 3), while different letters indicate significant differences (Tukey's HSD test, $P < 0.05$) between clones and sampling points within (lower case letters) and roots (upper case letters).

SUPPLEMENTARY MATERIALS FOR CHAPTER IV

Table Supplementary 1. Height, grafting height and mortality after inoculation with *Phytophthora cinnamomi* Sample size (n) and morphological characteristics at the time of the six *Castanea* spp. scion/rootstock combinations used in this study. Combinations are formed according to the inherent resistance of scion and rootstock to *Pc*. Accumulated mortality in each group at the end of the experiment (four months after inoculation) is shown. Errors are one standard deviation of the mean.

Rootstock	Group	n	Grafting height (cm)	Tree height before inoculation (cm)	Mortality after inoculation (%)
Susceptible	S	31	-	97.4 ± 33.8	83.8
	S/S	28	23.3 ±7.9	99.2 ± 33.9	85.7
	R/S	51	24.0 ± 7.9	95.3 ± 35.1	98.0
Resistant	R	33	-	114.8 ± 36.6	18.7
	R/R	26	37.6 ± 14.3	125.4 ± 31.8	42.3
	S/R	43	42.2 ± 18.2	125.5 ± 30.5	33.3

R and S: resistant and susceptible own-rooted controls; R/R and S/S: resistant and susceptible autografts; S/R: susceptible scion onto resistant rootstock; R/S: resistant scion onto susceptible rootstock.

Figure Supplementary 1. Graft junction of *Castanea* spp. at the time of inoculation, not totally fused.

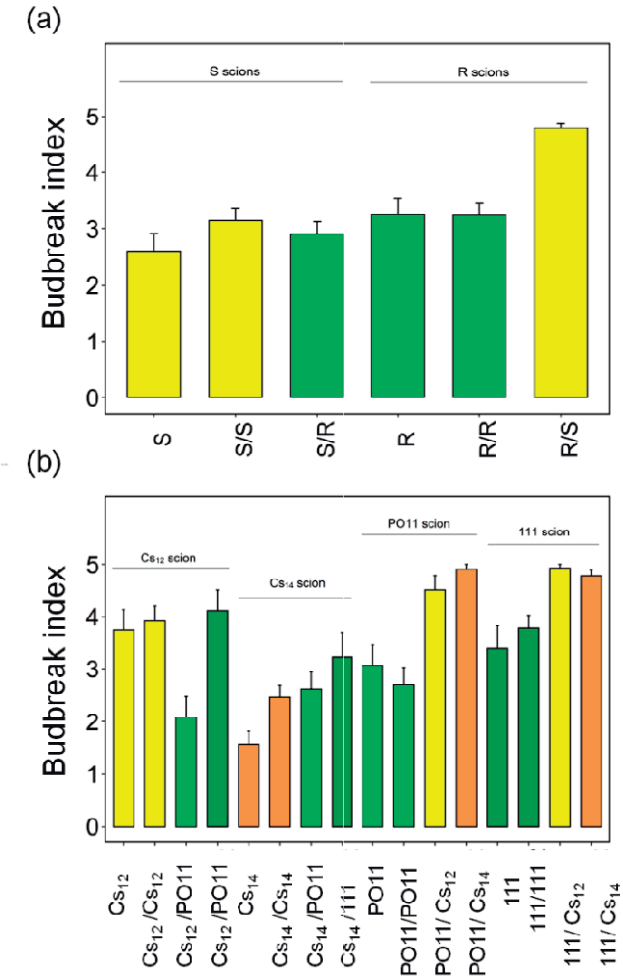

Figure Supplementary 2. Mean values ± se of vegetative budburst scores recorded in April 2017 in susceptible (S) and resistant (R) non-grafted, self-grafted (S/S and R/R) and heterografts (R/S and S/R) of *Castanea* spp. (a). Results are also presented considering *Pc* susceptible (Cs_{12} and Cs_{14}) and resistant (PO11 and 111-1) *Castanea* spp. clones (b).

SUPPLEMENTARY MATERIALS FOR CHAPTER V

Table Supplementary 1. 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*	–	X_1/ H_1*	X_2/ H_1
	H_2	–	H_2/ H_2	X_1/ H_2	X_2/ H_2
	X_1	H_1/ X_1*	H_2/ X_1	X_1/ X_1*	–
	X_2	H_1/ X_2	H_2/ X_2	–	X_2/ X_2

Scion/rootstock combinations with '–' were not used and those combinations selected for hormone and proline analysis are denoted with '*'.

Figure Supplementary 1. (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.

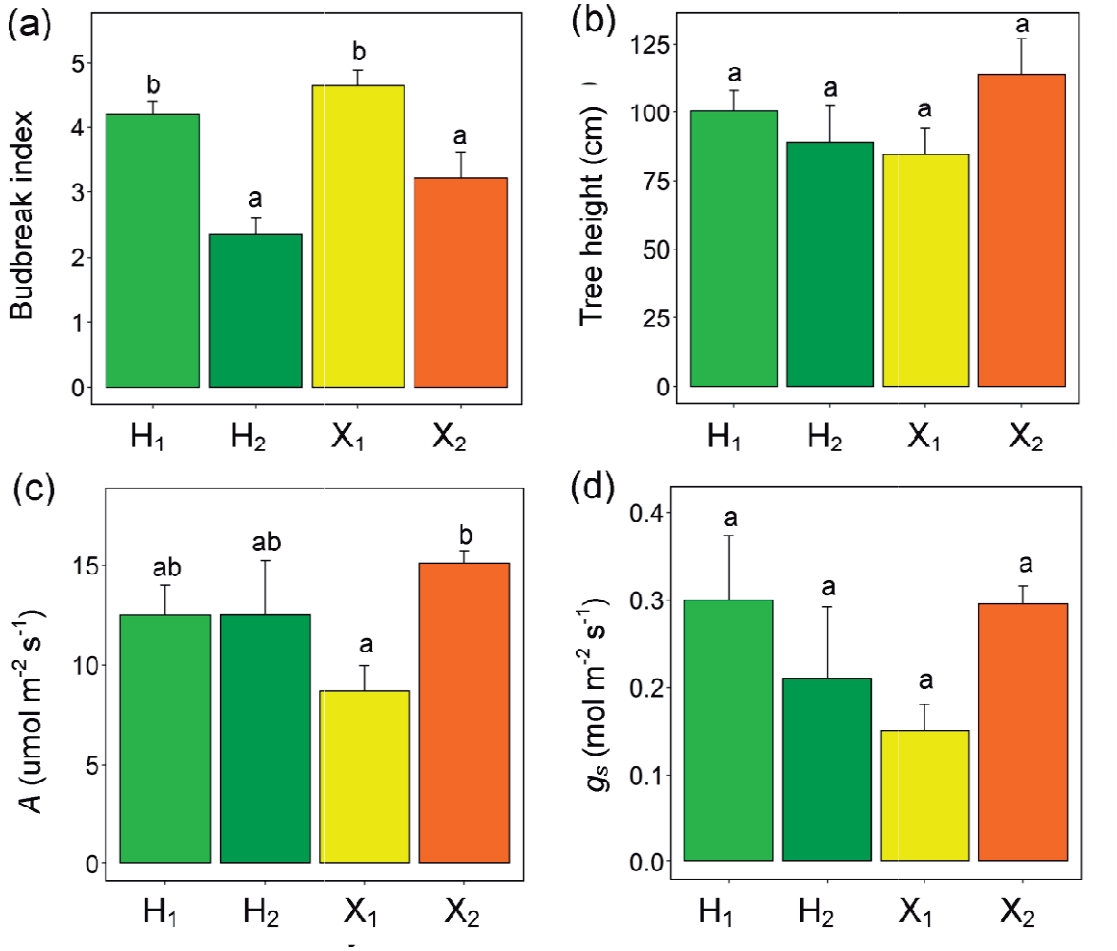

Figure Supplementary 2. Mean values of (a) budbreak index in April, (b) tree height, (c) carbon assimilation (A), and (c) stomatal conductance (g_s) in non-grafted controls of the *Castanea sativa* families X_1, X_2, H_1 and H_2 under optimal watering conditions. Error bars indicate one standard error of the mean (n = 5-7) and different letters indicate significant differences between means ($P < 0.05$; Tukey HSD).

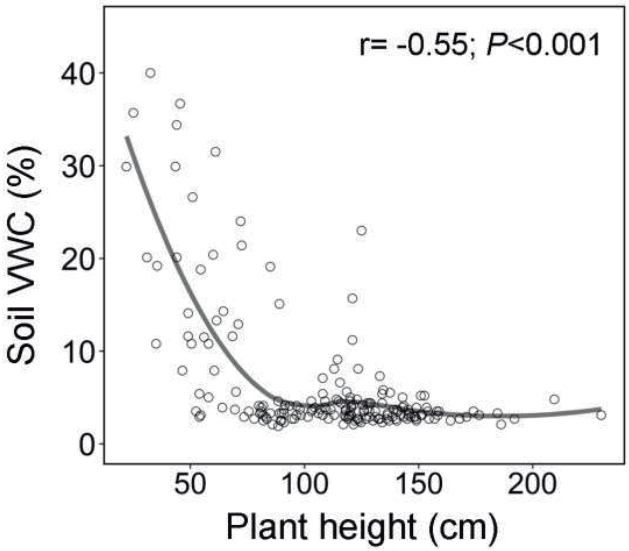

Figure Supplementary 3. Relationship between soil volumetric water content (soil VWC) in pots two weeks after water deprivation (when leaf wilting was assessed) and tree height measured just before water deprivation. Pearson correlation coefficient (r) and its significance (*P*) are shown.

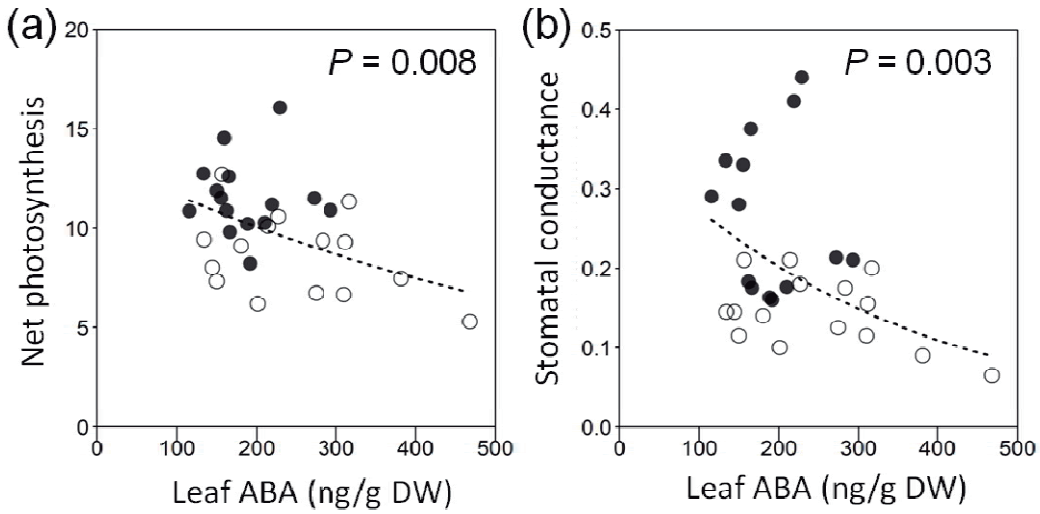

Figure Supplementary 4. Relationship between the constitutive content of ABA in leaves and (a) net photosynthesis (*A*, μmol CO_2 m^{-2} s^{-1}) and (b) stomatal conductance (g_s, mol m^{-2} s^{-1}) measured under optimum watering in *Castanea sativa* trees from xeric (open circles) and humid (closed circles). Negative exponential fits and their significance (*P*) are shown.