UNIVERSIDAD DE EXTREMADURA

# TESIS DOCTORAL

# Resiliencia de *Castanea sativa* Mill. ante factores de estrés relacionados con el cambio global

Francisco Javier Dorado Reyes

**Programa de Doctorado en Ciencia y Tecnología de los Sistemas Agroforestales**

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Conformidad de los Directores:

Fdo.: Dr. Alejandro Solla Hach

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Año 2023

Los doctores D. **Alejandro Solla Hach**, Catedrático de Universidad del Departamento de Ingeniería del Medio Agronómico y Forestal de la Universidad de Extremadura, y Dña. **M.ª Ángela Martín Cuevas** (Codirectora), Profesora Titular de Universidad del Departamento de Genética de la Universidad de Córdoba **certifican**:

Que la presente Tesis Doctoral titulada "Resiliencia de *Castanea sativa* Mill. ante factores de estrés relacionados con el cambio global", presentada por D. Francisco Javier Dorado Reyes para la obtención del título de Doctor, ha sido realizada bajo su dirección y cumple con los requisitos necesarios para ser juzgada por el correspondiente tribunal.

Y para que conste y surta los efectos oportunos, firman la presente en Plasencia, a 21 de diciembre de 2022.

Fdo.: Dr. Alejandro Solla Hach

Fdo.: Dra. M.ª Ángela Martín Cuevas

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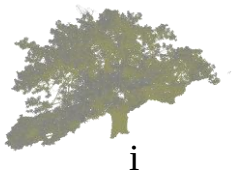

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# ÍNDICE DE CONTENIDO

# RESUMEN

El castaño (*Castanea sativa* Mill.) es un árbol termófilo de clima templado que se distribuye por toda la cuenca mediterránea y apreciado por sus múltiples aprovechamientos. Sin embargo, el aumento de las temperaturas, las olas de calor cada vez más frecuentes y el ataque de agentes bióticos como *Phytophthora cinnamomi* Rands. (*Pc*), están diezmando su productividad y supervivencia. El objetivo general de esta tesis ha sido contribuir, mediante un enfoque multidisciplinar, al conocimiento de las respuestas de *C. sativa* a factores relacionados con el cambio global, incluyendo el incremento de temperatura, las olas de calor y la combinación del incremento de temperatura y la infección por *Pc*. La respuesta del castaño a la temperatura y a las olas de calor (de tres a siete días consecutivos con temperaturas superiores a los 40 $^{o}$C), y su recuperación se evaluó desde un enfoque multidisciplinar que incluyó respuestas morfológicas, fisiológicas y metabólicas. Los resultados mostraron que la respuesta a la temperatura depende de la intensidad y duración del incremento de la misma. Así, un incremento de cinco grados de la temperatura ambiente sostenido en el tiempo mejora el crecimiento en altura y la producción de raíces finas. Por el contrario, los eventos de ola de calor generan alteraciones cada vez más significativas a medida que la duración del episodio se incrementa, generando estrés térmico en plántulas sometidas a una ola de calor de siete días consecutivos con temperaturas superiores a los 40 $^{o}$C. El estrés térmico afectó significativamente al metabolismo primario de la parte aérea de las plántulas e incluyeron el agotamiento del almidón y un aumento de los azúcares solubles y el nitrógeno. Los cambios en el metabolismo secundario se observaron

en las raíces e incluyeron un aumento en el contenido total de compuestos fenólicos. Durante la fase de recuperación, procesos de aclimatación y memoria del estrés de las plantas podrían estar influyendo en la respuesta de las plántulas de castaño. Pero de forma general, las plántulas restauraron casi por completo todos los parámetros tras el estrés térmico. También se evaluó la transferibilidad y polimorfismo de un conjunto de marcadores moleculares microsatélites derivados de secuencia expresada (EST-SSR) asociados al estrés térmico y desarrollados en otras especies forestales. Siete EST-SSRs resultaron transferibles a *C. sativa*, mostraron polimorfismo y han sido útiles para evaluar la diversidad genética adaptiva de poblaciones de la especie. Además, se identificaron dos marcadores, *POR016* y *VIT099*, bajo selección positiva que podrían utilizarse en programas de mejora genética de la especie para la selección asistida por marcadores. Se exploró el potencial adaptativo de las poblaciones de *C. sativa* en relación a su tolerancia al estrés térmico mediante la comparación de su diversidad genética funcional y la variabilidad fenotípica de sus progenies. Los resultados han mostrado que el uso combinado de ambos permite determinar variación en la plasticidad fenotípica relacionada con la respuesta al estrés térmico en castaño. Por último, se exploró el impacto que el efecto combinado de escenarios de calor y la infección posterior con *Pc* puede tener sobre la fisiología, bioquímica y supervivencia de las plántulas de *C. sativa*. Los resultados mostraron que los escenarios de elevada temperatura ambiente mejoran la tolerancia a *Pc*, probablemente por una mejora del fitness de la planta y por posibles modificaciones en su maquinaria defensiva frente al patógeno. En resumen, esta Tesis Doctoral ha permitido conocer la elevada resiliencia que tiene el castaño frente a eventos climáticos extremos como las olas de calor prolongadas, lo que proporciona información muy valiosa a la hora de establecer programas de mejora encaminados a producir planta tolerante a los escenarios futuros de cambio global.

# ABSTRACT

Chestnut (*Castanea sativa* Mill.) is a temperate thermophilic tree distributed throughout the Mediterranean basin and appreciated for its multiple uses. However, rising temperatures, increasingly frequent heat waves and biotic agents attacks, such as *Phytophthora cinnamomi* Rands (*Pc*), are decimating its productivity and survival. The overall objective of this Thesis has been to contribute, through a multidisciplinary approach, to the understanding of the responses of *C. sativa* to factors related to global change, including temperature increase, heat waves and the combination of temperature increase and *Pc* infection. Chestnut response to temperature and heat waves (three to seven consecutive days with temperatures above 40 ºC), and its recovery was evaluated from a multidisciplinary approach including morphological, physiological and metabolic responses. Thus, a five-degree increase in ambient temperature sustained over time improves height growth and fine root production. In contrast, heat wave events generate increasingly significant alterations as the duration of the episode increases, generating heat stress in seedlings subjected to a heat wave of seven consecutive days with temperatures above 40 ºC. Heat stress significantly affected the primary metabolism of the aerial part of the seedlings and included starch depletion and an increase in soluble sugars and nitrogen. Secondary metabolism changes were observed in the roots and included an increase in the total content of phenolic compounds. During the recovery phase, processes of acclimation and memory of plant stress might be influencing the response of chestnut seedlings. In general, the seedlings restored almost completely all parameters after heat stress. During recovery, processes of

acclimation and plant stress memory might be influencing the response of the chestnut seedlings. In general, seedlings restored almost completely all parameters after heat stress. Transferability and polymorphism of a set of expressed sequence-derived microsatellite molecular markers (EST-SSRs) associated with heat stress and developed in other forest species were also assessed. Seven EST-SSRs were found to be transferable to *C. sativa*, showed polymorphism and have been useful for assessing the adaptive genetic diversity of populations of the species. In addition, two markers, *POR016* and *VIT099*, were identified under positive selection that could be used in breeding programmes of the species for marker-assisted selection. *C. sativa* adaptive potential of populations was explored in relation to their tolerance to heat stress by comparing their adaptive genetic diversity and phenotypic variability of their progenies. The results have shown that the combined use of both allows determining variation in phenotypic plasticity related to the response to heat stress in chestnut. Finally, the impact that the combined effect of heat scenarios and subsequent infection with *Pc* can have on the physiology, biochemistry and survival of *C. sativa* seedlings was explored. The results showed that high ambient temperature scenario improve tolerance to *Pc*, probably due to an improvement in plant fitness and possible modifications in the plant's defensive machinery against the pathogen. In summary, this Doctoral Thesis has provided knowledge of the high resilience of chestnut to extreme climatic events such as prolonged heat waves, which provides valuable information for establishing breeding programs aimed at producing plants tolerant to future global change scenarios.

# INTRODUCCIÓN GENERAL

## Factores de cambio global: cambio climático y patógenos exóticos invasores forestales

El cambio global hace referencia al impacto de las actividades humanas sobre los procesos claves que rigen el funcionamiento de la biosfera (IPCC, 2013). El crecimiento exponencial de la población mundial y su concomitante expansión de las redes de comercio mundial, el aumento del volumen de bienes comercializados y emisiones de gases de efecto invernadero, ha provocado un cambio del clima y un incremento de los organismos exóticos invasores (Jiang & Hardee, 2011; Ramsfield et al., 2016). La influencia humana ha contribuido a los cambios observados a escala mundial en la frecuencia e intensidad de las temperaturas extremas diarias desde mediados del siglo XX, y es probable que su influencia haya duplicado la probabilidad de que se produzcan olas de calor en algunos lugares (IPCC, 2013). A su vez, el cambio climático está alterando las formas de interactuar de los patógenos con las especies forestales y la capacidad de éstas para resistir y tolerar los ataques (Linnakoski et al., 2019). Todo esto supone un reto para poder predecir el impacto de las interacciones clima-patógeno-árbol, y cómo repercutirán dichas interacciones en la persistencia de los ecosistemas forestales (Alberto et al., 2013; Pautasso et al., 2015).

El cambio climático está aumentando la temperatura de la superficie terrestre a un ritmo sin precedentes. Se prevé que el aumento de las temperaturas medias globales en superficie para 2081-2100 en relación con 1986-2005 se sitúe probablemente en los intervalos de 0.3 ºC a 1.7 ºC (RCP2.6) en el escenario más prometedor, y de 2.6 ºC a 4.8 ºC (RCP8.5) bajo el escenario de emisiones más pesimista (IPCC, 2013). Además, el cambio climático está incrementando la frecuencia e intensidad de los fenómenos meteorológicos extremos como las olas de calor (IPCC, 2013). Una ola de calor se define, según la Organización Meteorológica Mundial, como un fenómeno meteorológico extremo con un marcado calentamiento del aire en una zona extensa que suele durar de unos días a unas semanas. Otras definiciones son más precisas, e indican que una ola de calor se define como un periodo en el que al menos de 3 a 6 días consecutivos superan su respectivo percentil de día natural de temperatura máxima diaria (Molina et al., 2020).

La región Mediterránea está considerada como un "punto caliente" del cambio climático debido a preocupantes proyecciones para la zona (Lionello & Scarascia, 2018). En las últimas décadas, se ha producido un gran aumento de las temperaturas extremas y las olas de calor (intensidad, número y duración) y se prevé un calentamiento estival a un ritmo de un 40% superior a la media mundial (Lionello & Scarascia, 2018, 2020).Todo esto está ocasionando que la diversidad y la supervivencia de los bosques mediterráneos se esté viendo amenazada (Peñuelas et al., 2017).

Las perturbaciones sobre los ecosistemas forestales, derivadas de la acción del hombre, y la intensificación del comercio internacional han propiciado que a lo largo del último siglo se hayan notificado nuevas enfermedades a un ritmo cada vez mayor (Stenlid et al., 2011). Existen numerosos ejemplos de enfermedades que se han propagado fuera de sus rangos de distribución natural y que han tenido repercusiones catastróficas para los bosques. Por ejemplo, *Ophiostoma ulmi* y *O.*

*novo-ulmi*, agentes causales de la grafiosis del olmo, han devastado las poblaciones de la especie por todo el hemisferio norte debido probablemente al comercio de madera infestada (Brasier & Kirk, 2010). El comercio de material vegetal infestado a través de viveros, también, ha propiciado que el patógeno de origen asiático, *Phytophthora* cinnamomi, esté presente en gran parte de los castañares de Europa (Jung et al., 2016).

Las plantas están continuamente expuestas a factores bióticos y abióticos que actúan en combinación, lo que las ha llevado a realizar ajustes y remodelaciones de su maquinaria defensiva, así como a la reconfiguración de su metabolismo (Nephali et al., 2020). Cada vez más datos científicos indican que existe un solapamiento en las respuestas defensivas de las plantas a múltiples estreses bióticos y abióticos y que, aunque la respuesta es única en la combinación de estreses, existen patrones comunes con el estrés aplicado individualmente (Nejat & Mantri, 2017). Por ejemplo, uno de los mecanismos que tienen las plantas para tolerar el calor es la inducción de la síntesis de proteínas pertenecientes a la familia de las proteínas de choque térmico (HSP) (Bourgine & Guihur, 2021; Serrano et al., 2019). Las HSPs también son importantes en la señalización de defensa durante el ataque de patógenos, modulando la estabilidad y acumulación de proteínas de resistencia, y se ha comprobado que ante la infección por *Pc* estas proteínas son silenciadas en *C. sativa* (Saiz-Fernández et al., 2020). Condiciones de clima cambiante en combinación con la presencia de un patógeno exótico invasor (factores de cambio global) pueden poner en jaque la supervivencia de los árboles.

## Plasticidad fenotípica, diversidad genética y adaptación local

En respuesta al cambio climático, las poblaciones de árboles forestales tienen tres destinos posibles: migración, adaptación o desaparición (Aitken et al., 2008). La capacidad de los árboles para adaptarse a los cambios medioambientales depende de la diversidad genética de sus poblaciones y de la capacidad de mostrar respuestas fenotípicas plásticas (Aitken y Whitlock, 2013; Alberto et al., 2013).

Niveles bajos de diversidad genética pero elevados de plasticidad fenotípica podrían constituir una importante vía de persistencia frente a los cambios medioambientales. Por el contrario, una baja plasticidad fenotípica combinada con una baja diversidad genética podría suponer una combinación de potencial migratorio y adaptativo limitado que podría conducir a la desaparición (Boyd et al., 2022). La plasticidad fenotípica es el fenómeno por el cual un genotipo puede producir diferentes fenotipos en respuesta a las condiciones ambientales (Ghalambor et al., 2007). La plasticidad fenotípica puede ser adaptativa si tiene un efecto positivo en la aptitud de la planta, desadaptativa si tiene un efecto negativo, o neutra si no tiene ningún efecto (Scheiner, 1993). Por lo general, las plantas presentan altos grados de plasticidad fenotípica, pero la variación en el grado de plasticidad adaptativa puede hacer variar drásticamente las respuestas de las poblaciones o especies vegetales a los cambios ambientales (Boyd et al., 2022). Un conocimiento profundo de las respuestas fenotípicas plásticas de los árboles y de su ocurrencia entre poblaciones es esencial para predecir su potencial de adaptación al cambio climático (Valladares et al., 2014; Castellana et al., 2021). Cuando un fenotipo particularmente adaptado confiere una ventaja bajo el cambio climático puede invadir la población local a través de la migración y la selección, surgiendo una adaptación local (Villemereuil et al., 2018). Una de las herramientas más interesantes de las que disponen los investigadores para evaluar la adaptación local es el uso combinado de campos experimentales de plántulas y estudios genómicos (Alberto et al., 2013; Sork et al., 2013).

La genómica proporciona información útil sobre cómo la selección puede dar forma a los patrones de diversidad genética en poblaciones forestales (Allendorf et al., 2010). Las técnicas moleculares ofrecen nuevas posibilidades para los estudios genéticos y ambientales, permitiendo valorar la variabilidad existente entre moléculas resultantes de la acción directa de los genes, como isoenzimas o proteínas de reserva, o detectarla directamente en el material hereditario (ADN). Dentro de los marcadores de ADN, los marcadores microsatélites derivados de secuencia

expresada (EST-SSRs) asociados a regiones expresadas del genoma en diferentes condiciones fisiológicas de las plantas proporcionan una fuerte evidencia de selección que es más informativa que los SSRs genómicos (Kalia et al., 2011). El comportamiento no neutro de los loci anotados por marcadores EST-SSR puede indicar genes asociados a un rasgo fenotípico implicado en la adaptación (Cortés et al., 2020). Además, la detección de *outlier* loci (loci con niveles significativamente altos o bajos de variación y diferenciación) permite separar los efectos causados por procesos demográficos de los efectos adaptativos específicos de locus (Alcaide et al., 2019; Luikart et al., 2003). La creciente disponibilidad de secuencias de genes de muchos organismos, incluidos los árboles, está ayudando a aumentar la viabilidad de los estudios de asociación entre la diversidad genética adaptativa y la variación en los rasgos fenotípicos (Gailing et al., 2009; Mittler & Shulaev, 2013). Varios estudios han asociado la diversidad genética adaptativa con la variación en rasgos fenotípicos en especies arbóreas relevantes de los géneros *Pinus*, *Eucalyptus*, *Quercus*, *Alnus* y *Castanea* (Alcaide et al., 2019, 2020; Bradbury et al., 2013b; De Kort et al., 2014; Grivet et al., 2011; Sullivan et al., 2013).

## Impacto de las temperaturas sobre la vegetación

La temperatura es una de las variables más importantes que influyen en el crecimiento de las plantas (Gray & Brady, 2016). En árboles de regiones templadas, mejora el crecimiento en altura y la biomasa aérea probablemente debido a una mejora de la fotosíntesis y de la respiración por una función enzimática más rápida (Way & Oren, 2010). La fotosíntesis puede verse favorecida por un incremento de temperatura o por un proceso de aclimatación inducido por el propio incremento de temperatura que desplace el óptimo térmico para la cual el ratio fotosintético neto es máximo (Crous et al., 2022; Way & Oren, 2010; Way & Yamori, 2014). Además, puede cambiar el patrón de atribución de carbono entre la parte aérea y radicular, lo que repercute en el comportamiento de la especie ante otros factores de estrés abiótico como el estrés hídrico (Way & Oren, 2010). La temperatura puede

modificar la estructura del sistema radicular de las plantas, desempeñando un papel clave en la adaptación de las plantas a temperaturas elevadas (Luo et al., 2020). Por otro lado, se ha demostrado que la arquitectura del sistema radicular no solo mejora la adaptación de las plantas al incremento de temperatura, sino que también ayuda al rendimiento de las mismas, en condiciones de campo, por una mejora en la competencia en las interacciones subterráneas (Caffaro et al., 2011; de Dorlodot et al., 2007; Luo et al., 2020; Román-Avilés et al., 2004). Sin embargo, si las temperaturas superan un umbral de temperatura, que va a depender de la especie, paran su crecimiento. En general, un incremento por encima de los 10-15 $^{o}$C sobre la temperatura ambiente es suficiente para poder causar estrés térmico (Wahid et al., 2007)

El estrés térmico, por tanto, se define como un aumento de la temperatura por encima de un umbral suficiente para causar daños irreversibles a la planta (Wahid, 2007). Los daños visibles derivados del estrés térmico incluyen quemaduras de hojas y ramas, senescencia y abscisión de hojas, o inhibición del crecimiento de brotes y raíces (Hasanuzzaman et al., 2013; He et al., 2021; Wahid et al., 2007), lo que repercute en la productividad y vitalidad de las plantas. Múltiples mecanismos fisiológicos y bioquímicos subyacen al daño visible causado por el estrés térmico en las plantas (Wahid et al., 2007). Por lo general, las especies vegetales reaccionan al calor cerrando los estomas (Marchin et al., 2022). Cuando esto ocurre, las plantas mantienen la función hidráulica a expensas del sobrecalentamiento de las hojas (debido a la reducción de la transpiración) y a la reducción de la asimilación de carbono (debido a la reducción de la fotosíntesis) (Ruehr et al., 2015). El calor puede impactar directamente en las tasas fotosintéticas reduciendo la actividad enzimática, principalmente Rubisco, y dañando el fotosistema II (Bhagat et al., 2014; Birami et al., 2018; Nievola et al., 2017) a través de la acumulación de especies reactivas de oxígeno (ROS). Para protegerse del estrés oxidativo, las plantas utilizan maquinaria antioxidante tanto enzimática (por ejemplo, superóxido dismutasa y ascorbato peroxidasa) como no enzimática (por ejemplo, ácidos

fenólicos y flavonoides) para descomponer y eliminar los radicales libres y/o interrumpir sus cadenas de reacción (Ahmad et al., 2010; Poudel & Poudel, 2020; Shivashankara et al., 2016).

Las plantas sintetizan una gran diversidad de compuestos de bajo peso molecular como carbohidratos, metabolitos secundarios (ácidos fenólicos, flavonoles y ligninas) y hormonas que son esenciales para la aclimatación, supervivencia y recuperación de las plantas bajo estrés abiótico (Zandalinas et al., 2017). Los carbohidratos no estructurales (NSC), incluyendo azúcares solubles y almidón, son una pequeña fracción de los carbohidratos adquiridos por la planta vía fotosíntesis (Martínez-Vilalta et al., 2016). Sin embargo, los NSC desempeñan un papel crucial en la supervivencia de las plantas bajo estrés ambiental, amortiguando la asincronía entre la oferta y la demanda de sustratos (Dietze et al., 2014; Galiano et al., 2011; Hartmann et al., 2013; Li et al., 2018; O'Brien et al., 2014; Wiley & Helliker, 2012). La mayoría de los compuestos de metabolitos secundarios se sintetizan a partir de productos del metabolismo del carbono primario (Wahid & Ghazanfar, 2006). Los compuestos fenólicos son una clase importante de metabolitos secundarios, y su acumulación en los tejidos vegetales se considera una respuesta adaptativa de las plantas a condiciones ambientales adversas (Akhi et al., 2021). En especies agronómicas, se ha reportado que la acumulación de metabolitos secundarios fotoprotectores y antioxidantes podría ser un mecanismo adaptativo para prevenir daños por estrés térmico (Rivero et al., 2001; Zandalinas et al., 2017). En especies forestales, la producción de metabolitos secundarios inducida por el calor parece depender de la especie, del compuesto o incluso de la forma en la que se aplique el calor (Berini et al., 2018). En el escenario actual de cambio climático, comprender el impacto del estrés térmico y las olas de calor en la vegetación es un reto crucial para anticipar pérdidas socioeconómicas y daños a los ecosistemas naturales (Della-Marta et al., 2007; Rita et al., 2020; Spinoni et al., 2014).

## Especie de estudio: *Castanea sativa* Mill.

El castaño (*Castanea sativa* Mill.) es un árbol termófilo de clima templado que forma masas naturales, seminaturales y gestionadas de gran valor ambiental, cultural y económico (Conedera et al., 2004; Fernández-López et al., 2021; Fernández-López & Alía, 2003). Es una especie apreciada por su producción maderera y frutícola, su contribución al paisaje y al medio ambiente y sus subproductos, como la miel y las setas comestibles. Su área de distribución abarca desde el sur de Europa (Península Ibérica, Italia, Balcanes, islas mediterráneas) y el norte de África (Marruecos), hasta el noroeste de Europa (Inglaterra, Bélgica) y el este de Asia occidental (noreste de Turquía, Armenia, Georgia, Azerbaiyán, Siria), cubriendo alrededor de 1700 000 ha en Europa (Conedera et al., 2016; Fernández-López & Alía, 2003). Encuentra su hábitat óptimo en zonas donde la temperatura media anual oscile entre 8 y 15 ºC y las temperaturas medias mensuales sean superiores a 10 ºC durante 6 meses. La especie necesita una pluviosidad mínima que oscila entre 600 y 800 mm según su distribución e interacción con las temperaturas (Conedera et al., 2016). En climas atlánticos y continentales, *C. sativa* aparece como especie dominante en zonas de media ladera. Sin embargo, en zonas donde la disponibilidad de agua es baja, comparte su hábitat con especies ribereñas como *Alnus spp.*, *Ulmus* spp. y *Fraxinus angustifolia* (Camisón et al., 2020). En los valles del sur de Portugal, España, Italia y Grecia, los castañares que habitan en las laderas superiores están expuestos a la sequía y olas de calor en verano (Camisón et al., 2020). En España, la superficie ocupada por la especie según datos del Tercer y Cuarto Inventario Forestal Nacional ronda las 300 000 ha de masa pura, y se distribuye principalmente por la región húmeda norte-noroeste, encontrándose manchas dispersas en el centro y sur en condiciones de mayor sequía y olas de calor extremas (Fernández-López et al., 2021). Presenta una elevada diversidad genética tanto intra- como inter-poblacional (Fernández-López et al., 2021; Martín et al., 2009, 2012; Míguez-Soto et al., 2019), lo que permite ser optimistas en la capacidad de adaptación de la especie a los cambios globales. Además, se ha recuperado el

interés por el cultivo dedicado a la producción de fruto debido a la necesidad de tener una mayor producción para poder competir con la castaña de Asia, lo que ha repercutido en un incremento de los precios de la castaña.

## Impacto del cambio climático en *C. sativa*

Los bosques de castaño de la región Mediterránea están amenazados por el calentamiento global. Los modelos de distribución de especies predicen que el castaño se verá desplazado de la cuenca mediterránea hacia Europa central (Buras & Menzel, 2019). Sin embargo, la amplia distribución del castaño a lo largo de regiones con marcadas diferencias climáticas y la elevada diversidad genética entre y dentro de las poblaciones podría estar ayudando a la especie a adaptarse al cambio climático (Martín et al., 2010; Mattioni et al., 2017; Castellana et al., 2021). De hecho, las diferencias en la pluviometría entre las poblaciones del norte y el centro-sur de España han dado lugar a la diferenciación de dos ecotipos de castaño (mesófilo en el norte de España y xérico en el centro-sur de España) con diferentes respuestas adaptativas al estrés hídrico (Alcaide et al., 2019; Míguez-Soto et al., 2019; Míguez-Soto & Fernández-López, 2015). La sequía es considerada como una fuerza selectiva que moldea la diferenciación de las poblaciones de castaño (Díaz et al., 2009; Míguez-Soto y Fernández-López, 2015). El conocimiento de los efectos del estrés hídrico en *C. sativa* es muy amplio y se conocen los cambios morfológicos, fisiológicos, metabólicos y hormonales en respuesta a la sequía (Camisón et al., 2020; Camisón et al., 2021; Ciordia et al., 2012; Gomes-Laranjo et al., 2012; Maurel et al., 2004). Otros factores de estrés abiótico como el encharcamiento o las heladas también han sido abordados en la especie (Camisón et al., 2020; Díaz et al., 2009). Sin embargo, poco se sabe sobre la respuesta de *C. sativa* al estrés térmico o si el contraste de hábitats climáticos implica respuestas diferenciales a temperaturas más altas. Se ha estudiado la eficacia de los fertilizantes en la tolerancia del castaño a las altas temperaturas (Carneiro-Carvalho et al., 2021) y el efecto de la temperatura (Gomes-Laranjo et al., 2006) en algunos

cultivares de castaño. Sin embargo, ningún estudio ha abordado hasta el momento los efectos de las altas temperaturas sobre la fisiología y la bioquímica de esta especie.

## Impacto de *P. cinnamomi* en *C. sativa*

El castaño se enfrenta a múltiples factores de estrés biótico que han repercutido o están repercutiendo en la persistencia de sus bosques. Entre las enfermedades más importantes que sufre la especie se encuentran: el chancro del castaño, cuyo agente causal es *Cryphonectria parasitica*, y la tinta del castaño causada por especies del género *Phytophthora*. La tinta, causada principalmente por *Phytophthora cinnamomi* Rands (*Pc*), se considera la enfermedad más extendida y destructiva de *C. sativa* (Hardham & Blackman, 2018; Jung et al., 2018; Serrazina et al., 2015; Vettraino et al., 2005). Los síntomas incluyen pudrición de las raíces finas, necrosis del cuello de la raíz, obstrucción de los vasos del xilema, clorosis foliar, marchitez rápida o gradual de las hojas y muerte (Fernandes et al., 2022; Gomes-Laranjo et al., 2004). *Pc* es un patógeno transmitido por el suelo (Stramenopila, Oomycota) que infecta cerca de 5000 especies de plantas, incluyendo muchas de importancia en agricultura, silvicultura y horticultura (Hardham & Blackman, 2018). En castaño, se han estudiado recientemente los antecedentes genéticos, la adaptación, los efectos maternos y la histología de los árboles en respuesta a la infección por *Pc* (Alcaide et al., 2020; Camisón et al., 2019a; Fernandes et al., 2021; Santos et al., 2017; Serrazina et al., 2015). Además, se están dilucidando los cambios metabólicos, proteómicos y hormonales del castaño durante la infección con *Pc* (Camisón et al., 2019b; Dinis et al., 2011; Saiz-Fernández et al., 2020). *Pc* parece tener un gran impacto en especies de clima de tipo mediterráneo, donde las condiciones suaves y húmedas de otoño y primavera, ideales para la esporulación y la infección del hospedante, se alternan con veranos calurosos y secos, desfavorables para las plantas. De hecho, el impacto de la enfermedad de la tinta en el castaño depende del ambiente, siendo la enfermedad

más severa en zonas con altas temperaturas, baja humedad relativa y veranos secos (Martins et al., 1999). En castaño, se ha realizado un estudio sobre el impacto de *Pc* en combinación con estreses abióticos como la sequía o el encharcamiento (Martín et al., 2016). Sin embargo, no se ha estudiado el efecto combinado del incremento de temperatura y *Pc* en la especie. Ante el calentamiento global, es urgente estudiar si la temperatura influye en la fisiología, bioquímica y supervivencia del castaño en respuesta al *Pc*.

# OBJETIVOS

El objetivo general de esta Tesis Doctoral fue contribuir, mediante un enfoque multidisciplinar, al conocimiento de las respuestas de *Castanea sativa* a los factores relacionados con el cambio global, incluyendo el incremento de temperatura, las olas de calor y combinación del incremento de temperatura y la infección por *Phytophthora cinnamomi* (*Pc*). Los objetivos específicos fueron:

i) Analizar el impacto del incremento de las temperaturas y de los eventos de ola de calor en la fisiología y la bioquímica de plántulas de *C. sativa*; y cuantificar su respuesta al incremento de temperatura y eventos de ola de calor cuando las temperaturas vuelven a ser más bajas (Artículos I y II).

ii) Identificar respuestas fenotípicas y variación genética en la plasticidad fenotípica en respuesta al estrés térmico en plántulas de *C. sativa* de diferentes regiones climáticas (Artículo II).

iii) Seleccionar marcadores EST-SSR desarrollados en *Quercus* spp., *Fagus* spp. y *Eucalyptus* spp. asociados con el estrés térmico y confirmar su transferibilidad y polimorfismo en material español de *C. sativa* (Artículo III).

iv) Explorar el potencial adaptativo de las poblaciones de *C. sativa* en relación a su tolerancia al estrés térmico mediante la comparación de su diversidad genética funcional y la variabilidad fenotípica de sus progenies (Artículos II y III).

v) Explorar el impacto del efecto combinado de los escenarios térmicos y la infección posterior con *Pc* puede tener sobre la fisiología, bioquímica y supervivencia de las plántulas de *C. sativa* (Artículo I)

# MATERIAL Y MÉTODOS

En este apartado se expone una breve explicación sobre los materiales y métodos utilizados en la Tesis Doctoral. La información detallada se encuentra en los artículos originales.

## Material vegetal

Los estudios se han llevado a cabo en árboles madre procedentes de tres poblaciones silvestres de castaño (Artículos II y III.) y en plántulas de seis meses de edad (Artículos I, II y III). Las semillas se obtuvieron específicamente de: cuatro árboles madre localizados en Castañar de Ibor, Cáceres, Extremadura (39º37′23.6″ N, 5º23′1.6″ W) (Artículo I); 16 árboles madre por población en tres poblaciones localizadas en Puebla de Sanabria, Zamora, Castilla y León (42°02’05.4’’ N, 6°40’19.5’’ W), Valle de Matamoros, Badajoz, Extremadura (38°22’28.5’’ N, 6°48’00.1’’ W) y Paterna del Río, Almería, Andalucía (37°01’54.0’’ N, 2°57’15.5’’ W) (Artículos II y III). De estas tres últimas poblaciones, se recogieron muestras de cinco hojas verdes sanas por árbol madre. Las muestras de hojas se secaron y almacenaron en gel de sílice a temperatura ambiente para los análisis genéticos. En cada población de castaños, se seleccionaron los árboles madre separados, al menos, 70 m para minimizar las posibilidades de muestreo de individuos

entrecruzados.

Las semillas recogidas de cada uno de los árboles madre por población (Artículos I, II y III) se sumergieron en una solución fungicida (2 g $L^{-1}$ Thiram 80GD, ADAMA Inc., España) durante 5 min y las que flotaron se descartaron como no viables. Posteriormente, se enjuagaron con agua esterilizada y se almacenaron a 4 ºC durante dos semanas. Tras esto, se pesaron individualmente y se sembraron a 1 cm de profundidad en bandejas forestales de 48 alveolos con una semilla por alveolo. Los alveolos individuales tenían un volumen de 330 mL, 18 cm de altura, 5.3 × 5.3 cm de superficie superior y contenían turba (PKN1 Florava® Peat Substrate; pH = 4.5) como substrato. Se consideró que la germinación había tenido éxito cuando la parte aérea que emergía del embrión era verde. La emergencia aérea de las plantas se evaluó semanalmente. Las plantas se mantuvieron a la luz natural del día bajo la sombra del invernadero que reducía la radiación solar en un 50%, y se regaron a mano cada cuatro días a capacidad de campo hasta que estuvieron bien establecidas. El invernadero se encontraba en la Facultad de Ciencias Forestales de Plasencia (40º02′ N, 6º04′ W; 374 m s.n.m., región de Extremadura, España). Cuando las semillas habían germinado y las plántulas tenían unos 10-20 cm de altura, se recogieron dos hojas por plántula, se secaron y se conservaron en gel de sílice a temperatura ambiente para los análisis genéticos (Artículo III).

## Breve caracterización climática de las poblaciones

Castañar de Ibor se encuentra en el centro de España, en la comarca cacereña de Las Villuercas, se caracteriza por una pluviosidad media de unos 750 mm anuales, una temperatura media de 13.8 ºC y una temperatura máxima que ronda los 37 ºC (Artículo I). Puebla de Sanabria se encuentra en el norte de España, cerca de la región transmontana del noreste de Portugal, conocida como Tierra Fría ('Terra Fria' en portugués), y se caracteriza por una pluviosidad de unos 1.000 mm anuales (régimen pluviométrico húmedo, una temperatura media de 9.7 ºC y una temperatura máxima de 32.8 ºC. Valle de Matamoros se encuentra en las

estribaciones de Sierra Morena (centro-sur de España), se caracteriza por una pluviometría de 740 mm anuales (régimen pluviométrico continental), una temperatura media de 15 ºC y una temperatura máxima de 39.5 ºC. Paterna del Río está en las Alpujarras, en la vertiente sur de Sierra Nevada, en el sureste de España y se caracteriza por unos 650 mm de precipitación anual (régimen pluviométrico xérico), una temperatura media de 12.4 ºC y una temperatura máxima de 33.6 ºC. Para mayor claridad en la exposición de los resultados de los artículos, las poblaciones se denominaron como norte, centro y sur, respectivamente en el Artículo II; o húmeda, continental y xérica, respectivamente en el Artículo III. En la presente Tesis Doctoral, me referiré a ellas como población norte, centro o sur. La población del norte de España se localizaba en zona bioclimática supramediterránea mientras que el resto se encuentran en la zona mesomediterránea.

## Aislado fúngico

Se empleó una única cepa agresiva A2 (codificada como Ps-1683) de *Phytophthora cinnamomi* Rands. (*Pc*) aislada de un castaño adulto decaído en Galicia (norte de España) y cuya virulencia había sido probada previamente en plántulas de *C. sativa* (Camisón et al., 2019) (Artículo I).

## Diseño experimental

Se plantearon dos experimentos manipulativos de invernadero. En el primero (Artículo I), las plántulas se dispusieron siguiendo un diseño aleatorio de parcelas divididas replicado en tres bloques, en el que los tratamientos térmicos actuaron como factor principal (tres categorías: temperatura ambiente, temperatura ambiente elevada y episodios de olas de calor; parcelas enteras) y los árboles madre como factor dividido (cuatro categorías). Se utilizaron dos bandejas forestales por bloque y tratamiento térmico. En los tres bloques, los árboles madre estaban representados

en cada parcela entera por 24 plántulas de los cuatro árboles madre. Las plántulas se colocaron aleatoriamente dentro de los bloques. El experimento comprendía 864 plantas correspondientes a tres bloques × tres escenarios de calentamiento × cuatro familias × 24 individuos. Para la inoculación de *Pc*, se utilizó una bandeja por bloque y escenario de calentamiento. Los tratamientos térmicos duraron treinta días. El primer grupo de plántulas se colocó en un invernadero en el que la temperatura de 11 a 17 h se fijó en 30 ºC (temperatura ambiente). Según la quinta evaluación del IPCC, se prevé que la temperatura media global del aire aumente entre 0.3 y 4.8 °C a finales de este siglo (IPCC, 2013). Basándose en esta proyección, el segundo grupo de plántulas se colocó en un invernadero diferente en el que la temperatura de 11 a 17 h se fijó en 35 ºC (temperatura ambiente elevada). Ambos invernaderos eran similares en tamaño y exposición al sol (50% de radiación solar). El tercer grupo de plántulas se colocó en el primer invernadero durante 15 días (30 ºC de 11 a 17 h), en una cámara climática durante tres días (45 ºC de 11 a 17 h; primera ola de calor), de nuevo en el primer invernadero durante nueve días (30 ºC de 11 a 17 h), y de nuevo en la cámara climática durante tres días (45 ºC de 11 a 17 h; segunda ola de calor). La cámara climática tenía paredes translúcidas y estaba bajo un 50% de radiación solar. Treinta días después del inicio de los tratamientos y coincidiendo con el día después de la segunda ola de calor, la mitad de las plántulas se sometieron a temperatura ambiente para evaluar la recuperación, y la otra mitad de las plántulas se inocularon con *Pc*.

Para el segundo experimento (Artículos II y III), las plántulas se dispusieron siguiendo un diseño aleatorio de parcelas divididas replicado en tres bloques, en el que los tratamientos térmicos actuaron como factor principal (dos categorías: control y estrés térmico; parcelas enteras) y las poblaciones como factor dividido (tres categorías: norte, centro y sur; parcelas divididas). En los tres bloques, las tres poblaciones estaban representadas en cada parcela entera por cinco plántulas por árbol madre. Las plántulas se colocaron aleatoriamente dentro de los bloques. El experimento incluyó 1440 plántulas correspondientes a 3 bloques × 2 tratamientos

× 3 poblaciones × 16 árboles madre × 5 plántulas. El tratamiento de estrés térmico consistió en colocar las plantas dentro de una cámara climática de paredes translúcidas, en el mismo invernadero y aplicar una temperatura media de 42.5 °C desde las 11 de la mañana hasta las 5 de la tarde durante siete días seguidos, mientras que las plántulas en situación control estuvieron a 30.2 °C en esa franja horaria. Al finalizar el séptimo día, las plántulas del estrés térmico fueron llevadas a la situación control para evaluar su recuperación.

En ambos experimentos (Artículos I, II y III), la franja horaria que se seleccionó para aplicar el incremento de temperaturas simulaba las condiciones naturales de aumento diario de la temperatura durante el verano en la cuenca mediterránea, con una temperatura máxima desde el mediodía hasta las primeras horas de la tarde. Durante los tratamientos, todos los grupos de plántulas estuvieron bien regados y las hojas de las plántulas correspondientes al tratamiento de temperatura ambiente/control se mantuvieron verdes y turgentes en el transcurso los dos experimentos.

### Método de inoculación con *Pc*

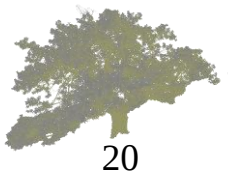
El inóculo de *Pc* se preparó según Jung et al. (1996), y se incubó a 20-25 ºC en oscuridad total, durante cuatro semanas. La infestación del suelo se realizó mezclando 12 mL de inóculo con los tres primeros centímetros de sustrato en cada alveolo individual, evitando dañar las raíces de las plántulas durante este proceso. Para favorecer un mejor establecimiento del patógeno, se mezclaron aproximadamente 50 g de hojas recién formadas con el sustrato y el inóculo en cada bandeja forestal de 48 alveolos. Tras la inoculación, las plántulas se regaron y, al día siguiente, se inundaron durante dos días en agua no clorada para promover la producción de esporangios y la liberación de zoosporas.

## Mediciones y muestreo de plántulas

Las mediciones y el muestreo para el análisis bioquímico se realizaron el primer día de los tratamientos (Artículo II), el último día de los tratamientos térmicos (Artículos I, II y III) y 10 días después del cese de los tratamientos térmicos (Artículos I y II), cuando las plántulas se encontraban en fase de recuperación. También, a los diez días del cese de los tratamientos térmicos, los grupos de plántulas que habían sido inoculados con *Pc* (diez días post inoculación) fueron medidos y muestreados (Artículo I).

El día 1, se midieron los parámetros de intercambio gaseoso de las hojas para observar la respuesta fotosintética de las poblaciones al aumento de la temperatura (Artículo I). En las fases de tratamientos térmicos y recuperación (o inoculadas con *Pc*), los efectos del calor en las plántulas se evaluaron mediante (i) síntomas foliares, marchitamiento de las hojas, crecimiento de la planta y mortalidad (Artículos I, II y III); (ii) intercambio de gases foliares (Artículos I y II); (iii) cuantificación de los parámetros del metabolismo primario (carbohidratos no estructurales, NSC; y prolina) y el contenido de nitrógeno (N) (Artículo II); y (iv) cuantificación de los parámetros del metabolismo secundario (compuestos fenólicos totales y compuestos ortofenólicos y flavonoides), eliminación de radicales libres 2,2-difenil-1-picrilhidrazilo (DPPH) (Artículo II) y perfiles de metabolitos no específicos (Artículos I y II).

Los parámetros de intercambio gaseoso foliar -tasa fotosintética neta ($P_n$), tasa de transpiración ($E$) y conductancia estomática ($g_s$)- se midieron utilizando un analizador de gases infrarrojo diferencial portátil (IRGA; Li-6400, Li-Cor INC., Lincoln, NE, EE.UU.) conectado a una cámara de hoja ancha y a un sensor de temperatura. La eficiencia instantánea e intrínseca en el uso del agua se calculó a partir del intercambio de gases foliares, utilizando las fórmulas WUE instantánea = Pn/E y WUE intrínseca = Pn /gs, respectivamente (Artículo II).



La biomasa seca (Artículos I, II y III), el N y los NSC (Artículo II) se determinaron a partir de plántulas recolectadas de forma destructiva y separadas en hojas, tejidos leñosos aéreos (tallo más ramitas pequeñas, "tallos") y raíces (raíz pivotante más raíces finas, "raíces"). Las muestras se secaron en estufa, se pesaron y se molieron hasta obtener un polvo fino en un molino de bolas (Mixer Mill MM 400, Retsch, Alemania).

Para los análisis de prolina (Artículos II y III), metabolitos secundarios (compuestos fenólicos totales y compuestos ortofenólicos y flavonoides), eliminación de radicales libres 2,2-difenil-1-picrilhidrazilo (DPPH) (Artículo II) y perfiles de metabolitos no dirigidos (Artículos I y II), se tomaron muestras de hojas y raíces de plántulas sometidas a los diferentes tratamientos (Artículos I, II y III). Cuando no había suficiente material, una muestra (hoja o raíz) estaba formada por material procedente de tres plántulas diferentes (Artículo II). En el caso de las hojas, se tomaron muestras de las que estaban completamente desarrolladas cerca del ápice de la planta. En las raíces, se recogieron las raíces finas más externas tras levantar con cuidado el cepellón de la bandeja forestal. Las muestras se congelaron inmediatamente en nitrógeno líquido y se almacenaron a -80 °C.

## Evaluación de síntomas externos, crecimiento y mortalidad

La evaluación de los síntomas externos comprendió la caracterización visual de la decoloración foliar observada en las plántulas (Artículos I, II y III). El porcentaje de marchitamiento se determinó mediante estimación visual del porcentaje de hojas marchitas en plantas sometidas a estrés térmico, en intervalos del 10% de acuerdo con Alcaide et al. (2019) (Artículos II y III). El crecimiento en altura de las plántulas se determinó midiendo la altura al inicio de los tratamientos y al final los mismos (Artículo I). La biomasa seca total de la planta se utilizó como indicador del crecimiento de la planta (Artículos I, II y III). La mortalidad inducida por *Pc* se evaluó periódicamente durante 30 días (Artículo I).

## Metabolismo primario

Los carbohidratos no estructurales (NSC) totales incluyeron la suma de azúcares solubles y almidón. Los azúcares solubles y el almidón en los tejidos de hoja, tallo y raíz se determinaron siguiendo el protocolo adaptado para el castaño por Camisón et al. (2020). Se calcularon los ratios azúcar soluble:almidón en cada tejido vegetal y se utilizaron como proxy de la movilización de almidón a azúcares solubles (Piper, 2011) ((Artículo II).

El nitrógeno (N) total en tejidos de hoja, tallo y raíz se determinó mediante el método Dumas (DUMATHERM® CN, C-Gerhardt Analytical Systems) y se expresó como porcentaje del peso seco (Artículo II). El contenido en prolina se determinó siguiendo el protocolo de Bates et al. (1973), con las modificaciones propuestas por Camisón et al. (2021) para el castaño (Artículos II y III). La concentración de NSC o N a nivel de planta entera se calculó mediante una media ponderada, teniendo en cuenta la concentración total de NSC o N de cada tejido y la contribución relativa de la biomasa de cada tejido a la biomasa seca total de la planta, siguiendo: NSC o N de toda la planta (%) = ((Xl × al + Xs × bs + Xr × cr) / (al + bs + cr)); donde Xl, Xs y Xr son las concentraciones totales de NSC o N en hojas, tallos y raíces, respectivamente, y al, bs y cr son las proporciones en que cada tejido contribuye a la biomasa seca de la planta (Artículo II).

## Metabolismo secundario

*Compuestos fenólicos y eliminación de radicales libres 2,2-difenil-1-picrilhidrazilo (DPPH)*

Las muestras de hojas o raíces pulverizadas se homogeneizaron con metanol acuoso y se agitaron en un agitador orbital. Después se centrifugaron, se recogió el sobrenadante y se repitió el proceso tres veces. El extracto se almacenó a -80 °C para su posterior determinación de compuestos fenólicos totales, compuestos

ortofenólicos y flavonoides, y eliminación de radicales libres DPPH (Artículo II).

Los contenidos fenólicos totales y ortofenólicos se estimaron mediante el método de Folin-Ciocalteu y el ensayo colorimétrico de molibdato sódico, respectivamente, ambos adaptados de Singleton et al. (1999). El contenido total de flavonoides se midió siguiendo el ensayo colorimétrico con cloruro de aluminio adaptado de Chang et al. (2002). La capacidad antioxidante total se midió mediante el método de eliminación de radicales libres 2,2-difenil-1-picrilhidrazilo (DPPH) adaptado de Xu & Chang (2007) (Artículos I y II).

### *Perfil de metabolitos no dirigido*

El polvo de hoja o de raíz fina se homogeneizó con etanol acuoso y se sometió a ultrasonidos. Los extractos se maceraron en frío, se centrifugaron y el sobrenadante se filtró. Los extractos filtrados se almacenaron a -80 °C hasta su análisis (Artículos I y II).

El análisis, la identificación y la cuantificación se realizaron con el sistema LC-MS (HPLC 1260 - QTOF 6520, Agilent Technologies, Santa Clara, CA, Estados Unidos). Se inyectó extracto filtrado de cada muestra en una columna analítica de fase inversa Spherisorb C-18 5 µ 250 × 4,6 mm a una velocidad de 1 mL/min. La fase móvil utilizada fue un gradiente de agua (ácido fórmico al 2%)/metanol del 1% de agua al 100% de metanol en 100 min para las hojas y 50 min para las raíces. Los cromatogramas se registraron a 350 nm y 280 nm de longitud de onda. Las concentraciones de los compuestos se estimaron a partir de una curva estándar utilizando los patrones ácido gálico, ácido elágico, quercetina y quercetina 3-O-rutinósido dependiendo de la naturaleza del compuesto a cuantificar.

## Análisis genético

Se utilizaron hojas de los 48 árboles adultos y de sus progenies (Artículo III). Se seleccionaron al azar hojas de ~35 plántulas (progenies) sometidas a estrés térmico por población. Se extrajo ADN genómico de hojas liofilizadas según el protocolo de Qiagen DNeasy™ Plant mini kit. Se preseleccionaron 20 marcadores EST-SSR asociados a genes relacionados con el estrés térmico desarrollados en *Quercus* spp, *Fagus* spp y *Eucalyptus* spp (Bradbury et al., 2013a; Burger et al., 2018; Durand et al., 2010; Müller & Gailing, 2018). Solo se analizaron los loci vinculados a secuencias con funciones conocidas.

Para probar la transferibilidad y el polimorfismo de los EST-SSRs, se amplificó el ADN de cuatro individuos en reacciones individuales siguiendo las indicaciones de los autores para las diferentes especies (Bradbury et al., 2013a; Burger et al., 2018; Durand et al., 2010; Müller & Gailing, 2018). Siete de los 20 EST-SSRs amplificaron y mostraron polimorfismo en todas las muestras. Basándose en el tamaño de los productos, se diseñaron tres mezclas multiplex-PCR, la primera incluyendo los cebadores *PIE125* y *VIT099* (A), la segunda incluyendo los cebadores *FIR035, POR016* y *GOT022* (B) y la tercera incluyendo los cebadores *FIR089* y *Qr6783* (C). Los cebadores *forwards* se marcaron con un fluorocromo (FAM; Merck Life Science S.L.U., Madrid, España; y VIC, NED; Applied Biosystems, Foster City, CA, EE.UU.). Las amplificaciones se llevaron a cabo siguiendo el protocolo Multiplex kit de Qiagen en un volumen total de 20 µL siguiendo el programa siguiente: 95 °C durante 15 min, 30 ciclos de 30 s a 94 °C, 90 s a 57 °C y 60 s a 72 °C, y un paso final de 30 min a 72 °C. Los productos de PCR resultantes de la amplificación (1 µL) se analizaron empleando un secuenciador automático capilar ABI 3010 Genetic Analycer Applied Biosystems/HITACHI. Para este análisis las muestras amplificadas se desnaturalizaron a 95 ºC durante 5 minutos y los alelos se definieron en función de su tamaño en pares de bases comparándolos con un marcador de tamaño estándar

(ROX-500). Los resultados obtenidos se procesaron mediante el programa Genotyper 3.7 (Applied Biosystems, UK).

## Análisis estadístico

Previo a los análisis estadísticos de los parámetros fisiológicos y bioquímicos, se comprobaron las asunciones de normalidad y homogeneidad de varianzas. Cuando los datos fueron normales, se emplearon modelos lineales generales (GLM) o ANOVAs (de una o dos vías) para determinar si las medias entre grupos fueron diferentes estadísticamente. Se utilizaron las pruebas de comparación múltiple de Tukey para identificar diferencias significativas entre grupos ($P < 0,05$). Estos procedimientos estadísticos y las representaciones gráficas se llevaron a cabo con el software STATISTICA v10 (StatSoft *Inc.*, 2011) (Artículos I, II y III).

Se empleó una matriz de correlaciones de Pearson para analizar las relaciones entre los parámetros mediante el paquete 'corrplot' del software R (R Core Team, 2018) (Artículo II).

Se aplicaron análisis de componentes principales (PCA) para reducir la dimensionalidad de los datos preservando la mayor parte de la varianza (Artículos I y II). También, se aplicaron análisis de función discriminante (DFA) (Artículo II). Los análisis DFA se realizaron con el software STATISTICA v10 (StatSoft *Inc.*, 2011). Los análisis PCA y la representación gráfica de los PCAs y DFAs se realizaron con el software R (R Core Team, 2018).

Para cada parámetro y población, la plasticidad fenotípica en respuesta al estrés térmico se expresó como porcentaje relativo a las plantas control siguiendo la ecuación propuesta por Rehschuh et al. (2020) en la fase de calor: $Plasticidad\ fenotípica\ (\%) = ((x - Cpop) / Cpop) \times 100$; donde $x$ es el valor inducido por el calor del parámetro de una determinada plántula de la población, y $Cpop$ es el valor medio constitutivo (control) del parámetro de las plántulas de la población (Artículo

II).

Los índices de diversidad genética intra e interpoblacional se calcularon con GenAlEx 6 (Peakall & Smouse, 2006): número de alelos por locus (A); heterocigosidad observada (Ho), esperada (He) y esperada corregida (uHe); y número de alelos privados en las poblaciones (Pa). El coeficiente de endogamia $F_{IS}$ (Weir & Cockerham, 1984) y el de diferenciación entre poblaciones $F_{ST}$ (Weir & Cockerham, 1984) y $R_{ST}$ (Slatkin, 1995)se calcularon con Arlequin 3.11 (Excoffier et al., 2005). La riqueza alélica (Ar) se calculó mediante el método de rarefacción con el software HP-rare (Kalinowski, 2005). Para cada locus, se calculó la frecuencia alélica nula estimada con el software Micro-Checker (Van Oosterhout et al., 2004). Se utilizó el software LOSITAN (Antao et al., 2008) para detectar loci atípicos, es decir, marcadores en los que la diversidad genética dentro de las poblaciones (heterocigosidad) y entre poblaciones ($F_{ST}$) no se ajusta a la predicción de selección neutra. Para determinar si los marcadores EST-SSR seleccionados asociados a la tolerancia al estrés térmico eran capaces de diferenciar estadísticamente las poblaciones de *C. sativa*, se utilizó un DFA empleando como variables las componentes principales de un análisis de coordenadas principales (PCoA) de la matriz de covarianza de distancia genética. El PCoA se realizó utilizando datos estandarizados y GenAlEx 6 (Peakall & Smouse, 2006). El DFA se realizó utilizando el software STATISTICA v10 (StatSoft *Inc.*, 2011) (Artículo III).

La estructura genética de las poblaciones de *C. sativa* se analizó aplicando un enfoque bayesiano implementado en el software STRUCTURE v.2.3.4 (Pritchard et al., 2000). Para identificar el número de grupos que mejor explicaba los datos se utilizó STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Las simulaciones se promediaron utilizando el software CLUMPP (Jakobsson & Rosenberg, 2007) y se representaron gráficamente con DISTRUCT (Rosenberg, 2004) (Artículo III).

Para analizar el tiempo hasta la muerte de las plántulas y determinar las

probabilidades de tiempo de supervivencia tras la inoculación con *Pc*, se utilizó la estimación de Kaplan-Meier (Solla et al., 2011) (Artículo I).

# RESULTADOS Y DISCUSIÓN

A continuación, se exponen los principales resultados e ideas de debate derivados de la Tesis. La información detallada se encuentra en los artículos originales.

## 1. Respuestas generales de *C. sativa* al incremento de temperatura y su recuperación

La presente Tesis Doctoral ha pretendido estudiar la respuesta de las plántulas de *C. sativa* a incrementos de temperatura de diferente intensidad y duración. Según la quinta evaluación del IPCC, se prevé que la temperatura media global del aire aumente entre 0.3 y 4.8 °C a finales de este siglo (IPCC, 2013). Además, los eventos climáticos extremos como las olas de calor se han incrementado en intensidad, duración y número en las últimas décadas en la región Mediterránea (Lionello & Scarascia, 2020). Existen numerosas definiciones para una ola de calor que varían en los límites mínimos de duración entre los tres y los seis días en los que las temperaturas superan su respectivo percentil de día natural de temperatura máxima diaria (Molina et al., 2020). Así en el Artículo I, se estudió los efectos en la fisiología y compuestos fenólicos del castaño de dos escenarios de calentamiento: i) temperatura ambiente elevada durante 30 días con cinco grados por encima de la

temperatura ambiente (temperatura ambiente elevada de aquí en adelante) y, ii) dos eventos de ola de calor de tres días de duración separados por un periodo de recuperación (evento de ola de calor de corta duración de aquí en adelante). En el Artículo II, en cambio, se estudió detalladamente los efectos de un único evento de ola de calor de una duración de siete días (evento de ola de calor de larga duración) en la fisiología y en los metabolismos primario y secundario de las plántulas de *C. sativa*.

No se observó mortalidad en las plántulas de *C. sativa* sometidas a los diferentes escenarios simulados. Sin embargo, los síntomas foliares observados difirieron entre los escenarios de calentamiento. Así, las plántulas de castaño sometidas a los escenarios de temperatura ambiente elevada y ola de calor de corta duración no sufrieron marchitamiento ni quemaduras. De hecho, las plántulas del escenario de temperatura ambiente elevada crecieron en altura y biomasa de raíces finas significativamente más que las de los escenarios de ola de calor de corta duración y temperatura ambiente. El estrés térmico se define como un aumento de la temperatura por encima de un umbral suficiente para causar daños irreversibles a la planta (Wahid, 2007). En regiones templadas se ha observado que si este umbral no es superado, el incremento de temperatura produce un incremento en la altura de los árboles y una alteración de la arquitectura de las raíces (Luo et al., 2020; Way & Oren, 2010). Por el contrario, las hojas de las plántulas del escenario de ola de calor de larga duración mostraron márgenes marrones, amarilleamiento y pardeamiento de las zonas interiores, quemaduras y, además, una menor biomasa foliar, radicular y total en comparación con las plántulas sometidas a temperatura ambiente. Estas observaciones coinciden con los síntomas morfológicos comunes derivados del estrés térmico en plantas, como son las quemaduras en hojas y ramas, senescencia y abscisión de hojas e inhibición del crecimiento de brotes y raíces (Wahid et al., 2007).

Los parámetros de intercambio gaseoso de las hojas no difirieron

significativamente con respecto a la temperatura ambiente en ninguno de los escenarios de calentamiento. Tras la aplicación de los escenarios térmicos de los artículos I y II, el ratio fotosintético neto no se alteró, coincidiendo con los resultados obtenidos por Ameye et al. (2012) en *Quercus rubra*. Esto sugiere que en las plántulas de castaño la tasa de fotosíntesis neta podría ser más sensible a las temperaturas diarias que a la exposición prolongada al calor, y también podría indicar la adaptación de la planta a temperaturas elevadas.

El estrés térmico inducido por la ola de calor de larga duración indujo cambios considerables en compuestos derivados del metabolismo primario. Las plántulas respondieron al estrés aumentando los azúcares solubles (hojas y tallo), la relación azúcar soluble:almidón (hojas y toda la planta) y disminuyendo el almidón (hojas y tallo) y los NSC (hojas) ($P < 0.05$). La reserva de NSC en los árboles constituye el principal recurso de suministro energético, y generalmente se reduce cuando los árboles experimentan estrés abiótico (Dietze et al., 2014; Galiano et al., 2011; Hartmann et al., 2013; O'Brien et al., 2014; Wiley & Helliker, 2012). Se observó una correlación fenotípica positiva entre el contenido de azúcares solubles y el contenido de prolina en las hojas ($P < 0.05$). La prolina protege la estructura de proteínas y enzimas manteniendo la integridad de las membranas y eliminando las especies reactivas de oxígeno (ROS) (Hayat et al., 2012). Las vías de señalización de la prolina y los azúcares solubles interactúan sinérgicamente, formando parte del sistema antioxidante de la planta en situaciones de estrés (Moustakas et al., 2011). Los resultados del aumento de azúcares solubles, nitrógeno, prolina y capacidad antioxidante (DPPH) observado en las hojas ($P < 0.05$), junto con la ausencia de cambios en los compuestos fenólicos de las mismas, sugiere que los castaños utilizan los carbohidratos como compuestos antioxidantes. Además, la disminución del almidón, el aumento de los azúcares solubles y la conductancia estomática inalterada entre tratamientos sugieren que los castaños utilizan azúcares para mantener los estomas abiertos a pesar del estrés térmico.

En relación con el metabolismo secundario, se observó una alteración del perfil de compuestos fenólicos dependiente del escenario de calentamiento aplicado. De forma general, los compuestos fenólicos que mostraron una mayor variación en las plántulas sometidas a los escenarios de temperatura ambiente elevada y ola de calor de corta duración disminuyeron de forma significativa o marginalmente significativa con respecto a las plántulas sometidas a temperatura ambiente. Por el contrario, las plántulas sometidas a un escenario de ola de calor de larga duración sufrieron una acumulación de compuestos fenólicos totales y compuestos ortofenólicos y flavonoides en las raíces. En las raíces, el ácido hidroxibenzoico, el kaempferol 3-O-(6"-O-acetil) glucósido-7-O-ramnósido y el hiperósido participaron significativamente en la discriminación entre plántulas estresadas y no estresadas por el calor. En los árboles, la biosíntesis de metabolitos secundarios inducida por el calor parece depender de la especie, del compuesto o incluso en la forma que se aplique el calor (Berini et al., 2018). La acumulación de compuestos fenólicos en las raíces se ha asociado con la pérdida de biomasa de las mismas en las plantas (Rhodes et al., 1987), como ocurrió en las plántulas sometidas al escenario de ola de calor de larga duración. Compuestos derivados de dos de los compuestos implicados en la separación entre los tratamientos de temperatura ambiente y ola de calor de larga duración en el análisis discriminante (kaempferol 3-O-(6"-O-acetil) glucósido-7-O-ramnósido e hiperósido) se acumularon en las raíces y se asociaron con la inhibición del transporte polar de auxina (PAT) en este órgano de la planta (Yin et al., 2014). La acumulación fenólica, especialmente de compuestos con funciones antioxidantes, se ha reportado como frecuente cuando se aplica estrés abiótico y puede ocurrir como un mecanismo adaptativo (Bhattacharya & Kundu, 2020; Carneiro-Carvalho et al., 2021; Rivero et al., 2001; Zandalinas et al., 2017). Sin embargo, se han descrito disminuciones de los compuestos fenólicos totales en hojas y raíces en plantas de vid sometidas a estrés hídrico prolongado, estrés osmótico o estrés por frío en relación con las plantas control. De forma similar, plántulas de *Quercus suber* sometidas a bajas temperaturas han mostrado una mayor concentración de flavonoides que a altas temperaturas (Chaves et al.,

2011).

La recuperación de los valores normales de las plántulas tras los escenarios de calentamiento dependió también de la forma de aplicación de los mismos y de la naturaleza del parámetro medido. Las plántulas de los escenarios de temperatura ambiente elevada y ola de calor de corta duración disminuyeron significativamente los valores de la tasa de fotosíntesis neta con respecto a la temperatura ambiente ($P < 0.05$). Aunque los cambios fueron similares en ambos escenarios, las causas podrían haber sido muy diferentes. Las condiciones de las plántulas durante un mes a una temperatura más alta (escenario de temperatura ambiente elevada) podría haber provocado una aclimatación de las plántulas y desplazado la temperatura óptima para la que la tasa de fotosíntesis neta es máxima, causando una disminución de la tasa cuando las plántulas volvieron a las condiciones de temperatura ambiente. Por el contrario, las plántulas sometidas al escenario de ola de calor de corta duración podrían haber disminuido la fotosíntesis por efecto memoria de la planta o por daños producidos en el fotosistema II por sucesivos aumentos de temperatura. Por ejemplo, el efecto memoria del estrés de la planta después de la recuperación ha sido reportado en plantas de *Arabidopsis thaliana* que continuaron manteniendo los estomas cerrados durante la fase de recuperación de un estrés hídrico (Virlouvet & Fromm, 2015). En cuanto al metabolismo secundario, las plántulas sometidas al escenario de temperatura ambiente elevada siguieron mostrando valores inferiores para muchos de los compuestos fenólicos en comparación con las plántulas de temperatura ambiente. Por el contrario, las plántulas del escenario de ola de calor de corta duración cambiaron la tendencia y aumentaron los contenidos foliares de casi todos los compuestos que mostraron variación. Se han descrito cambios de tendencia en los compuestos fenólicos cuando las plantas han entrado en una fase de recuperación del estrés (Amarowicz et al., 2010; Król et al., 2014; Weidner et al., 2011). Sin embargo, la tendencia general durante esta fase es restaurar los contenidos a los niveles de plántulas a temperatura ambiente cuando no se ha superado el límite de daño (Correia et al., 2018; Escandón et al., 2016). De hecho,

durante la recuperación de las plántulas de castaño al estrés térmico inducido por la ola de calor de larga duración la mayoría de los parámetros volvieron a los niveles iniciales. Sin embargo, el contenido de azúcares solubles en hoja, almidón y flavonoides, y la capacidad antioxidante de la hoja permanecieron alterados. La acumulación duradera de azúcares solubles, flavonoides y capacidad antioxidante coincide con el papel de estos compuestos en la tolerancia, adaptación y recuperación del estrés abiótico (Bhattacharya & Kundu, 2020; Singh et al., 2017). Estudios previos en castaño han mostrado una acumulación duradera de carbohidratos en respuesta al estrés hídrico (Camisón et al., 2020). Además, no podemos descartar que el aumento de estos compuestos en las plántulas sometidas a los escenarios de las olas de calor (corta y larga) pueda estar relacionado con la memoria de la planta al estrés. De hecho, hay estudios que indican que la memoria de la planta está vinculada a niveles elevados de enzimas antioxidantes durante varias semanas después de la aplicación del estrés (Lukić et al., 2020).

## 2. Evaluación de la plasticidad fenotípica poblacional de *C. sativa* en respuesta al estrés térmico

Las respuestas fisiológicas y bioquímicas de los árboles al estrés térmico pueden variar en función del clima de origen, y las poblaciones pueden mostrar respuestas variadas, desde una alta tolerancia en climas cálidos a una baja tolerancia en climas fríos (Marias et al., 2017). El conocimiento sobre las respuestas plásticas fenotípicas y su control genético es necesario para prever todo el potencial de una especie para adaptarse y/o evolucionar a condiciones cambiantes (Blumstein & Hopkins, 2021; Zas et al., 2020). Del mismo modo, debido al cambio climático, cada vez hay más interés en cuantificar la plasticidad fenotípica y la adaptación local (es decir, los cambios genéticos adaptativos) al estrés por calor y sequía (Castellana et al., 2021). En el Artículo II, se encontraron diferencias inter-poblacionales en la respuesta de las plántulas de castaño al estrés térmico para

parámetros morfológicos, fisiológicos y bioquímicos. Las plántulas cuyas madres procedían de condiciones de calor menos expuestas en su zona de origen, es decir, de la población del norte, fueron las más afectadas por el calor en términos de reducción de biomasa en hojas, raíces y a nivel de toda la planta ($P < 0.05$). Durante las primeras horas de calor, con la temperatura en aumento, las plántulas de la población supramediterránea (norte) aumentaron exponencialmente sus tasas de transpiración ($R^2 = 0.81$; $P < 0.001$) en comparación con las plántulas de las poblaciones mesomediterráneas (centro y sur; sin variación con el incremento de temperatura). Estos resultados sugieren que las plántulas de la población norte utilizan la transpiración inmediata como estrategia para enfriar las hojas y evitar las consecuencias del estrés térmico, como se ha observado en *Quercus rubra* (Ameye et al., 2012) y *Acer rubrum* (Weston & Bauerle, 2007). En esta población, la tasa fotosintética neta ($R^2 = -0.90$; $P < 0.001$) disminuyó mientras que la conductancia estomática ($R^2 = -0.21$; $P < 0.390$) se mantuvo sin cambios, lo que sugiere una alteración de la capacidad fotosintética del mesófilo de la hoja inducida por el incremento repentino de la temperatura (Camejo et al., 2005). La disminución de Pn debida a limitaciones no estomáticas se ha observado en plantas no adaptadas al calor (Camejo et al., 2005). Sin embargo, al final de los tratamientos térmicos, no se observaron diferencias inter-poblacionales en los parámetros de intercambio gaseoso foliar. Esto podría sugerir que, independientemente de la población, el castaño es capaz de adaptar su aparato fotosintético al estrés térmico sostenido.

En el metabolismo primario de las plántulas se detectaron diferentes respuestas inter-poblacionales al estrés térmico. Los NSC fueron los parámetros que mostraron la mayor variación genética en plasticidad fenotípica, de acuerdo con un estudio reciente realizado en *Populus trichocarpa* (Blumstein & Hopkins, 2021). Esta variación genética en la respuesta al calor podría proporcionar un mecanismo clave a través del cual las poblaciones pueden desarrollar una mejor respuesta adaptativa al futuro calentamiento global. Las plántulas de la población sur fueron las que presentaron un mayor porcentaje de plasticidad fenotípica, seguidas de las

de la población central y, por último, de las de la población norte. Las plántulas de la población sur se caracterizaron por un agotamiento casi total del almidón ($P < 0.05$) y un aumento de los azúcares solubles y de la relación azúcares solubles:almidón ($P < 0.05$) que, como se ha comentado anteriormente, están asociados con la tolerancia al estrés térmico (Xiong et al., 2015). La acumulación de prolina en las hojas de las plantas sometidas a estrés térmico solo se produjo en las plántulas de la población del norte ($P < 0.05$), siendo la principal variable explicativa de la separación de poblaciones en la componente principal dos (PC2) del análisis de componentes principales de plántulas sometidas a estrés térmico. La baja acumulación de prolina en castaño se ha asociado con la tolerancia al estrés térmico en plantas tratadas con silicato potásico (Carneiro-Carvalho et al., 2021), mientras que la acumulación de prolina inducida por el calor en plantas de Arabidopsis se ha considerado perjudicial y responsable de una menor termotolerancia (Lv et al., 2011). Así, la acumulación de este osmolito en las plántulas de *C. sativa* de la población del norte podría indicar una menor termotolerancia.

En cuanto al metabolismo secundario, las poblaciones del sur y del norte también mostraron la mayor y menor respuesta al estrés térmico, respectivamente. La respuesta de las plántulas de la población sur fue una elevada acumulación de compuestos fenólicos totales y de compuestos ortofenólicos y flavonoides en las raíces ($P < 0.05$). Sin embargo, el análisis de perfiles de metabolitos no dirigidos mostró que las plántulas de la población central, expuestas a temperaturas máximas de casi 40 $^{o}$C en la zona de origen, experimentaron los cambios más pronunciados, incluyendo un aumento de ácido elágico, acetil-xilósido de ácido elágico, derivados de quercetina, kaempferol-3-O-glucósido e isorhamnetina en hojas. Los enfoques selectivos y no selectivos proporcionaron información complementaria y podrían ser utilizados conjuntamente para caracterizar mejor la respuesta de los árboles al estrés térmico.

Diez días después de acabar el tratamiento de estrés térmico, las plántulas de las tres poblaciones alcanzaron los niveles de control (sometidas a temperatura ambiente) en prácticamente todos los parámetros fisiológicos y bioquímicos. Sólo las de la población sur se mantuvieron separadas del control en el análisis de componentes principales debido al agotamiento del almidón y a su conversión a azúcares solubles. En España, las diferencias en la pluviometría de los hábitats del castaño han dado lugar a la diferenciación de dos ecotipos de *C. sativa* con diferentes respuestas adaptativas al estrés por sequía (Alcaide et al., 2019; Míguez-Soto et al., 2019; Míguez-Soto & Fernández-López, 2015). En base a los resultados obtenidos en esta Tesis Doctoral, las diferencias en el régimen térmico de los hábitats del castaño pueden haber dado lugar a diferentes ecotipos asociados con la tolerancia al estrés térmico. Teniendo en cuenta que la tolerancia al estrés ambiental puede cambiar a lo largo de la vida de los árboles (Niinemets, 2010; Solla et al., 2005), y que estos resultados se han obtenido a nivel de plántula, podría ser esperable que la plasticidad fenotípica y la aclimatación del castaño al estrés térmico también pueda variar dependiendo de la edad.

## 3. Evaluación de la diversidad genética adaptativa al estrés térmico

De los 20 marcadores analizados en el Artículo III, siete de los desarrollados en *Eucalyptus* spp. no amplificaron, los dos de *Fagus sylvatica* dieron una amplificación inconsistente y tres de los diez desarrollados en *Quercus* spp. no mostraron polimorfismo. De los ocho marcadores que mostraron un patrón repetible y polimórfico, el marcador *EMG37* de *Eucalyptus* spp. se descartó porque el tamaño del amplicón (en pares de bases, pb) no coincidió con el tamaño descrito (437 pb y 261 pb, respectivamente). Finalmente, se seleccionaron los siete marcadores desarrollados en *Quercus* spp. para evaluar la diversidad genética adaptativa al estrés térmico de *C. sativa*. Estos resultados concuerdan con estudios previos que

reportan una alta transferibilidad entre especies de *Castanea* y *Quercus* (Alcaide et al., 2019; Castellana et al., 2021; Martín et al., 2017) agrupadas en la familia *Fagaceae*.

La diversidad genética para la tolerancia al estrés térmico no se ha estudiado previamente en *C. sativa*. Los resultados han mostrado que, las poblaciones norte y sur mostraron la mayor y menor diversidad genética, respectivamente, en términos de número medio de alelos, riqueza alélica y heterocigosidad esperada, tanto para los árboles madre como para su descendencia. Estos valores coinciden con la diversidad genética descrita previamente para las poblaciones de castaño del norte de España (Fernández-Cruz & Fernández-López, 2016; Pereira-Lorenzo et al., 2010). También se obtuvieron resultados similares cuando se utilizaron EST-SSRs para evaluar la tolerancia a la sequía en poblaciones de castaño (Alcaide et al., 2019). Varios estudios han indicado la alta eficiencia de los marcadores funcionales para evaluar la respuesta adaptativa de las plantas al estrés (Dounavi et al., 2016; Khodwekar & Gailing, 2017; Lind & Gailing, 2013; Sullivan et al., 2013; B. Wang et al., 2017; Wang et al., 2014). En *C. sativa*, por ejemplo, se notificaron respuestas adaptativas a la brotación (Cuestas et al., 2017; Martín et al., 2010, 2017), a la sequía (Alcaide et al., 2019; Castellana et al., 2021) y al patógeno *P. cinnamomi* (Alcaide et al., 2020) tras la detección de *outlier* loci a partir de EST-SSR.

El análisis bayesiano identificó un K = 2 como la división más probable, separando las plántulas de *C. sativa* en dos grupos, con mezcla entre ellos, y cierto efecto clinal. Según el coeficiente de pertenencia estimado (*Q*), el Grupo I incluyó en mayor proporción a los individuos pertenecientes a las poblaciones más termófilas o mesomediterráneas (81% de las plántulas de la población sur, 72% de la población centro y 52% de la población norte). Esta variación geográfica fue menos pronunciada que la variación registrada en poblaciones de *C. sativa* de ambientes climáticos contrastantes para la respuesta a la brotación y la sequía (Alcaide et al., 2019; Martín et al., 2010; Míguez-Soto et al., 2019). Cabe destacar

que, entre las variables climáticas, la temperatura puede explicar mucho menos sobre la variación genética adaptativa que el fotoperiodo y la disponibilidad de agua. Sin embargo, en bosques de *Quercus suber*, los estudios de asociación han revelado que la temperatura se asociaba con mayor frecuencia que la precipitación a la variación genética , siendo el rango de temperatura anual la variable ambiental que más influye en esta variación (Aronson et al., 2009; Cox et al., 2011; De Kort et al., 2014; Pina-Martins et al., 2019). En especies como *Alnus glutinosa* y *Pinus* spp., también se ha detentado diferenciación adaptativa relacionada con la temperatura (De Kort et al., 2014; Grivet et al., 2009). Los cambios a través de gradientes térmicos a pequeña escala también pueden influir en los resultados. En línea con el análisis bayesiano, los resultados del análisis discriminante empleando como variables las componentes principales del análisis de coordenadas principales de la matriz de covarianza de distancia genética detectó un patrón geográfico de las poblaciones de *C. sativa* en respuesta al estrés térmico (más del 76 por ciento de la varianza explicada; prueba lambda de Wilks, $P < 0.01$). Los árboles y plántulas de la población supramediterránea se discriminaron significativamente de los árboles y plántulas de las poblaciones mesomediterráneas.

Los resultados de los análisis discriminante y bayesiano concuerdan con las respuestas fenotípicas mencionadas en el apartado previo y detalladas en el Artículo II, y son congruentes con la temperatura media anual de cada población. La población supramediterránea, más sensible al estrés térmico, tiene las temperaturas medias y máximas absolutas más bajas, mientras que las poblaciones mesoediterráneas tienen temperaturas medias y máximas similares, ambas superiores a las de la población supramediterránea. Las poblaciones mesomediterráneas están sometidas a frecuentes olas de calor en verano, con temperaturas máximas similares a las utilizadas en el experimento de los Artículos II y III.

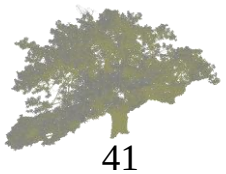
## 4. Firmas génicas de selección positiva al estrés térmico en *C. sativa* y validación de marcadores EST-SSRs

El software LOSITAN identificó a los marcadores *POR016* ($F_{ST}$ = 0,45, P < 0,05) y *VIT099* ($F_{ST}$ = 0,33, P < 0,05) como marcadores *outlier* bajo selección positiva. El marcador *VIT099* tiene una probable función *NAC domain-containing protein 78*, implicada en la regulación de la biosíntesis de flavonoides y de los proteasomas 20S y 26S en respuesta al estrés fotooxidativo (Morishita et al., 2009). La fotooxidación se observa a temperaturas extremas y, por tanto, está relacionada con el estrés térmico (Guo et al., 2006). El marcador *POR016* está asociado a una función relacionada con una proteína de choque térmico 70 k (HSP70), importante para el cierre estomático, y también modula las respuestas fisiológicas dependientes del ácido abscísico (ABA) (Clément et al., 2011). Se ha sugerido que la inducción de varias proteínas HSP (incluida la HSP70) por el ABA puede ser uno de los mecanismos que confieren termotolerancia a las plantas (Pareek et al., 1998). El ABA también desempeña un papel clave en la regulación del metabolismo de la prolina en plantas sometidas a estrés abiótico (Kumar et al., 2012; Marcińska et al., 2013; Pesci, 1992). La diferente acumulación de prolina y flavonoides inducida por el estrés térmico en las poblaciones estudiadas y la detección de marcadores EST-SSR bajo selección positiva relacionados con respuestas fisiológicas dependientes de la biosíntesis de estos compuestos hacen que *POR016* y *VIT099* sean candidatos interesantes para la selección temprana de individuos tolerantes al estrés térmico.

## 5. Los escenarios de calentamiento global modulan la respuesta de las plántulas de C. sativa a la infección por *Pc*

El efecto combinado de los escenarios de temperatura ambiente elevada u ola de calor de corta duración e infección por *Pc* no mostró síntomas foliares diferentes

de los observados en las plántulas sometidas a temperatura ambiente e infección por *Pc*. Todas las plántulas desarrollaron síntomas de daños aéreos por *Pc* (marchitamiento y desprendimiento de hojas) que precedieron a la muerte de la plántula. De igual modo, no se observaron diferencias en los parámetros de intercambio gaseoso entre las plántulas no inoculadas (en fase de recuperación) y las inoculadas con *Pc* de los diferentes escenarios térmicos. Sólo las plántulas sometidas a temperatura ambiente disminuyeron significativamente los valores de estos parámetros (la conductancia estomática de forma marginalmente significativa) con la inoculación. El perfil de compuestos fenólicos y la variación del contenido de los mismos fueron únicos para cada combinación de escenario de calentamiento y *Pc*. Algunos de estos compuestos están implicados en la biosíntesis de lignina o participan en la eliminación de especies reactivas de oxígeno (ROS) como antioxidantes (Humphreys & Chapple, 2002; Singh et al., 2021). La inducción de algunos compuestos en las hojas de las plántulas sugiere una comunicación parte radicular-aérea en la interacción planta-microbio influida por los escenarios de calentamiento (Mandal et al., 2010). El análisis de componentes principales de los compuestos fenólicos mostró solamente separación entre plántulas inoculadas y no inoculadas en el escenario que incluía plántulas sometidas a temperatura ambiente (efecto aislado de *Pc*). Esto sugiere que las plántulas de los escenarios de calentamiento (sin *Pc*) generaron respuestas comunes en los compuestos fenólicos a la combinación del escenario de calentamiento e inoculación de forma combinada. Las plantas, debido a su estilo de vida sésil, están continuamente expuestas a factores bióticos y abióticos que actúan en combinación, lo que las ha llevado a realizar ajustes y remodelaciones de su maquinaria defensiva, así como a la reconfiguración de su metabolismo (Nephali et al., 2020). Cada vez hay más pruebas de que existe un solapamiento en las respuestas defensivas de las plantas a múltiples estreses bióticos y abióticos y que, aunque la respuesta es única en la combinación, existen patrones comunes con el estrés aplicado individualmente (Nejat & Mantri, 2017). Por ejemplo, uno de los mecanismos que tienen las plantas para tolerar el calor es la inducción de la síntesis de proteínas pertenecientes a la



familia de las proteínas de choque térmico (HSP) (Bourgine & Guihur, 2021; Serrano et al., 2019). De hecho, uno de los marcadores EST-SSR identificado como marcador bajo selección positiva (*POR016*) tiene una función probable como un tipo de proteína HSP. Las HSPs también son importantes en la señalización de defensa durante el ataque de patógenos, modulando la estabilidad y acumulación de proteínas de resistencia, y se ha comprobado que en *C. sativa* estas proteínas son silenciadas ante la infección por *Pc* (Saiz-Fernández et al., 2020). El análisis de las curvas de supervivencia durante 30 días de infección por *Pc* reveló una mortalidad significativamente menor en las plántulas sometidas a temperatura ambiente frente a las sometidas a un escenario de temperatura ambiente elevada. La mortalidad de las plántulas sometidas al escenario de evento de ola de calor de corta duración no difirió del resto de escenarios y su porcentaje final de mortalidad se situó entre los escenarios de temperatura ambiente y temperatura ambiente elevada. El mayor contenido de raíces finas observado en las plántulas sometidas a temperatura ambiente elevada en el momento de la inoculación podría haber influenciado la mayor tolerancia al patógeno observada. De hecho, es bien sabido que las zoosporas de *Pc* infectan y descomponen primero las raíces finas (Jung et al., 2018). Una mayor biomasa de raíces finas en el momento de la inoculación puede ser una ventaja para sobrevivir al patógeno durante más tiempo. Además, la modulación de los mecanismos de resistencia al patógeno inducidas por el calor moderado podrían haber tenido también un papel importante en la mayor tolerancia a *Pc* en plántulas de castaño.

# CONCLUSIONES

i) La respuesta de *C. sativa* a la temperatura depende de la intensidad y duración del incremento de la misma. Así, un incremento leve de la temperatura sostenido en el tiempo mejora el crecimiento en altura y la producción de raíces finas, sin repercusiones negativas en la morfología, fisiología y metabolismo de la planta. Por el contrario, los eventos de ola de calor generan alteraciones cada vez más significativas a medida que la duración del episodio se incrementa, generando estrés térmico en plántulas sometidas a una ola de calor de siete días consecutivos.

ii) El estrés térmico afectó significativamente al metabolismo primario y secundario de las plántulas de *C. sativa*. Los cambios en el metabolismo primario se observaron principalmente en la parte aérea e incluyeron el agotamiento del almidón y un aumento de los azúcares solubles y el nitrógeno. Los cambios en el metabolismo secundario se observaron en las raíces e incluyeron un aumento en el contenido total de compuestos fenólicos.

iii) Los perfiles de compuestos fenólicos en respuesta al estrés térmico, estudiados por primera vez en castaño, han sido relevantes para distinguir plantas estresadas por calor de plantas no estresadas. Isorhamnetin ha sido el más discriminante en hojas, y ácido hidroxibenzoico y kaempferol 3-O-(6"-O-acetil) glucósido-7-O-rhamnósido en raíces.

iv) De forma general, las plántulas de castaño restauraron casi por completo

todos los parámetros fisiológicos y bioquímicos tras el estrés térmico, indicando una elevada resiliencia de la especie frente a eventos climáticos extremos como las olas de calor prolongadas. Futuros trabajos tendrán que abordar la respuesta de *C. sativa* frente al efecto combinado del estrés hídrico y térmico.

v) Se han identificado siete marcadores EST-SSR asociados al estrés térmico que han resultado útiles para evaluar la diversidad genética adaptiva en la especie. Los marcadores, *POR016* y *VIT099*, identificados como marcadores bajo selección positiva podrían utilizarse en programas de mejora genética para predecir la tolerancia al estrés térmico en árboles de *C. sativa*.

vi) El uso combinado de rasgos fenotípicos y marcadores genéticos funcionales ha permitido determinar la variación genética en la plasticidad fenotípica en respuesta al estrés térmico en castaño. Así, se ha puesto de manifiesto que las poblaciones mesomediterráneas suplen la menor diversidad genética adaptativa con una elevada plasticidad fenotípica.

vii) El escenario de temperatura ambiente elevada mejora la tolerancia a *Phytophthora cinnamomi*, probablemente por una mejora del fitness de la planta y por modificaciones en la maquinaria defensiva frente al patógeno. Sin embargo, son necesarios más estudios que combinen factores abióticos y la infección con *Pc* para poder predecir mejor la persistencia de los castañares, sometidos frecuentemente a una combinación de múltiples factores de estrés.

# CONCLUSIONS

i) *C. sativa* response to temperature depends on the intensity and duration of the temperature increase. Thus, a slight increase in temperature sustained over time improves height growth and fine root production, without negative repercussions on plant morphology, physiology and metabolism. In contrast, heat wave events generate increasingly significant alterations as the duration of the episode increases, generating heat stress in seedlings subjected to a heat wave lasting seven consecutive days.

ii) Heat stress significantly affected *C. sativa* seedling primary and secondary metabolism. Changes in primary metabolism were mainly observed in the aerial part and included starch depletion and an increase in soluble sugars and nitrogen. Changes in secondary metabolism were observed in the roots and included an increase in total phenolic compound content.

iii) Phenolic compound profiles in response to heat stress, studied for the first time in chestnut, have been relevant to distinguish heat-stressed plants from non-stressed plants. Isorhamnetin has been the most discriminant in leaves, and hydroxybenzoic acid and kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside in roots.

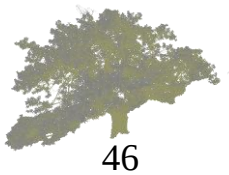
iv) In general, chestnut seedlings restored almost completely all physiological and biochemical parameters after heat stress, indicating a high resilience of the species to extreme climatic events such as prolonged heat waves. Future work will have to address the response of *C. sativa* under water and

heat stress in combination.

v) Seven EST-SSR markers associated with heat stress have been identified and proved useful for assessing adaptive genetic diversity in the species. The markers, *POR016* and *VIT099*, identified as markers under positive selection could be used in breeding programs to predict heat stress tolerance in *C. sativa* trees.

vi) The combined use of phenotypic traits and functional genetic markers has allowed determining genetic variation in phenotypic plasticity in response to heat stress in chestnut. Thus, it has been shown that mesomediterranean populations compensate for lower adaptive genetic diversity with high phenotypic plasticity.

vii) High ambient temperature scenario improves tolerance to *Phytophthora cinnamomi*, probably due to an enhancement of plant fitness and modifications in the defensive machinery against the pathogen. However, more studies combining abiotic factors and *Pc* infection are needed to better predict the persistence of chestnut trees, which are often subjected to a combination of multiple stress factors.



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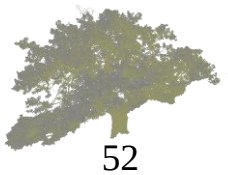

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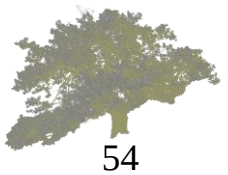

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# PRODUCCIÓN CIENTÍFICA

**Artículo I:** Dorado, F. J., Alías, J. C, Chaves, N., & Solla, A. (2023). Warming scenarios and *Phytophthora cinnamomi* infection in chestnut (Castanea sativa Mill.). *(*submitted to *Plants* journal*)*.

**Artículo II:** Dorado, F. J., Pinto, G., Monteiro, P., Chaves, N., Alías, J. C., Rodrigo, S., Camisón, Á., & Solla, A. (2023). Heat stress and recovery effects on the physiology and biochemistry of *Castanea sativa* Mill. *Frontiers in Forests and Global Change*. (print proofs).

**Artículo III:** Dorado, F. J., Solla, A., Alcaide, F., & Martín, M. Á. (2022). Assessing heat stress tolerance in *Castanea sativa*. *Forestry*, *95*(5), 667-677.

- Publicaciones adicionales resultantes de la colaboración en otras tareas fuera del objetivo de esta Tesis Doctoral fueron:

Dorado, F. J., Pujade-Villar, J., Muñoz-Adalia, E. J., Vinagrero, J. C., Diez-Casero, J. J., & Fernández-Fernández, M. M. (2020). Characterization of native parasitoid community associated with the invasive pest *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae) in Cantabria (northern Spain). *Scandinavian Journal of Forest Research*, *35*(7), 334-340.

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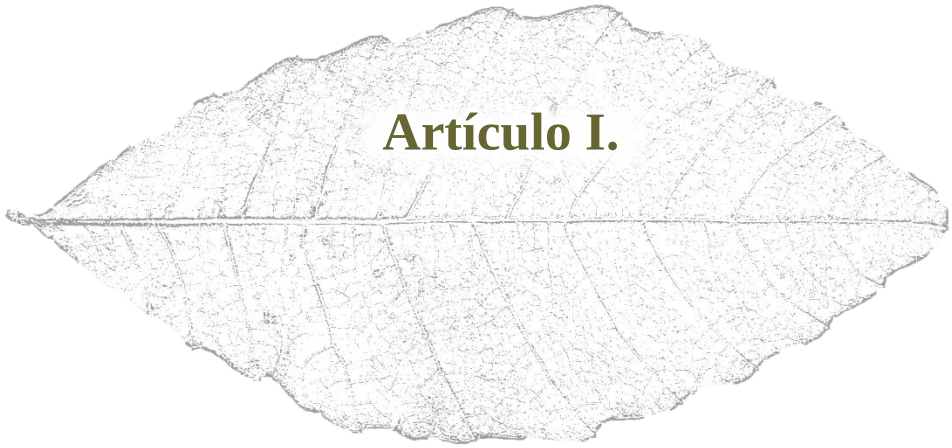
# ARTÍCULOS ORIGINALES



# Artículo I.

# Warming scenarios and *Phytophthora cinnamomi* infection in chestnut (*Castanea sativa* Mill.)

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## ABSTRACT

The main threats of chestnut tree in Europe are climate change and emerging pathogens. The impact of elevated temperatures and *Phytophthora cinnamomi* Rands (*Pc*) infection on *Castanea sativa* Miller has been studied in depth separately, but not simultaneously. The objectives of this work were to describe the physiology, secondary metabolism and survival of 6-month-old *C. sativa* seedlings after exposure of plants to ambient temperature, high ambient temperature, and heat wave event, and subsequent infection by *Pc*. Ten days after the warming scenarios ceased, the biochemistry of leaves and roots of plants was quantified and the recovery effect assessed. Growth and root biomass of plants under high ambient temperature was significantly higher than that of plants under ambient temperature and heat wave event. Seven secondary metabolite compounds in leaves and three in roots significantly changed with temperature. In general, phenolic compounds decreased in response to increased temperature. However, ellagic acid in roots was significantly more abundant in plants subjected to ambient and high ambient temperatures than in plants subjected to the heat wave event. At recovery, in plants subjected to high ambient temperature, leaf procyanidin and catechin remained down-regulated. Mortality by *Pc* was fastest and highest in plants subjected to ambient temperature, and lowest in plants subjected to high ambient temperature. Changes in the secondary metabolite profile of plants in response to *Pc* were dependent on the warming scenarios previously experienced by plants, in particular the amount of five compounds in leaves and three compounds in roots showed a significant 'warming scenario' × '*Pc*' interaction. The group of trees which survived better *Pc* infection were characterized by an increase of quercetin 3-O-glucuronide, 3-feruloylquinic acid, gallic acid ethyl ester, and ellagic acid. This is the first study on the combined effects of global warming and *Pc* infection in chestnut.

## INTRODUCTION

Plants are exposed to a multitude of stresses that often occur simultaneously (Ben Rejeb et al., 2014; Kissoudis et al., 2014; Suzuki et al., 2014). The response mechanisms of plants to an individual stress (Chaves *et al.*, 2011; Drake *et al.*, 2015; Conrad *et al.*, 2017; Almeida *et al.*, 2020; Corcobado *et al.*, 2022) often differs from the response mechanisms of plans to combined stress, mostly because of complex synergistic or antagonistic interactions occurring between hosts and stressors. To easily identify response mechanisms of plants to combined stress, preliminary studies should be performed best under controlled conditions, (Atkinson & Urwin, 2012; Rizhsky et al., 2004). Regardless of type of stress or combination, plants accumulate secondary metabolites in response to stress, some of which allow plants to mitigate damage (Jan et al., 2021). Secondary metabolites are a diverse group of compounds of low molecular weight, mostly synthesized from products of primary carbon metabolism. Temperature has been shown to alter the secondary metabolism of plants and this response seems to be specific, or compound- and context-dependent (Berini et al., 2018). Compounds from the secondary metabolism of plants may directly interact with pathogens (e.g., as antifungal) or participate in the immune response of plants (Zaynab et al., 2018).

Chestnut (*Castanea sativa* Mill.) is a Fagaceae tree species of great economic and environmental importance that inhabits the Mediterranean region. Climate models predict that the Mediterranean region will suffer an increase in temperature, heat wave events and extreme drought (IPCC, 2022). Therefore, studies on the impact of climate change on chestnut, including tolerance, adaptation and genetic variability have increased enormously in recent years (Dorado et al., 2020; Freitas et al., 2021). The development of this species in contrasting climatic conditions involved different evolutionary pressures on the genome of the species, giving rise to ecotypes adapted to different climates (Alcaide et al., 2019; Dorado et al., 2022; Fernandes et al., 2022; Míguez-Soto et al., 2019; Míguez-Soto & Fernández-López,

2015). The genetic variability, physiology and biochemistry of chestnut in response heat and drought stress has been studied (Alcaide et al., 2019; Camisón et al., 2020; Camisón et al., 2021; Ciordia et al., 2012; Dorado et al., 2023; Lauteri et al., 2004; Maurel et al., 2004), and this knowledge is helping breeders to obtaining plant material resilient to the new conditions of climate change. However, no information about the response of *C. sativa* to combined stress is available.

Ink disease, caused mainly by *Phytophthora cinnamomi* Rands, is considered the most widespread and destructive disease of *C. sativa* (Jung et al., 2018) (Hardham & Blackman, 2018; Serrazina et al., 2015; Vettraino et al., 2005). Symptoms include fine root rot, root collar necrosis, obstruction of xylem vessels, leaf chlorosis, rapid or gradual wilting of leaves and dieback (Fernandes et al., 2022; Gomes-Laranjo et al., 2004). *Phytophthora cinnamomi* (*Pc*) is a soil-borne pathogen (Stramenopila, Oomycota) which infects close to 5000 species of plants, including many of importance in agriculture, forestry and horticulture (Hardmand and Blackman 2018). In chestnut, genetic background, adaptation, maternal effects and histology of trees in response to *Pc* infection have been recently studied (Camisón et al., 2019; Fernandes et al., 2021; Santos et al., 2017; Serrazina et al., 2015). Moreover, during pathogenesis, metabolic, proteomic and hormonal changes of chestnut are being elucidated (Camisón et al., 2019; Saiz-Fernández et al., 2020). The impact of Ink disease in chestnut depends on the environment, being the disease more severe in areas with high temperature, low relative humidity and dry summers (Martins et al., 1999). *Pc* appears to have a huge impact in species from the Mediterranean-type climate, where mild and wet conditions of autumn and spring, ideal for sporulation and host infection, alternate with hot and dry summers, unfavorable for plants. A combination of abiotic and biotic factors is thought to be behind the decline of oak or jarrah trees (Davison, 1997; Robin et al., 2001). Studies on the impact of *Pc* diseases in combination with abiotic stress such as drought have been carried out in several tree species (Gómez et al., 2018; Umami et al., 2021). However, in *C. sativa* the combined effect of abiotic and *Pc* stress was not studied.

In the face of global warming there is an urgent need to study if temperature influence the physiology, biochemistry and survival of chestnut in response to *Pc*. The objectives of this work were to test the following hypotheses: (i) high ambient temperature and heat wave event induce physiological and biochemical changes in *C. sativa*, (ii) infection of *C. sativa* seedlings by *Pc* increases the abundance of secondary metabolites in leaf and root tissues, and (iii) the combined effect of warming scenarios and *Pc* infection increases the susceptibility of *C. sativa* to *Pc*.

## MATERIALS AND METHODS

### *Plant material*

In October 2017, seeds from four wild *C. stativa* trees originating from central Spain (39º37′23.6″ N, 5º23′1.6″ W; 870 m a.s.l.; Castañar de Ibor, Las Villuercas, Extremadura) were collected. About 200 nuts per tree were collected. Trees belong to of a group of 15 chestnuts (>500 years old) known as "Castaños de Calabazas", which is protected by regional law. Because giant and centenary trees represent a reservoir of genetic diversity (Pereira-Lorenzo et al. 2019), their nuts were used. Trees were selected at least 70 m apart to minimize the chances of sampling intercrossed individuals.

Immediately after seed collection, seeds were immersed in a fungicide solution (2 g $L^{-1}$ Thiram 80GD, ADAMA Inc., Spain) for 5 min and those that floated were discarded as non-viable. Viable seeds were then washed with sterile water and stored at 4 ºC for two weeks.

In November 2017, viable seeds were individually weighed and sown at 1 cm depth in 48-cell plastic root trainers with one seed per cell. Individual cells were 330 mL in volume, 18 cm high, 5.3 × 5.3 cm upper surface, and contained peat (PKN1 Florava® Peat Substrate, pH 5-6). Germination was evaluated weekly and until plants were well established, they were maintained at natural light conditions under shade (50% of solar radiation) and field capacity. The greenhouse was located

at the Faculty of Forestry of Plasencia (40º02′ N, 6º04′ W; 374 m a.s.l., Extremadura region, Spain).

### *Treatments and experimental design*

To assess the secondary metabolism of chestnut in response to different thermal and *Pc* scenarios, two greenhouses and one climatic chamber were used. At the end of May 2018, when seedlings were six months old, they were divided into three groups and the following treatments were applied: (i) ambient temperature, (ii) high ambient temperature, and (iii) ambient temperature plus two separate heat waves. Treatments lasted thirty days (Figure 1). The first group of plants was placed in a greenhouse in which the temperature from 11 to 17 h was set to 30 ºC (ambient temperature), simulating the midday temperature of a meso-Mediterranean bioclimatic zone during June. According to the IPCC's fifth assessment, global average air temperature is projected to increase by 0.3 to 4.8 °C by the end of this century (IPCC, 2013). Based on this projection, the second group of plants was placed in a different greenhouse in which the temperature from 11 to 17 h was set to 35 ºC (high ambient temperature). Both greenhouses were similar in size and sun exposure (50% of solar radiation). The third group of plants was placed in the first greenhouse for 15 days (30 ºC from 11 to 17 h), in a climatic chamber for 3 days (45 ºC from 11 to 17 h; first heat wave), again in the first greenhouse for 9 days (30 ºC from 11 to 17 h), and again in the climatic chamber for 3 days (45 ºC from 11 to 17 h; second heat wave). This treatment simulated two identical heat waves such as the recent ones over Europe (Molina et al., 2020). The climatic chamber had translucent walls and was under 50% of solar radiation. Irrespective of treatments, plants were well watered, maintaining the volumetric soil water content (VWC) at values close to 30%, optimal for the correct growth of chestnut (Camisón et al., 2020). VWC was verified using a TDR 100 soil moisture meter (Spectrum Technologies Inc., Plainfield, Illinois, USA). The relative humidity conditions were the same in the three scenarios. At the end of June 2018, thirty days after treatments started and coinciding with the day after the second heat

wave, half of seedlings were subjected to ambient temperature to assess the recovery (non-epidemic scenario in Figure 1), and half of seedlings were inoculated with *Pc* (epidemic scenario in Figure 1).

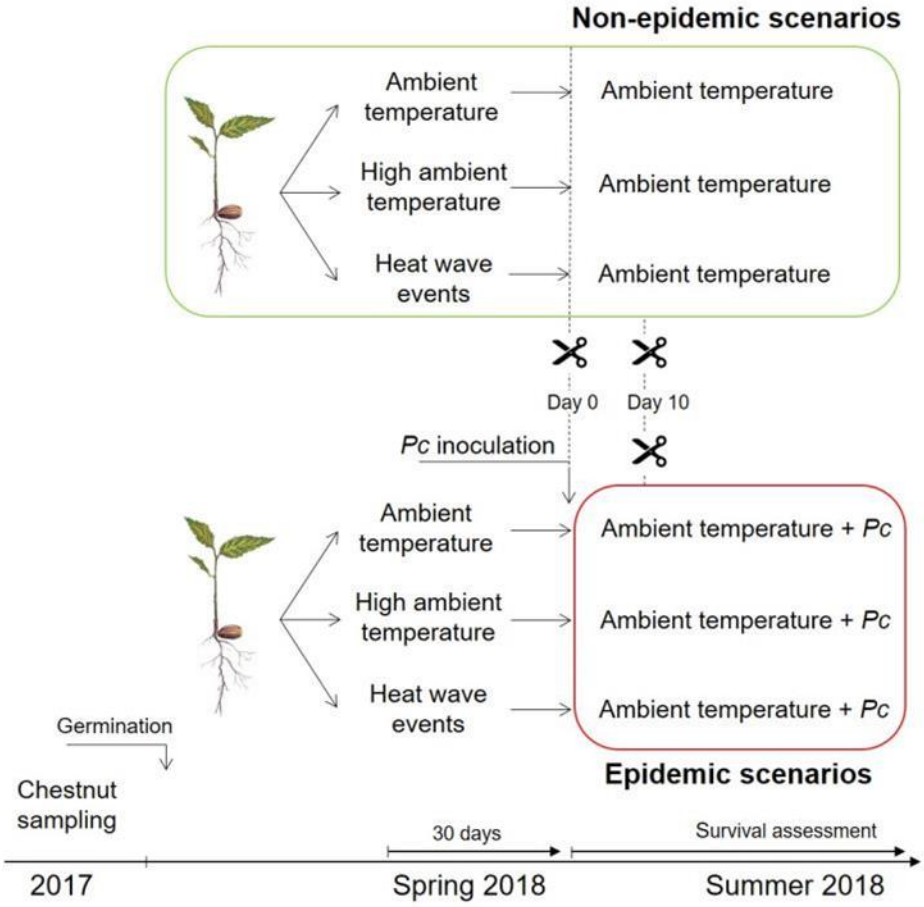



**Figure 1.** Experimental design and sampling plan.

Plants were arranged following a split-plot random design replicated in three blocks, with the warming scenarios acting as the main factor (three categories: ambient temperature, high ambient temperature and heat wave events; whole plots) and the mother trees as the split factor (four categories). Two root trainers per block and warming scenario were used. In the three blocks, the mother trees were

represented in each whole plot by 24 individuals from the four open-pollinated families. Individuals were randomly positioned within the blocks. The experiment comprised 864 plants corresponding to three blocks × three warming scenarios × four families × 24 individuals. For *Pc* inoculation, one root trainer per block and warming scenario was used.

### *Inoculation of Pc and mortality assessment*

A single A2 strain of *Pc* (coded Ps-1683) isolated from a diseased *C. sativa* tree in Galicia (43º18′32″N 153 8º13′57″W, northern Spain) was used. The strain was proven to be highly virulent in *C. sativa* (Camisón et al., 2019; Alcaide et al., 2020). *Pc* inoculum was prepared according to Jung et al. (1996), and incubated at 20-25 ºC, in total darkness, for four weeks. Soil infestation was carried out by mixing 12 mL of inoculum with the first three centimetres of substrate in each individual cell, avoiding damaging the roots of the seedlings during this process. To promote better establishment of the pathogen, approximately 50 g of freshly formed leaves of *C. sativa* were mixed with the substrate and inoculum in each 48-cell plastic root trainer. After inoculation, seedlings were irrigated and, the following day, flooded for two days in non-chlorinated water to promote sporangia production and zoospore release. Symptom and mortality assessment induced by *Pc* was monitored for 30 days. In August 2018, to fulfil Koch's postulates, *Pc* was successfully re-isolated on PARPH selective medium from the roots of inoculated seedlings.

### *Plant measurements and sampling*

Measurements and sampling for biochemical analysis were conducted the last day of treatments (day 0) and 10 days after cessation of treatments (Figure 1). This happened 0 and 10 days post-infection, respectively. The effects of treatments and *Pc* infection on plants were evaluated by (i) leaf symptoms, plant growth, plant biomass and plant mortality, (ii) leaf gas exchange, and (iii) quantification of phenolic compounds. At the end of the experiment (day 30), plant growth and

biomass were evaluated only in plants under the non-epidemic scenario.

The evaluation of external symptoms involved visual characterization of leaf discoloration observed on seedlings. Plant growth was expressed as the difference in seedling height before and after the warming treatments (30-day period).

Leaf gas exchange parameters-net photosynthetic rate ($P_n$), transpiration rate ($E$) and stomatal conductance ($g_s$) were determined at days 0 and 10 in 10-15 seedlings per scenario and treatment (Figure 1). Measurement of leaf gas exchange parameters of seedling was performed using a portable differential infrared gas analyzer (IRGA; Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf chamber. Measurements were performed from 9 a.m. to 2 p.m., with temperatures ranging from 28-30 $^{o}$C, 33-35 $^{o}$C, and 42-45 $^{o}$C in the ambient temperature, high ambient temperature, and heat wave event scenarios, respectively, and photosynthetically active radiation (PAR) ranging from 500 to 800 μmol photons $m^{-2}$ $s^{-1}$ (daylight and variable PAR conditions). In the recovery and inoculation phase, temperatures ranged from 28 to 30 $^{o}$C in all seedling groups.

At days 0 and 10, leaves and roots of six seedlings per treatment and scenario (from two mother trees) were sampled to obtain a non-targeted phenolic compound profiling of plants. Destructive sampling was carried out by removing the seedling from the alveolus, collecting the leaves and carefully separating the peat from the roots. Leaves and roots were dried at room temperature in complete darkness by using silica gel. Once the samples were dry, they were ground to a fine powder in a ball mill (Mixer Mill MM 400, Retsch, Germany) to pass through a 0.42 mm screen.

Plant biomass was assessed by destructive sampling of 32 seedlings per warming scenario (8 seedlings per family) that were separated by organs (leaf, stem, fine root and coarse root) and dried in an oven at 60 $^{o}$C to a constant weight on a precision scale.

### *Non-targeted phenolic compound profiling*

Two out of four chestnut families, selected randomly, were used. Fine leaf and root powder (0.3 g and 1 g, respectively) was homogenised with 70% ethanol (5 mL and 8 mL, respectively) and sonicated for 15 min. The extracts were cold macerated at 4 °C for 24h, centrifuged at 3500 rpm for 15 min, and the supernatant was filtered by using a 0.45 µm filter. The filtered extracts were stored at -80 °C until analysis.

Analysis, identification and quantification of phenolic compounds was performed with LC-MS system (HPLC 1260 - QTOF 6550, Agilent Technologies, Santa Clara, CA, United States). A total of 20 µL of filtered extract of each sample was injected onto a Spherisorb C 18 (250 × 4.6 mm ID, 5 µ) reversed-phase column at a rate of 1 mL/min. The mobile phase consisted of a gradient elution (water with 2% formic acid in methanol) from 1% water to 100% methanol of 100 min for leaves and 50 min for roots. Chromatograms were recorded at a wavelength of 350 nm for flavonoids and 280 nm for phenols. Concentrations of the compounds were estimated from a standard curve (0.00, 0.01, 0.05, 0.10, 0.20 mg/mL) by using gallic acid, ellagic acid, quinetin or quercetin 3-O-rutinoside. The results were expressed in mg of equivalents per g of dry weight.

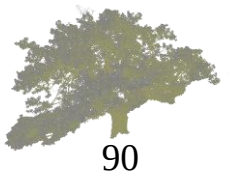
### *Statistical analysis*

To evaluate the effect of the thermal treatments on leaf gas exchange, growth and biomass of *C. sativa* seedlings, a one-way analysis of variance (ANOVA) was performed using "warming scenario" as a single factor. To analyse the combined response of seedlings to the thermal treatments and *Pc*, a two-way ANOVA was performed, including "warming scenario" and "presence of *Pc*" as main effects, and their interaction. To identify significant differences between means, Tukey's multiple comparison tests were used at $P < 0.05$.

**Table 1.** List of secondary metabolite compounds detected, identified and quantified in leaves and roots of six-month-old *Castanea sativa* plants.

| Class | Subclass | Leaf | Root |
|---|---|---|---|
| Phenols | Hydroxybenzoic acids | Ethyl gallate | Hydroxibenzoic acid |
| | | Ellagic acid acety-xyloside | Gallic acid |
| | | Ellagic acid | Ellagic acid |
| | Hydroxycinnamic acid | 3-Feruloylquinic acid | |
| | Hydroxyphenylacetic acid | | 4-Hydroxyphenylacetic acid |
| Lignans | | Lariciresinol | |
| Flavonoids | Flavanols | Procyanidyn | |
| | | Catechin | |
| | Flavonols | Miquelianin (Quercetin 3-O-glucuronide) | Hyperoside (Quercetin 3-O-galactoside) |
| | | Rutin (Quercetin 3-O-rutinoside) | Kaempferol-3-O-(6'' acetyl) glucoside 7-O rhamnoside |
| | | Quercetin 3-O-rhamnoside | |
| | | Hyperoside (Quercetin 3-O-galactoside) | |
| | | Astragalin (Kaempferol 3-O-glucoside) | |
| | | Isorhamnetin | |
| | | Isorhamnetin 3-O-glucoronide | |
| Other polyphenols | | Hydroxytyrosol | Tyrosol |
| | | Hydroxytyrosol acetate | Coniferyl aldehyde (4-hydroxy-3-metoxycinnamaldehyde) |

To detect the effect of warming scenarios and/or *Pc* on the phenolic compound profile of *C. sativa* plants, general linear models (GLM) that included the amount of a particular compound as a dependent variable were used. "Warming scenario" and/or " presence of *Pc*", "mother tree" and their interactions were used as fixed factors, and "seed weight", " time to emerge" and "plant height" as covariates. To estimate variation in time of phenolic compounds in response to warming scenarios and/or *Pc*, the fixed factors "time" was added in the previous models. The residuals of the models were tested for normality and means were compared using Tukey's HSD test.



To obtain patterns of variation in the significant compounds detected in the GLMs, three principal component analyses (PCA) were performed: for warming scenarios at day 0, for warming scenarios at day 10, and for warming and epidemic scenarios toghether. PCAs were performed using the R statistical package 'factoextra' (R Core Team, 2018). Prior to PCAs, data were centered and standardized to reduce scale effects.

To analyse time to death of inoculated seedlings subjected to different warming scenarios and compare survival probabilities to *Pc*, survival time analysis based on the Kaplan-Meier estimate was used.

Before analyses, data were checked for normality and homogeneity of variances. ANOVAs, GLMs and Tukey's tests were performed with STATISTICA v10 software (StatSoft Inc., 2011).

## RESULTS

### *Warming scenarios in chestnut*

Under the non-epidemic scenarios, *C. sativa* seedlings did not show wilting and mortality. Plant growth was highest in non-inoculated seedlings subjected to high ambient temperature, and total fine root biomass was significantly higher in non-inoculated seedlings subjected to high ambient temperature than in non-inoculated

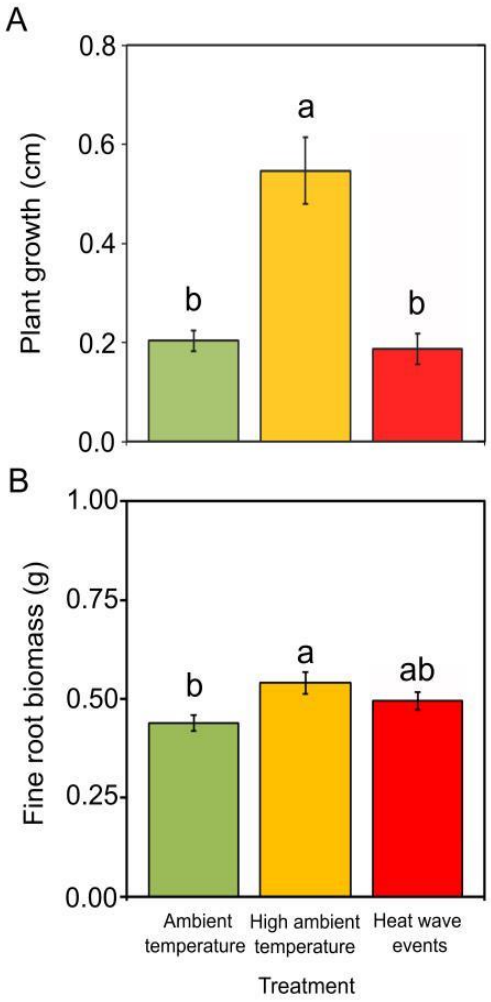



**Figure 2.** Plant growth (A) and fine roots biomass (B) of six-month-old *Castanea sativa* seedlings exposed to ambient temperature (green), high ambient temperature (orange), and two heat waves (red). Vertical bars are standard errors and different letters indicate significant differences between means (Tukey's HSD tests at $P < 0.05$).

seedlings subjected to ambient temperature (Figure 2).

On day 0, $P_n$ and $E$ values were similar irrespective of thermal treatments (Figure 3A and B), while $g_s$ values increased significantly in plants under high ambient temperature compared to values in plants under ambient temperature (Figure 3C). On day 10, $P_n$, $E$ and $g_s$ values of seedlings under high ambient temperature and heat wave event scenarios were significantly lower in comparison to those of seedlings under ambient temperature (Figure 3). No differences in plant growth, root biomass and leaf exchange parameters between families were observed (results not shown). The phenolic compound profiling of seedlings changed depending on the warming scenarios, the mother tree and the sampling time. Eleven out of 23 compounds identified in leaves and roots (Table 1), changed depending on the warming scenario (Table 2). More phenolic compounds in leaves showed changes than in roots (eight *vs* three, respectively; Table 2). Two and four compounds significantly changed depending on the mother tree and sampling date (Table 2). On day 0, the PCA plot clearly separated plants under ambient temperature from plants under high ambient temperature (Figure 4A). Disaggregation of groups occurred mainly throughout PC2 (Figure 4A), and the compounds that contributed significantly to this separation (in order of highest to lowest contribution) were catechin, hydroxytyrosol acetate, ellagic acid acetyl-xyloside and lariciresinol. These four compounds were foliar and decreased with increasing temperature (Table 3). The tenth day after subjecting seedlings from the warming scenarios to ambient temperature (recovery), the PCA revealed a clear separation between the three groups of seedlings (Figure 4B). Compounds that contributed significantly to disaggregation in PC1 were catechin and procyanidin from leaves, while compounds contributing to disaggregation in PC2 were hydroxybenzoic acid, ellagic acid, and coniferyl aldehyde from roots. In relation to ambient temperature, high ambient temperature frequently implied a decrease of phenolic compounds in plants, while heat wave events frequently implied an

increase of phenolic compounds in plants (Table 3).

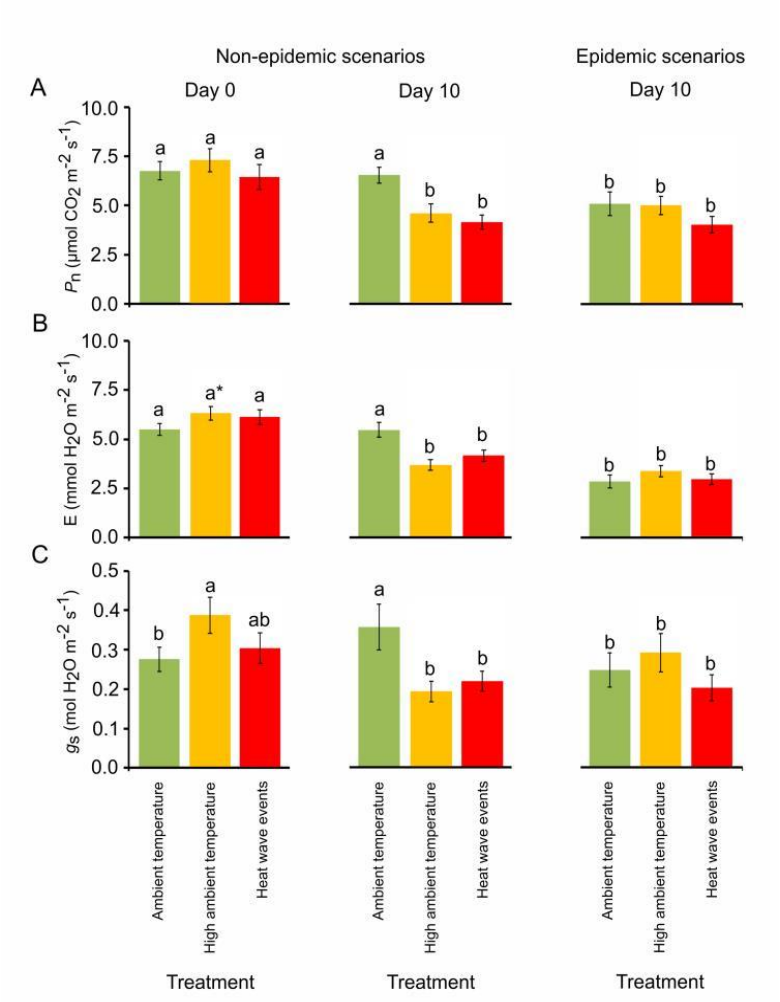



**Figure 3.** Photosynthetic rate values ($P_n$) (A), transpiration rates ($E$) (B), and stomatal conductance ($g_s$) (C) of *Castanea sativa* seedlings exposed to ambient temperature (green), high ambient temperature (orange), and two heat waves (red). Measurements were obtained 0 and 10 days after exposure of plants to treatments (non-epidemic scenarios) and 10 days after exposure of plants to treatments and *Phytophthora cinnamomi* (*Pc*) infection (epidemic scenarios). Vertical bars are standard errors, different letters indicate significant differences ($P < 0.05$), and asterisks indicate marginally significant differences ($P < 0.10$) (Tukey's HSD tests).

**Table 2.** Results of general linear models for analyses of chemical changes in seedlings of two *Castanea sativa* mother trees in response to warming scenarios. Significant *P*-values are indicated in bold ($P < 0.05$), asterisks indicate marginally significant differences ($P < 0.10$), and ns indicates not significant.

| | | Leaf | | | | | | Root | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Effects** | **Df** | **Ethyl gallate** | **Ellagic acid acetyl-xyloside** | **Ellagic acid** | **Laricirесinol** | **Procyanidin** | **Catechin** | **Quercetin 3-O-glucuronide** | **Hydroxytyrosol acetate** | **Ellagic acid** | **Hydroxibenzoic acid** | **Coniferyl aldehyde** |
| Scenario [S] | 2 | **0.012** | **0.004** | **0.006** | **0.019** | **0.001** | **0.002** | **0.009** | **0.039** | **0.015** | **<0.001** | **<0.001** |
| Mother tree [M] | 1 | ns* | ns | ns | ns | ns | ns | **0.034** | ns | ns | ns | **0.007** |
| Time [T] | 1 | **0.012** | **0.022** | ns | ns | **0.035** | ns | ns | **0.014** | ns | ns | ns |
| S × M | 2 | ns* | ns | ns | ns | ns | ns | **0.007** | ns | ns | ns | ns |
| S × T | 2 | **0.029** | **0.037** | ns* | ns | **0.006** | ns | **<0.001** | **0.015** | **0.002** | **0.002** | **0.022** |
| M × T | 1 | ns* | ns | ns | ns | **0.046** | ns | ns | ns | ns* | ns | ns |
| S × M × T | 2 | ns | ns | ns | ns | ns | ns | ns | ns | **0.005** | **0.005** | **0.022** |
| Seed weight (g) | 1 | **0.028** | ns | ns | ns | **0.014** | ns | ns | ns | ns | ns | ns |
| Time to emerge (d) | 1 | ns | ns | ns | ns | ns | ns | **0.017** | **0.017** | ns | ns | ns |
| Plant height (cm) | 1 | **0.021** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |

Ten days after inoculation, $P_n$, $E$ and $g_s$ parameters significantly decreased in infected plants previously subjected to ambient temperature (Figure 3). Meanwhile, $P_n$, $E$ and $g_s$ remained unchanged in infected plants previously subjected to high ambient temperature and heat wave events. At day 15, regardless of the warming treatment, most seedlings inoculated with *Pc* showed leaf chlorosis and wilting. Defoliation frequently occurred prior to plant death, which was faster and higher in seedlings subjected to ambient temperature than in seedlings subjected to high ambient temperature (Figure 5). Thirty days after inoculation, overall mortality of *Pc*-inoculated seedlings was 60.7%, and final mortalities of plants previously subjected to ambient temperature, high ambient temperature and heat wave events were 55, 60 and 69%, respectively. The two chestnut families used for phenolic compound profiling significantly differed in susceptibility to *Pc* (Figure S1).

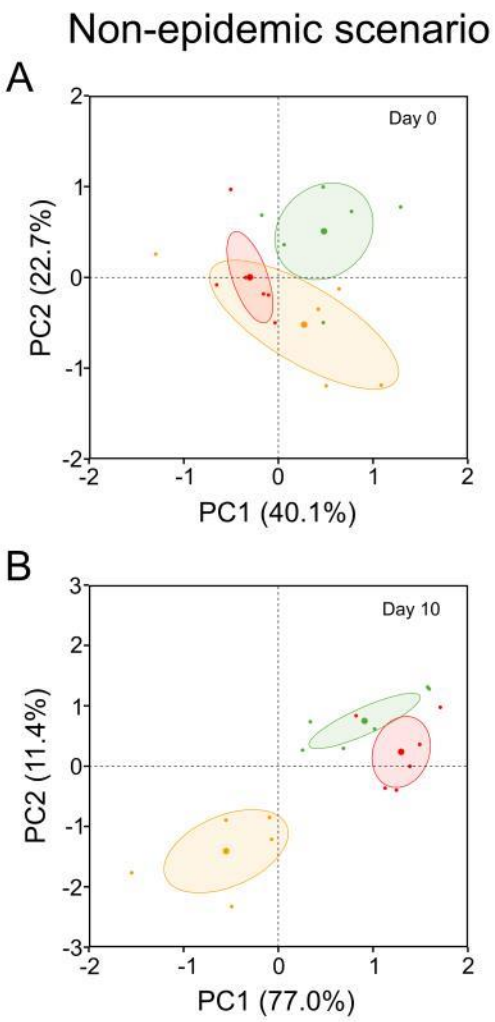



**Figure 4.** PCA of phenolic compounds included in Table 2 of non-inoculated *Castanea sativa* seedlings exposed to ambient temperature (green circles), high ambient temperature (orange circles), and two heat waves (red circles).

Four compounds of the secondary metabolism of plants showed significant or marginally significant changes in response to *Pc* (Table 4). Ethyl gallate in leaves

was significantly highest in *Pc*-inoculated seedlings (Figure 6A), and hydroxybenzoic acid, 4-hydroxyphenylacetic acid and coniferyl aldehyde in roots were lowest in *Pc*-inoculated seedlings (Figure 6B-D). However, ethyl gallate increased in plants exposed to ambient and high ambient temperatures but decreased in plants exposed to heat waves, 4-hydroxyphenylacetic acid decreased irrespective of the scenario, and coniferyl aldehyde decreased in plants exposed to ambient temperatures but increased in plants exposed to heat waves (Figure 7). Eight out of the 23 compounds identified in leaves and roots (Table 1) changed differently depending on the warming scenario and *Pc* infection (significant *Pc* × scenario interaction in Table 4) (Figure 7). Miquelianin and hydroxytyrosol acetate changed differently depending on the family and *Pc* infection (significant *Pc* × mother tree interaction in Table 4) (Figure SM2), and the previous changed differently depending on the family, scenario and *Pc* infection (significant *Pc* × S × mother tree interaction in Table 4) (Figure SM3).

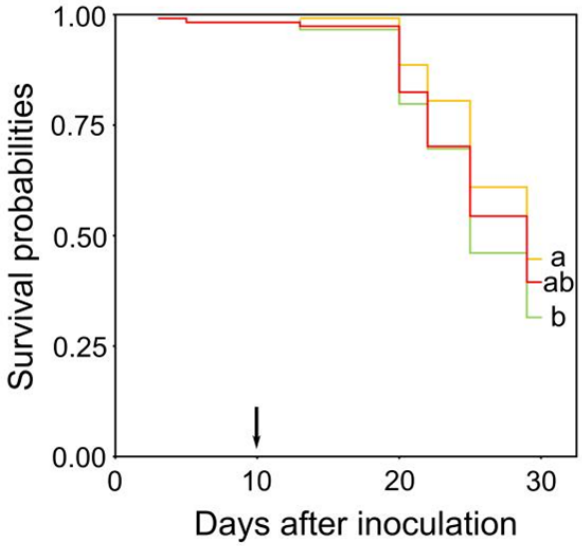



**Figura 5.** Survival probabilities of *Castanea sativa* seedlings exposed to ambient temperature (green), high ambient temperature (orange), and heat waves (red) and inoculated at day 0 with *Phytophthora cinnamomi*. Global log-rank test was significant at $P = 0.080$. Different letters indicate significant differences between survival curves ($P < 0.05$), and the arrow indicate the dates of phenol assessment.

**Table 3.** Changes of phenolic compounds in leaves and roots of *Castanea sativa* seedlings, 0 and 10 days after exposure of plants to high ambient temperature and heat wave events, in relation to plants exposed to ambient temperature. Red and blue colours indicate increase and decrease, respectively. Two arrows indicates significant variation ($P < 0.05$), one arrow indicates marginally significant variation ($P < 0.10$), and ns indicates not-significant variation (Tukey's HSD test).

| | | Non-epidemic scenario | | | |
|---|---|---|---|---|---|
| | | Day 0 | | Day 10 | |
| **Organ** | **Compound** | **High ambient temperature** | **Heat wave events** | **High ambient temperature** | **Heat wave events** |
| **Leaf** | Ethyl gallate (mg gallic acid/g DW) | ns | ns | ns | ↑↑ |
| | Ellagic acid acetyl-xyloside (mg gallic acid/g DW) | ↓↓ | ↓↓ | ↓↓ | ns |
| | Lariciresinol (mg gallic acid/g DW) | ↓ | ↓ | ↓↓ | ns |
| | Procyanidin (mg procyanidin /g DW) | ns | ns | ↓↓ | ↑↑ |
| | Catechin (mg catechin /g DW) | ↓↓ | ns | ↓↓ | ns |
| | Miquelianin (quercetin 3-O-glucuronide) (mg quercetin/g DW) | ns | ns | ns | ↑↑ |
| | Hydroxytyrosol acetate (mg gallic acid /g DW) | ↓↓ | ↓↓ | ns | ↑ |
| | Ellagic acid (mg ellagic acid /g DW) | ns | ns | ns | ↑↑ |
| **Root** | Ellagic acid (mg ellagic acid /g DW) | ns | ns | ↑↑ | ns |
| | Hydroxybenzoic acid (mg gallic acid/g DW) | ns | ns | ↓↓ | ↓↓ |
| | Coniferyl aldehyde (mg gallic acid/g DW) | ns | ns | ↓↓ | ns |

As observed in the non-epidemic scenarios, changes of compounds in leaves were more relevant in the epidemic scenarios than changes of compounds in roots (Table 4).

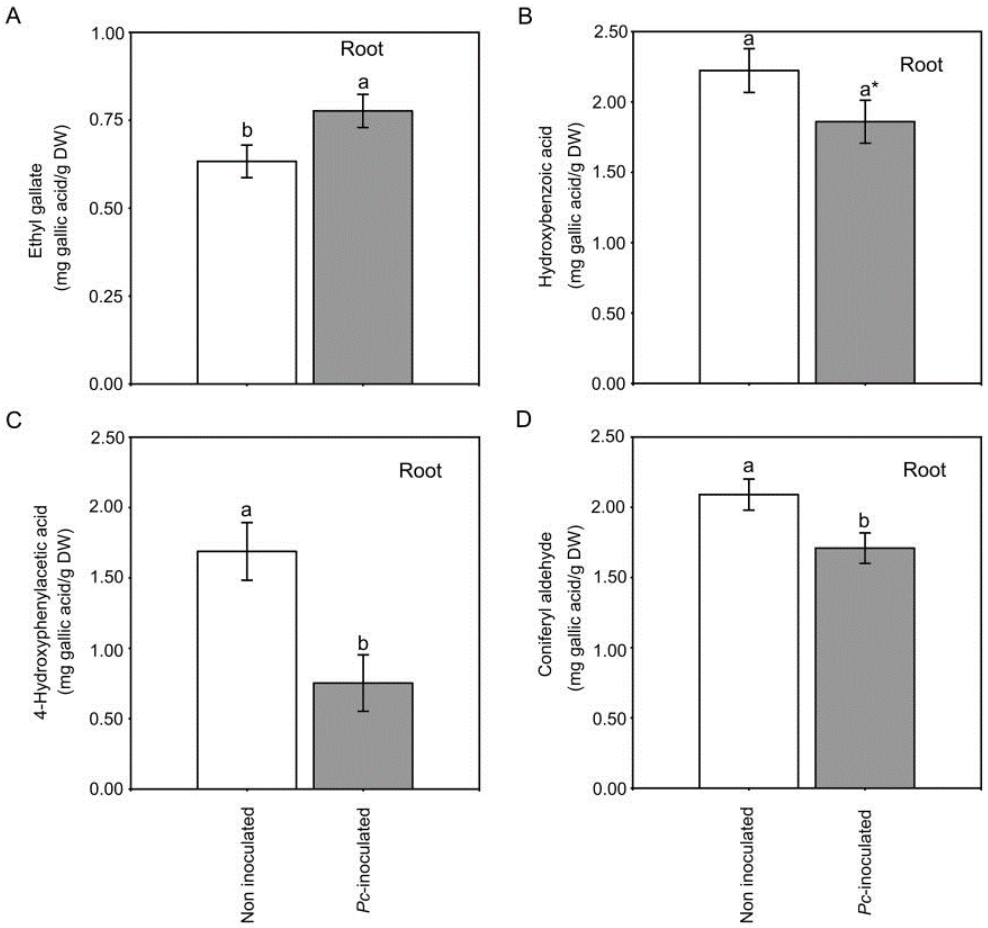



**Figure 6.** Mean values of phenolic compounds of *Castanea sativa* seedlings not inoculated or inoculated with *Phytophthora cinnamomi* (*Pc*). According to the linear mixed models shown in Table 4, the four compounds were significantly affected by *Pc* infection. Measurements were taken 10 days post inoculation.Vertical bars are standard errors, different letters indicate significant differences ($P < 0.05$), and the asterisk indicates marginally significant difference ($P < 0.10$) (Tukey's HSD tests).

At day 10, the PCA obtained from the compounds that changed in response to the heat treatments (Table 3) allowed visual differentiation of plants under non-

epidemic and epidemic scenarios (Figure 8). A clear separation between non-infected and *Pc*-infected plants was observed within plants subjected to ambient temperature, only (Figure 8). The compounds that contributed most to the PC1 and PC2 axes were 3-feruloylquinic acid from leaves (81.3%), 4-hydroxyphenylacetic acid (8.7%), coniferyl aldehyde (3.9%), and hydroxybenzoic acid (3.6%) from roots, and ellagic acid from leaves (0.9%). The phenolic compound profile and variation in contents was unique for each combination of warming scenario and *Pc* (Table 5).

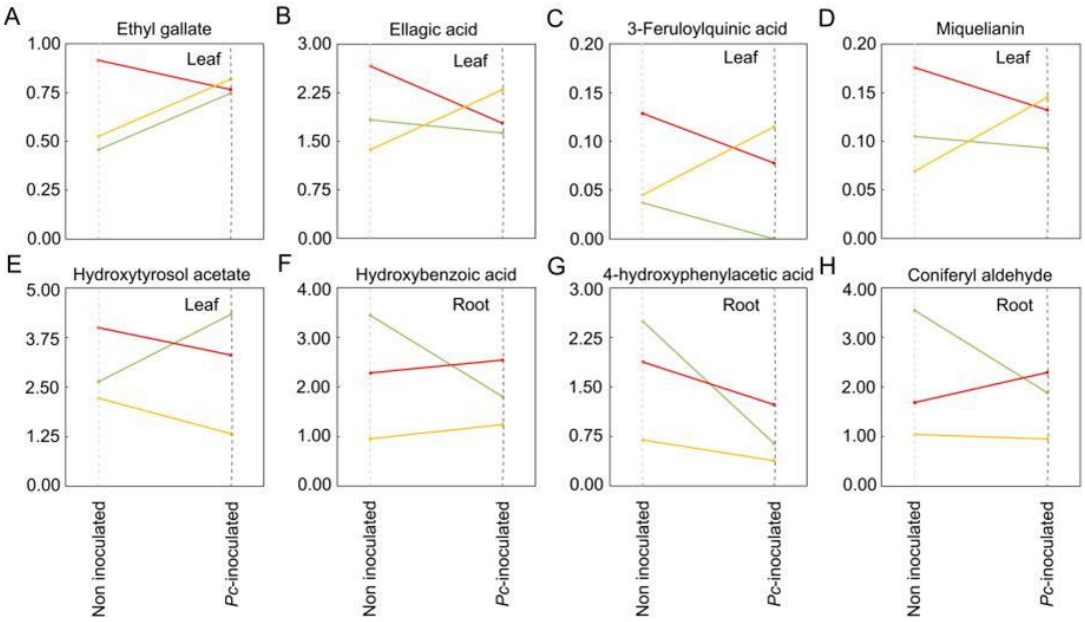



**Figure 7.** Phenolic compounds of *Castanea sativa* plants that differently changed their contents in response to *Phytophthora cinnamomi* infection and the scenario experienced by plants before inoculation (significant *Pc* × scenario interactions in Table 4; $P < 0.05$). Phenolic contents were obtained at day 10 after inoculation in plants previously exposed to ambient temperature (green lines), high ambient temperature (orange lines), and two heat waves (red lines).

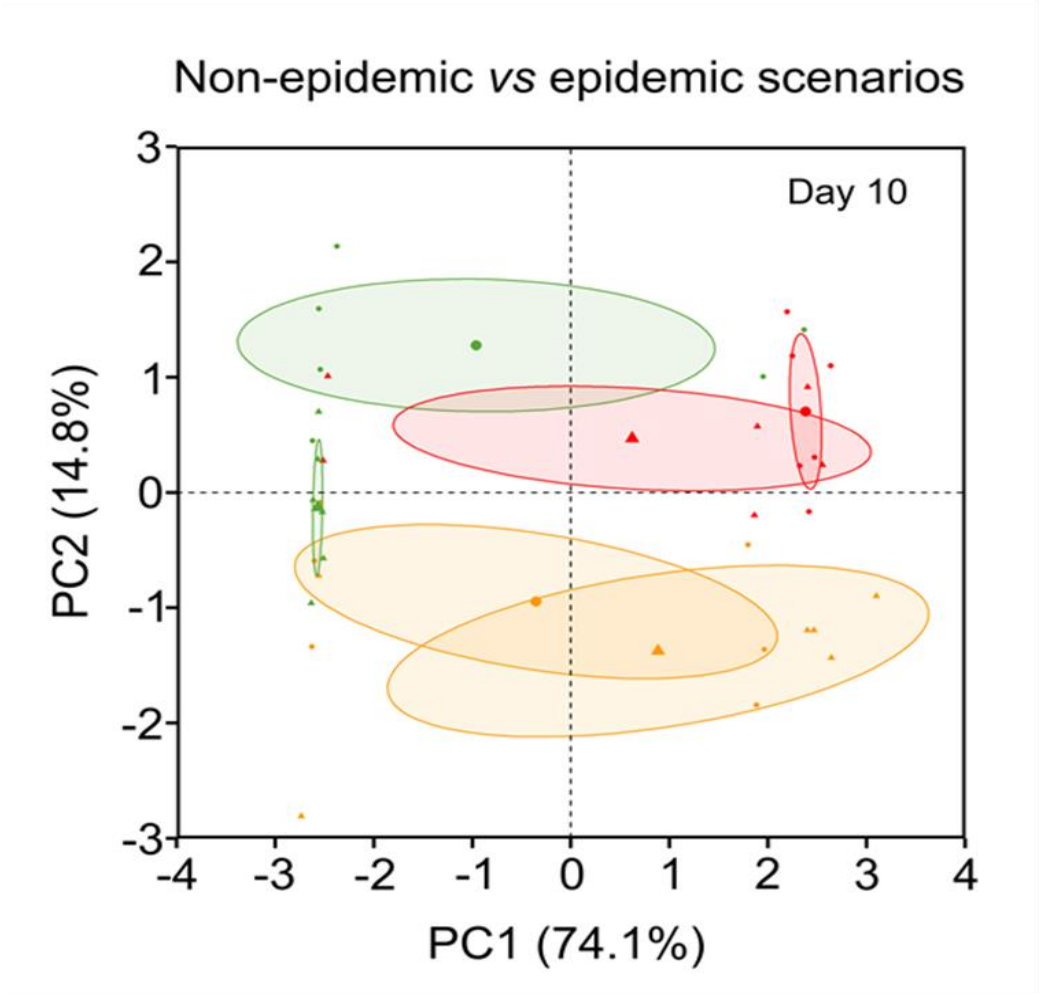



**Figure 8.** PCA of phenolic compounds included in Table 4 of non-inoculated *Castanea sativa* seedlings exposed to ambient temperature (green circles), high ambient temperature (orange circles), and two heat waves (red circles) (non-epidemic scenario) and *Phytophthora cinnamomi*-inoculated *C. sativa* seedlings previously exposed to ambient temperature (green triangles), high ambient temperature (orange triangles), and two heat waves (red triangles) (epidemic scenario). The five compounds contributing most to the PC1 and PC2 axes were leaf 3-feruloylquinic acid (81.3%), root 4-hydroxyphenylacetic acid (8.7%), root coniferyl aldehyde (3.9%), root hydroxybenzoic acid (3.6%), and leaf ellagic acid (0.9%).

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**Table 4.** Results of general linear models for analyses of chemical changes in seedlings of two *Castanea sativa* mother trees in response to warming scenarios and *Phytophthora cinnamomi* infection. Significant *P*-values are indicated in bold ($P < 0.05$), asterisks indicate marginally significant differences ($P < 0.10$), and ns indicates not significant.

| | | **Leaf** | | | | **Root** | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Effect** | **Df** | **Ethyl gallate** | **Ellagic acid** | **3-Feruloylquinic acid** | **Miquelianin** | **Hydroxytyrosol acetate** | **Hydroxybenzoic acid** | **4-hydroxyphenylacetic acid** | **Coniferyl aldehyde** |
| *Phytophthora* [*Pc*] | 1 | **0.032** | ns | ns | ns | ns | ns* | **0.006** | **0.014** |
| Scenario [S] | 2 | **0.018** | ns | **0.038** | **0.015** | **<0.001** | **<0.001** | **0.009** | **<0.001** |
| Mother tree [M] | 1 | ns | ns | ns | ns* | ns | ns | ns | **0.031** |
| *Pc* × S | 2 | **0.016** | **0.014** | **0.044** | **0.005** | **<0.001** | **0.003** | ns* | **<0.001** |
| *Pc* × M | 1 | ns | ns | ns | **0.037** | **0.015** | ns | ns | ns |
| S × M | 2 | ns | ns | ns | ns | **<0.001** | ns | ns | ns |
| *Pc* × S × M | 2 | ns | ns | ns | ns | ns | **0.031** | ns | ns* |
| Seed weight (g) | 1 | ns | ns | ns | ns | ns | ns | ns | ns |
| Time to emerge (d) | 1 | ns | ns | ns* | ns | **0.002** | ns | ns | ns |
| Plant height (cm) | 1 | ns | ns | ns | ns | ns | ns | ns | ns* |

**Table 5.** Changes of phenolic compounds in leaves and roots of *Phytopththora cinnamomi*-infected *Castanea sativa* seedlings, 0 and 10 days after exposure of plants to high ambient temperature and heat wave events, in relation to plants exposed to ambient temperature. Red and blue colours indicate increase and decrease, respectively. Two arrows indicates significant variation ($P < 0.05$), one arrow indicates marginally significant variation ($P < 0.10$), and ns indicates not-significant variation (Tukey's HSD test)

| Organ | Compound | Epidemic scenario (day 10) | |
|---|---|---|---|
| | | **High ambient temperature + *Pc*** | **Heat wave events+ *Pc*** |
| **Leaf** | Ethyl gallate (mg gallic acid/g DW) | ns | ns |
| | Ellagic acid (mg gallic acid/g DW) | ns | ns |
| | 3-Feruloylquinic acid ( mg gallic acid /g DW) | ↑↑ | ↑ |
| | Miquelianin (quercetin 3-O-glucuronide) (mg quercetin/g DW) | ↑↑ | ns |
| | Hydroxytyrosol acetate ( mg gallic acid /g DW) | ↓↓ | ↓↓ |
| **Root** | Hydroxybenzoic acid (mg gallic acid/g DW) | ns | ↑ |
| | 4-Hydroxyphenylacetic acid (mg gallic acid/g DW) | ns | ns |
| | Coniferyl aldehyde (mg gallic acid/g DW) | ↓↓ | ns |

## DISCUSSION

Achievements of this study were providing, for the first time in *C. sativa*, information about (i) growth rates, biomass, leaf gas exchange parameters and phenolic compounds of seedlings under altered scenarios of high ambient temperature and heat waves, (ii) recovery of seedlings to altered scenarios, (iii) reduced susceptibility if *Pc*-infected well-irrigated plants were previously exposed for 30 days to high ambient temperature, and (iv) the phenolic compound profiling of seedlings exposed to altered scenarios and *Pc* infection

### *Effects of temperature increase in chestnut*

Increased growth and fine root biomass of *C. sativa* seedlings exposed to high ambient temperature is in agreement with studies of trees from the temperate regions exposed to increasing temperature (Way & Oren, 2010). Root architecture is also influenced by increased temperature, although it can be highly variable between species (Luo et al., 2020). Our altered scenarios did not significantly affect $P_n$ and $E$ at the end of the 30-day period (day 0), although there was a tendency to increase in plants exposed to high ambient temperature (Figure 3). Warm temperatures in temperate regions may enhance $P_n$ of trees by an increase of their photosynthetic pigments, although it depends on the species (Saxe et al., 2001). In general, $P_n$ reaches its maximum between 25 and 40 $^{o}$C, and higher temperatures make them fall due to a deterioration in protein function (Berry & Bjorkman, 1980). After 10 days of recovery, seedlings from the two altered scenarios had significantly decreased $P_n$, $E$ and $g_s$ values with respect to ambient temperature. The causes of this reduction could have been different depending on the scenario. At high ambient temperature, plants could have experienced heat acclimation (Tarvainen et al., 2022) by shifting their optimum temperature for which $P_n$ was maximum. Conversely, seedlings subjected to the two heat waves could had their PSII damaged and/or stress memory induced (Virlouvet & Fromm, 2015).

Decrease in total phenolic compounds has been reported in leaves and roots of grapevine plants subjected to water, osmotic and cold stress (Amarowicz et al., 2010; Król et al., 2014; Weidner et al., 2011). Similarly, in *Quercus suber* seedlings subjected to low temperatures, higher concentration of flavonoids were reported in relation to control plants, being suggested that the biosynthesis of these compounds was favoured by low temperatures (Chaves et al., 2011). In *C. sativa* seedlings of the same age subjected to heat stress, an increase of phenolic compounds was observed in the roots (Dorado et al., 2023), although this response was variable depending on the origin of trees. In trees, biosynthesis of secondary metabolites in response to heat appears to be specific and compound-dependent (Berini et al., 2018). In this work, phenolic changes in chestnut in response to altered scenarios were genotype dependant (Figure SM3).

During recovery, seedlings previously subjected to high ambient temperature continued to show, at day 10, decreased values of several phenolic compounds in comparison to seedlings previously exposed to ambient temperature (Table 3). However, seedlings experiencing the heat waves turned from phenolic compound decrease at day 0 to phenolic compound reestablishment, or increase, at day 10. Delayed changes or changes in trends of phenolic content occur when plants have entered a recovery phase after stress (Amarowicz et al., 2010; Król et al., 2014; Weidner et al., 2009; Lukić et al., 2020). During the recovery phase, the general tendency of plants is to restore the phenolic content to levels occurring at ambient temperature unless physiological damage is produced (Correia et al., 2018; Dorado et al., 2023). The increase of several phenolic compounds in our heat wave-stressed plants may probably have occurred as consequence of stress memory, as observed for *Alopecurus pratensis* (Lukić et al., 2020), but this assumption needs further study to be confirmed.

### *Phenolic profile of chestnut seedlings after infection by Pc*

Among studies on phenolic quantification in *C. sativa* plants infected by *Pc*,

only Camisón et al. (2019) used roots, which is the natural way of pathogen entry. Nine days after inoculation of susceptible *C. sativa* individuals with the same *Pc* strain used here, total phenolics decreased in roots and did not change in leaves (Camisón et al., 2019). In the present study, we went a step further by obtaining a non-targeted phenolic profile of infected and non-infected plants and detected significant variation of three compounds in response to *Pc* (one in leaves and two in roots; Figure 6). Phenolic compounds are involved in many plant defense mechanisms of plants against abiotic and biotic stress ( Kumar et al., 2020). In response to pathogens, these compounds can act as elicitors and trigger systemic acquired resistance (SAR) for long lasting immunity (Goupil et al., 2017). Ethyl gallate, which was significantly highest in leaves of *Pc*-inoculated seedlings, acts as antimicrobial and elicitor, being capable of activating SAR pathway and inducing pathogenesis-related (PR) resistance gene expression (Goupil et al., 2017). Hydroxybenzoic acid, better known as salicylic acid (SA), was significantly lowest in roots of *Pc*-inoculated seedlings. SA is a stress induced hormone and one of the key molecules in the signal transduction pathway involved in both local defence reactions at infection sites and the induction of SAR (Widhalm & Dudareva, 2015). SA participates in the early stages of the defensive response of plants to infection (Huang et al., 2020) when *P. cinnamomi* acts as a biotrophic pathogen. Changes of ethyl gallate and hydroxybenzoic acid detected in our *Pc*-infected plants were probably involved in the mechanisms described above, but this needs to be tested.

Phenolic compounds also participate in the lignification of cell walls, this process being an additional defensive response to pathogens (Fernandes et al., 2021). Lignin enrichment of root cell walls has been reported as a plant defensive response in early stages (hours) after infection, although in susceptible plants it not sufficient to stop the penetration of pathogen hyphae into the roots (Redondo et al., 2015; van den Berg et al., 2021). Coniferyl aldehyde has been reported to be used by plants for hydroxylation of the enzyme ferulate-5-hydroxylase as a downstream substrate in the lignin pathway (Humphreys et al., 1999; Boudet, 2000). The lower

levels of coniferyl aldehyde in *Pc*-infected plants could indicate this compound was used for lignin biosynthesis. Finally, 4-hydroxyphenylacetic acid has been reported to be metabolized by plant pathogens and used as sources of carbon and energy (Dodge & Wackett, 2005; Lanoue et al., 2010).

### *Enhanced resistance of chestnut to Pc after high ambient temperature*

Within the epidemic scenarios, high ambient temperature with subsequent *Pc* infection was the best combination for chestnut in terms of survival. This information may be useful for modelling purposes when comparing regions of the world characterized by different climates. Based on climate change predictions for Mediterranean countries, the effects of temperature on the impact of *Phytophthora* species on trees have been addressed before. In *Alnus* glutinosa in north-eastern France, hot summers and cold winters allow trees to survive better alder decline induced by *Phytophthora* x*alni*, probably because extreme temperatures limit the pathogen's survival (Aguayo et al., 2014). In *Quercus ilex* in central Spain, increasing temperatures due to climate change are not expected to increase the impact of *Phytophthora* species on acorn germination (Martín-García et al., 2015). In several ornamental plant in California, *P. ramorum* was most pathogenic at 20 °C, had intermediate pathogenicity at 25 °C and lowest pathogenicity at 12 °C (Garbelotto et al., 2021).

Why seedlings subjected to high ambient temperature had a reduced and delayed mortality? A plausible explanation could have been that increased temperatures allowed high photosynthetic rates in plants which in turn lead to increased availability of soluble sugars (e.g. glucose and sucrose) for plant defence. However, non-structural carbohydrates were not assessed here, and from measurements of $P_n$ performed on day 0 there is no evidence that differences in photosynthesis occurred. Under the epidemic scenarios, in response to *Pc* infection, plants at ambient temperature were the only group showing reduced $P_n$, $E$ and $g_s$ values, in agreement with what has been observed in previous studies of susceptible

*C. sativa* individuals (Dinis et al., 2011; Maurel et al., 2001). In addition, seedlings subjected to ambient temperature showed a less developed root system than seedlings subjected to high ambient temperature (Figure 2B), which probably provided them a disadvantage in terms of survival, given that *Pc* zoospores infect and rot fine roots first (Jung et al., 2018). It is also importance to remark that seedlings under high ambient temperature showed the strongest alterations in phenolic contents in response to *Pc*, which is in agreement with the more dynamic response of hormones and metabolites in resistant rather than in susceptible chestnut clones (Camisón et al., 2019). There is additional explanation of why seedlings subjected to high ambient temperature had a reduced and delayed mortality. Among the mechanisms of plants to tolerate heat, the synthesis of Heat Shock Proteins (HSPs) (Bourgine & Guihur, 2021; Zhou et al., 2022) occur at temperatures near to the one used for the plants at high ambient temperature (Lindquist & Craig, 1988). HSPs are also important players in defense signalling during pathogen attack, although some Phytophthora species, including Pc, are able to decrease their levels on the host by targeting their promoters (Saiz-Fernández et al., 2020). Further studies are needed to explore if high ambient temperature involved enhanced levels of HSPs in chestnut at the time of inoculation, and if HSPs were less silenced if plants encountered a warm scenario before Pc infection.

## CONCLUSIONS

The following conclusions can be drawn:

1. Chestnut seedlings exposed to high ambient temperature (35 ºC from 11 to 17 h) showed the highest vigour in terms of plant growth, fine root biomass, and dynamic response of phenolic compounds to biotic stress. Plant mortality induced by *Pc* was 20% lower in chestnuts previously exposed to high ambient temperature (for 30 days) than in chestnuts previously exposed to ambient temperature.
2. Two heat waves of 45 ºC for three days did not alter plant growth, fine

root biomass, and susceptibility of chestnut trees to *Pc*.

3. In response to heat, changes on the phenolic compound profile of chestnut plants exposed to high ambient temperature and heat waves were similar. However, during recovery, most phenolic compounds of plants exposed to high ambient temperature continued to be low, whereas most phenolic compounds of plants exposed to heat waves increased. Changes of compounds were more relevant in leaves than in roots.
4. Three phenolic compounds, ethyl gallate in leaves, 4-hydroxyphenylacetic acid in roots, and coniferyl aldehyde in roots showed significant variation in chestnut in response to *Pc* infection. Five additional phenolic compounds differently changed their contents in response to *Pc* and the scenario experienced by plants before inoculation.
5. Variation in plasticity at the familiar level of several phenolic compounds in response to altered warming scenarios was observed. Such variation would be an opportunity for *C. sativa* to respond and probably adapt to climate change.

## AUTHOR CONTRIBUTIONS

FJD and AS conceived and planned the experiment. FJD carried out the experiment. FJD carried out the measurements and sampling. NC and JCA performed the determination and quantification of non-targeted phenolic compound profiles. FJD took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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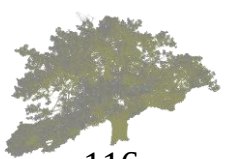

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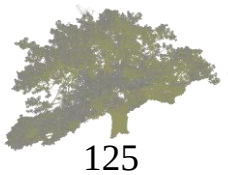


# Supplementary material

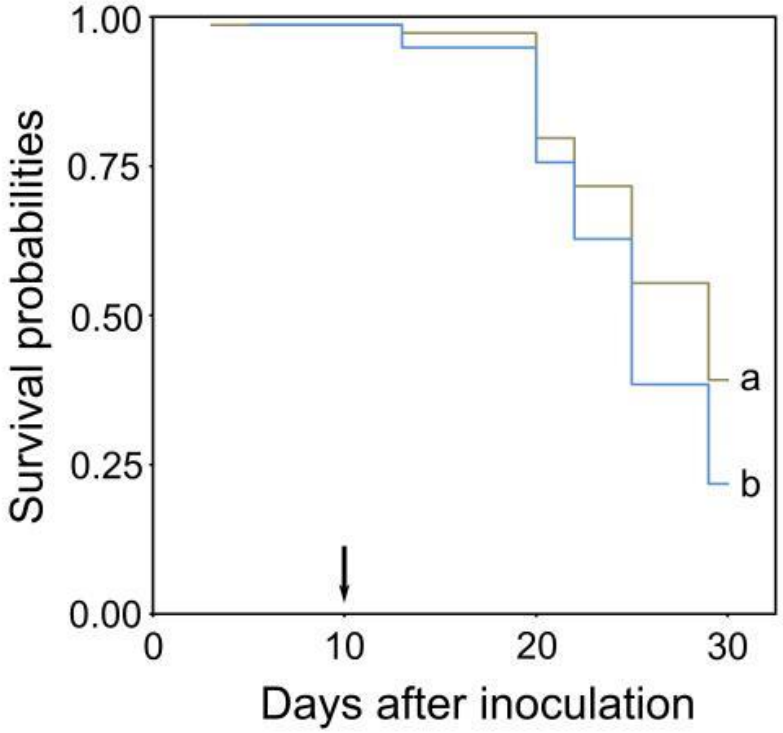



**Figure SM1**. Survival probabilities of *Castanea sativa* seedlings from two mother trees (family 1 in blue and family 4 in brown) inoculated at day 0 with *Phytophthora cinnamomi*. Global log-rank test was significant at $P < 0.041$. Different letters indicate significant differences between survival curves ($P < 0.05$).

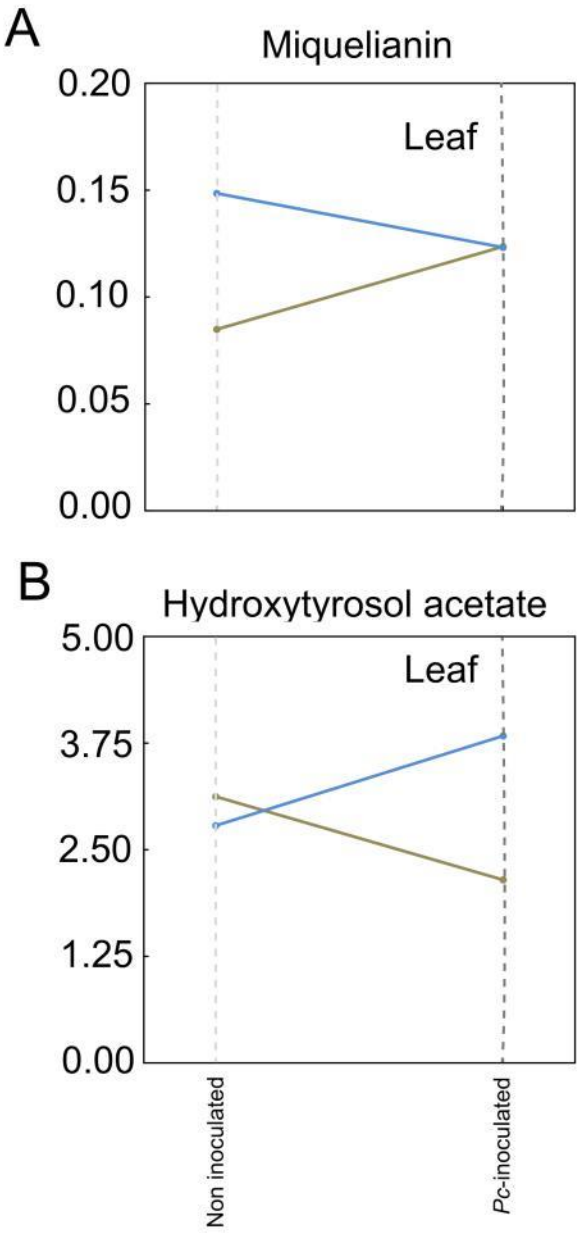



**Figure SM2.** Phenolic compounds of *Castanea sativa* seedlings from two mother trees (family 1 in blue and family 4 in brown) that differently changed their contents in response to *Phytophthora cinnamomi* (significant *Pc* × mother tree interactions in Table 4; $P < 0.05$).

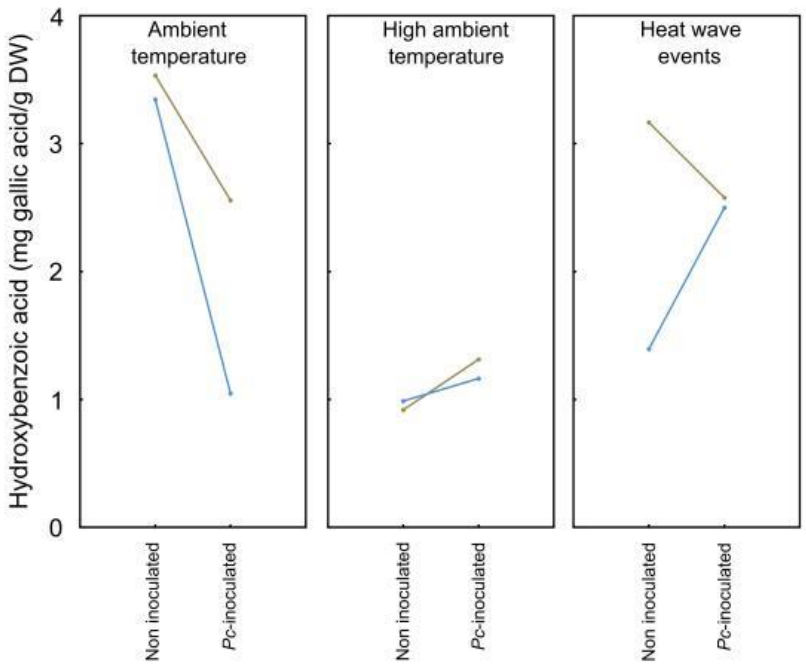
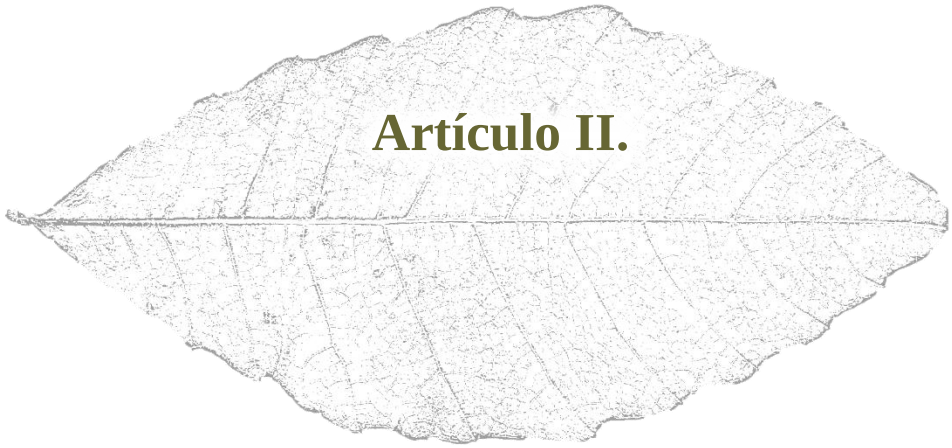



**Figure SM3**. Phenolic compound of *Castanea sativa* seedlings from two mother trees (family 1 in blue and family 4 in brown) that differently changed it content in response to *Phytophthora cinnamomi* and the scenario experienced by plants before inoculation (significant *Pc* × S × mother tree interaction in Table4; $P < 0.05$).



# Artículo II.

# Heat stress and recovery effects on the physiology and biochemistry of *Castanea sativa* Mill.

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## ABSTRACT

Chestnut forests are undergoing increasing heat stress due to the current global warming, but little is known about the physiology and biochemistry responses of *Castanea sativa* Mill. to heat or whether differences exist between populations. Six-month-old seedlings from three climatically contrasting populations of *C. sativa* (from the north, centre and south of Spain) were subjected to control and heat stress conditions for seven days. The effects of heat stress on seedlings and their recovery (10 days after heat stress) were described by assessment of visible symptoms, growth, mortality and leaf gas exchange of plants, quantification of compounds involved in the primary and secondary metabolism, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging. In response to stress, plant biomass decreased and plant biochemistry altered depending on the tissue and the population. Major alterations in the primary metabolism of stressed plants occurred in leaves, characterised by increased levels of soluble sugars, nitrogen and proline, and depletion of starch. Increased levels of soluble sugars and starch depletion occurred mostly in seedlings from the southern population, while proline increase occurred only in the northern population. Secondary metabolism of seedlings experienced the highest variation below ground, and roots of heat-stressed plants increased the content of phenolic compounds. LC-MS analysis permitted identification and quantification of six compounds induced by heat, five of which were detected in the roots. Differential biochemistry responses to heat stress were observed among populations. At recovery, most of the altered parameters had returned to control conditions, suggesting high resilience to heat stress in this Mediterranean tree species. This is the first study to address the effects of heat stress on the physiology and biochemistry of *C. sativa* and their interpopulation variability. Most parameters were significantly influenced by the interaction of population and heat treatment, indicating that genetic differentiation controlled the phenotypic differences of *C. sativa* in response to heat stress. Extensive genetic variation in plasticity in physiological and biochemical

parameters in response to heat stress reveals an opportunity for chestnut for global warming-mediated selection.

## INTRODUCTION

Heat stress is defined as an increase in temperature above a threshold sufficient to cause irreversible damage to the plant (Wahid, 2007). Visible damage observed from heat stress includes leaf and branch burn, leaf senescence and abscission, or inhibition of shoot and root growth (Wahid et al., 2007; Hasanuzzaman et al., 2013; He et al., 2021), impacting plant productivity and vitality. Multiple physiological and biochemical mechanisms underlie the visible damage caused by heat stress in plants (Wahid et al., 2007). Plant species generally react to heat by closing stomata (Marchin et al., 2022). When this occurs, plants will maintain hydraulic function at the expense of leaves overheating (because of reduced transpiration) and carbon assimilation reduction (because of reduced photosynthesis) (Ruehr et al., 2015). Heat can directly impact photosynthetic rates by reducing enzyme activity, mainly Rubisco, and damaging photosystem II (Bhagat et al., 2014; Birami et al., 2018; Nievola et al., 2017) through accumulation of reactive oxygen species (ROS). To protect themselves from oxidative stress, plants use both enzymatic (e.g. superoxide dismutase and ascorbate peroxidase) and non-enzymatic (e.g. phenolic acids and flavonoids) antioxidant machinery to break down and scavenge free radicals and/or disrupt their reaction chains (Ahmad et al., 2010; Poudel & Poudel, 2020; Shivashankara et al., 2016).

Plants synthesise a large diversity of low molecular weight compounds such as carbohydrates, secondary metabolites (phenolic acids, flavonols and lignins) and hormones that are essential for plant acclimatisation, survival and recovery under abiotic stress (Zandalinas et al., 2017; Martín et al. 2018a). Non-structural carbohydrates (NSCs), including soluble sugars and starch, are a small fraction of the carbohydrates acquired by the plant via photosynthesis (Martínez-Vilalta et al.,

2016). However, NSCs play a crucial role in plant survival under environmental stress by buffering the asynchrony of substrate supply and demand (Galiano et al., 2011; Wiley and Helliker, 2012; Hartmann et al., 2013; Dietze et al., 2014; O'Brien et al., 2014; Li et al., 2018). Most secondary metabolite compounds are synthesised from products of primary carbon metabolism (Wahid & Ghazanfar, 2006). Phenolic compounds are an important class of secondary metabolites, and their accumulation in plant tissues is considered an adaptive response of plants to adverse environmental conditions (Akhi et al., 2021). In crop plants, it has been reported that the accumulation of photoprotective and antioxidant secondary metabolites could be an adaptive mechanism to prevent damage from heat stress (Rivero et al., 2001; Zandalinas et al., 2017). In trees, heat-induced production of secondary metabolites appears to be species-, compound- or even context-dependent (Berini et al., 2018). Under the current climate change scenario, understanding the impact of heat stress and heat waves on vegetation is a crucial challenge (Della-Marta et al., 2007; Rita et al., 2020; Spinoni et al., 2014) to anticipate socioeconomic losses and harm to natural ecosystems.

In response to climate change, forest tree populations have three possible fates: migration, adaptation or extirpation (Aitken et al., 2008). The ability of trees to adapt to environmental changes depends on the genetics of their populations and on the ability to show plastic phenotypic responses (Aitken & Whitlock, 2013; Alberto et al., 2013). Phenotypic plasticity is the phenomenon by which a genotype can produce different phenotypes in response to environmental conditions (Ghalambor et al., 2007). Phenotypic plasticity can be adaptive if it has a positive effect on plant fitness, maladaptive if it has a negative effect, or neutral if it has no effect (Scheiner, 1993). A thorough understanding of the plastic phenotypic responses of trees and their occurrence among populations is essential to predict their potential for adaptation to climate change (Valladares et al., 2014; Castellana et al., 2021). When a particularly adapted phenotype confers an advantage under climate change, this variant may invade the local population through migration and

selection, and a local adaptation may emerge (Villemereuil et al., 2018). There is considerable evidence for local adaptation and genetic variation in plasticity (i.e., variation in phenotypic plasticity between tree populations) in Mediterranean tree species (Matesanz and Valladares, 2014; Zas et al., 2020; Blumstein and Hopkins, 2021; Vázquez-González 2022), but there are no studies for some important forest species.

Chestnut (*Castanea sativa* Mill.) is a temperate thermophilic tree distributed throughout the Mediterranean basin in natural, semi-natural, and managed stands of considerable environmental, cultural and economic value (Conedera et al., 2004; Fernández-López et al., 2021; Fernández-López & Alía, 2003). Its main area of distribution in Spain is in the humid northwest region, as well as scattered stands in central and southern Iberia, where high temperatures are frequent (Fernández-López et al., 2021). Chestnut forests in Spain are threatened by global warming (e.g. May and July air temperatures hit a record high in 2022), providing an ideal testbed for studying heat stress effects. Differences in rainfall between northern and south-central Spanish populations have resulted in differentiation of two chestnut ecotypes (mesophytic in northern Spain and xeric in south-central Spain) with different adaptive responses to water stress (Alcaide et al., 2019; Míguez-Soto et al., 2019; Míguez-Soto & Fernández-López, 2015). Drought is therefore a selective force shaping the differentiation of chestnut populations (Díaz et al., 2009; Míguez-Soto & Fernández-López, 2015) and heat stress is also responsible for this differentiation (Dorado et al., 2022). Little is known about the response of *C. sativa* to heat stress or whether contrasting climate habitats involve differential responses to higher temperatures. The efficacy of fertilisers in chestnut tolerance to high temperatures (Carneiro-Carvalho et al., 2021) and the effect of temperature (Gomes-Laranjo et al., 2006) on some chestnut cultivars has been studied. However, no studies addressed the effects of high temperatures on the physiology and biochemistry of this species. The objectives of this study were to (i) analyse the impact of heat stress on the physiology and biochemistry of *C. sativa* seedlings from

contrasting climate conditions, (ii) quantify the response of chestnut seedlings to heat stress when temperatures lower, and (iii) to identify genetic variation and phenotypic plastic responses to heat stress in *C. sativa* seedlings from different climatic regions.

## MATERIALS AND METHODS

### *Plant material*

In November 2018, *C. sativa* seeds were collected from 16 adult trees per population in three wild populations in Spain (Puebla de Sanabria, Valle de Matamoros and Paterna del Río). The three populations had contrasting climates in terms of precipitation and absolute maximum temperature (Table 1). Puebla de Sanabria is in northern Spain, close to the Transmontane region of northeast Portugal, known as Cold Land ('Terra Fria'), and has ca. 1,000 mm rainfall per year and belongs to the supra-Mediterranean bioclimatic zone (Table 1). Valle de Matamoros is in the foothills of Sierra Morena (centre-south of Spain), is characterised by 740 mm annual rainfall and belongs to the meso-Mediterranean bioclimatic zone (Table 1). Paterna del Río is in the Alpujarras, on the southern slopes of Sierra Nevada, has ca. 650 mm rainfall per year and belongs to the meso-Mediterranean bioclimatic (Table 1). For clarity, the populations were named northern, central and southern populations, respectively. In each chestnut population, 16 fruit-bearing trees were selected at least 70 m apart to minimise the chances of sampling intercrossed individuals.

In November 2018, about 100 seeds collected from each of the 16 trees per population were immersed in a fungicide solution (2 g $L^{-1}$ Thiram 80GD, ADAMA Inc., Spain) for 5 min, and those that floated were discarded as non-viable. Seeds were rinsed with sterilised water and stored at 4 $^{o}$C for two weeks. In December 2018, they were individually weighed and sown at 1 cm depth in 48-cell plastic root trainers with one seed per cell.

**Table 1.** Description of the three Spanish populations of *Castanea sativa* assessed.

T- Mean annual temperature; Tmax- Absolute maximum temperature; P- Cumulative annual rainfall; No. trees- Number of mother trees sampled

| Population | Bioclimatic zone | Location | Coordinates | Altitude (m asl) | T (ºC) | Tmax (ºC) | P (mm) |
|---|---|---|---|---|---|---|---|
| Northern | Supra-Mediterranean | Puebla de Sanabria, Zamora, Castilla y León | 42°02'05.4''N 6°40'19.5''W | 1003 | 9.7 | 32.8 | 1013 |
| Central | Meso-Mediterranean | Valle de Matamoros, Badajoz, Extremadura | 38°22'28.5''N 6°48'00.1''W | 624 | 15 | 39.5 | 740 |
| Southern | Meso-Mediterranean | Paterna del Río, Almería, Andalusia | 37°01'54.0''N 2°57'15.5''W | 1380 | 12.4 | 33.6 | 654 |

Individual cells were 330 mL in volume, 18 cm high, 5.3 × 5.3 cm upper surface, and contained peat (PKN1 Florava® Peat Substrate; pH=4.5). Germination was considered successful when the aerial part emerging from the embryo was green. Aerial emergence of plants was assessed weekly. Plants were kept in natural daylight under greenhouse shade that reduced solar radiation by 50% and hand watered every four days to field capacity until they were well established. The greenhouse was at the Faculty of Forestry of Plasencia (40º02′ N, 6º04′ W; 374 m a.s.l., Extremadura region, Spain).

### *Experimental design and treatments*

The experiment included seedlings from three populations, obtained from 16 mother trees in each population. Thirty seedlings per mother tree were used, of which half were subjected to ambient temperature (control), and half were subjected to a heat stress treatment. To reduce spatial thermal variability, the plants were arranged following a split-plot random design replicated in three blocks, with the heat treatments acting as the main factor (two categories: control and heat-stressed; whole plots) and the populations as the split factor (three categories: northern, central and southern, as shown in Table 1; split plots). In the three blocks, the three populations were represented in each whole plot by five seedlings per mother tree. Seedlings were randomly positioned within the blocks. The experiment comprised 1,440 seedlings corresponding to 3 blocks × 2 treatments × 3 populations × 16 mother trees × 5 seedlings.

In May 2019, when the plants were six months old, 720 seedlings were subjected to ambient temperature, and 720 seedlings were subjected to the heat treatment. From 11 a.m. to 5 p.m., mean temperatures of control and heat-stressed plants were 30.2 and 42.5 °C, respectively. The time frame selected for applying heat stress simulated natural conditions of daily temperature increase during summer in the Mediterranean Basin, with a maximum temperature from midday to early afternoon. The heat stress treatment comprised placing the plants inside a

climate chamber with translucent walls in the same greenhouse. During treatments, both groups of plants were well irrigated. Volumetric soil water content (VWC), a relative measure of soil moisture, was maintained at values around 30% by irrigating once a day the control plants and by irrigating twice a day the heat stressed plants. Previous work indicated that 30% VWC was optimal for chestnut growth (A. Camisón et al., 2020). VWC was verified using a TDR 100 soil moisture meter (Spectrum Technologies Inc., Plainfield, Illinois, USA) and 12-cm-long rods in 10 cells per block per treatment. The leaves of control plants were green and turgid throughout the experiment (Figure 1A). During treatments, the relative air humidity and light conditions of both groups of plants were similar (≈ 80% and PAR ≈ 850 μmol photons $m^{-2}s^{-1}$). The heat treatments lasted seven days (day 7, 'heat', Figure S1). After the heat treatments ended, heat-stressed seedlings were kept under control conditions for 10 days to assess their recovery (day 17, 'recovery phase', Figure S1).

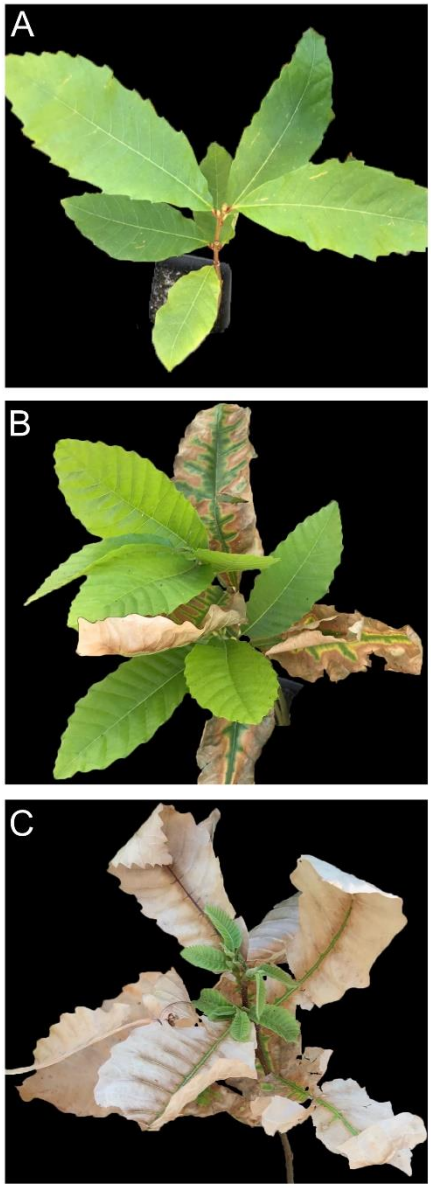



**Figure 1.** Six-month-old *Castanea sativa* seedlings exposed to ambient conditions (A), heat stress conditions for seven days (B), and ambient conditions for 10 days after heat stress ceased (C). Heat scorching is observed on the foliage of stressed plants, starting with browning of the leaf margins and/or yellowing or darkening of the areas between the main leaf veins. Ten days after heat treatment ceased, the foliage of the scorch-affected plant finally wilted, but the buds were viable, and the plant started to resprout.

### *Plant measurements and sampling*

Measurements and sampling for biochemical analysis were performed on the

first day of treatments (day 1), the last day of treatments (day 7, heat; Figure S1), and 10 days after treatments ceased (day 17, recovery phase; Figure S1) when plants were in recovery from stress (Figure 1C). On day 1, leaf gas exchange parameters were measured to observe the photosynthetic response of the populations to the increase in temperature. In the heat and recovery phases, the effects of heat on seedlings were evaluated by (i) foliar symptoms, leaf wilting, plant growth and mortality; (ii) leaf gas exchange; (iii) quantification of primary metabolism parameters (NSC and proline) and N content; and (iv) quantification of secondary metabolism parameters (total phenolic compounds and ortho-phenolic and flavonoid compounds), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, and non-targeted metabolite profiling.

Leaf gas exchange parameters - net photosynthetic rate (Pn), transpiration rate (E) and stomatal conductance ($g_s$) - were determined on the first day of treatments (day 1) in 15 seedlings per population (five per block) and treatment. In the heat and recovery phases, leaf gas exchange parameters were measured in 10 seedlings per population using a portable differential infrared gas analyser (IRGA; Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf chamber and a temperature sensor. On day 1, measurements were performed from 9 a.m. to 2 p.m., with temperature ranging from 32 to 48 ºC, photosynthetically active radiation (PAR) ranging from 500 to 800 μmol photons $m^{-2}$ $s^{-1}$ (daylight and variable PAR conditions), and ambient $CO_2$ concentration ranging from 390 to 410 μmol $mol^{-1}$. In the heat phase, temperatures ranged from 23 to 27 ºC and 37 to 42 ºC in the control and heat treatments, respectively. In the recovery phase, temperatures ranged from 23 to 27 ºC for both treatments. To reduce errors associated with $CO_2$ concentration and PAR changes over time, leaf gas exchange measurements were carried out by systematically alternating seedlings from the three populations (day 1) or by systematically alternating seedlings from the three populations and two treatments (heat and recovery phases). Instantaneous and intrinsic water use efficiencies were calculated based on leaf gas exchange, using the formulae

*instantaneous WUE* = Pn/E and *intrinsic WUE* = Pn /$g_s$, respectively.

Eighteen plants per population and treatment were destructively harvested for primary and secondary metabolite analysis. After collection, samples from three different seedlings were pooled to obtain a final sample size of six pools per population and treatment. Samples were immediately frozen in liquid nitrogen and stored at -80 °C. To account for the diurnal oscillation of NSC content in plant tissues (Tixier et al., 2018) and to ensure the results were comparable, plants were sampled from 2.00 p.m. to 3.00 p.m. Leaves, aerial woody tissues (stem plus small twigs, 'stems') and roots (taproot plus fine roots, 'roots') of each seedling were used to determine dry biomass and analyse N and NSC. Within 30 minutes of harvesting, excised organs were subjected to microwave treatment (80 s, 800 W) to inactivate the enzymes and fix the actual NSC content (Hoch et al., 2002). Samples were oven dried (48 h, 60 °C), weighed on a precision balance for dry biomass quantification, and ground to a fine powder in a ball mill (Mixer Mill MM 400, Retsch, Germany) to pass through a 0.42 mm mesh screen.

For analyses of proline, secondary metabolites (total phenolic compounds and ortho-phenolic and flavonoid compounds), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and non-targeted metabolite profiling, leaves and roots of seedlings from the two treatments were sampled. For leaves, those that were fully developed near the shoot tip were sampled. For roots, the outermost fine roots were collected after carefully lifting the root ball from the tray.

### *Assessment of external symptoms, wilting, growth and mortality*

External symptom evaluation comprised visual characterisation of the foliar discolouration observed on seedlings. Wilting percentage was determined by visual estimation of the percentage of wilted leaves in plants subjected to heat stress in 10% intervals following Alcaide et al. (2019). Total plant dry biomass, comprising the dry weight of tissues used for NSC and nitrogen determination, was used as an indicator of plant growth.

### *Primary metabolism*

Total NSCs included the sum of soluble sugars and starch. Soluble sugars and starch in leaf, stem and root tissues were determined following the protocol adapted for chestnut by Camisón et al. (2020). Soluble sugar:starch ratios were calculated in each plant tissue and used as a proxy for mobilisation of starch to soluble sugars (Piper, 2011).

Total N in leaf, stem and root tissues was determined by the Dumas method (DUMATHERM® CN, C-Gerhardt Analytical Systems) and expressed as a percentage of dry weight. Proline content was determined using the protocol of Bates et al. (1973), with slight modifications for chestnut by Camisón et al. (2021). NSC or N concentration at the whole plant level was calculated by a weighted mean, taking into account the total NSC or N concentration of each tissue and the relative contribution of each tissue's biomass to plant total dry biomass, following: *Whole plant NSC or N (%)* $= ((X_l \times a_l + X_s \times b_s + X_r \times c_r) / (a_l + b_s + c_r))$; where $X_l$, $X_s$ and $X_r$ are the total NSC or N concentrations in leaves, stems and roots, respectively, and $a_l$, $b_s$ and $c_r$ are the proportions in which each tissue contributes to plant dry biomass.

### *Secondary metabolism*

*Phenolic compounds and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging.* Powdered leaf or root samples (50 mg) were homogenised with 1.5 mL of 70% aqueous methanol and shaken in an orbital shaker for 1 h (700 rpm at 25 °C). After centrifugation (15 min at 4 °C and 10,000 g), the supernatant was collected, and the process was repeated three times (to 6 mL volume). The extract was stored at -80 °C for further determination of total phenolic compounds, ortho-phenolic and flavonoid compounds, and DPPH free radical scavenging.

Total phenolic and ortho-phenolic contents were estimated by the Folin-Ciocalteu method and the sodium molybdate colourimetric assay, respectively, both

adapted from Singleton et al. (1999). Total flavonoid content was measured following the aluminium chloride colourimetric assay adapted from Chang et al. (2002). Total antioxidant capacity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method adapted from Xu and Chang (2007).

*Non-targeted metabolite profiling.* Leaf or fine root powder (0.2 g) was homogenised with 70% ethanol (5 mL) and sonicated for 15 min. The extracts were cold macerated (4 °C, 24 h), centrifuged (3500 rpm, 15 min), and the supernatant was filtered (0.45 µm filter). The filtered extracts were stored at -80 °C until analysis.

Analysis, identification and quantification were performed with an LC-MS system (HPLC 1260 - QTOF 6520, Agilent Technologies, Santa Clara, CA, United States). A total of 20 µL of filtered extract of each sample was injected onto a Spherisorb C-18 5 µ 250 × 4.6 mm reversed-phase analytical column at a rate of 1 mL/min. The mobile phase used was a water (2% formic acid)/methanol gradient from 1% water to 100% methanol in 100 min for leaves and 50 min for roots. Chromatograms were recorded at 350 nm and 280 nm wavelengths. Concentrations of the compounds were estimated from a standard curve (0.00, 0.01, 0.05, 0.10, 0.20 mg/mL) using gallic acid, ellagic acid, quercetin and quercetin 3-O-rutinoside depending on the nature of the compound to be quantified.

### *Statistical analysis*

To determine how the increase in temperature affected gas exchange parameters, a global and population-based regression analysis was performed. To assess the effect of heat stress on the physiological and biochemical parameters of *C. sativa* seedlings, one-way analysis of variance (ANOVA) was performed using treatment as the only factor. To analyse the differential response of populations, two-way ANOVAs were performed, this time including the main effects of treatment, population, and their interaction as factors. Within each date (heat and

recovery phase), Tukey's multiple comparison tests were used to identify significant differences between treatments and populations ($P < 0.05$). Lack of differences between treatments for physiological and biochemical parameters indicated no phenotypic plasticity in response to heat stress, whereas significant differences between treatments indicated phenotypic plasticity in response to heat stress. A significant treatment × population interaction was interpreted as genetic variation in phenotypic plasticity of a parameter in response to heat stress.

Discriminant function analysis (DFA) was performed with compounds from the non-targeted metabolite profiling to differentiate the three *C. sativa* populations and their response to heat in the heat and recovery phases. The population × treatment interaction was used as a grouping variable, and the concentrations of the compounds (identified and unidentified) were used as independent variables. The number of compounds initially used as independent variables in the DFA was 18. The independent variables finally selected were obtained from a sequential variable forward stepwise method. The DFA analysis was performed with STATISTICA v10 software (StatSoft, *Inc.* 2011), and the graphical representation was performed with R software (R Core Team, 2018).

Principal component analysis (PCA) was applied to detect patterns of variation in physiological and biochemical parameters due to origin (population), response to heat, and recovery. The PCA was performed using the R software 'factoextra' statistical package (R Core Team, 2018). Before the PCA, the data were centred and standardised to reduce scale effects (Martín et al., 2008b). Relationships between leaf and root parameters used to perform PCA were assessed for the heat phase, separated into control and heat stress conditions, using a Pearson's correlation matrix performed with the R software 'corrplot' package (R Core Team, 2018).

For each parameter and population, phenotypic plasticity in response to heat stress was expressed as a percentage relative to control plants following the

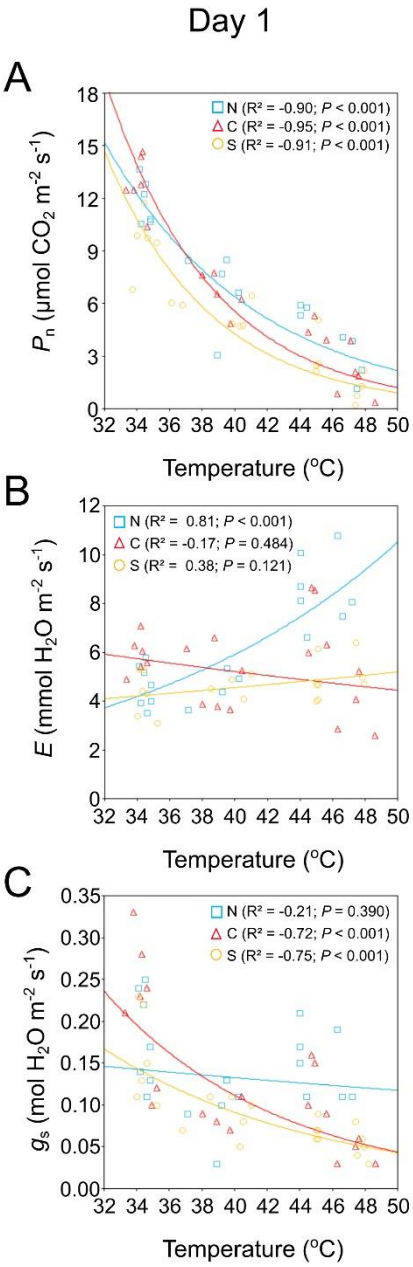



**Figure 2.** Effect of temperature on net photosynthetic rate (A), transpiration rate (B), and stomatal conductance (C) of six-month-old *Castanea sativa* seedlings from the northern (N, blue squares), central (C, red triangles) and southern (S, yellow circles) populations. The variance explained ($R^2$), and the significance (*P*) of the models are shown.

equation proposed by Rehschuh et al. (2020) in the heat phase: *Phenotypic plasticity (%) = ((x - $C_{pop}$) / $C_{pop}$) × 100*; where *x* is the heat-induced value of the parameter of a particular seedling of the population, and $C_{pop}$ is the mean constitutive (control) value of the parameter of seedlings of the population. To check whether the effect of treatment differed between populations, one-way ANOVAs were performed using the population as the only factor. Before all analyses, data were checked for normality and homogeneity of variances. ANOVAs and Tukey's tests were performed using STATISTICA v10 software (StatSoft, *Inc.* 2011).

## RESULTS

### *Rapid response of leaf gas exchange parameters to temperature increase in C. sativa*

On day 1, Pn and $g_s$ values in seedlings decreased exponentially as temperature increased ($R^2$ = -0.90 and $R^2$ = -0.59, respectively, *P* <0.001; Supplementary Table 1), while E values increased as temperature increased ($R^2$ = 0.28, *P* = 0.037). Gas exchange response to temperature increase depended on population (Figure 2).

**Table 2.** Significant changes in leaves and roots of six-month-old *Castanea sativa* seedlings subjected to seven days of heat stress (n=18; heat phase) and 10 days after application (n=12; recovery phase). Red indicates accumulation, and blue indicates decrease compared to controls. + + indicates highly significant accumulation (Tukey's HSD, $P < 0.05$); + indicates marginally significant accumulation (Tukey's HSD, $0.05 < P < 0.10$); - - indicates highly significant decrease (Tukey's HSD, $P < 0.05$); - indicates marginally significant decrease (Tukey's HSD $0.05 < P < 0.10$).

| Organ | Trait | Heat effect | |
|---|---|---|---|
| | | Heat phase | Recovery phase |
| Leaf | $P_n/E$ | - - | - - |
| | $P_n/ g_s$ | - - | ns |
| | Biomass | - - | ns |
| | Soluble sugars | + + | + + |
| | Starch | - - | - - |
| | NSC | - - | ns |
| | SS:starch ratio | + + | + + |
| | Nitrogen | + + | ns |
| | Proline | + + | ns |
| | Flavonoids | ns | + + |
| | DPPH | - - | - - |
| Stem | Soluble sugars | + | + + |
| | Starch | - - | ns |
| | Nitrogen | + + | ns |
| Root | Biomass | - - | ns |
| | Nitrogen | + + | ns |
| | Phenolic compounds | + + | - - |
| | Ortho-phenols | + + | ns |
| | Flavonoids | + + | ns |
| Whole plant | Biomass | - - | ns |
| | Soluble sugars | + | ns |
| | SS:starch ratio | + + | ns |
| | Nitrogen | + + | ns |

In seedlings from the northern population, E increased with temperature, while in seedlings from the central and southern populations, E was not significantly altered (Figure 2B). In seedlings from the northern population, $g_s$ did not change, while in seedlings from the central and southern populations, stomata closed as temperature increased (Figure 2C).

### *General response and recovery of C. sativa from heat stress*

During the heat phase, the leaves of stressed seedlings showed brown margins, yellowing and browning of inner areas, and scorching (Figure 1B). Leaf damage was observed only in about 10% of seedlings, but heat-stressed seedlings showed lower leaf, root and total biomass compared to control plants (Tables 2 and Supplementary Table 2). Heat stress did not lead to plant mortality. In the recovery phase, seedlings that had wilted more started to resprout (Figure 1C), although significant changes in seedling biomass were not detected (Supplementary Table 2).

Heat stress did not affect plant Pn, E and $g_s$ but induced a significant decrease in *instantaneous* and *intrinsic WUE* compared to control seedlings (Tables 2 and Supplementary Table 2). During the recovery phase, E and *intrinsic WUE* remained altered in heat stressed seedlings compared to control seedlings (Table 2 and Supplementary Table 2).

Major alterations observed during the heat phase in primary metabolism parameters occurred mainly in leaves (Table 2 and Supplementary Table 2). Heat-stressed seedlings responded by increasing soluble sugars and decreasing starch in leaves and stems (Table 2 and Supplementary Table 2). Soluble sugar:starch ratio was significantly increased by heat stress in leaves and the whole plant. Total NSC content in leaves decreased significantly with heat stress, while total NSC in stems and roots did not change significantly (Table 2 and Supplementary Table 2).

**Table 3.** List of secondary metabolite compounds detected, identified and quantified in leaves and roots of six-month-old *Castanea sativa* seedlings.

| Group | Organ | Compound |
|---|---|---|
| Phenolic acid | Leaf | Ellagic acid acetyl-xyloside |
| | | Ellagic acid |
| | Root | Ellagic acid |
| | | Hydroxibenzoic acid |
| | | 4-hydroxyphenylacetic acid |
| Lignan | Leaf | Lariciresinol |
| Flavonoid | Leaf | Miquelianin (quercetin 3-O-glucuronide) |
| | | Rutin (quercetin 3-O-glucuronide) |
| | | Astragalin (kaempferol-3-O-glucoside) |
| | | Isorhamnetin |
| | | 6´´-O-manolylglycitin |
| | | Hyperoside (quercetin-3-O-galactoside) |
| | Root | Hyperoside (quercetin-3-O-galactoside) |
| | | Kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside |
| Other polyphenols | Root | Coniferyl aldehyde (4-hydroxy-3-metoxycinnamaldehyde) |

In heat-stressed seedlings, N content significantly increased in all organs and at the whole plant level, and proline content in leaves doubled (Table 2 and Supplementary Table 2). Recovering seedlings continued to have higher soluble sugar concentration, lower starch concentration in leaves and stems and higher soluble sugar:starch ratios in leaves than control seedlings (Table 2 and Supplementary Table 2). In contrast, N and proline concentration returned to the levels of control seedlings (Supplementary Table 2).

Secondary metabolism of heat-stressed seedlings was most altered in the roots (Table 2, 3, 4 and Supplementary Table 2). Chestnut seedlings subjected to heat stress significantly increased total phenolic compounds, ortho-phenolic, and flavonoid compounds only in roots (Table 2 and Supplementary Table 2). Given

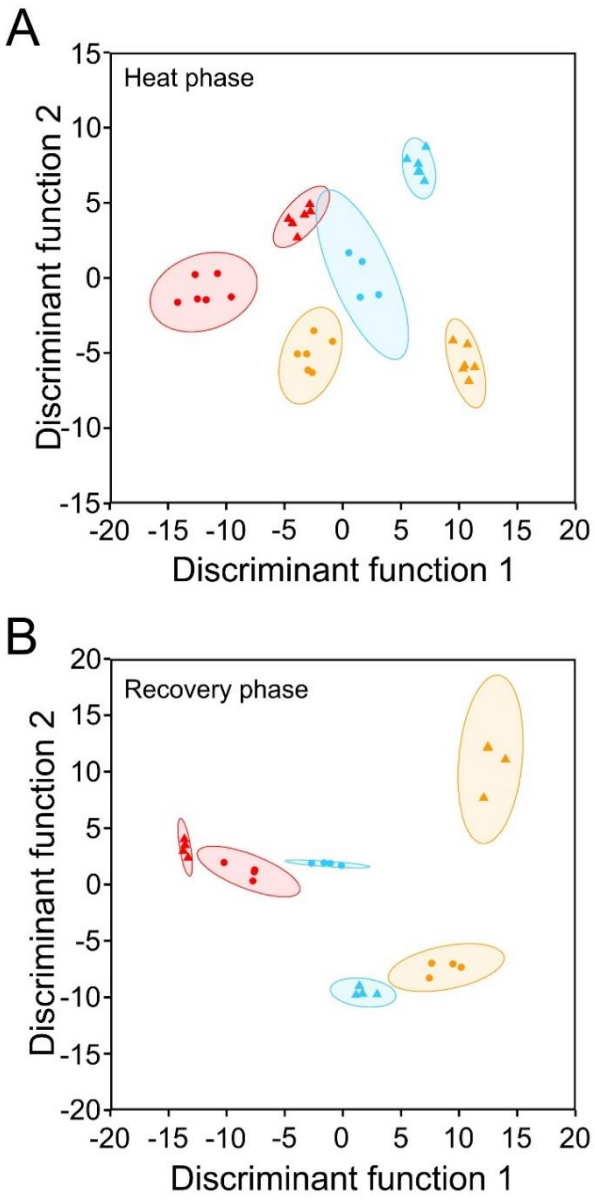



**Figure 3.** DFA of six-month-old *Castanea sativa* seedlings showing differences between control (circles) and heat-stressed (triangles) seedlings from the northern (blue symbols), central (red symbols) and southern (yellow symbols) populations based on the 18 compounds detected in roots and leaves by non-targeted metabolite profiling. In A, heat-stressed plants were at the time of maximum stress; in B, heat-stressed plants were left for 10 days to recover (Fig. SM1).

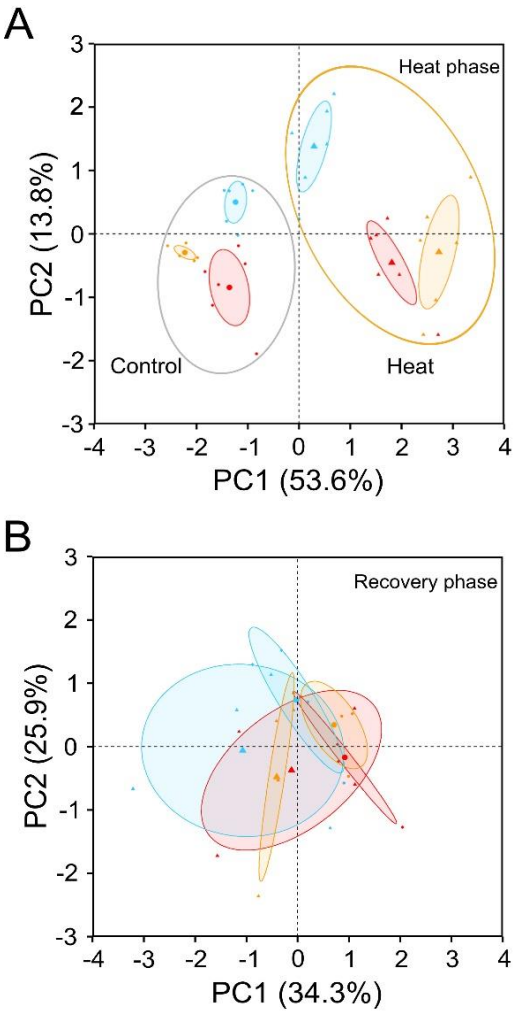



**Figure 4.** PCA of six-month-old *Castanea sativa* seedlings showing differences between control (circles) and heat-stressed (triangles) seedlings at heat (A) and recovery (B) phases from northern (blue symbols), central (red symbols) and southern (yellow symbols) populations according to the physiological variables included in Figure 5. In A, heat-stressed plants were at the time of maximum stress; in B, heat-stressed plants were left for 10 days to recover (Fig. SM1). In A, the 10 variables contributing most to the PC1 and PC2 axes were leaf starch (28.4%), leaf soluble sugars:starch ratio (27.9%), root proline (12.1%), whole plant soluble sugars:starch ratio (8.1%), root flavonoids (3.1%), root ortho-phenols (2.8%), leaf proline (2.6%), root phenols (1.9%), instantaneous water use efficiency (1.5%) and root biomass (1.4%).

the heat-induced alterations in leaf and root phenolic compounds, non-targeted metabolite profiling was applied, revealing 18 compounds, 15 of which were identified (Table 3). Six identified compounds increased in heat-stressed plants (Table 4). Five of the six compounds showing variation were in the roots and belonged to the phenolic acid (hydroxybenzoic acid subclass) and flavonoid (flavonol subclass) families (Tables 3 and 4). DFA revealed different secondary metabolism profiles between treatments in chestnut (Figure 3A). Isorhamnetin in leaves, and hydroxybenzoic acid, kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside and hyperoside in roots, were involved in this discrimination ($P < 0.001$). However, under heat stress, antioxidant capacity increased significantly only in leaves (Table 2 and Supplementary Table 2). During the recovery phase, only flavonoids in leaves remained altered by heat stress (Table 2 and Supplementary Table 2). DFA of non-targeted metabolite profiling showed differences between recovery and control seedlings (Figure

3B). Astragalin and hydroxybenzoic acid decreased significantly in recovering seedlings compared to controls (Table 4 and Supplementary Table 4).

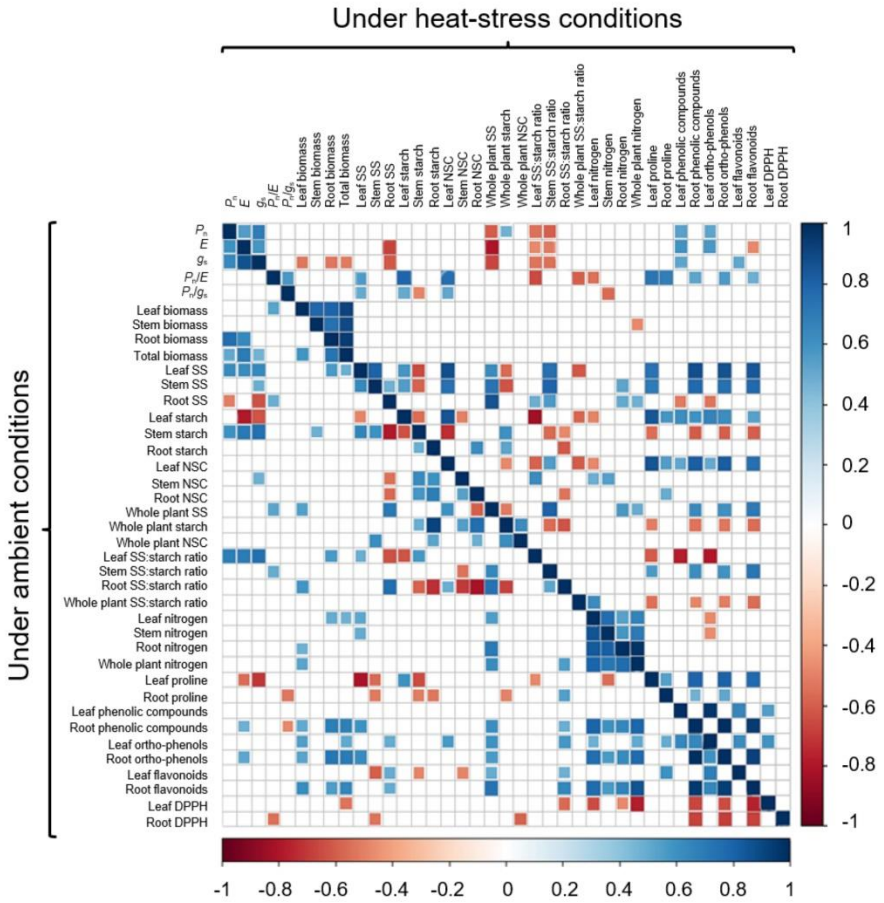



**Figure 5.** Matrix of significant ($P < 0.05$) Pearson correlation coefficients between gas exchange parameters, plant biomass, NSCs, nitrogen and proline content, total phenolic compounds, ortho-phenolic and flavonoid compounds and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging of six-month-old *Castanea sativa* seedlings subjected to ambient (below the main diagonal) and heat-stress conditions (above the main diagonal). Coloured correlations are significant. Colour gradient from blue to red indicates the gradient from positive to negative correlation (1,-1).

**Table 4.** Significant changes in leaves and roots of secondary metabolite-derived compounds of six-month-old *Castanea sativa* seedlings from three populations (N-Northern; C-Central; S-Southern; n=6 per population in heat phase; n=4 per population in recovery phase) subjected to heat stress for seven days (n=18; heat phase) and 10 days after application (n=12; recovery phase). Red indicates accumulation, and blue indicates decrease compared to controls. + + indicates highly significant accumulation (Tukey HSD $P < 0.05$); + indicates marginally significant accumulation ($0.05 < P < 0.10$); - - indicates highly significant decrease ($P < 0.05$); - indicates marginally significant decrease ($0.05 < P < 0.10$).

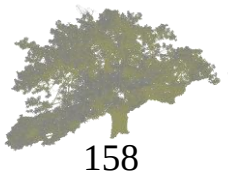
| | | Heat effect | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Heat phase | Recovery phase | Heat phase | | | Recovery phase | | |
| **Compound** | **Organ** | | | **N** | **C** | **S** | **N** | **C** | **S** |
| Ellagic acid acetyl-xyloside | Leaf | ns | ns | ns | + + | ns | ns | ns | ns |
| Miquelianin (quercetin 3-O-glucuronide) | Leaf | ns | ns | ns | ns | ns | ns | + + | ns |
| Rutin (quercetin 3-O-rutinoside) | Leaf | ns | ns | ns | + + | ns | ns | ns | ns |
| Astragalin (kaempferol-3-O-glucoside) | Leaf | ns | - | ns | + + | ns | ns | - - | - - |
| Isorhamnetin | Leaf | + + | ns | + + | + + | ns | ns | ns | ns |
| 6´´-O-manolylglycitin | Leaf | ns | ns | ns | ns | - - | ns | ns | ns |
| Ellagic acid | Leaf | ns | ns | ns | + + | ns | ns | ns | - - |
| | Root | + + | ns | + + | ns | + + | ns | ns | ns |
| Hyperoside (quercetin-3-O-galactoside) | Leaf | ns | ns | ns | + + | ns | ns | ns | ns |
| | Root | + + | ns | + + | + + | + + | ns | ns | ns |
| Hydroxybenzoic acid | Root | + + | - - | ns | ns | ns | ns | - - | - - |
| Kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside | Root | + + | ns | + + | ns | ns | ns | ns | ns |
| Coniferyl aldehyde (4-hydroxy-3-metoxycinnamaldehyde) | Root | + + | ns | ns | ns | + + | ns | - - | - - |

PCA revealed a clear difference in the physiological and biochemical status of *C. sativa* seedlings under control and heat treatments (Figure 4A). During the heat phase, the separation between control and heat was mainly associated with starch depletion in leaves (28.4%), high ratios of soluble sugars:starch in leaves (27.9%) and at the whole plant level (8.1%), proline accumulation in leaves and roots (2.6% and 12.1%, respectively), and accumulation of phenols (1.9%), ortho-phenols (2.8%) and flavonoids (3.1%) in roots (Figure 4A). After recovery, these differences mostly disappeared, and groups of plants clustered close to each other (Figure 4B). Correlation analysis between PCA variables revealed a biologically interesting correlation between proline content in leaves and soluble sugars in leaves and stems (Figure 5). This correlation changed from negative under control conditions to positive under heat stress conditions (Figure 5).

### *Heat stress response of C. sativa depends on climate origin*

During the heat phase, no interpopulation differences were observed in external symptoms and foliar damage. However, seedlings from the northern population were the most adversely affected by heat stress in terms of biomass reduction in leaves, roots and at the whole plant level (Supplementary Table 3). Biomass of seedlings of southern origin was unaltered during and after heat stress (Supplementary Table 3). At recovery, no differences in biomass between heat-stressed and control seedlings were found for any population (Supplementary Table 3). Among leaf gas exchange parameters, E showed the highest interpopulation variability in response to heat stress (Supplementary Figure 2), increasing ca. 50 and 25% in the southern and central populations and decreasing ca. 25% in the northern population compared to controls (Supplementary Figure 2).

Interpopulation differences in primary metabolism were mostly observed in leaves and stems (Figure 6 and Supplementary Table 3). Leaf starch and soluble sugars:starch ratio were the parameters with the greatest interpopulation variability, each contributing approximately 45% to the separation between populations in the



PCA (Supplementary Figure 3A). The southern population was the most reactive population to heat stress, reducing starch concentration in leaves by almost 100% and markedly increasing the soluble sugars:starch ratio relative to the control (Figure 6A and Supplementary Table 3). Variations in leaves for starch and the soluble sugars:starch ratio of seedlings from the northern population were low (Supplementary Table 3). In the stem, starch and NSC content decreased in the northern and central populations but increased in the southern population. Soluble sugar changes were similar in the northern and southern populations (Figure 6B).

Central and southern heat-stressed seedlings significantly increased their N content in leaves and stems compared to control seedlings (Supplementary Table 3). Heat-stressed seedlings from the northern population had a four-fold increase in leaf proline content, while no change was observed in the proline content of seedlings from the central and southern populations (Supplementary Table 3). After recovery, only southern seedlings had lower total starch and NSC content in leaves compared to the control (Supplementary Table 3). Both N and proline in recovering seedlings returned to control levels, regardless of the population (Supplementary Table 3).

Regarding secondary metabolism compounds, the populations most adapted to heat stress (the meso-Mediterranean central and southern populations) showed the highest variations in total phenylpropanoids and antioxidant capacity in leaves and roots after heat treatment (Figure 7). Non-targeted metabolite profiling in response to heat showed different changes in the contents of the compounds identified depending on the population of origin (Figure 4A and Table 4). Heat-stressed seedlings from the central population showed the greatest alteration of secondary metabolism, with six compounds (hyperoside, rutin, isorhamnetin, astragalin, ellagic acid and ellagic acid acetyl-xyloside) accumulating in leaves (Supplementary Table 5). Seedlings from the southern and northern populations showed an altered metabolomic profile in roots (Supplementary Table 4).

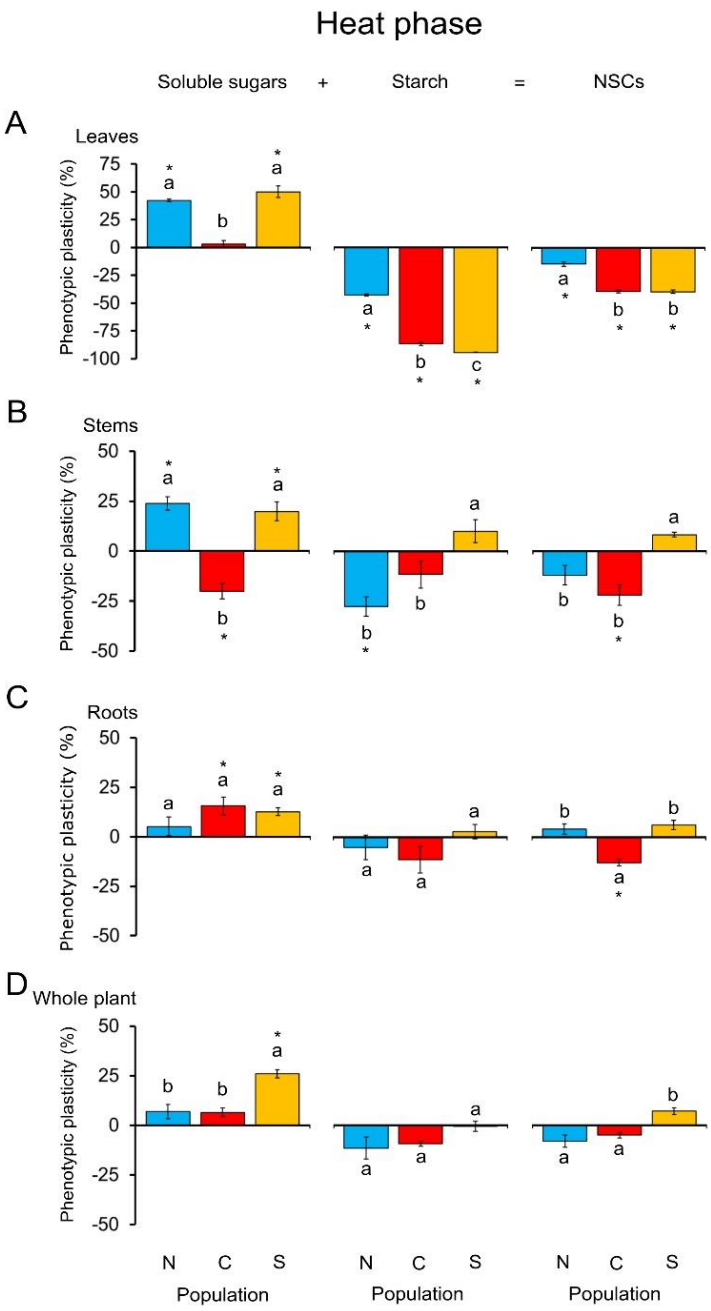



**Figure 6.** Phenotypic plasticity in soluble sugars, starch and total NSCs in leaves (A), stems (B), roots (C) and whole plant (D) of six-month-old *Castanea sativa* seedlings from the northern, central and southern populations in Spain (N, C and S, respectively) after they were subjected to heat stress for seven days. Heat treatment effects are shown in percentages relative to the values obtained for control plants. Vertical lines indicate standard errors of the mean (n=6). Asterisks indicate significant differences from the control treatment (Tukey's HSD test, $P < 0.05$). Different letters indicate different significant effects between populations within each variable (Tukey's HSD test, $P < 0.05$).

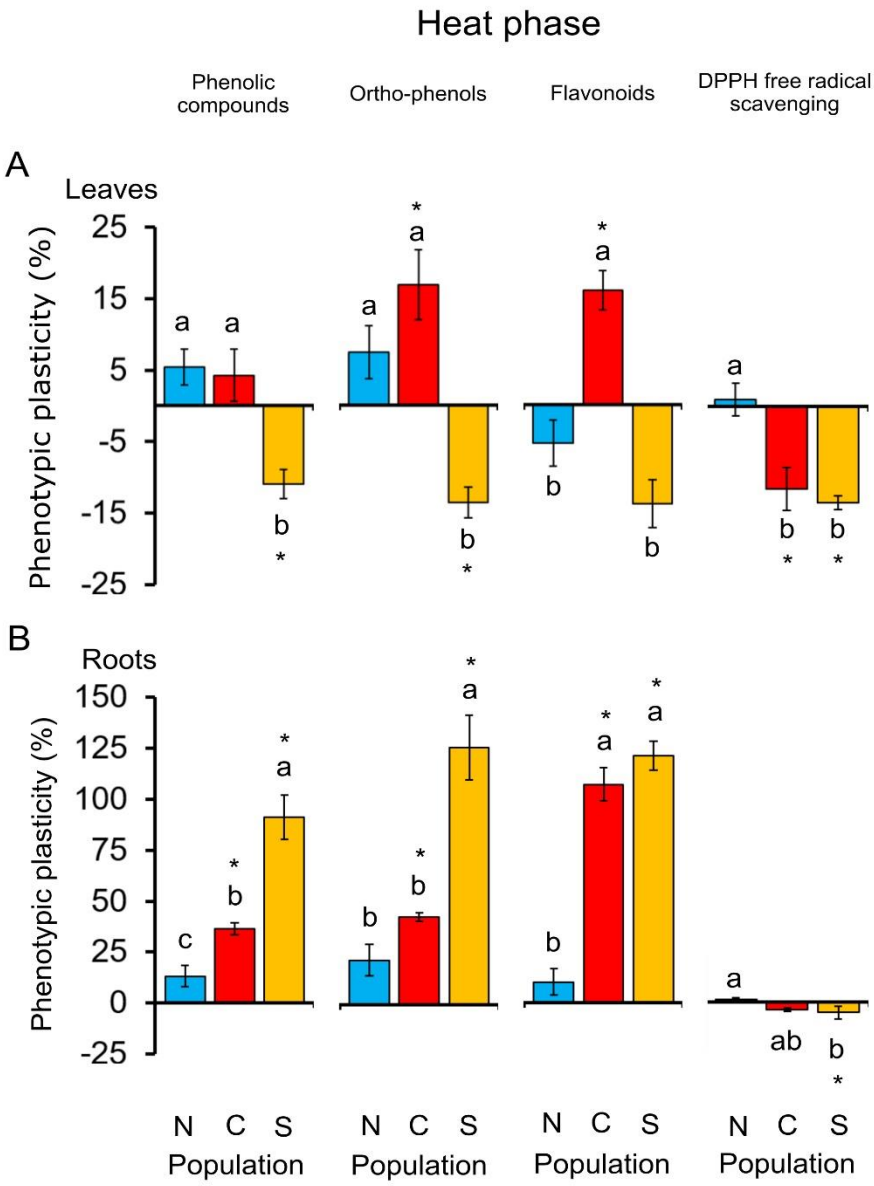



**Figure 7.** Phenotypic plasticity in total phenolic compounds and ortho-phenolic and flavonoid compounds, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging in leaves (A) and roots (B) of six-month-old *Castanea sativa* seedlings from the northern, central and southern populations in Spain (N, C and S, respectively) after they were subjected to heat stress for seven days. Heat treatment effects are shown in percentages relative to the values obtained for control plants. Vertical lines indicate standard errors of the mean (n=6). Asterisks indicate significant differences from the control treatment (Tukey's HSD test, $P < 0.05$). Different letters indicate different significant effects between populations within each variable (Tukey's HSD test, $P < 0.05$).

DFA of the recovery phase showed different metabolite profiles depending on the seedling origin and pretreatment (Figure 3B). PCA analysis also showed a differential interpopulation response during the heat phase (Figure 4A). The greatest response to heat stress by PCA analysis was in seedlings from the southern population, associated with higher starch depletion and a higher soluble sugars:starch ratio in leaves and at the whole plant level, and higher accumulation of proline, total phenolic compounds and ortho-phenolic and flavonoid compounds in roots (Figure 4A). After recovery, only seedlings from the heat-stressed southern population were separated from their respective controls, mainly due to starch depletion in leaves (Figure 4B).

Genetic variation in phenotypic plasticity of *C. sativa* in response to heat stress was observed for ten physiological and biochemical parameters (Figure 8). The southern population was not responsive in terms of biomass in comparison to the northern and central populations. However, the southern population was the most responsive in terms of soluble sugars:starch ratios and N content (Figure 8). NSC always declined for the central population, but behaved differently for the other populations, being the parameter showing the highest genetic variation in phenotypic plasticity (Figure 8).

## DISCUSSION

For the first time in chestnut, the impact of heat stress and recovery effects on the physiology and biochemistry of seedlings was assessed. Phenotypic plastic responses in plant material from different climatic regions were quantified, and genetic variation in phenotypic plasticity in response to heat stress was observed.

### *Heat stress impact on C. sativa*

Observations reported here in *C. sativa* seedlings subjected to 42.5 °C for seven days confirm that heat stress caused leaf and branch scorch, leaf senescence and abscission, and inhibition of shoot and root growth (Wahid et al., 2007).

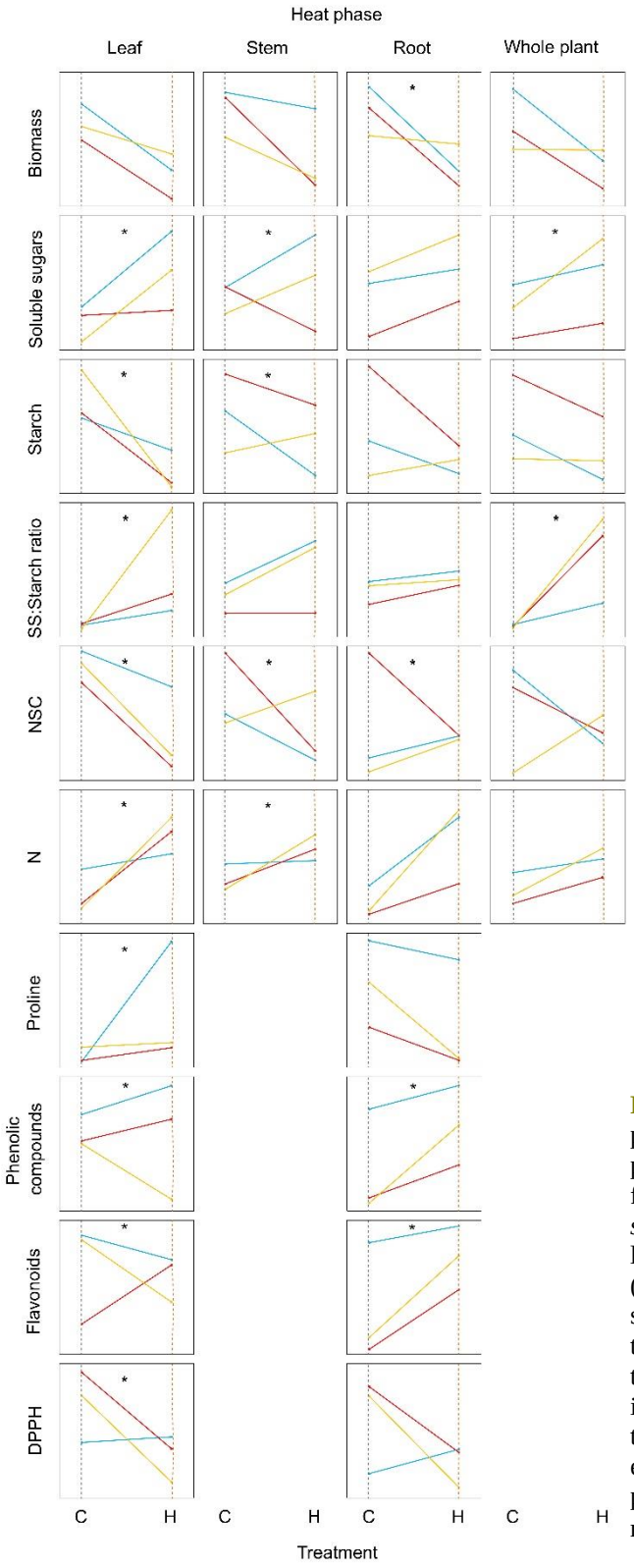



**Figure 8.** Phenotypic values of ten physiological and biochemical parameters obtained from the leaf, stem, fine root and whole plant of *Castanea sativa* seedlings from the northern (blue line), central (red line) and southern (yellow line) populations in Spain after seedlings were subjected to ambient temperature (C) and heat stress (H) treatments for seven days. Asterisks indicate significant population × treatment interactions, and provide evidence for genetic variation in phenotypic plasticity of parameters in response to heat stress.

However, differentiation across populations in genetic variation of biomass was minimal. Leaf gas exchange parameters were different on the first and seventh days after heat stress. During the first episode of temperature increase, seedlings decreased net photosynthetic ratio, but after a heat wave (seven days of heat stress), this parameter did not alter, agreeing with results obtained by Ameye et al. (2012) in *Quercus rubra*. This suggests that in chestnut seedlings Pn is more sensitive to daily temperatures than to prolonged heat exposure and could also indicate plant adaptation to elevated temperatures.

Heat stress induced considerable changes in compounds derived from primary and secondary metabolisms. NSC was especially plastic above ground, and at the whole plant level, NSC decreased in response to heat stress except in the southern population. The NSC reserve pool in trees constitutes the primary resource supply for energetic disparities and generally draws down when trees experience abiotic stress (Galiano et al., 2011; Wiley and Helliker, 2012; Hartmann et al., 2013; Dietze et al., 2014; O'Brien et al., 2014). The high genetic variation in phenotypic plasticity of NSC (Figure 8) is in accordance with a recent study performed in *Populus trichocarpa* trees (Blumstein and Hopkins, 2021). This genetic variation in heat response could provide a key mechanism through which populations can evolve a more adaptive response to future global warming.

NSCs act as protective molecules against heat stress (Wani & Kumar, 2020). Soluble sugars stabilise proteins and cell membranes, maintain cell turgor, participate in stress signalling pathways, and scavenge excess ROS (Bhattacharya & Kundu, 2020), and starch degrades to aid plant fitness and survival under harsh growing conditions (Thalmann & Santelia, 2017), e.g. a rapid degradation of starch generates organic acids and sugars that maintain stomatal pore guard cell turgor and promote stomatal opening (Horrer et al., 2016). In response to heat stress, glucose and fructose increase has been reported in conifer needles (Marias et al., 2017). Rapid starch degradation and soluble sugars accumulation has been reported in

*Populus tremula* leaves at elevated temperatures as a strategy of trees to protect respiratory membranes and buffer consumption in respiratory processes (Hüve et al., 2012). In a drought-tolerant *Eucalyptus globulus* clone subjected to heat stress, a decrease in leaf starch content and an increase in soluble sugars (mannitol, sorbitol, inositol and several amino acids) were observed (Correia et al., 2018). In our heat-stressed chestnuts, plastic variation in the amount of NSCs also had a genetic component.

A positive phenotypic correlation was observed between soluble sugar content and proline content in leaves. Proline protects the structure of enzymes and proteins by maintaining membrane integrity and eliminating ROS (Hayat et al., 2012). Proline and soluble sugar signalling pathways interact synergistically, forming part of the plant antioxidant system under stress situations (Moustakas et al., 2011). The increase in soluble sugars, N, proline and antioxidant capacity observed here in leaves, together with the absence of changes in phenolic compounds in leaves, suggests chestnut trees use carbohydrates as antioxidant compounds. In addition, the decrease in starch, increase in soluble sugars, and unaltered stomatal conductance between treatments suggest chestnut trees use sugars to keep stomata open despite heat stress.

In relation to the secondary metabolism, an accumulation of total phenolic compounds and ortho-phenolic and flavonoid compounds was observed below ground in chestnut seedlings. We identified and quantified 15 phenolic compounds, some of which (ellagic acid, 4-hydroxyphenylacetic acid, hydroxybenzoic acid, quercetin 3-O-galactoside, kaempferol 3-O-(6"acetyl) glucoside 7-O-rhamnoside, tyrosol, and 4-hydroxy-3-methoxycinnamaldehyde) are reported here for the first time in chestnut roots. Moreover, hydroxybenzoic acid, kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside and hyperoside in roots were significantly involved in discriminating between heat-stressed and non-stressed seedlings. Accumulation of phenolic compounds in roots has been associated with plant root

biomass loss (Rhodes et al., 1987), as occurred here. Derivatives of two compounds involved in the DFA separation between treatments (kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside and hyperoside) accumulated in roots and were associated with inhibition of polar auxin transport (PAT) in roots (Yin et al., 2014). Phenolic accumulation, especially of compounds with antioxidant functions, has been reported as frequent when applying abiotic stress and may occur as an adaptive mechanism (Rivero et al., 2001; Zandalinas et al., 2017; Bhattacharya and Kundu, 2020; Carneiro-Carvalho et al., 2021).

Regarding the recovery of chestnut seedlings from heat stress, most parameters returned to initial levels, agreeing with observations in other forest species (Correia et al., 2018; Escandón et al., 2016). However, leaf soluble sugar, starch and flavonoid content, and leaf antioxidant capacity remained altered. The durable accumulation of soluble sugars, flavonoids, and antioxidant capacity concur with the role of these compounds in tolerance, adaptation and recovery from abiotic stress (Bhattacharya & Kundu, 2020; B. Singh et al., 2017). Previous studies in chestnut reported durable accumulation of carbohydrates in response to water stress (A. Camisón et al., 2020).

### *Interpopulation variability of C. sativa in response to heat stress*

Physiological and biochemical heat stress responses of trees can vary depending on the climate of origin, and different populations can show varied responses, from high tolerance in warm climates to low tolerance in cold climates (Marias et al., 2017). Knowledge about phenotypic plastic responses and their genetic control is necessary to forecast the full potential of a species to adapt and/or evolve to changing conditions (Zas et al., 2020; Blumstein and Hopkins, 2021). Likewise, because of the increasing impact of global warming on plants, there is increasing interest in quantifying phenotypic plasticity and local adaptation (i.e., adaptive genetic changes) to heat and drought stress (Castellana et al., 2021). We found interpopulation differences in chestnut seedlings in morphological,

physiological and biochemical parameters in response to heat stress. Seedlings whose mother trees originated from less exposed heat conditions in their area of origin, i.e., from the northern population, were the most adversely affected by heat in terms of biomass reduction. During the first hours of heat, plants from the supra-Mediterranean (northern) population increased their transpiration rates exponentially compared to seedlings from the meso-Mediterranean (central and southern) populations. We hypothesise that seedlings from the northern population use immediate transpiration as a strategy to cool leaves and avoid heat stress consequences, as observed in *Q. rubra* (Ameye et al., 2012) and *Acer rubrum* (Weston & Bauerle, 2007). In the northern population, the decrease in Pn while $g_s$ remained unchanged suggests an alteration of the photosynthetic capacity of the mesophyll (Camejo et al., 2005). Decreased Pn due to non-stomatic limitations has been observed in plants not adapted to heat (Camejo et al., 2005). However, during the heat phase, no interpopulation differences were observed in gas exchange parameters. This could suggest that regardless of the population, chestnut is able to adapt its photosynthetic apparatus to sustained heat stress.

Different interpopulation responses to heat stress were detected in the primary metabolism of seedlings. Seedlings from the southern population were the most responsive, followed by those from the central population and, finally, by those from the northern population. Seedlings from the southern population were characterised by almost total starch depletion and an increase in soluble sugars and the soluble sugars:starch ratio and, as discussed above, starch mobilisation and accumulation of soluble sugars are associated with tolerance to heat stress (Xiong et al., 2015). Proline accumulation in leaves of heat-stressed plants occurred only in seedlings from the northern population, and proline was the main variable explaining the separation of populations in the PCA of heat-stressed seedlings (Figure 3A). Low proline accumulation in chestnut has been associated with tolerance to heat stress in plants treated with SiK (Carneiro-Carvalho et al., 2021), and heat-induced proline accumulation in *Arabidopsis* plants has been considered

detrimental and responsible for reduced thermotolerance (Lv et al., 2011). Thus, the accumulation of this osmolyte in *C. sativa* seedlings from the northern population could indicate reduced thermotolerance.

Regarding secondary metabolism, the southern and northern populations also showed the highest and lowest response to heat stress, respectively. The response of seedlings from the southern population was a high accumulation of total phenolic compounds and ortho-phenolic and flavonoid compounds in roots. However, analysis of non-targeted metabolite profiling showed that seedlings from the central population, exposed to maximum temperatures of almost 40 $^{o}$C in the area of origin, experienced the most pronounced changes, comprising an increase in ellagic acid, ellagic acid acetyl-xyloside, derivatives of quercetin, kaempferol-3-O-glucoside and isorhamnetin in leaves. Targeted and non-targeted approaches provided complementary information and should be used together, when possible, to better characterise tree response to heat stress.

Ten days after the heat stress treatment ceased, seedlings from the three populations reached levels of control seedlings in practically all physiological and biochemical parameters. Only those from the southern population remained separated from the control in the PCA analysis due to starch depletion and mobilisation to soluble sugars. In Spain, differences in the pluviometry of chestnut habitats have resulted in differentiation of two *C. sativa* ecotypes with different adaptive responses to drought stress (Alcaide et al., 2019; Míguez-Soto et al., 2019; Míguez-Soto & Fernández-López, 2015). These ecotypes were also differentiated by EST-SSRs associated with heat stress using the same populations of the present study (Dorado et al., 2022). Based on the results obtained here, differences in the thermal regime of chestnut habitats may also have resulted in different ecotypes associated with heat stress tolerance. It should be acknowledged that our results were obtained in seedlings. Since tolerance to environmental stress may change throughout the lifetime of trees (Solla et al., 2005; Niinemets, 2010), variation of

phenotypic plasticity and acclimatization to heat stress may also change depending on the age of trees and forests.

## CONCLUSIONS

Heat stress significantly impacted the primary and secondary metabolism of *C. sativa* seedlings. Changes in the primary metabolism were observed mainly above ground and included depletion of starch and an increase in soluble sugars, N, and proline. Changes in the secondary metabolism were observed below ground and included an increase in total phenolic, ortho-phenolic and flavonoid content. For the first time in chestnut, metabolite profiles in response to heat stress have been analysed, and several compounds, including isorhamnetin in leaves, and hydroxybenzoic acid, kaempferol 3-O-(6''-O-acetyl) glucoside-7-O-rhamnoside in roots, have been identified as relevant for distinguishing between heat-stressed and non-stressed plants. Ten days after the heat stress treatment ceased, the physiology and biochemistry of *C. sativa* recovered, indicating the high heat stress tolerance of European chestnut. This work reveals different patterns of heat stress tolerance depending on the climate conditions in the region of origin of mother trees. The division of *C. sativa* into two ecotypes, previously demonstrated for water stress tolerance, is reinforced here by differences in the physiology and biochemistry of populations in response to heat stress. Phenotypic plasticity in response to heat stress was higher in the meso-Mediterranean than in the supra-Mediterranean populations. Genetic variation in plasticity in several physiological and biochemical parameters in response to heat stress was also observed. If proven to be adaptive, such variation would be an opportunity for *C. sativa* to respond to global warming-mediated selection.

## AUTHOR CONTRIBUTIONS

FJD and AS conceived and planned the experiment. FJD carried out the experiment. FJD and AC carried out the sampling. GP provided the necessary

methods for the determination of proline and total phenolic compounds. PM and SR assisted in sample processing and analysis. NC and JCA performed the determination and quantification of non-targeted metabolite profiles. FJD took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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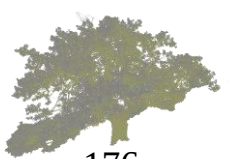

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# Supplementary material

**Supplementary Table 1.** Effect of temperature on net photosynthetic rate ($P_n$), transpiration rate (E), and stomatal conductance ($g_s$) of six-month-old *Castanea sativa* seedlings on day 1 of the assay. Explained variance ($R^2$) and significance (*P*) of the models are shown.

| **Traits** | **Equation** | **R** | ***P* value** |
|---|---|---|---|
| $P_n$ | $P_n = 1527.72 \times e^{(-0.14 \times \text{Temperature})}$ | -0.90 | <0.001 |
| E | $E = 3.03 \times e^{(0.01 \times \text{Temperature})}$ | 0.28 | 0.037 |
| $g_s$ | $g_s = 0.04 \times e^{(0.13 \times \text{Temperature})}$ | -0.59 | <0.001 |

**Supplementary Table 2.** Changes in leaves and roots of six-month-old *Castanea sativa* seedlings subjected to seven days of heat stress (n=18; heat phase) and 10 days after application (n=12; recovery phase). Values are mean ± SE, and different letters indicate significant differences within a time point (Tukey's HSD test, $P < 0.05$) between treatments. Asterisks indicate differences between treatments within a time point that are marginally significant (Tukey's HSD test, $P = 0.05 - 0.1$). Significant responses of a population to heat treatment relative to its own control for heat and recovery phase are highlighted in bold. Red indicates accumulation, and blue indicates a decrease compared to controls.

| Traits | Organ | Sampling time | | | |
|---|---|---|---|---|---|
| | | Heat phase | | Recovery phase | |
| | | Control | Heat | Control | Heat recovery |
| *Pn* (µmol $CO_2$ $m^{-2}$ $s^{-1}$) | Leaf | 5.8 ± 0.4a | 6.1 ± 0.5a | 10.9 ± 0.9a | 10.0 ± 1.0a |
| *E* (mmol $H_2O$ $m^{-2}$ $s^{-1}$) | Leaf | 3.2 ± 0.3a | 3.6 ± 0.2a | 2.8 ± 0.4a | 3.6 ± 0.4a |
| $g_s$ (mol $H_2O$ $m^{-2}$ $s^{-1}$) | Leaf | 0.1 ± 0.0a | 0.1 ± 0.0a | 0.2 ± 0.0a | 0.2 ± 0.0a |
| *Pn/E* | Leaf | **2.5 ± 0.2a** | **1.9 ± 0.1b** | **5.1 ± 0.7a** | **3.0 ± 0.4b** |
| *Pn/ $g_s$* | Leaf | **79.1 ± 5.9a** | **59.4 ± 4.5b** | 109.9 ± 22.2a | 72.4 ± 9.6a |
| Biomass (g) | Leaf | **1.6 ± 0.0a** | **1.2 ± 0.1b** | 1.1 ± 0.1a | 1.0 ± 0.1a |
| | Stem | 1.4 ± 0.1a | 1.2 ± 0.1a | 1.9 ± 0.2a | 1.4 ± 0.2a |
| | Root | **3.3 ± 0.1a** | **2.2 ± 0.2b** | 3.0 ± 0.2a | 2.9 ± 0.4a |
| Total biomass | - | **6.0 ± 0.3a** | **4.7 ± 0.4b** | 6.0 ± 0.4a | 5.3 ± 0.6a |
| Soluble sugars (% dry matter) | Leaf | **5.7 ± 0.2a** | **7.2 ± 0.4b** | **6.3 ± 0.2a** | **7.1 ± 0.3b** |
| | Stem | 4.5 ± 0.1a | 5.2 ± 0.3a (*) | **4.1 ± 0.2a** | **5.4 ± 0.2b** |
| | Root | 6.4 ± 0.2a | 6.5 ± 0.3a | 5.8 ± 0.2a | 5.3 ± 0.2a |
| Whole plant | - | 5.8 ± 0.1a | 6.2 ± 0.2a (*) | 5.3 ± 0.1a | 5.7 ± 0.2a |

**Supplementary Table 2.** Continued.

| Traits | Organ | Sampling time | | | |
|---|---|---|---|---|---|
| | | Heat phase | | Recovery phase | |
| | | Control | Heat | Control | Heat recovery |
| Starch (% dry matter) | Leaf | **5.0 ± 0.6a** | **1.5 ± 0.3b** | **6.6 ± 0.4a** | **4.0 ± 0.7b** |
| | Stem | **6.9 ± 0.3a** | **5.3 ± 0.5b** | 5.9 ± 0.8a | 5.6 ± 0.6a |
| | Root | 21.3 ± 0.8a | 20.5 ± 0.6a | 21.2 ± 0.9a | 21.4 ± 1.0a |
| | Whole plant | 13.7 ± 0.6a | 12.8 ± 0.5a | 13.8 ± 0.9a | 13.8 ± 0.9a |
| NSC (% dry matter) | Leaf | **10.7 ± 0.6a** | **8.3 ± 0.6b** | 12.9 ± 0.5a | 11.2 ± 0.8a |
| | Stem | 11.6 ± 0.4a | 10.2 ± 0.5a (*) | 9.9 ± 0.8a | 11.0 ± 0.5a |
| | Root | 28.2 ± 0.7a | 27.5 ± 0.5a | 26.9 ± 0.8a | 26.9 ± 0.9a |
| | Whole plant | 19.3 ± 0.6a | 19.2 ± 0.4a | 19.2 ± 0.9a | 19.6 ± 0.8a |
| SS:starch ratio | Leaf | **1.6 ± 0.3a** | **7.1 ± 1.5b** | **1.0 ± 0.1a** | **2.5 ± 0.5b** |
| | Stem | 0.7 ± 0.1a | 1.1 ± 0.3a | 0.8 ± 0.1a | 1.1 ± 0.2a |
| | Root | 0.3 ± 0.0a | 0.4 ± 0.0a | 0.3 ± 0.0a | 0.3 ± 0.0a |
| | Whole plant | **0.7 ± 0.0a** | **1.8 ± 0.3b** | 0.6 ± 0.1a | 0.9 ± 0.2a |

**Supplementary Table 2.** Continued.

| Traits | Organ | Sampling time | | | |
|---|---|---|---|---|---|
| | | Heat phase | | Recovery phase | |
| | | Control | Heat | Control | Heat recovery |
| Nitrogen (% dry matter) | Leaf | **1.2 ± 0.0a** | **1.7 ± 0.0b** | 1.2 ± 0.0a | 1.2 ± 0.1a |
| | Stem | **0.6 ± 0.0a** | **0.7 ± 0.0b** | 0.5 ± 0.0a | 0.5 ± 0.0a |
| | Root | **0.6 ± 0.0a** | **0.8 ± 0.1b** | 0.6 ± 0.0a | 0.6 ± 0.1a |
| Whole plant | - | **0.7 ± 0.0a** | **1.0 ± 0.1b** | 0.7 ± 0.0a | 0.7 ± 0.0a |
| Proline (µg/g FW) | Leaf | **14.7 ± 0.8a** | **25.9 ± 4.1b** | 18.8 ± 1.2a | 21.5 ± 2.0a |
| | Root | 6.5 ± 0.8a | 4.7 ± 0.9a | 27.6 ± 2.7a | 25.4 ± 1.8a |
| Phenolic compounds (mg gallic acid/g FW) | Leaf | 30.4 ± 0.74a | 30.6 ± 0.9a | 33.2 ± 0.9a | 31.6 ± 1.2a |
| | Root | **14.3 ± 1.6a** | **20.0 ± 1.2b** | **26.4 ± 1.2b** | **22.2 ± 0.8a** |
| Ortho-phenols (mg gallic acid/g FW) | Leaf | 63.8 ± 1.9a | 65.9 ± 2.5a | 71.9 ± 2.5a | 69.9 ± 4.2a |
| | Root | **21.8 ± 2.8a** | **34.2 ± 2.6b** | 42.2 ± 1.9a | 37.9 ± 2.2a |
| Flavonoids (mg catechin/g FW) | Leaf | 6.4 ± 0.3a | 6.2 ± 0.2a | **4.4 ± 0.2a** | **5.2 ± 0.3b** |
| | Root | **2.7 ± 0.4a** | **4.4 ± 0.3b** | 3.9 ± 0.2a | 3.6 ± 0.1a |
| DPPH (mg trolox/g FW) | Leaf | **18.9 ± 0.3a** | **17.3 ± 0.3b** | **72.8 ± 0.7a** | **69.4 ± 0.8b** |
| | Root | 18.9 ± 0.2a | 18.4 ± 0.2a | 69.5 ± 1.5a | 67.1 ± 1.4a |

**Supplementary Table 3.** Physiological variables measured in the study at the end of the application of the treatments (n=6; heat phase) and 10 days after application (n=4; recovery phase) in six-month-old *Castanea sativa* seedlings subjected to control and heat treatments. Values are mean ± SE, and different letters indicate significant differences within a time point (Tukey's HSD test, $P < 0.05$) between populations (H-Humid; C-Continental; X-Xeric) and treatments. Significant responses of a population to heat treatment relative to its own control for heat and recovery phase are highlighted in bold. Red indicates accumulation, and blue indicates a decrease compared to controls.

| | | **Sampling time** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Heat phase** | | | | | | **Recovery phase** | | | | | |
| **Traits** | **Organ** | **Control** | | | **Heat** | | | **Control** | | | **Heat recovery** | | |
| | | H | C | X | H | C | X | H | C | X | H | C | X |
| *Pn* (µmol $CO_2$ $m^{-2}$ $s^{-1}$) | Leaf | 6.4 ± 0.4b | 6.9 ± 0.5ab | 4.2 ± 0.5c | 6.7 ± 0.8b | 8.2 ± 0.3a | 4.5 ± 0.4c | 14.5 ± 2.4a | 9.3±0.6b | 11.0±1.0ab | 14.9±0.8a | 8.9±1.2b | 9.9±1.0b |
| *E* (mmol $H_2O$ $m^{-2}$ $s^{-1}$) | Leaf | 4.4 ± 0.5ab | 3.9 ± 0.4ab | 1.8 ± 0.2d | 3.6 ± 0.4bc | 4.7 ± 0.2a | 2.6 ± 0.1cd | 4.1 ± 0.9a | 2.5±0.6a | 3.0±0.4a | 4.6±1.0a | 3.7±0.6a | 3.7±0.7a |
| $g_s$ (mol $H_2O$ $m^{-2}$ $s^{-1}$) | Leaf | 0.13 ± 0.0a | 0.15 ± 0.0a | 0.05 ± 0.0b | 0.14 ± 0.0a | 0.18 ± 0.0a | 0.07 ± 0.0b | 0.3 ± 0.1a | 0.1±0.0a | 0.2±0.0a | 0.3±0.1a | 0.2±0.0a | 0.2±0.0a |
| *Pn/E* | Leaf | **3.0 ± 0.4a** | **2.3 ± 0.1bc** | **2.8 ± 0.2ab** | **2.2 ± 0.1c** | **1.6 ± 0.1d** | **1.5 ± 0.1d** | 3.6 ± 0.2a | 4.8±1.7a | 3.9±0.8a | 4.2±1.6a | 2.5±0.2a | 2.8±0.3a |
| *Pn/* $g_s$ | Leaf | 67.7 ± 7.6ab | **81.9 ± 9.1a** | **81.6 ± 3.3a** | 77.4 ± 6.1a | **56.9 ± 5.5b** | **64.3 ± 5.5ab** | 61.2 ± 15.3a | 104.7±42.5a | 77.4±17.9a | 81.4±.35.5a | 60.6±6.9a | 67.7±10.5a |
| Biomass (g) | Leaf | **1.8 ± 0.0a** | 1.5 ± 0.1abc | 1.6 ± 0.1ab | **1.3 ± 0.3bc** | 1.1 ± 0.1c | 1.4 ± 0.3abc | 1.2 ± 0.2a | 1.0±0.1a | 1.1±0.2a | 1.0±0.2a | 1.1±0.3a | 0.9±0.1a |
| | Stem | 1.5 ± 0.1a | 1.5 ± 0.1a | 1.3 ± 0.1a | 1.4 ± 0.4a | 1.1 ± 0.2a | 1.1 ± 0.2a | 2.3 ± 0.5a | 1.6±0.3a | 1.7±0.2a | 1.5±0.3a | 1.2±0.3a | 1.4±0.4a |
| | Root | **3.7 ± 0.1a** | **3.3 ± 0.0ab** | 2.8 ± 0.2bc | **2.2 ± 0.4cd** | **1.9 ± 0.1d** | 2.7 ± 0.4bc | 3.3 ± 0.4a | 2.8±0.3a | 3.0±0.2a | 2.9±0.5a | 2.5±0.7a | 3.3±0.9a |
| Total biomass | - | **7.0 ± 0.3a** | **5.7 ± 0.3ab** | 5.2 ± 0.3bc | **4.9 ± 1.0bc** | **4.0 ± 0.3c** | 5.2 ± 0.8bc | 6.7 ± 1.0a | 5.4±0.5a | 5.9±0.4a | 5.4±0.9a | 4.9±1.2a | 5.6±1.3a |

**Supplementary Table 3.** Continued.

| Traits | Organ | Sampling time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Heat phase | | | | | | Recovery phase | | | | | |
| | | Control | | | Heat | | | Control | | | Heat recovery | | |
| | | H | C | X | H | C | X | H | C | X | H | C | X |
| Soluble sugars (% dry matter) | Leaf | **5.9 ± 0.1c** | 5.7 ± 0.1c | **4.8 ± 0.3d** | **8.5 ± 0.1a** | 5.8 ± 0.2c | **7.2 ± 0.2b** | 6.5 ±0.2abc | **5.9±0.5c** | 6.5±0.2abc | 7.4±0.2ab | **7.6±0.4a** | 6.4±0.2bc |
| | Stem | **4.9 ± 0.2b** | **4.9 ± 0.2b** | **4.3 ± 0.1c** | **6.1 ± 0.2a** | **3.9 ± 0.2c** | **5.2 ± 0.2b** | **4.7± 0.2b** | **3.5±0.2c** | **4.0±0.4bc** | **5.9±0.3a** | **4.7±0.2b** | **5.6±0.3a** |
| | Root | 7.2 ± 0.2bc | **5.9 ± 0.2d** | **7.5 ± 0.1b** | 7.6 ± 0.3b | **6.8 ± 0.3c** | **8.4 ± 0.1a** | 5.9 ± 0.3a | 5.8±0.3ab | 5.6±0.4ab | 5.8±0.1ab | 5.2±0.2ab | 4.9±0.5b |
| | Whole plant | - | 6.5 ± 0.1b | 5.3 ± 0.1d | **5.9 ± 0.2c** | 6.9 ± 0.2b | 5.6 ± 0.1cd | **7.5 ± 0.1a** | 5.6 ± 0.2ab | 5.1±0.1b | 5.3±0.2b | 6.1±0.2a | 5.6±0.2ab | 5.3±0.3b |
| Starch (% dry matter) | Leaf | **4.5 ± 0.3b** | **4.8 ± 0.5b** | **7.3 ± 0.7a** | **2.6 ± 0.0c** | **0.6 ± 0.1d** | **0.4 ± 0.0d** | 6.0 ± 0.9abc | 6.5±1.0ab | **7.2±0.2a** | 4.0±1.5bc | 4.8±1.4abc | **3.3±0.8c** |
| | Stem | **6.9 ± 0.2b** | 8.1 ± 0.2a | 5.7 ± 0.2cd | **5.0 ± 0.3d** | 7.1 ± 0.5ab | 6.3 ± 0.3bc | 5.6 ± 1.1a | 5.5±1.0a | 6.5±2.0a | 4.0±0.7a | 7.2±1.0a | 5.7±0.7a |
| | Root | 20.8 ± 0.9ab | 23.2 ± 1.5a | 19.6 ± 0.9b | 19.7 ± 1.3b | 20.6 ± 1.6ab | 20.1 ± 0.7ab | 19.3± 2.0a | 22.4±1.4a | 21.8±1.2a | 20.2±1.1a | 22.4±2.3a | 21.8±1.5a |
| | Whole plant | - | 13.0 ± 0.7ab | 15.0 ± 1.3a | 12.2 ± 0.7b | 11.5 ± 0.7b | 13.6 ± 0.2ab | 12.1 ± 0.3b | 12.6 ± 1.9a | 14.4±1.6a | 14.5±1.4a | 12.6±0.5a | 14.2±1.9a | 14.9±1.4a |
| NSC (% dry matter) | Leaf | **12.7 ± 0.5a** | **11.1 ± 0.2b** | **12.1 ± 1.0ab** | **10.9 ± 0.3b** | **6.7 ± 0.1c** | **7.3 ± 0.2c** | 12.5 ± 1.1ab | 12.5±1.2ab | **13.7±0.3a** | 11.4±1.9ab | 12.3±1.6ab | **9.7±0.7b** |
| | Stem | 11.5 ± 0.6bc | **13.3 ± 0.5a** | 11.2 ± 0.7bc | 10.1 ± 0.6c | **10.4 ± 0.7c** | 12.2 ± 0.1ab | 10.3 ± 1.0a | 9.0±1.1a | 10.5±2.3a | 9.9±0.6a | 11.9±1.1a | 11.3±0.9a |
| | Root | 27.7 ± 1.0b | **33.1 ± 1.1a** | 27.0 ± 0.8b | 28.8 ± 0.7b | **28.9 ± 0.5b** | 28.6 ± 0.6b | 25.2 ± 1.8a | 28.2±1.1a | 27.4±1.0a | 26.0±0.9a | 27.6±2.1a | 27.1±1.4a |
| | Whole plant | - | 20.5 ± 1.0a | 20.1 ± 1.0ab | 18.2 ± 0.6b | 18.8 ± 0.6ab | 19.1 ± 0.3ab | 19.5 ± 0.3ab | 18.2 ± 1.8a | 19.5±1.6a | 19.8±1.3a | 18.7±0.5a | 19.8±1.8a | 20.5±1.4a |

**Supplementary Table 3.** Continued.

| Traits | Organ | Sampling time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Heat phase | | | | | | Recovery phase | | | | | |
| | | Control | | | Heat | | | Control | | | Heat recovery | | |
| | | H | C | X | H | C | X | H | C | X | H | C | X |
| SS:starch ratio | Leaf | **1.8 ± 0.1a** | **1.4 ± 0.2a** | **0.7 ± 0.0a** | **3.2 ± 0.1c** | **5.6 ± 0.4b** | **17.4 ± 1.3a** | 1.1 ± 0.1a | 1.0±0.2a | 0.9±0.0a | 2.9±1.1a | 2.1±0.6a | 2.6±1.0a |
| | Stem | **0.7 ± 0.0b** | 0.6 ± 0.0b | **0.7 ± 0.1b** | **0.9 ± 0.0a** | 0.6 ± 0.0b | **0.8 ± 0.1a** | **1.0 ± 0.2b** | 0.7±0.2b | 0.8±0.2b | **1.7±0.4a** | 0.7±0.1b | 1.0±0.1b |
| | Root | **0.4 ± 0.0b** | **0.2 ± 0.0c** | 0.4 ± 0.0b | **0.5 ± 0.0a** | **0.4 ± 0.0b** | 0.4 ± 0.0ab | 0.3 ± 0.1a | 0.3±0.0a | 0.3±0.0a | 0.3±0.0a | 0.2±0.0a | 0.2±0.0a |
| Whole plant | - | 0.8 ± 0.0b | **0.7 ± 0.1b** | **0.7 ± 0.1b** | 1.3 ± 0.1b | **2.8 ± 0.2a** | **3.1 ± 1.0a** | 0.7 ± 0.1ab | 0.6±0.1b | 0.5±0.1b | 1.2±0.4a | 0.8±0.2ab | 0.7±0.1ab |
| Nitrogen (% dry matter) | Leaf | 1.4 ± 0.0c | **1.2 ± 0.0d** | **1.1 ± 0.0d** | 1.5 ± 0.1bc | **1.7 ± 0.0ab** | **1.8 ± 0.1a** | 1.3 ± 0.0a | 1.2±0.0a | 1.2±0.1a | 1.3±0.0a | 1.2±0.1a | 1.2±0.0a |
| | Stem | 0.6 ± 0.0bc | **0.5 ± 0.0cd** | **0.5 ± 0.0d** | 0.6 ± 0.1bc | **0.7 ± 0.0ab** | **0.7 ± 0.0a** | 0.5 ± 0.0abc | **0.4±0.0d** | 0.5±0.0ab | 0.5±0.0bc | **0.5±0.0c** | 0.6±0.0a |
| | Root | **0.6 ± 0.0b** | 0.5 ± 0.0b | **0.5 ± 0.0b** | **0.9 ± 0.1a** | 0.6 ± 0.0b | **0.9 ± 0.1a** | 0.6 ± 0.0a | 0.5±0.0a | 0.6±0.0a | 0.7±0.0a | 0.6±0.1a | 0.6±0.1a |
| Whole plant | - | 0.9 ± 0.0b | **0.7 ± 0.0d** | **0.7 ± 0.0cd** | 1.0 ± 0.1ab | **0.8 ± 0.0bc** | **1.1 ± 0.1a** | 0.7 ±0.0a | 0.6±0.0a | 0.7±0.0a | 0.7±0.0a | 0.7±0.1a | 0.7±0.1a |
| Proline (µg/g FW) | Leaf | **12.8 ± 0.2b** | 13.1 ± 0.4b | 17.2 ± 1.6b | **49.4 ± 6.4a** | 17.0 ± 1.3b | 18.6 ± 0.7b | 20.2 ± 1.0ab | 15.4±1.2b | 20.7±2.6ab | 25.4±5.1a | 17.9±1.1ab | 22.1±1.1ab |
| | Root | 8.7 ± 1.3a | 4.8 ± 1.1bc | **6.8 ± 0.4ab** | 7.8 ± 1.6ab | 3.3 ± 0.9c | **3.4 ± 1.0c** | **31.8 ±1.7a** | 28.2±5.7ab | 22.6±2.6ab | **22.0±1.0b** | 26.5±3.5ab | 27.7±1.7ab |
| Phenolic compounds (mg gallic acid/g FW) | Leaf | 31.7 ± 1.3ab | 30.2 ± 1.3b | **30.0 ±0.6b** | 33.5 ± 0.8a | 31.5 ± 1.1ab | **26.7 ± 0.6c** | 34.9 ± 1.7ab | **31.9±0.6bc** | 32.6±1.3abc | 35.4±0.9a | **27.5±1.0d** | 30.5±0.1cd |
| | Root | **22.0 ± 1.3b** | **11.1 ± 0.4d** | **10.5 ± 0.1d** | **24.9 ± 1.2a** | **15.2 ± 0.3c** | **20.0 ± 1.1b** | **27.6 ± 1.0a** | 22.3±1.7bc | **29.4±1.3a** | **23.9±0.9b** | 22.6±0.9bc | **19.6±1.0c** |

**Supplementary Table 3.** Continued.

| Traits | Organ | Sampling time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Heat phase | | | | | | Recovery phase | | | | | |
| | | Control | | | Heat | | | Control | | | Heat recovery | | |
| | | H | C | X | H | C | X | H | C | X | H | C | X |
| Ortho-phenols (mg gallic acid/g FW) | Leaf | 69.3 ± 2.5ab | **58.3 ± 1.4cd** | **63.0 ± 2.8bc** | 74.4 ± 2.6a | **68.2 ± 2.9ab** | **54.4 ± 1.4d** | **74.5 ± 5.1b** | **70.3 ± 2.5bc** | 70.6 ± 4.5bc | **85.9 ± 2.0a** | **60.8 ± 1.6d** | 62.9 ± 1.5cd |
| | Root | **36.5 ± 2.6b** | **17.0 ± 0.3d** | **14.9 ± 0.3d** | **44.5 ± 2.9a** | **24.3 ± 0.4c** | **33.7 ± 2.4b** | 45.3 ± 1.9a | 35.6 ± 3.2b | **45.7 ± 2.4a** | 44.8 ± 2.6a | 36.8 ± 1.9b | **30.0 ± 1.1b** |
| Flavonoids (mg catechin/g FW) | Leaf | 6.8 ± 0.6a | 5.5 ± 0.2b | 6.7 ± 0.4a | 6.4 ± 0.2ab | 6.3 ± 0.2ab | 5.8 ± 0.2ab | 4.9 ± 0.2b | 3.9 ± 0.1c | **4.5 ± 0.3bc** | 5.1 ± 0.2b | 4.7 ± 0.6bc | **6.0 ± 0.1a** |
| | Root | **4.8 ± 0.1b** | **1.7 ± 0.1d** | **2.0 ± 0.0d** | **5.3 ± 0.3a** | **3.4 ± 0.1c** | **4.4 ± 0.1b** | 4.1 ± 0.2ab | **3.1 ± 0.2d** | **4.5 ± 0.2a** | 3.7 ± 0.1bc | **3.8 ± 0.1b** | **3.2 ± 0.2cd** |
| DPPH (mg trolox/g FW) | Leaf | 17.7 ± 0.3b | **19.7 ± 0.3a** | **19.0 ± 0.3a** | 17.8 ± 0.4b | **17.5 ± 0.6bc** | **16.5 ± 0.2c** | 71.0 ± 1.1ab | **73.5 ± 1.2a** | 73.9 ± 1.1a | 69.3 ± 0.4b | **68.6 ± 2.2b** | 70.3 ± 0.8ab |
| | Root | 18.3 ± 0.3bc | 19.2 ± 0.1a | **19.1 ± 0.2ab** | 18.5 ± 0.1abc | 18.5 ± 0.2abc | **18.1 ± 0.6c** | 70.2 ± 2.1a | 68.0 ± 3.6a | 70.2 ± 2.8a | 64.9 ± 1.6a | 68.1 ± 3.6a | 68.3 ± 1.9a |

**Supplementary Table 4.** Changes in secondary metabolite-derived compounds in leaves and roots of six-month-old *Castanea sativa* seedlings subjected to seven days of heat stress (n=18; heat phase) and 10 days after application (n=12; recovery phase). Values are mean ± SE, and different letters indicate significant differences within a time point (Tukey's HSD test, $P < 0.05$) between treatments. Asterisks indicate differences between treatments within a time point that are marginally significant (Tukey's HSD test, $P = 0.05 - 0.1$). Significant responses of a population to heat treatment relative to its own control for heat and recovery phase are highlighted in bold. Red indicates accumulation, and blue indicates a decrease compared to controls.

| Compound | Organ | Sampling time | | | |
|---|---|---|---|---|---|
| | | Heat phase | | Recovery phase | |
| | | Control | Heat | Control | Heat |
| Ellagic acid (mg ellagic acid/g DW) | Leaf | 1.34 ± 0.18a | 1.69 ± 0.19a | 1.96 ± 0.24a | 1.91 ± 0.20a |
| | Root | **1.07 ± 0.06a** | **1.41 ± 0.06b** | 1.53 ± 0.06a | 1.48 ± 0.12a |
| Hyperoside (quercetin-3-O-galactoside) (mg quercetin/g DW) | Leaf | 0.86 ± 0.18a | 0.96 ± 0.12a | 1.01 ± 0.21a | 0.75 ± 0.15a |
| | Root | **0.19 ± 0.02a** | **0.42 ± 0.03b** | 0.43 ± 0.23a | 0.49 ± 0.03a |
| Lariciresinol (mg gallic acid/g DW) | Leaf | 4.16 ± 0.56a | 4.52 ± 0.51a | 2.79 ± 0.43a | 2.59 ± 0.51a |
| Ellagic acid acetyl-xyloside (mg gallic acid/g DW) | Leaf | 1.51 ± 0.27a | 1.62 ± 0.14a | 1.68 ± 0.33a | 1.22 ± 0.27a |
| Miquelianin (quercetin 3-O-glucuronide)(mg quercetin/g DW) | Leaf | 0.0012 ± 0.0012a | 0.011 ± 0.006a | 0.0128 ± 0.0055a | 0.0426 ± 0.0238a |
| Rutin (quercetin 3-O-rutinoside)(mg rutin/g DW) | Leaf | 0.81 ± 0.09a | 0.86 ± 0.05a | 0.91 ± 0.09a | 0.79 ± 0.11a |
| Astragalin (kaempferol-3-O-glucoside)(mg quercetin/g DW) | Leaf | 2.80 ± 0.54a | 3.18 ± 0.33a | 2.84 ± 0.50a | 1.81 ± 0.29a* |
| Isorhamnetin (mg quercetin/g DW) | Leaf | **0.12 ± 0.01a** | **0.34 ± 0.04b** | 0.38 ± 0.08a | 0.35 ± 0.06a |
| 6´´-O-manolylglycitin (mg quercetin/g DW) | Leaf | 0.07 ± 0.01a | 0.05 ± 0.01a | 0.14 ± 0.07a | 0.04 ± 0.01a |
| Hydroxybenzoic acid (mg gallic acid/g DW) | Root | **0.94 ± 0.09a** | **1.22 ± 0.09b** | **1.21 ± 0.06a** | **0.89 ± 0.08b** |

**Supplementary Table 4.** Continued.

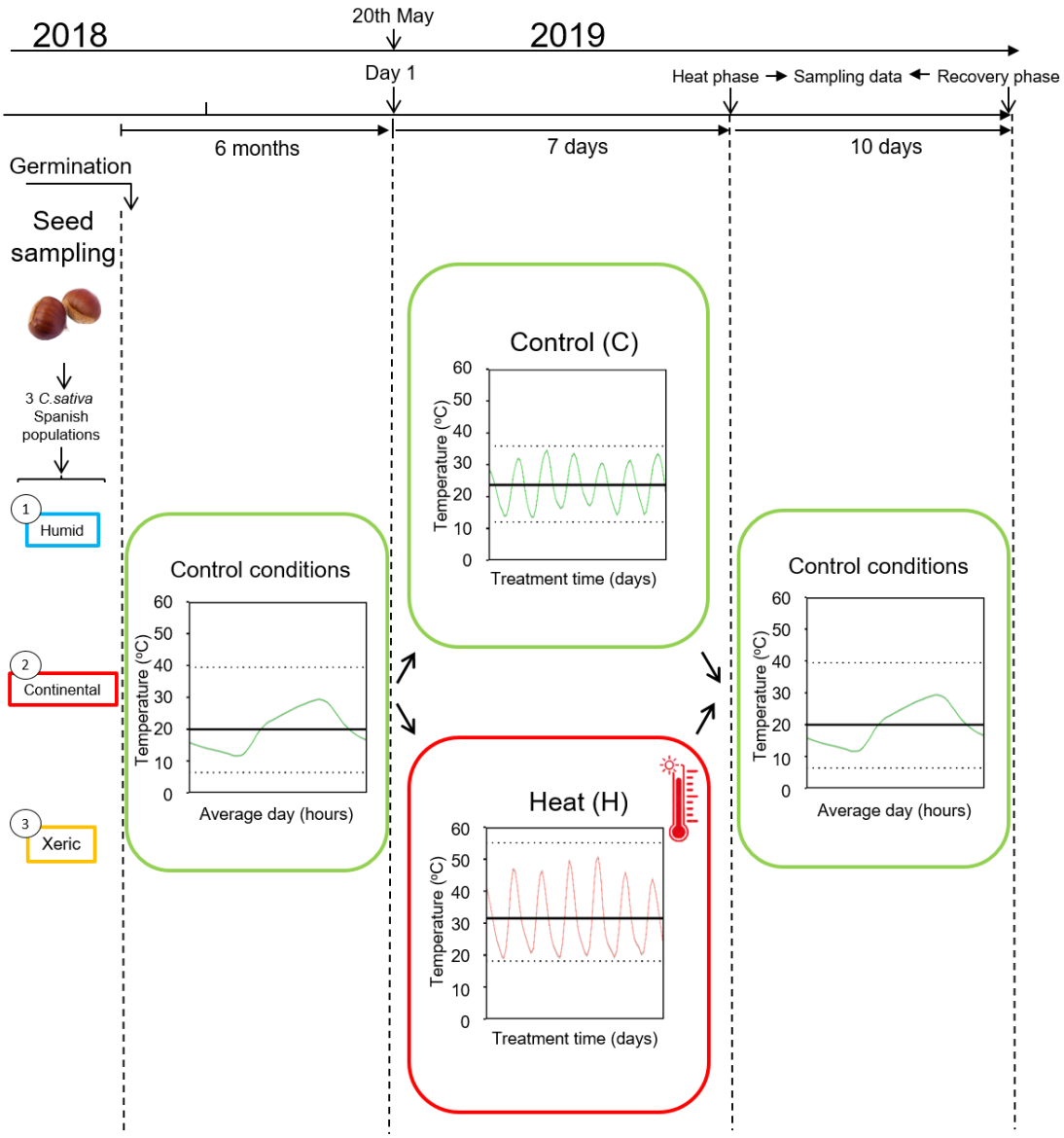
| Compound | Organ | Sampling time | | | |
|---|---|---|---|---|---|
| | | Heat phase | | Recovery phase | |
| | | Control | Heat | Control | Heat |
| 4-hydroxyphenylacetic acid<br><br>(mg gallic acid/g DW) | Root | 0.42 ± 0.10a | 0.47 ± 0.09a | 0.81 ± 0.07a | 0.71 ± 0.09a |
| Kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside<br><br>(mg quercetin/g DW) | Root | **0.06 ± 0.01a** | **0.11 ± 0.01b** | 0.10 ± 0.01a | 0.11 ± 0.01a |
| Coniferyl aldehyde (4-hydroxy-3-metoxycinnamaldehyde)(mg gallic acid/g DW) | Root | **1.10 ± 0.11a** | **2.06 ± 0.19b** | 1.73 ± 0.14a | 0.95 ± 0.12b |

**Supplementary Table 5.** Changes in leaves and roots of secondary metabolite-derived compounds of six-month-old *Castanea sativa* seedlings subjected to seven days of heat stress (n=18; heat phase) and 10 days after application (n=12; recovery phase). Values are mean ± SE, and different letters indicate significant differences within a time point (Tukey's HSD test, P < 0.05) between populations (H-Humid; C-Continental; X-Xeric) and treatments. Significant responses of a population to heat treatment relative to its own control for heat and recovery phase are highlighted in bold. Red indicates accumulation, and blue indicates a decrease compared to controls.

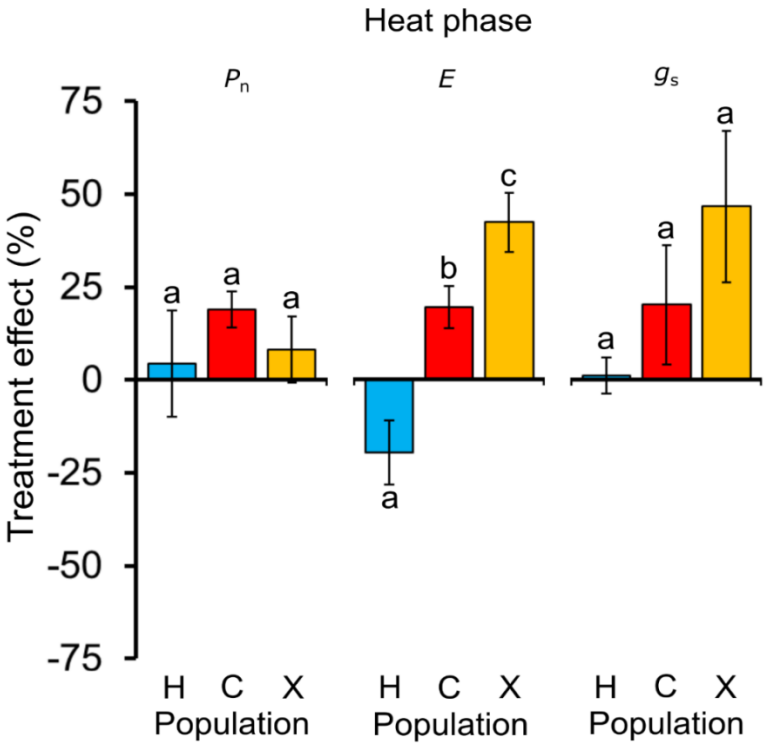
| Compound | Organ | Sampling time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Heat phase | | | | | | Recovery phase | | | | | |
| | | Control | | | Heat | | | Control | | | Heat | | |
| | | H | C | X | H | C | X | H | C | X | H | C | X |
| Ellagic acid(mg ellagic acid/g DW) | Leaf | 1.25 ± 0.13 ab | **0.79 ± 0.21b** | 1.95 ± 0.25a | 1.48 ± 0.11 ab | **2.07 ± 0.38a** | 1.53 ± 0.43ab | 1.20 ± 0.27c | 2.14 ± 0.33ab | **2.53 ± 0.39ab** | 1.77 ± 0.29bc | 2.66 ± 0.12a | **1.31 ± 0.09c** |
| | Root | **1.17 ± 0.16bc** | 0.97 ± 0.10c | **1.12 ± 0.08c** | **1.58 ± 0.11a** | 1.18 ± 0.10bc | **1.45 ± 0.06ab** | 1.44 ± 0.09b | 1.50 ± 0.15ab | 1.65 ± 0.08ab | 1.32 ± 0.16b | 1.26 ± 0.16b | 1.86 ± 0.17a |
| Hyperoside (quercetin-3-O-galactoside) (mg quercetin/g DW) | Leaf | 0.50 ± 0.16 bc | **0.40 ± 0.11c** | 1.57 ± 0.26a | 0.79 ± 0.13bc | **0.99 ± 0.28ab** | 1.09 ± 0.18ab | 0.43 ± 0.12bc | 1.00±0.24abc | 1.59 ± 0.46a | 1.11 ± 0.36ab | 0.29 ± 0.06c | 0.85 ± 0.06abc |
| | Root | **0.27 ± 0.06c** | **0.14 ± 0.02d** | **0.19 ± 0.01d** | **0.53± 0.01a** | **0.29 ± 0.02c** | **0.45 ± 0.02b** | 0.45 ± 0.05abc | 0.38±0.06c | 0.45 ± 0.04abc | 0.52 ± 0.06ab | 0.39 ± 0.03bc | 0.55 ± 0.02a |
| Ellagic acid acetyl-xyloside (mg galic acid/g DW) | Leaf | 0.97 ± 0.29cd | **0.79 ± 0.16d** | 2.59 ± 0.40a | 1.42 ± 0.14bcd | **1.59 ± 0.36bc** | 1.85 ± 0.19ab | 0.76 ± 0.22b | 1.63±0.33ab | 2.64 ± 0.69a | 1.74 ± 0.64ab | 0.48 ± 0.17b | 1.44 ± 0.23ab |
| Lariciresinol (mg gallic acid/g DW) | Leaf | 3.69 ± 0.82a | 4.31 ± 1.12 a | 4.31 ± 0.98a | 5.42 ± 1.07a | 4.12 ± 0.84 a | 4.02 ± 0.77a | 1.89 ± 0.43a | 2.77±0.71a | 3.72 ± 0.87a | 3.51 ± 1.47a | 2.37 ± 0.29a | 1.89 ± 0.45a |
| Miquelianin (quercetin 3-O-glucuronide) (mg quercetin /g DW) | Leaf | 0.0000 ± 0.0000a | 0.0033 ± 0.0033 a | 0.0000±0.0000 a | 0.0171±0.0171 a | 0.01 ±0.001a | 0.0058 ± 0.0037a | 0.0214±0.0124b | **0.0089±0.0089b** | 0.00792±0.00792b | 0.0141±0.00141b | **0.1135±0.0589a** | 0.0000±0.0000b |
| Rutin (quercetin 3-O-rutinoside) (mg rutin /g DW) | Leaf | 0.77 ± 0.11bcd | **0.52 ± 0.09d** | 1.14 ± 0.09a | 0.73±0.07cd | **0.85 ± 0.09bc** | 0.99 ± 0.05ab | 0.68 ± 0.05bc | 0.86±0.09abc | 1.18 ± 0.20a | 0.96 ± 0.23ab | 0.50 ± 0.18c | 0.89 ± 0.08abc |
| Astragalin (kaempferol-3-O-glucoside) (mg quercetin/g DW) | Leaf | 1.67 ± 0.36cd | **1.36 ± 0.34d** | 4.98 ± 0.78a | 2.33±0.62cd | **2.88 ± 0.51bc** | 4.34 ± 0.21ab | 1.39 ± 0.43bc | **2.74±0.56b** | **4.38 ± 0.84a** | 2.34 ± 0.56bc | **0.91 ± 0.39c** | **2.18 ± 0.14bc** |

**Supplementary Table 5.** Continued.

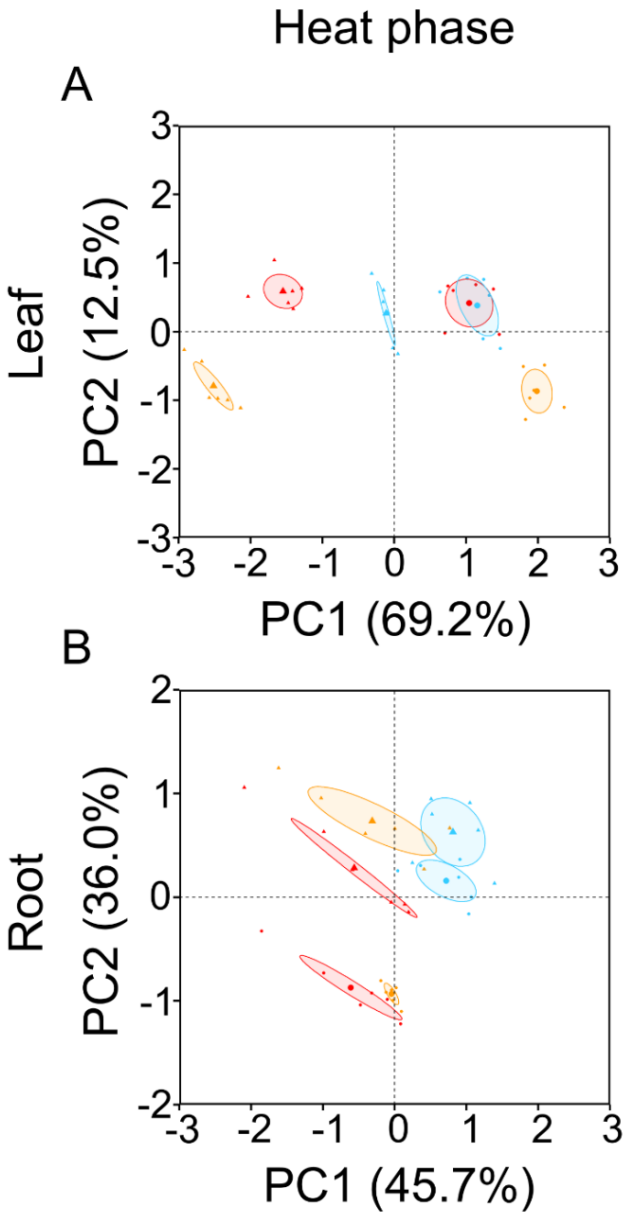
| Compound | Organ | Sampling time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Heat phase | | | | | | Recovery phase | | | | | |
| | | Control | | | Heat | | | Control | | | Heat | | |
| | | H | C | X | H | C | X | H | C | X | H | C | X |
| Isorhamnetin (mg quercetin/g DW) | Leaf | **0.14 ± 0.01cd** | **0.10 ± 0.02d** | 0.12 ± 0.01cd | **0.47±0.08a** | **0.30 ± 0.06b** | 0.24 ± 0.04bc | 0.55 ± 0.20a | 0.29±0.08ab | 0.29 ± 0.09ab | 0.45 ± 0.09ab | 0.39 ± 0.12ab | 0.19 ± 0.04b |
| 6´´-O-manolylglycitin(mg quercetin/g DW) | Leaf | 0.0505 ± 0.0172b | 0.0421 ± 0.0203b | **0.1103 ± 0.0044a** | 0.0494±0.0163 b | 0.0505 ± 0.0159b | **0.0602 ± 0.0195b** | 0.2557±0.1958 a | 0.0447±0.0261 a | 0.1133±0.0031 a | 0.0232±0.0232 a | 0.02712±0.019 1a | 0.0742±0.0021 a |
| Hydroxybenzoic acid (mg gallic acid/g DW) | Root | 1.22 ± 0.20ab | 0.99 ± 0.16bc | 0.72 ± 0.05c | 1.59±0.14a | 1.07 ± 0.09bc | 1.01 ± 0.13bc | 1.19 ± 0.15ab | **1.29 ± 0.05a** | **1.14 ± 0.07ab** | 1.07 ± 0.15abc | **0.87 ± 0.12bc** | **0.75 ± 0.10c** |
| 4-hydroxyphenylacetic acid (mg gallic acid/g DW) | Root | 0.95 ± 0.28a | 0.23 ± 0.06b | 0.26 ± 0.04b | 0.83±0.21a | 0.27 ± 0.03b | 0.32 ± 0.05b | 1.01 ± 0.08a | 0.64 ± 0.11ab | 0.76 ± 0.13ab | 0.82 ± 0.15ab | 0.82 ± 0.21ab | 0.50 ± 0.08b |
| Kaempferol 3-O-(6"-O-acetyl) glucoside-7-O-rhamnoside(mg quercetin/g DW) | Root | **0.074 ± 0.018bc** | 0.049 ± 0.006c | 0.063 ± 0.004bc | **0.14±0.03a** | 0.080 ± 0.007bc | 0.108 ± 0.009ab | 0.11 ± 0.02ab | 0.09 ± 0.01ab | 0.09 ± 0.00ab | 0.07 ± 0.01b | 0.13 ± 0.01a | 0.12 ± 0.01a |
| Coniferyl aldehyde (4-hydroxy-3-metoxycinnamaldehyde)(mg gallic acid/g DW) | Root | 1.38 ± 0.21bcd | 1.11 ± 0.16cd | **0.91 ± 0.19d** | 2.17±0.31ab | 1.71 ± 0.01abc | **2.31 ± 0.40a** | 1.51 ± 0.25abc | **2.12 ± 0.09a** | **1.57 ± 0.28ab** | 1.14 ± 0.21bcd | **0.92 ± 0.16cd** | **0.79 ± 0.09d** |



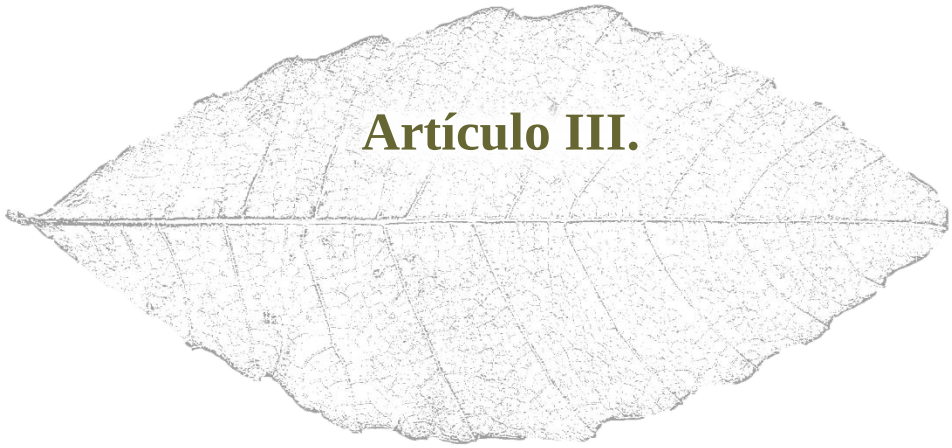

**Supplementary Figure 1.** Experimental design and sampling planning. Solid lines in the temperature graphs refer to the mean temperature, and dashed lines to the maximum and minimum temperatures of each period.




**Supplementary Figure 2.** Changes in net photosynthetic rate ($P_n$), transpiration rate ($E$) and stomatal conductance ($g_s$) of six-month-old *Castanea sativa* seedlings from the humid (H), continental (C) and xeric (X) populations in Spain after they were subjected to heat stress for seven days. Heat treatment effects are shown in percentages relative to the values obtained for control plants. Vertical lines indicate standard errors of the mean (n=6). Asterisks indicate significant differences from the control treatment (Tukey's HSD test, $P < 0.05$). Different letters indicate different significant effects between populations within each variable (Tukey's HSD test, $P < 0.05$).




**Supplementary Figure 3.** PCA of six-month-old *Castanea sativa* seedlings showing differences between control (circles) and heat-stressed (triangles) seedlings during the heat phase in leaves (A) and roots (B) of humid (blue symbols), continental (red symbols) and xeric (yellow symbols) populations according to the physiological variables included in Figure 5. In A, leaves variables were used; in B, root variables were used.



# Artículo III.

# Assessing heat stress tolerance in *Castanea sativa*

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## ABSTRACT

Increasing temperatures and heat waves decimate the productivity and survival of valuable trees like the European chestnut (*Castanea sativa* Miller). The main objectives of this study were to assess variation in heat stress tolerance within and between chestnut populations, select molecular markers associated with heat stress tolerance, and to use the selected markers to explore the adaptive potential of *C. sativa* to tolerate heat stress. Forty-eight trees from three Spanish wild chestnut populations of contrasting climate characteristics were used. Seven out of 20 expressed sequence tag- simple sequence repeat (EST-SSR) markers were selected. To validate the markers, progenies of the 48 trees were subjected to heat stress for 7 days and their heat tolerance was assessed through morphological and physiological changes. Leaf proline content induced by heat was highest in the least thermophilic population. Genetic structure analysis of populations revealed a cluster that included 81 per cent of the seedlings of the most thermophilic population. Signatures of positive selection for heat stress tolerance were detected using *VIT099* and *POR016* markers, associated with the antioxidant response of plants to heat damage. These markers should be included as candidates for their use in early selection of individuals tolerant to heat stress in *C. sativa*. Molecular and physiological findings converged in this study to better understand chestnut adaptation to global warming.

## INTRODUCTION

In recent decades, the Mediterranean basin has been heavily impacted by increasing temperature extremes and heat-waves, and decreasing precipitation (Petit *et al.*, 2005; Lionello and Scarascia, 2020). Mediterranean habitats are considered among the most threatened by the consequences of rising temperatures and aridity, and their survival will largely depend on the capacity of trees to tolerate temperature and precipitation changes (Alberto *et al.*, 2013). In the past decade, forest mortality induced by rising temperatures and increased drought has increased rapidly worldwide (Niu *et al.*, 2014). Trees subjected to high temperatures may have their growth, phenology and physiology altered (Hasanuzzaman *et al.*, 2013). To protect themselves from oxidative stress caused by heat, trees employ enzymatic (e.g. superoxide dismutase and ascorbate peroxidase) and non-enzymatic antioxidant (e.g. flavonoids or proline) tolerance mechanisms (Shivashankara *et al.*, 2016). In particular, the accumulation of osmolytes such as proline in plant tissues has been documented under environmental stress. This metabolite provides stress tolerance by maintaining cell turgor and osmotic balance and preventing electrolyte leakage and oxidative burst in plants (Hayat *et al.*, 2012).

Genomic approaches can be used to detect allelic variants associated with tree adaptation to different environments and identify genes under selection (Cortés *et al.*, 2020; Isabel *et al.*, 2020). Microsatellite markers derived from expressed sequence tags (EST-SSRs) associated with transcribed regions of the genome provide strong evidence of selection that is more informative than genomic SSRs. The non-neutral behaviour of the loci annotated by EST-SSR markers may indicate associated genes for a phenotypic trait involved in adaptation (Cortés *et al.*, 2020). Moreover, detection of outlier loci enables the separation of genome-wide effects caused by demographic processes from locus-specific adaptive effects (Luikart *et al.*, 2003; Alcaide *et al.*, 2019). The increasing availability of gene sequences from many organisms, including trees, is helping to increase the feasibility of association

studies between adaptive genetic diversity and variation in phenotypic traits (Gailing *et al.*, 2009; Mittler *et al.*, 2013). Several studies have associated adaptive genetic diversity with variation in phenotypic traits in relevant tree species of the genera *Pinus*, *Eucalyptus*, *Quercus*, *Alnus* and *Castanea* (Grivet *et al.*, 2011; Bradbury *et al.*, 2013b; Sullivan *et al.*, 2013; De Kort *et al.*, 2014; Alcaide *et al.*, 2019, 2020).

The European chestnut (*Castanea sativa* Mill.) is a highly valued tree species that forms multipurpose ecosystems, often agroforestry systems, covering an area of 2.5 million hectares in Europe (Conedera *et al.*, 2004). It is appreciated for its timber and fruit production, contribution to the landscape and the environment and by-products such as honey and edible mushrooms. *C. sativa* inhabits regions with markedly variable environmental conditions (Pazianoto *et al.*, 2019) and many studies have assessed adaptation and genetically based differentiation of traits related to bud burst, drought and tolerance to *Phytophthora cinnamomi* (Lauteri *et al.*, 1998; Martín *et al.*, 2010; Alcaide *et al.*, 2019, 2020; Camisón *et al.*, 2021; Castellana *et al.*, 2021).

In chestnut, genetic markers have been reported for assisted selection related to global change such as drought (Alcaide *et al.*, 2019) and resistance to *P. cinnamomi* (Santos *et al.*, 2015; Alcaide *et al.*, 2020). However, no markers have been reported to assist selection of chestnut trees for heat stress tolerance. The objectives of the study were to:

i) Explore variation in heat stress tolerance within and between chestnut populations with contrasting climates.
ii) Select EST-SSR markers associated with heat stress tolerance.
iii) Use the selected EST-SSR markers to explore the adaptive potential of *C. sativa* populations to heat stress tolerance.

## MATERIALS AND METHODS


**Figure 1.** *Castanea sativa* adult trees sampled in November 2018 from (a) humid, (b) continental and (c) xeric forests.

### *Plant material*

In November 2018, 48 adult *C. sativa* trees from three wild populations in Spain were sampled (Figure 1). According to the location and contrasting climate characteristics (Figure 2; Table S1), the populations were classified as humid, continental and xeric. Within each chestnut population, 16 trees were selected at least 70 m apart to minimize the chances of sampling intercrossed individuals. Samples of five healthy green leaves per tree were collected. Leaf samples were dried and stored in silica gel at room temperature for genetic analyses.

About 100 seeds from each of the 48 trees were collected by hand and immersed in a fungicide solution (2 g LThiram 80GD, ADAMA Inc., Spain) for 5 min, discarding those that floated as non-viable. Seeds were rinsed with sterilized water and stored at 4°C for 2 weeks.

*Seed germination*

In December 2018, a greenhouse experiment with chestnut seedlings was designed to assess the heat tolerance. The greenhouse was located at the Faculty of Forestry of Plasencia (40°02′ N, 6°04′ W; 374 m a.s.l., Extremadura region, Spain). For this purpose, previously collected and stratified seeds from the three populations were individually weighed and sown at 1 cm depth in 48-cell plastic root trainers with one seed per cell. Individual cells were 330 mL in volume, 18 cm high, 5.3 × 5.3 cm upper surface and contained peat (PKN1 Florava® Peat Substrate, pH 5–6). Plants were arranged following a split-plot random design replicated in three blocks, with the heat treatments acting as the main factor (2 categories: heat-stressed and control; whole plots) and the populations as the split factor (3 categories: humid, continental and xeric, as shown in Figure 1; split plots). In the three blocks, the three populations were represented in each whole plot by five individuals from the 16 open-pollinated families. Individuals were randomly positioned within the blocks. The experiment comprised 1440 plants corresponding to 3 blocks × 2 heating treatments × 3 populations × 16 families × 5 individuals, thus including 480 plants per population and 30 plants per family. Germination was considered successful when the aerial part emerging from the embryo was green. Aerial emergence of plants was assessed weekly. Plants were kept in natural daylight under greenhouse shade that reduced solar radiation by 50 per cent, and hand watered every 4 days to field capacity until they were well established. In April 2019, when the seeds had germinated and the seedlings were about 10–20 cm high, two leaves per seedling were collected, dried and kept in silica gel at room temperature for genetic analyses.

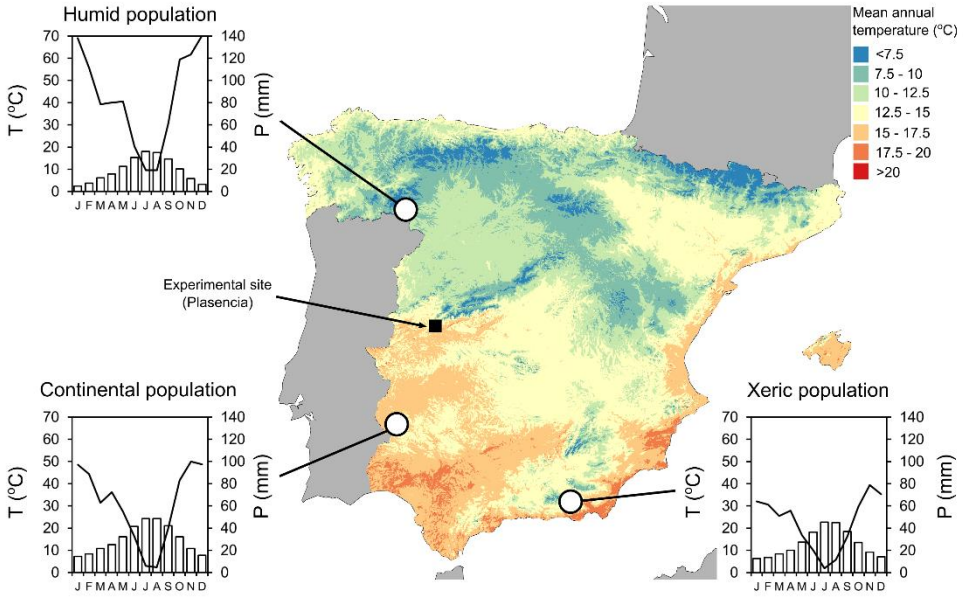



**Figure 2.** Mean annual temperature in Spain 1940–2005 (MAPAMA) and location and climographs of the three *Castanea sativa* populations studied.

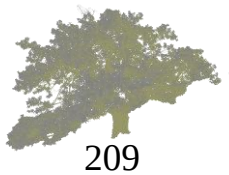
### *Heat tolerance assessment*

The manipulative greenhouse experiment consisted of subjecting seedlings of the three chestnut populations to heat stress and assessing the heat stress tolerance of families. In May 2019, when seedlings were 6 months old, they were divided into two groups (n = 720) and two treatments were applied: ambient temperature (24 h a day, control) and ambient plus elevated temperature (17 h ambient temperature + 7 h elevated temperature, heat stress). The mean temperature of control plants was 25°C. The elevated temperature of heat-stressed plants was 42.5°C from 11 a.m. to 5 p.m. The heat stress treatment was applied using a climate chamber with translucent walls placed inside the greenhouse. During treatments, both groups of plants were well irrigated. Soil moisture, checked in 10 cells per block and treatment using a TDR 100 soil moisture meter (Spectrum Technologies Inc., Plainfield, Illinois, USA) and 12-cm-length rods, revealed soil water content values around 30 per cent in volume for both plant groups. During treatments, the

relative air humidity and light conditions of both groups were similar (~60 per cent and PAR ~ 850μmol photons $m^{-2}s^{-1}$, respectively). The thermal treatments lasted 7 days.

Heat tolerance of each family was assessed by (i) visual estimation of the leaf wilting percentage in heat-stressed plants at the end of treatments, (ii) determining leaf proline content of plants at the end of treatments and (iii) comparing plant biomass values in control and heat-stressed plants at the end of the vegetative period. Leaf wilting percentage of plants was estimated using 10 per cent intervals and considering leaves and portions of leaves wilted if they were dry. Leaf proline content of plants was determined after proline extraction following Bates et al. (1973). Briefly, 80 mg of leaf tissue was homogenized with 750 μL sulfosalicylic acid (3 per cent, w/v). After centrifugation (10 min, 10 000 g at 4°C), 500 μL supernatant was collected and 500 μL ninhydrin acid and 500 μL glacial acetic acid were added. After incubation for 1 h at 100°C and cooling for 15 min on ice, 1 mL toluene was added to the solution, mixed vigorously and left at room temperature (25°C) until clear two-phase separation. Absorbance of the chromophore-containing toluene (upper phase) was read at 520 nm using a spectrophotometer (Genesys 10-uv S, Thermo Fisher Scientific Inc., Waltham, MA, USA). Proline concentration was obtained from a standard curve using L-proline. The stock solution of L-Proline (1 mg $mL^{-1}$) for the standard curve was prepared in sulfosalicylic acid in 3 per cent. Six leaf samples per population and treatment were assessed and each sample comprised a pool of three seedlings.

In October 2019, the biomass of leaves, stem, fine roots (FRs, diameter $< 2$ mm) and coarse roots (CRs, diameter $\geq 2$ mm) (Cubera et al., 2012) of all seedlings was determined. After harvesting, the tissues were oven dried (2 days at 60°C) and weighed, and total biomass, belowground to above ground ratio (mg $mg^{-1}$) and FR to total root ratio (mg $mg^{-1}$) were obtained.



*Genetic assessment of heat stress tolerance*

Leaves from the 48 adult trees and the seedlings obtained were used. Leaves from ~35 heat-stressed seedlings per population were randomly selected. Genomic DNA was extracted from 18–20 mg lyophilized leaves according to the Qiagen DNeasy™ Plant mini kit protocol (Alcaide et al., 2022). Twenty EST-SSR markers associated with heat stress-related genes, 10 of which were developed in *Quercus* spp., 2 in *Fagus* spp. and 8 in *Eucalyptus* spp., were pre-selected (Durand et al., 2010; Bradbury et al., 2013a; Burger et al., 2018; Müller and Gailing, 2018). Only loci linked to known EST-SSRs genes were tested (Table S2).

To test transferability and polymorphism of loci, DNA from four *C. sativa* trees was amplified and the amplification products were run on an agarose gel. Single Polymerase Chain Reaction (PCR) reactions were performed in a total volume of 10 μL containing 10 ng genomic DNA template, 1 × PCR Polymerization Buffer containing dNTPs, 0.2 μM of each primer, 0.44 units Taq DNA polymerase and 2 mM $MgCl_2$ (reagents from Fisher Biotech, Perth, Western Australia, Australia). Amplifications were carried out following the authors' indications for the different species (Durand et al., 2010; Bradbury et al., 2013a; Burger et al., 2018; Müller and Gailing, 2018). Seven of the 20 EST-SSRs amplified and showed polymorphism in all samples (Table 1). Based on the size of the products, three multiplex-PCR mixes were designed, the first including *PIE125* and *VIT099* primers (A), the second including *FIR035*, *POR016* and *GOT022* primers (B) and the third including *FIR089* and *Qr6783* primers (C) (Table 1). The forward primers were labelled with a fluorochrome (FAM; Merck Life Science S.L.U., Madrid, Spain; and VIC, NED; Applied Biosystems, Foster City, CA, USA). Amplification was carried out in 20 μL total volume containing 20 ng genomic DNA following the Qiagen multiplex kit protocol. Cycling conditions were 15 min at 95°C, 30 cycles of 30 s at 94°C, 90 s at 57°C and 1 min at 72°C and a final step of 30 min at 72°C.

Amplification products (1µL) were added to 20 µL formamide and 0.3 µL GeneScan™ 500 LIZ dye (Size Standard, Applied Biosystems™) and denatured at 95°C for 5 min. Samples were run on an ABI Prism 3130xL Avant Genetic Analyzer DNA sequencer (Applied Biosystem™). The resulting raw data were collected using GeneMapper v. 4.0 software (Applied Biosystem™). Alleles were determined by automated binning and checked by visual inspection.

### *Statistical analysis*

To quantify variation in heat stress tolerance between and within *C. sativa* populations, several models considering leaf wilting, leaf proline content leaf, stem, FR, taproot and total biomass, belowground to above ground ratio and FR to total root ratio as dependent variables were run. Angular transformation of leaf wilting percentages ($x$) was performed to normalize data [$y = \text{arcsine}(x/100)^{1/2}$]. Linear mixed models were performed using 'leaf wilting' and 'leaf proline' as the dependent variables, 'treatment' and 'block' as the fixed factors, 'population', 'mother tree' and 'treatment × population' as random factors, and 'seed weight', 'time to emerge' and 'plant height' as covariates. The same models were used for the plant biomass parameters, excluding the covariate 'plant height'. Fisher's least significant difference (LSD) was used in post hoc tests. Analyses were performed with STATISTICA v10 (Stat Software Inc., Tulsa, OK, USA).

Intra- and inter-population genetic diversity indices were calculated using GenAlEx 6 (Peakall and Smouse, 2006): number of alleles per locus (A); observed (Ho), expected (He) and unbiased expected heterozygosity (uHe); and number of private alleles in populations (Pa). The inbreeding coefficient $F_{IS}$ (Weir and Cockerham, 1984) was calculated using Arlequin 3.11 (Excoffier *et al.*, 2005) and its deviation from zero was tested using 10 000 allele permutations. Differentiation between populations was calculated by $F_{ST}$ (Weir and Cockerham, 1984) and $R_{ST}$ (Slatkin, 1995). Allelic richness (Ar) was calculated by the rarefaction method with HP-rare software (Kalinowski, 2005). For each locus, estimated null allele

frequency was calculated using Micro-Checker software (van Oosterhout *et al.*, 2004). LOSITAN software (Antao *et al.*, 2008) was used to detect outlier loci, i.e. markers in which the genetic diversity within populations (heterozygosity) and between populations ($F_{ST}$) does not conform to the prediction of neutral selection. Simulation of neutral selection was conducted under the stepwise mutation model with 50 000 iterations at a confidence level of 95 per cent and a false discount rate of 0.1.

To determine whether the selected EST-SSR markers associated with heat stress tolerance were able to statistically differentiate the three *C. sativa* populations, two approaches were used. The first involved principal coordinate analyses (PCoA) of the genetic distance covariance matrices of the 48 adult trees and 105 seedlings selected at random from the 1440 seedlings assessed (~35 per population). The PCoA was performed using standardized data and GenAlEx 6 (Peakall and Smouse, 2006). This method extracts a set of uncorrelated variables as linear combinations of the original variables, reducing the dimensionality of data while preserving most of the variance. The new variables, called components, are arranged in order of decreasing variance such that those with the highest variance are termed PCs. The second approach comprised discriminant function analysis (DFA), a supervised projection method in which *a priori* information about sample grouping in the dataset is used to produce measures of within- and between-group variance (Martín *et al.*, 2008). This information is then applied to define discriminant functions that optimally separate the *a priori* groups (Alcaide *et al.*, 2019, 2020). In this study, 14 PCs from adult material and 21 PCs from seedling material were included in the independent variable list, and 'population' was used as the grouping variable. DFA was performed using the forward stepwise method in STATISTICA v10 software.

**Table 1.** Characteristics of the seven EST-SSRs grouped in multiplexes A, B and C and used to assess adaptive genetic diversity in 48 *Castanea sativa* mother trees from three Spanish populations

| Locus | Primer sequence (5′–3′) | Motif | Dye | Size (bp) | A | Ho | He | uHe | $F_{IS}$ |
|---|---|---|---|---|---|---|---|---|---|
| **Multiplex A** | | | | | | | | | |
| ***PIE125*** | F: AATACAAATCGCAGGAGGTG<br>R: CTAACCCATCGTTCATGGAG | $(GGAAGC)_3$ | VIC | 155-162 | 3 | 0.563 | 0.498 | 0.503 | -0.129 |
| ***VIT099*** | F: TGAGGTTGCTGATTCGTCAC<br>R: TGAAAATCCAAAACCCTAACC | $(TC)_{16}$ | NED | 127-131 | 3 | 0.042 | 0.486 | 0.491 | 0.914* |
| **Multiplex B** | | | | | | | | | |
| ***FIR035*** | F: GCTAAGGTTCCGTGTTCCAA<br>R: GGCCAGCAACTAAACCAAGA | $(AT)_6$ | FAM | 181-186 | 3 | 0.104 | 0.548 | 0.554 | 0.810* |
| ***POR016*** | F: AGCAACAGCAGAGCCAAAAT<br>R: CAGCGGCTTTGAGGTAATTC | $(GGT)_6$ | VIC | 115-117 | 2 | 0.063 | 0.317 | 0.321 | 0.803* |
| ***GOT022*** | F: GCCTTGCCAATCCATTAAAA<br>R: TACAAGGCTCTTGGCAGCTT | $(TA)_6$ | NED | 146-180 | 5 | 0.146 | 0.342 | 0.346 | 0.574* |
| **Multiplex C** | | | | | | | | | |
| ***FIR089*** | F: AGCGACTAACCCAACTTCCA<br>R: GCGGATTCGATAGCATTTTT | $(GA)_6$ | VIC | 183-199 | 4 | 0.167 | 0.193 | 0.195 | 0.136* |
| ***Qr6783*** | F: GAGAGCCCTGTTATCCTCCC<br>R: AATGAGTCTCAAAGCGGTGG | GTT | NED | 207-227 | 4 | 0.375 | 0.425 | 0.429 | 0.117 |

A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; uHe, unbiased expected heterozygosity; $F_{IS}$, inbreeding coefficient. Asterisks indicate signification at the 0.01 level.

Genetic structure of *C. sativa* populations was analysed by applying a model-based Bayesian approach implemented in STRUCTURE v.2.3.4 software (Pritchard *et al.*, 2000), using the admixture model on the whole dataset and the correlated allele frequencies (Hubisz *et al.*, 2009). The range of possible number of clusters (*K*) tested was 1 to 6 (putative number of populations plus 3), performing 20 independent runs for each *K* value, with a burn-in period of 10 000 steps followed by $10^5$ MCMC replicates. To identify the number of clusters (*K*) that best explained the data, the rate of change on L(*K*) ($\Delta K$) between successive *K* values was calculated using STRUCTURE HARVESTER (Earl and vonHoldt, 2012). The 20 runs for each simulation were averaged using CLUMPP software (Jakobsson and Rosenberg, 2007) and represented graphically with DISTRUCT (Rosenberg, 2004).

## RESULTS

### *Phenotypic response to heat stress*

Heat exposure (42.5°C) for 7 days induced $10.1 \pm 1.2$ per cent leaf wilting in 6-month-old *C. sativa* seedlings. Leaf wilting was $1.3 \pm 1.0$ in control plants, significantly lower than in plants exposed to heat stress ($P = 0.033$; Table S3). Tall early-germinating plants wilted more than small late-germinating plants (Table S3). Heat exposure did not cause plant mortality in the populations tested. No differences were observed in leaf wilting between populations and mother trees (data not shown). Leaf proline content was the parameter that best explained differences between populations in response to the heat treatment (Table 3; Figure 3). Constitutive content of proline in leaves did not vary between populations, whereas leaf proline content induced by heat was highest in the humid population ($P < 0.001$; Figure 3).

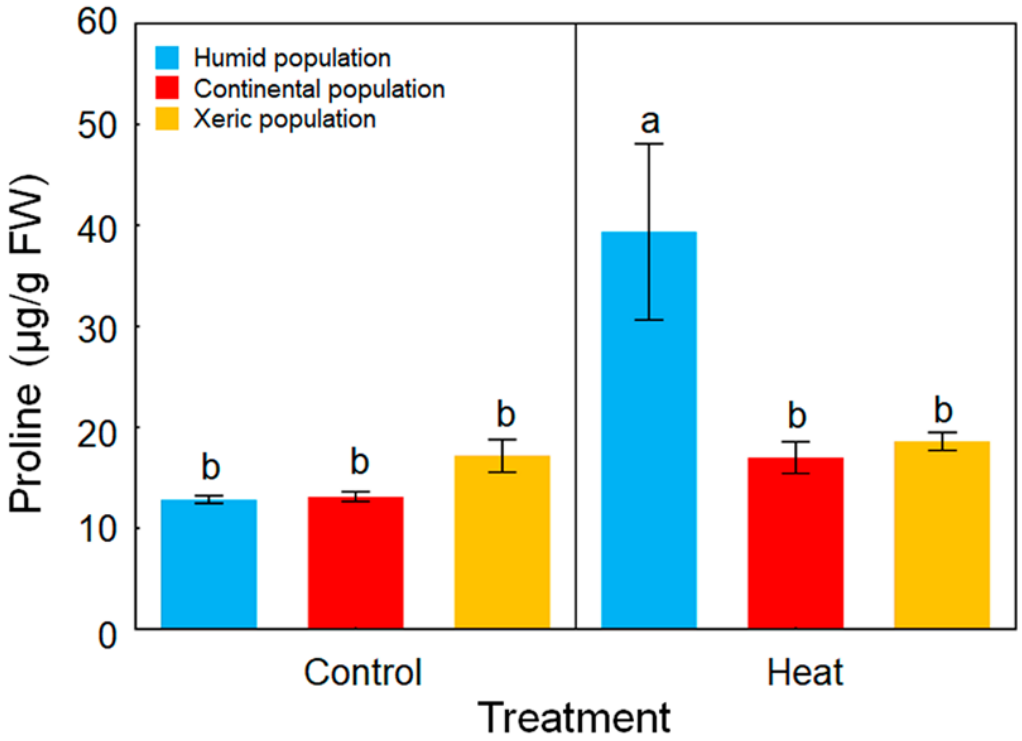



**Figure 3.** Proline content in leaves of 1-year-old *Castanea sativa* seedlings from humid (blue), continental (red) and xeric (yellow) populations subjected to control and heat stress treatments. Vertical bars indicate standard errors of means ($n = 6$) and different letters indicate significant differences at $P < 0.05$ (Fisher's LSD test) between all values.

At the end of the vegetative period, it was observed that exposure to heat stress induced a significant reduction in tap root biomass (3.0 vs. 2.6 g in control and heat-treated plants, respectively, $P = 0.035$) and belowground to aboveground ratio (1.37 vs. 1.17 in control and heat-treated plants, respectively, $P = 0.016$) of plants (Table S3). However, FR biomass and FR to total root ratio were higher in heat-treated plants than in control pants (marginally significant $P = 0.065$ and $P < 0.001$, respectively; Table S3). All plant biomass parameters covaried with 'seed weight' and 'germination time' variables. Plant biomass did not differ between populations (Table S3).

### *Validation of EST-SSRs and genetic diversity of populations*

Of the 20 loci tested, seven developed in *Eucalyptus* spp. failed to amplify, two developed in *Fagus sylvatica* gave inconsistent amplification and three developed in *Quercus* spp. showed no polymorphism. Of the eight loci that showed a repeatable and polymorphic pattern, *EMG37* from *Eucalyptus* spp. was discarded because the PCR product length did not match the length reported in *Eucalyptus* spp. (437 bp and 261bp, respectively; Figure S1). Seven loci developed in *Quercus* spp. were selected (Table S2). These 7 EST-SSRs detected 24 different alleles, of which 5 were rare (< 5 per cent frequency) and 10 were at frequencies of 5–20 per cent. Alleles per locus ranged from 2 (*POR016*) to 5 (*GOT022*), with a mean of 3.4 alleles (Table 1). The unbiased expected heterozygosity (uHe) was higher than the observed heterozygosity (Ho) at six of the seven loci, indicating a deficit in heterozygotes (Table 1). Likewise, the inbreeding coefficient ($F_{IS}$) was positive and significant for markers *VIT099*, *FIR035*, *POR016*, *GOT022* and *FIR089* and negative only for *PIE125* (Table 1). Although *VIT099* and *FIR035* were at the limit of statistical significance, Micro-Checker software showed no evidence of the null allele.

According to the genetic diversity parameters obtained (Table 2), genetic variation among mother trees and offspring populations was similar. However, in terms of the mean number of alleles, Ar and expected heterozygosity, the humid and xeric populations showed the highest and lowest diversity, respectively, for mother trees and offspring plant material (Table 2). One private allele was detected for the humid population and two private alleles were detected for the xeric population (Table 2). In mother trees of the three populations, $F_{ST}$ and $R_{ST}$ values were low (0.04 and 0.02, respectively), and in offspring they were slightly higher (0.06 and 0.02, respectively) (Table 2).

**Table 2.** Genetic diversity parameters for mother trees and offspring of *Castanea sativa* from three Spanish populations obtained from the seven EST-SSRs.

| Plant material | Population | N | A | $A_r$ | Ho | He | uHe | Pa | $F_{IS}$ | $F_{ST}$ | $R_{ST}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mother trees | Humid | 14 | 3.143 | 3.143 | 0.245 | 0.408 | 0.423 | 1 | 0.466* | - | - |
| | Continental | 18 | 2.714 | 2.693 | 0.262 | 0.395 | 0.406 | 0 | 0.358* | - | - |
| | Xeric | 16 | 2.429 | 2.388 | 0.116 | 0.334 | 0.345 | 2 | 0.528* | - | - |
| | | | | | | | | | | 0.04 | 0.02 |
| Offspring | Humid | 33 | 3.714 | 3.714 | 0.290 | 0.480 | 0.488 | 1 | 0.457* | - | - |
| | Continental | 37 | 3.429 | 3.409 | 0.228 | 0.478 | 0.484 | 0 | 0.558* | - | - |
| | Xeric | 35 | 2.571 | 2.562 | 0.139 | 0.309 | 0.313 | 2 | 0.472* | - | - |
| | | | | | | | | | | 0.06 | 0.06 |

N, sample size; A, mean number of alleles; $A_r$, allelic richness according to Kalinowski (2005); Ho, observed heterozygosity; He, expected heterozygosity; uHe, unbiased expected heterozygosity; Pa, private alleles; $F_{IS}$, inbreeding coefficient (asterisks indicate significance at the 0.01 level); $F_{ST}$, differentiation among populations according to Weir and Cockerham (1984); $R_{ST}$, differentiation among populations according to Slatkin (1995). Results of the general linear mixed model for the analysis of leaf proline content in the offspring of the three Spanish *Castanea sativa* populations subjected to heat and control treatments.

After PCoA of the genetic distance covariance matrices, examination of the PCo1–PCo2 score scatter plots did not allow clear distinction between the samples from the three populations. PCo3–PCo14 for mother trees and PCo3–PCo21 for seedlings were also examined, but no distinction between populations was detected. After DFA, however, the clusters of each group of trees were clearly separated in the DF1–DF2 score scatter plots (Figure 4; >76 per cent of variance explained; Wilks' lambda test, $P < 0.01$). The separation between mother trees from the humid versus continental and xeric populations was mainly characterized by the DF1 axis (Figure 4a). Trees from the continental and xeric populations were at the positive scoring gradient, whereas trees from the humid population were at the negative scoring gradient of the DF1 axis (Figure 4a). The separation between the continental and xeric populations was mainly characterized by the DF2 axis, which showed a positive score gradient for xeric mother trees and a negative score gradient for continental mother trees (Figure 4a). The separation between seedlings from the humid and xeric populations was mainly characterized by the DF1 axis, which showed a positive score gradient for trees from the xeric population and a

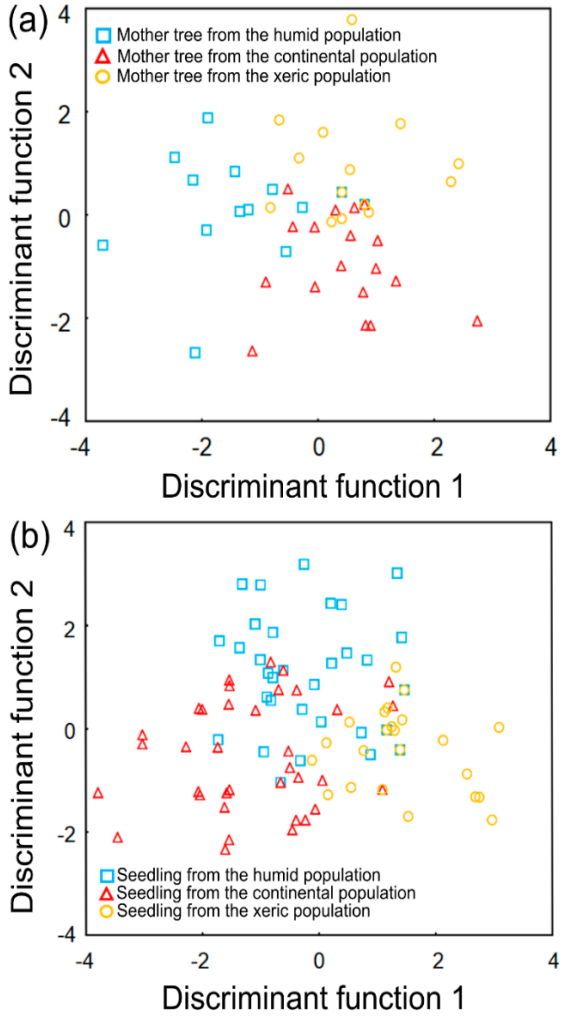



**Figure 4.** DFA of alleles of seven EST-SSRs of (a) 48 mother trees and (b) 105 seedlings from humid, continental and xeric *Castanea sativa* populations.

negative score gradient for trees from the humid population (Figure 4b). The separation between humid versus continental and xeric seedlings was mainly characterized by the DF2 axis (Figure 4b). Seedlings from the humid population were at the positive DF2 score gradient, whereas seedlings from the continental and xeric populations were at the negative score gradient (Figure 4b).

STRUCTURE software identified $K = 2$ as the most likely division with the highest support in terms of log-likelihood values (Figure 5). Based on $K = 2$, Bayesian clustering separated *C. sativa* seedlings into two groups with admixture among clusters. Based on the estimated membership coefficient or *Q*-value, Cluster I included 81 per cent of seedlings from the most thermophilic population (xeric), 72 per cent of seedlings from the continental population and 52 per cent of seedlings from the least thermophilic population (humid) (Figure 5). LOSITAN software identified *POR016* ($F_{ST} = 0.45$, $P < 0.05$) and *VIT099* ($F_{ST} = 0.33$, $P < 0.05$) as candidate outlier loci influenced by positive selection (Figure 6).

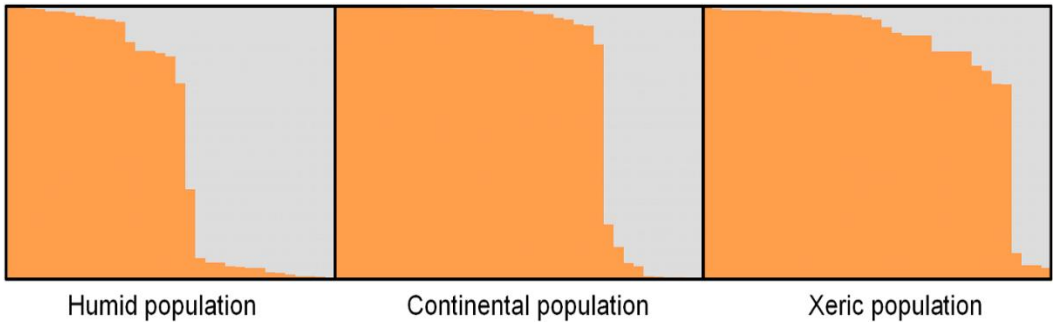



**Figure 5.** Population structure inferred for 105 *Castanea sativa* seedlings from three Spanish populations of contrasting climate characteristics. Genetic structure was estimated using STRUCTURE (Pritchard et al., 2000) and data of the seven EST-SSRs for K = 2. Vertical lines represent individuals and vertical black lines separate populations. Orange and grey shading indicate the probability of individuals belonging to clusters I and II, respectively.

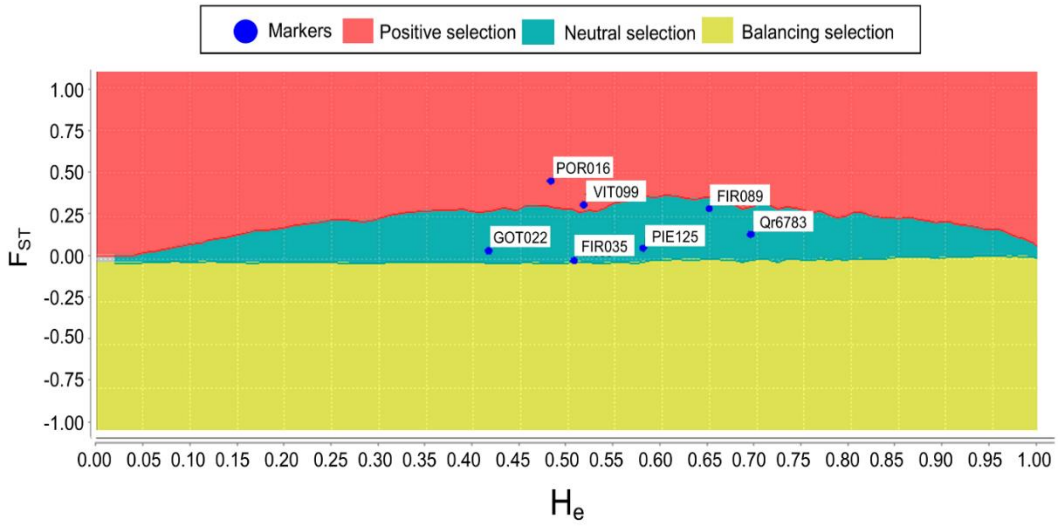



**Figure 6.** $F_{ST}$ and He comparisons in polymorphic loci to identify outliers and potential candidates for selection considering *Castanea sativa* offspring grouped into Cluster 1 and Cluster 2. Graphical output shows the simulated confidence area for neutral loci (blue area). LOSITAN software was used.

## DISCUSSION

This study contributes to the understanding of how wild chestnut populations of contrasting climate characteristics may respond to future global warming, reaching three significant results: firstly, it quantifies variation in *C. sativa* response to heat stress through a physiological trait (proline) and EST-SSR markers; secondly, it validates the use of adaptive molecular markers related to heat stress tolerance; and thirdly, it suggests loci for use in marker-assisted selection to identify heat-tolerant chestnut trees.

### *Two EST-SSR markers for assessing adaptive genetic diversity to heat stress in Fagaceae*

Of the 20 EST-SSR markers evaluated, only those developed in *Quercus* ssp. amplified in *C. sativa* and showed considerable polymorphism. These results agree with previous studies reporting high transferability between *Castanea* and *Quercus* species (Martín *et al.*, 2017; Alcaide *et al.*, 2019; Castellana *et al.*, 2021) grouped in the Fagaceae family. The genetic diversity indices were slightly higher for the humid compared to the xeric population (in both mother trees and offspring), in accordance with the diversity described in previous studies (Pereira-Lorenzo *et al*., 2010; Fernandez-Cruz and Fernandez-Lopez, 2016). Similar results were also obtained when EST-SSRs were used to evaluate the tolerance to drought of chestnut populations (Alcaide *et al.*, 2019). Likewise, several studies have indicated the high efficiency of functional markers to assess the adaptive response of plants to stress (Lind and Gailing, 2013; Sullivan *et al.*, 2013; Wang *et al.*, 2014; Dounavi *et al.*, 2016; Khodwekar and Gailing, 2017; Wang *et al.*, 2017). In *C. sativa*, for example, adaptive responses to bud burst (Cuestas *et al.*, 2017; Martín *et al.*, 2010, 2017), drought (Alcaide *et al.*, 2019; Castellana *et al.*, 2021) and the pathogen *P. cinnamomi* (Alcaide *et al.*, 2020) were reported after detection of outlier loci from EST-SSRs.

The study identified two markers under positive selection. Locus *VIT099* has a putative function as NAC domain-containing protein 78, involved in the regulation of flavonoid biosynthesis and 20S and 26S proteasomes in response to photooxidative stress (Morishita *et al.*, 2009). Photooxidation is observed at extreme temperatures and is therefore linked to heat stress (Guo *et al.*, 2006). Locus *POR016* has a putative function as heat shock protein 70 k (HSP70), important for stomatal closure, and also modulates abscisic acid (ABA)-dependent physiological responses (Clément *et al.*, 2011). It has been suggested that the induction of several HSPs (including HSP70) by ABA may be one of the mechanisms that confer

thermotolerance in plants (Pareek *et al.*, 1998). ABA also plays a key role in regulating proline metabolism in plants under abiotic stress (Pesci, 1992; Kumar *et al.*, 2012; Marcińska *et al.*, 2013). The different leaf proline accumulation of populations induced by heat stress observed here and the detection of one EST-SSR under positive selection related to ABA-dependent physiological responses makes the *POR016* locus an interesting candidate for early selection of heat stress-tolerant individuals. Because heat stress tolerance is probably under polygenic control and involves several resistance components regulated by sets of genes in different tissues and growth stages (Younis *et al.*, 2020), *VIT099* and *POR016* should be included as candidate markers for their use in early selection of individuals tolerant to heat stress.

Locus *EGM37* was satisfactorily amplified in our plant material, but was discarded from the analysis because the size range of the PCR products was higher than that reported elsewhere (Bradbury *et al.*, 2013a). In *Eucalyptus*, an exclusive association of allelic variation with temperature at this locus was reported (Bradbury *et al.*, 2013a). *EGM37* is homologous to quinone oxidoreductase proteins, which are located within chloroplast membranes, involved in photosynthesis and possibly function as reactive oxygen species (ROS)-scavenger enzymes (Müh *et al.*, 2012; Bradbury *et al.*, 2013b). In view of this, it would be interesting to conduct further studies in *C. sativa* to determine whether it is the same region.

### *Response of Spanish chestnut populations to heat stress*

Proline is an osmolyte that stabilizes and protects the structure of enzymes and proteins, maintains membrane integrity and scavenges ROS. Under stress conditions, proline accumulates to a high concentration in the cell cytoplasm (Lv *et al.*, 2011; Hayat *et al.*, 2012,Harsh *et al.*, 2016). However, it has also been suggested that excessive PRO accumulation could be toxic to the plant cell (Rizhsky *et al.*, 2004; Lv *et al.*, 2011). In *Arabidopsis thaliana*, proline accumulation in response

to heat has been suggested as being detrimental for the plant and responsible for reduced thermotolerance (Lv *et al.*, 2011). In our chestnut subjected to heat stress, seedlings from the humid population showed a clear and significant increase in leaf proline compared with control plants ($P < 0.001$; Figure 3), with no detrimental effects on plant performance (Table S3). In the two more thermophilic populations, no proline increase in response to heat stress was observed.

Plant individuals from natural populations cluster in groups according to their geographical origin and adaptation to the local climate environments (Hu *et al.*, 2021). Our DFA analysis detected a geographical pattern of the *C. sativa* populations in response to heat stress. Trees and seedlings from the humid population were significantly discriminated from trees and seedlings from the continental and xeric populations. DFA results are in line with the different increase observed in the leaf proline content in each population (significant 'treatment' × 'population' interaction, Table 3) and congruent with the mean annual temperature of each population. The most sensitive humid population has the lowest mean and absolute maximum temperatures, whereas the continental and xeric populations have similar highest mean and maximum temperatures that are both higher than the humid population (Figure 1; Table S1). The continental and xeric populations are subjected to frequent heat waves in summer, with maximum temperatures similar to that used here.

Bayesian clustering analysis revealed two main clusters with admixture within populations and some clinical effect: a larger proportion of trees belonging to cluster I in the more thermophilic populations (Figure 5). This geographical variation in response to heat stress was less pronounced than the geographical variation reported in *C. sativa* populations from contrasting climate environments in budburst and in response to drought (Martín *et al.*, 2010; Alcaide *et al.*, 2019; Míguez-Soto *et al.*, 2019). It should be noted that among the environment variables, temperature may explain much less than photoperiod and water availability about

adaptive genetic variation. Moreover, changes across small-scale thermal gradients may have influenced the results. In the Mediterranean climate, maximum temperatures have a decisive impact on stress induction, probably due to their interplay with water availability (Grivet *et al.*, 2011; Bradbury *et al.*, 2013b). In forests including *Quercus suber*, environmental association analyses revealed that temperature was more frequently associated with polymorphisms than precipitation, and the annual temperature range is the strongest environment variable shaping genetic variation (Aronson *et al.*, 2009; Cox *et al.*, 2011; De Kort *et al.*, 2014; Pina-Martins *et al.*, 2019). Additional evidence of temperature-related adaptive differentiation has been reported for *Alnus glutinosa* and *Pinus* spp. (Grivet *et al.*, 2009; De Kort *et al.*, 2014).

**Table 3.** Results of the general linear mixed model for the analysis of leaf proline content in the offspring of the three Spanish *Castanea sativa* populations subjected to heat and control treatments.

| **Effect** | **Df** | ***F*-ratio** | ***P* value** |
|---|---|---|---|
| Fixed factor | | | |
| Treatment [T] | 1 | 1.8 | 0.311 |
| Random factors | | | |
| Population [P] | 1 | 0.7 | 0.596 |
| T × P | 2 | 4.9 | 0.016 |

T × P, treatment by population interaction.

## CONCLUSION

This is a first study in which molecular and physiological approaches converge to allow a better understanding of heat stress tolerance in chestnut. We identified signatures of positive selection to heat stress by detecting two EST-SSR markers. *POR016* and *VIT099* should be included as candidate markers for their use in early selection of individuals tolerant to heat stress in *C. sativa*. In conjunction with previously described loci for water stress in the species, these two loci strengthen the division into two chestnut ecotypes, xeric and mesophytic, corresponding to trees inhabiting central-southern and northern Spain, respectively.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# Supplementary material

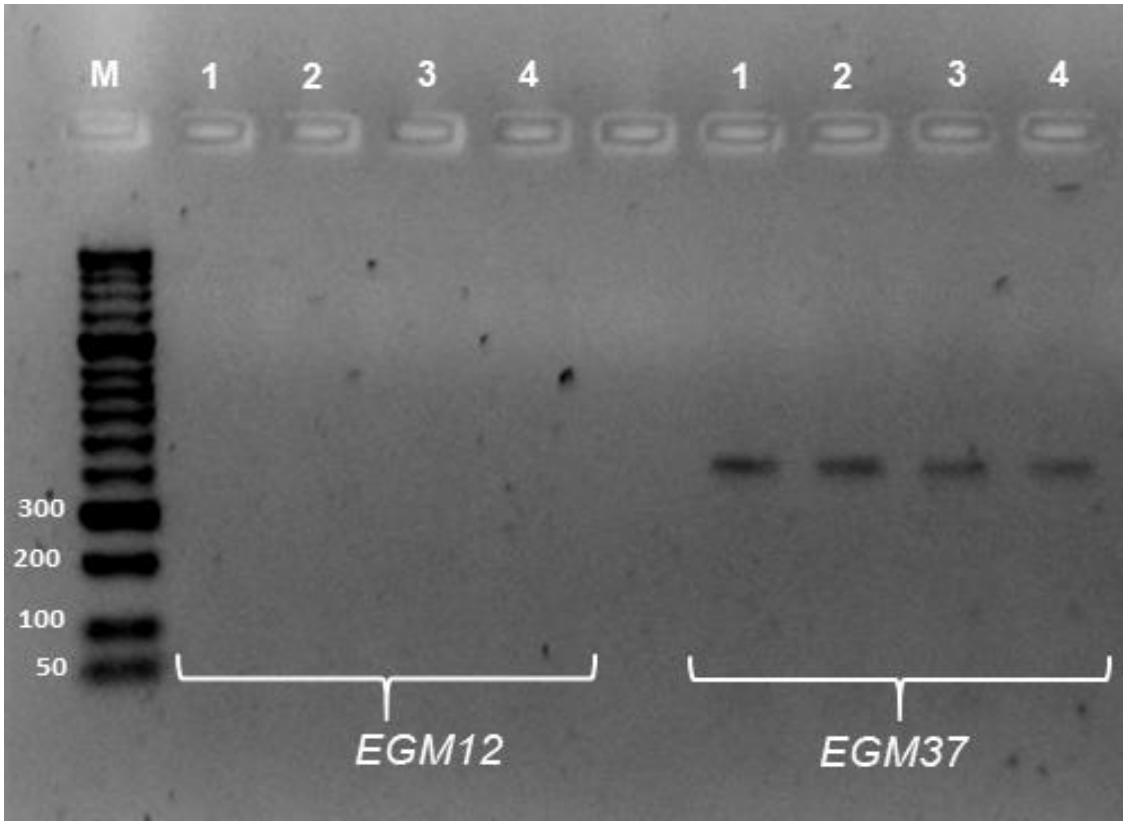


Figure S1. Amplification of loci *EGM12* and *EGM37* in four samples of *Castanea sativa*. The band size of the molecular weight marker (M) is expressed in bp.

**Table S1.** Description of the three Spanish populations of *Castanea sativa* assessed for adaptive genetic diversity to heat stress.

| Population | Location | Coordinates | Altitude (m asl) | T (ºC) | Tmax (ºC) | P (mm) | No. trees |
|---|---|---|---|---|---|---|---|
| Humid | Puebla de Sanabria, Zamora, Castilla y León | 42°02'05.4''N 6°40'19.5''W | 1003 | 9.7 | 32.8 | 1013 | 14 |
| Continental | Valle de Matamoros, Badajoz, Extremadura | 38°22'28.5''N 6°48'00.1''W | 624 | 15 | 39.5 | 740 | 18 |
| Xeric | Paterna del Río, Almería, Andalusia | 37°01'54.0''N 2°57'15.5''W | 1380 | 12.4 | 33.6 | 654 | 16 |

T- Mean annual temperature; Tmax- Absolute maximum temperature; P- Cumulative annual precipitation; No. trees- Number of mother trees sampled

**Table S2**. Characteristics of 20 EST-SSRs preselected and their putative functions

| Locus | Primer sequence (5′–3′) | Repeat motif | Expected size (bp) | Putative function (Protein) / Gene | Reported function, notes | References |
|---|---|---|---|---|---|---|
| EGM30 | F: AGTGCAGCACCTTTCAGACC<br>R: AAGATTGATTGCTAGATCAGTCACC | $(AG)_{18}$ | 225–255 | *Chlorophyll A/B binding protein* | The light-harvesting complex (LHC) functions as a light receptor, it captures and delivers excitation energy to photosystems with which it is closely associated. Response to light stimulus | Bradbury *et al.* (2013a) |
| EGM35 | F: ATACGCGTCCCAGTGATTTC<br>R: AGGAGCAGACGAACTTGCAT | $(AG)_{18}$ | 196–212 | *Fructose-1,6-bisphosphatase, cytosolic* | Catalyzes the first irreversible reaction from fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate and plays an important regulatory role in sucrose biosynthesis and metabolism. Its activity is essential to regulate starch levels. Functions in fructose-mediated signaling independently of its catalytic activity in sugar metabolism. May act downstream of ABA2/GIN1, which is involved in abscisic acid (ABA) synthesis to regulate autotrophic transition and modulate early seedling establishment after seed germination | Bradbury *et al.* (2013a) |
| EGM48 | F: TCACACTCCAATCTCCAACG<br>R: CCTGGTAGTCCTTCGACTGG | $(CT)_{12}$ | 141–155 | *Aquaporin PIP2;1* | Aquaporins highly expressed in roots, which make an important contribution to root water transport and its regulation by hormonal and abiotic factors. | Bradbury *et al.* (2013a) |

**Table S2**. Continued

| Locus | Primer sequence (5′–3′) | Repeat motif | Expected size (bp) | Putative function (Protein) / Gene | Reported function, notes | References |
|---|---|---|---|---|---|---|
| EGM30 | F: AGTGCAGCACCTTTCAGACC<br>R: AAGATTGATTGCTAGATCAGTCACC | $(AG)_{18}$ | 225–255 | *Chlorophyll A/B binding protein* | The light-harvesting complex (LHC) functions as a light receptor, it captures and delivers excitation energy to photosystems with which it is closely associated. Response to light stimulus | Bradbury *et al.* (2013a) |
| EGM35 | F: ATACGCGTCCCAGTGATTTC<br>R: AGGAGCAGACGAACTTGCAT | $(AG)_{18}$ | 196–212 | *Fructose-1,6-bisphosphatase, cytosolic* | Catalyzes the first irreversible reaction from fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate and plays an important regulatory role in sucrose biosynthesis and metabolism. Its activity is essential to regulate starch levels. Functions in fructose-mediated signaling independently of its catalytic activity in sugar metabolism. May act downstream of ABA2/GIN1, which is involved in abscisic acid (ABA) synthesis to regulate autotrophic transition and modulate early seedling establishment after seed germination | Bradbury *et al.* (2013a) |
| EGM48 | F: TCACACTCCAATCTCCAACG<br>R: CCTGGTAGTCCTTCGACTGG | $(CT)_{12}$ | 141–155 | *Aquaporin PIP2;1* | Aquaporins highly expressed in roots, which make an important contribution to root water transport and its regulation by hormonal and abiotic factors. | Bradbury *et al.* (2013a) |

**Table S2**. Continued

| Locus | Primer sequence (5′–3′) | Repeat motif | Expected size (bp) | Putative function (Protein) / Gene | Reported function, notes | References |
|---|---|---|---|---|---|---|
| FIR053 | F: AGTTTCCCCACATTTGTTGC<br>R: TACCATGCACCAAGCAATTC | $(GTG)_7$ | 136-150 | *Glutaredoxin-C9-like* | Cell redox homeostasis | Durand *et al.* (2010) |
| FIR089 | F: AGCGACTAACCCAACTTCCA<br>R: GCGGATTCGATAGCATTTTT | $(GA)_6$ | 159-181 | *Abscisic acid receptor PYL4* | Receptor for abscisic acid (ABA) required for ABA-mediated responses such as stomatal closure and germination inhibition. Abscisic acid-activated signalling pathway. Regulation of protein serine/threonine phosphatase activity. | Durand *et al.* (2010) |
| VIT107 | F: TGATCACAGATTGGAGCTTAACA<br>R: CCCCCACTTAGGAAAGAAGC | $(TA)_{13}$ | 124-142 | *Light-harvesting complex i protein LHCA2* | The light-harvesting complex (LHC) functions as a light receptor, it captures and delivers excitation energy to photosystems with which it is closely associated, here photosystem I. Response to high light intensity. Response to cold. Response to light stimulus. | Durand *et al.* (2010) |
| FS_C2361 | F: AGGTCCTTCAGTTTGGGAGC<br>R: ATTCCCATGCATCAAAATCC | GAA | 193-196 | *Light-harvesting complex-like protein OHP2* | May play a photoprotective role within PSI in response to light stress. | Müller and Gailing (2018) |

**Table S2**. Continued

| Locus | Primer sequence (5′–3′) | Repeat motif | Expected size (bp) | Putative function (Protein) / Gene | Reported function, notes | References |
|---|---|---|---|---|---|---|
| Qr6783 | F: GAGAGCCCTGTTATCCTCCC<br>R: AATGAGTCTCAAAGCGGTGG | GTT | 227-279 | *Superoxide dismutase [Cu-Zn] 2* | Destroys radicals normally produced in cells that are toxic to biological systems. It is involved in stress tolerance, including photo-oxidative stress. | Müller and Gailing (2018) |
| POR025 | F: CACACAAACCCATATGATCTGAA<br>R: TCTCTTTCGATCCCTTCTGC | $(TC)_{10}$ | 115-124 | *Heat shock cognate 70kDa protein 1* | Proteins important for stomatal closure and modulate abscisic acid dependent physiological responses. | Durand *et al.* (2010) |
| FgSI0016 | F: CGGAGAAGGACAAGGACAAG<br>R: TTCTTCGTAGAGCCTTGATGC | AAC | 157-172 | *Peptidyl-prolyl cis-trans Isomerase* | Catalyses the cis-trans isomerisation of proline imide peptide bonds in oligopeptides. Required for light-induced increase in thiol accumulation. Assists in the folding or assembly of the SAT1 enzyme to form the cysteine synthase complex. Links light and redox signals to the regulation of cysteine biosynthesis in response to stress. | Burger *et al.* (2018) |
| EGM14 | F: CACTGCCACTTACCAGAGTCG<br>R: CCTCCACCATCTCGAACG | $(CT)_{18}$ | 350 | *Heat shock factor protein HSF30* | Cellular response to heat. Positive transcriptional regulation from the RNA polymerase II promoter in response to heat stress. | Bradbury *et al.* (2013a) |

**Table S2**. Continued

| Locus | Primer sequence (5′–3′) | Repeat motif | Expected size (bp) | Putative function (Protein) / Gene | Reported function, notes | References |
|---|---|---|---|---|---|---|
| EGM155 | F: TAGACTTTCCCGAATCGCTTAC<br>R: GGTGCTTCGTTTGATCCTAAAA | $(AG)_{18}$ | 295 | *Small ubiquitin-like modifier 1 (SUM1)* | Stress conditions rapidly and substantially elevate the amount of SUMO1 and SUMO2 conjugates with a concomitant reduction in the amount of free SUMO proteins. The SUMO conjugation system plays an important role in stress protection and/or repair. Heat acclimation. Heat response. | Bradbury *et al.* (2013a) |
| EGM141 | F: CCATGAGATTCAACCACATCAT<br>R: TCTTCGCTTCGTAGAGCTTCTT | $(CCT)_{14}$ | 343 | *Cysteine protease inhibitor, cystatin* | Involved in the suppression of hypersensitive cell death triggered by avirulent pathogen or oxidative stress. Cellular response to heat. | Bradbury *et al.* (2013a) |
| EGM12 | F: GCGCCGAGAATCAATACG<br>R: GTAGCTGTTGGCAGCTTTGG | $(CAG)_{10}$ | 197 | *CONSTANS-like protein CO1* | Transcription factor that acts in the long day flowering pathway and may mediate between the circadian clock and the control of flowering. Plays a role in the regulation of flowering time. Involved in proline and ethylene biosynthesis. | Bradbury *et al.* (2013a) |
| EGM37 | F: TGAGGTCACTTCAAGCACCAAGA<br>R: GGAAGCGGCAACAACCTTAACA | $(GCTTA)_5$ | 269 | *Quinone oxidoreductase* | Associated with high summer temperature stress, seasonal temperature fluctuation, summer solar radiation and evaporation potential. Quinone oxidoreductases may function as ROS-scavenging enzymes. | Bradbury *et al.* (2013a) |

**Table S3**. Results of general linear mixed models for the analysis of leaf wilting and plant biomass parameters measured in the offspring of the three Spanish *Castanea sativa* populations subjected to control and heat stress treatments.

Mother tree (population) = Mother tree nested within population; na = Not applicable

| | | **Leaf wilting** | | **Leaf biomass** | | **Stem biomass** | | **Taproot biomass** | | **Fine root biomass** | | **Total biomass** | | **Belowground to above ground ratio** | | **Fine root to total root ratio** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Effect | Df | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value | *F*-ratio | *P*-value |
| Fixed factor | | | | | | | | | | | | | | | | | |
| Treatment [T] | 1 | 27.5 | **0.033** | 0.1 | 0.746 | 0.4 | 0.605 | 24.4 | **0.035** | 10.9 | 0.065 | 2.1 | 0.281 | 46.5 | **0.016** | 28.5 | **<0.001** |
| Random factors | | | | | | | | | | | | | | | | | |
| Population [P] | 2 | 0.1 | 0.910 | 45.6 | 0.718 | 1.5 | 0.334 | 1.4 | 0.352 | 3.0 | 0.168 | 3.3 | 0.171 | 4.0 | 0.064 | 4.2 | 0.191 |
| Mother tree (population) | 45 | 0.8 | 0.869 | 0.7 | 0.944 | 1.1 | 0.287 | 0.9 | 0.597 | 0.9 | 0.623 | 0.9 | 0.713 | 1.2 | 0.270 | 1.0 | 0.556 |
| P x T | 2 | 2.0 | 0.133 | 0.1 | 0.931 | 0.9 | 0.423 | 0.3 | 0.721 | 0.1 | 0.907 | 0.3 | 0.729 | 0.2 | 0.845 | 0.0 | 0.979 |
| Covariates | | | | | | | | | | | | | | | | | |
| Seed weight | 1 | 1.3 | 0.247 | 6.3 | **0.013** | 14.9 | **<0.001** | 27.3 | **<0.001** | 11.7 | **<0.001** | 22.9 | **<0.001** | 0.1 | 0.748 | 0.2 | 0.650 |
| Time to germination | 1 | 5.0 | **0.026** | 6.6 | **0.011** | 6.2 | **0.014** | 30.0 | **<0.001** | 15.4 | **<0.001** | 20.3 | **<0.001** | 1.4 | 0.243 | 3.4 | 0.068 |
| Plant height | 1 | 40.0 | **<0.001** | na | na | na | na | na | na | na | na | na | na | na | na | na | na |