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

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ABSTRACT

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

1. Introduction

Chestnuts (*Castanea* spp., *Fagaceae* family) are highly valuable trees of the temperate zone for their nutritious nuts, timber and ecosystem services. Ink disease caused by the invasive soil oomycete *Phytophthora cinnamomi* (*Pc*) is the most destructive disease affecting susceptible *Castanea* spp. globally (Jung et al., 2018), and has contributed to drastic reduction of chestnut distribution areas around the world (Martins et al., 2007; Sena et al., 2018). The root rot pathogen *Pc* is hemibiotrophic and able to infect around 5000 plant species worldwide (Hardham and Blackman, 2018). Sweet chestnut (*C. sativa* Mill.) is the most susceptible European tree to *Pc* in contrast to the Korean chestnut (*C. crenata* Sieb. & Zucc.), native to Japan and South Korea, which is considered a fully resistant species (Crandall et al., 1945). *Castanea crenata* germplasm has been used in chestnut breeding programs in several European countries as a source of resistance to *Pc* (López-Villamor et al., 2018) and several *Pc* resistant *C. crenata* × *C. sativa* hybrid clones are currently available in the market, used as rootstocks of traditional varieties of sweet chestnut, and cultivated in orchards for nut production (Miranda-Fontaíña et al., 2007; Fernández-López and Fernández-Cruz, 2015).

Considerable effort has been devoted to characterize the changes induced by *Pc* in susceptible and/or resistant chestnuts through studies involving root histology, plant water relations, root-to-shoot signalling, mineral nutrition and biochemical parameters (Maurel et al., 2001a, 2001b; Gomes-Laranjo et al., 2004; Maurel et al., 2004; Dinis et al.,

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2011; Medeira et al., 2012; Serrazina et al., 2015). Despite the substantial advances achieved recently at molecular level (Santos et al., 2017a, 2017b), there are still important knowledge gaps, which are crucial to link genotype to phenotype traits involved in plant defence and allow validation of resistant genotypes. More data and experiments are needed in relation to signalling occurring belowground, at the front of pathogen recognition, and more effort is needed to integrate this information with aboveground responses during *Pc* infection in chestnut trees.

Phytohormones are small signalling molecules known to be central regulators of plant responses to a wide range of biotic and abiotic stresses (de Torres Zabala et al., 2009; Martín et al., 2012; van den Berg et al., 2018; Pérez-Clemente et al., 2019). In general terms, salicylic acid (SA)-mediated signalling is important for plant defence against biotrophic pathogens (Spoel and Dong, 2008). The non-bioactive derivatives 2-O-β-d-glucoside (SAG) and salicylate glucose ester (SGE) contribute to the regulation and homeostais of the bioactive SA, and are worth to be quantified together with SA *in planta* in response to pathogen attack (Allasia et al., 2018). Jasmonates (JAs) are fatty acid-derived hormones which regulate defence against necrotrophic pathogens (Spoel and Dong, 2008). JAs include jasmonic acid (JA) and its amino acid conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile). Conjugation of phytohormones to amino acids is commonly associated with storage and inactivation, but JA-Ile is the bioactive form of JAs perceived by cells (Piotrowska and Bajguz, 2011). Other plant hormones such as abscisic acid (ABA) and auxins including indole-3-acetic acid (IAA), that have been thoroughly described to regulate plant development and growth, have recently emerged as key regulators of plant immunity (Denancé et al., 2013). So far, only two studies quantified hormones in trees after infection by *Pc*, reporting decreased cytokinins and ABA content in xylem sap of susceptible *Eucalyptus marginata* (Cahill et al., 1986) and *C. sativa* (Maurel at al., 2004) seedlings, respectively. No study so far has determined which hormones change and in which tissues they do upon *Pc* infection. An urgent call to investigate differences in hormonal responses between *Pc* susceptible and resistant *Castanea* spp. is derived from the work by Santos et al. (2017b), who found one QTLs for *Pc* resistance (designated *Pc_E*) associated to hormonal signalling processes.

Hormones work in a complex signalling network with other known stress-related metabolites. Soluble sugars are relevant in primary metabolism, providing plants with energy and structural material, and interacting as signal molecules with hormones (Ljung et al., 2015). Sugars enhance oxidative burst at early stages of infection by stimulating the synthesis of antioxidant phenolic compounds, which are also involved in plant signalling (*e.g.* flavonoids and SA) and defence (*e.g.* tannins) (Bolouri Moghaddam and Van den Ende, 2012). Plants accumulate the amino acid proline in response to a multitude of environmental stresses. Proline acts as a beneficial solute allowing plants to increase cellular osmolarity during water limitation. Moreover, proline metabolism has roles in redox buffering and energy transfer and is involved in plant-pathogen interactions and programmed cell death (Verslues and Sharma, 2010). Proline metabolism is regulated through a fine-tuned coupling with 1-pyrroline-5-carboxylic acid (P5C) (Cecchini et al., 2011; Qamar et al., 2015). With such an intricate toolbox, plants activate appropriate and effective defence responses against pathogens, and balance defence with growth (Bolouri Moghaddam and Van den Ende, 2012).

The main objectives of this study were to evaluate the impact of *Pc* infection on the main plant leaf physiological function, photosynthesis, and to quantify hormones and stress-related metabolites in leaves and roots of two *Castanea* spp. clonal genotypes of contrasted susceptibility to *Pc*. The resistant '111-1' clone, an F_2 *C. sativa* x *C. crenata* hybrid containing 67% of exclusive Asian alleles (González et al., 2011) was selected because is one of the most commonly planted *Pc*-resistant commercial rootstocks in Spain and Portugal (Miranda-Fontaíña et al., 2007). A pure *C. sativa* clone termed 'Cs14' native to the north-western coast of Spain was selected as susceptible material. This clone was previously used as 'susceptible control' when screening chestnuts for *Pc* resistance in Spain (Cuenca et al., 2009). We hypothesised that in these two clones there are both constitutive and *Pc*-induced differences in the content of hormones and other signalling metabolites in leaves and roots.

2. Material and methods

2.1. Plant material, growth conditions and experimental design

The plant material was obtained by *in vitro* micropropagation (Vidal et al., 2015) and acquired from a specialized chestnut supplier company (Grupo TRAGSA-SEPI, Maceda, Spain). In October 2016, one-year-old plantlets of each clone were planted in 2-liter pots containing a mixture of peat, vermiculite and perlite (1:1:1) and placed at the greenhouse of the Faculty of Forestry of Plasencia (40°02´N, 6°05´W; 374 m asl, western Spain). In January 2017, they were fertilized with Osmocote Pro 3–4 M (Osmocote® Pro) at 4 g L^{-1} and arranged in a complete randomized bi-factorial design considering 'susceptibility to *Pc*' (two categories: susceptible (Cs14) and resistant (111-1)) and 'inoculation with *Pc*' (two categories: yes and no) as factors. In total, there were 60 plantlets distributed over two susceptibilities × two treatments × 15 replicates. Plantlets were inoculated with *Pc* in July 2017, at the age of two years. Clones 111-1 and Cs14 were 107.7 ± 9.6 and 91.5 ± 10.3 cm in height ($P > 0.05$, *t*-test), and 1.1 ± 0.4 and 0.8 ± 0.4 cm in diameter ($P > 0.05$, *t*-test), respectively.

Five days before inoculation and nine days after inoculation the same individuals were sampled aboveground and belowground. Sampling at day nine after inoculation was done because at this stage of infection the first external symptoms (leaf wilting and yellowing) occurred in half of the plants within each chestnut clone. *In vivo* and immediate measurements of gas exchange-related parameters and chlorophyll fluorescence parameters (including maximum quantum yield of photosystem (PS) II and leaf chlorophyll content) were performed at each sampling date. Leaves and roots were collected, frozen in liquid nitrogen and kept at −80 °C for further quantification of hormones and metabolites.

2.2. Pc inoculation and symptom assessment

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

2.3. In vivo leaf physiological measurements

Gas exchange parameters such as stomatal conductance (g_s) and net leaf photosynthesis (A) were determined using a portable differential infrared gas analyser (IRGA) (Li-6400, Li-Cor INC., Lincoln, NE, USA) connected to a broadleaf chamber (Alcaide et al., 2019). Measurements were performed from 10.00 to 12.00 h at a photosynthetically active radiation (PAR) ranging from 300 to 500 μmol photons m^{-2} s^{-1}. Chlorophyll fluorescence F_v/F_m readings (the maximum quantum yield of PSII) were obtained from 8.00 to 10.00 h with a Multimode

Chlorophyll Fluorometer OS5p device (Opti-Science Inc., USA) after adapting leaves to the dark for 30 min. Leaf chlorophyll content was evaluated through SPAD readings that were obtained at noon with a chlorophyll fluorescence meter (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, Inc., USA). All parameters were assessed in the same leaves of plant. Two apical fully expanded leaves in about 12 plants per clone and treatment were used.

2.4. Leaf and root sampling

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

2.5. Hormone extraction and quantification

The plant hormones salicylic acid (SA), jasmonic acid (JA) and its conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), abscisic acid (ABA), indolacetic acid (IAA) and the non- active derivatives of SA, 2-O-β-d-glucoside (SAG) and salicylate glucose ester (SGE) were determined. Hormone extraction was performed from dried powdered plant tissue following Sanchez-Bel et al. (2016). One milliliter of 10% methanol aqueous solution containing a pool of deuterated and dehydrogenated hormonal internal standards was added to 50 mg of plant tissue. The mixture was vortexed and incubated (30 min, 4 °C) to allow for samples to rehydrate. After mixing in a mixer mill with glass beds (3 min, 30 Hz), samples were centrifuged (30 min, 4 °C, 13.000 rpm) and the supernatant was recovered. The pH of the supernatant was adjusted to 2.5–2.7 with acetic acid and partitioned twice against diethyl ether. The two organic fractions were joined and concentrated in a centrifuge evaporator at room temperature until dryness. Samples were suspended in 1 ml of 10% methanol aqueous solution with 0.01% of HCOOH leading to a final concentration of internal standards of 100 ng ml^{-1}.

Quantification was performed using external calibration curves with each pure chemical standard. Hormones were chromatographically separated in an Acquity Ultra Performance Liquid Chromatography system (UPLC) (Waters, Mildford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex) connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The chromatographic and mass spectrometry conditions were those used by Gamir et al. (2012).

2.6. Metabolite quantification

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

Total polyphenolics and condensed tannins were analyzed by the Folin-Ciocalteu and Porter methods, respectively. 50 mg of powdered tissue were extracted in 1 ml of 70% aqueous methanol by applying a sonic bath for 15 min followed by orbital shaking for one hour. After centrifugation (5 min, 4 °C, 10,000*g*), the supernatant was collected and used to determine total polyphenolics and condensed tannins. For the former, 0.2 ml of the 20-fold diluted extract was reacted with 1 ml Folin reagent and 0.8 ml sodium carbonate, and absorbance was read at 725 nm after 45 min in darkness. For condensed tannins, 45 µl of non-diluted extract were mixed with 1.5 ml Porter reagent, incubated (45 min, 70 °C) and cooled down on ice. Absorbance was measured at 550 nm and procyanidin B2 (Extrasynthese, GenayCedex, France) was used as standard. Flavonoids were analyzed with slight modification to the $AlCl_3$-$NaNO_2$-NaOH protocol described in Pękal and Pyrzynska (2014). Shortly, 20 mg of powdered tissue were washed 4 times with 1.25 ml of 70% aqueous methanol, supernatants were combined and then brought to 10 ml volume with 70% methanol, and frozen overnight (-80 °C). An aliquot was mixed with 5% $NaNO_2$ and left in dark for 6 min. Then 10% $AlCl_3$ was added and incubated for 6 min in dark, and 4% NaOH was added. The solution was shaken and absorbance read at 510 nm with a plate reader (Synergy HT, BioTek Instruments, USA). For quantification, a standard curve of catechin was used. Three technical replicates per pooled sample were analysed and then averaged. Concentrations were expressed on a dry weight (DW) basis.

2.7. Statistical analysis

To analyse time-to-death of plantlets and determine survival time probabilities after inoculation with *Pc*, the Kaplan–Meier estimate was used (Solla et al., 2011). Statistical differences between survival curves were tested by the log rank test. Metabolite and leaf physiological parameters were analyzed with generalized linear mixed models (GLMM), in which the factors 'susceptibility to *Pc*', 'inoculation with *Pc*' and their interaction were considered 'fixed' effects. To account for non independence of observations, the individual plant identity was included as 'random' factor in the models. For each organ, differences between susceptible and resistant interactions were tested through Tukey's HSD test with the Bonferroni correction. Principal component analysis (PCA) was applied to detect patterns of hormone and metabolite variation due to 'susceptibility to *Pc*' and 'inoculation with *Pc*'. Only data corresponding to the hormones showing significant differences between susceptible and resistant interactions in either leaves or roots were included in the analysis. Data were checked for normality and homocedasticity with Shapiro-Wilk and Levene tests, respectively, and statistical analyses were performed in R software environment version 3.4.2 (R Foundation for Statistical Computing).

3. Results

3.1. Symptom development, plant mortality and changes in leaf physiology

Disease progression and changes in leaf physiology were markedly different between the clones (Figs. 1–3). Both clones developed aerial symptoms (leaf wilting and shedding) indicating *Pc*-induced damage (Fig. 1A and B) but, while in plants of the susceptible Cs14 clone the symptoms preceded sudden or progressive plant death, most of plants of the resistant 111-1 clone were able to recover and survive. Accordingly, strong differences in plant mortality were observed between the susceptible and resistant clones: twelve and two out of fifteen plants died along the experiment, respectively (Fig. 2). In the susceptible clone, values of A and g_s 9 days after inoculation were similar as before

Fig. 1. Leaf symptoms of 2-year-old chestnut clones 9 days after soil infestation with *Phytophthora cinnamomi*. In the susceptible clone Cs14 (A), foliage turned yellow, wrinkled and buds dried out, the leaves remaining attached to twigs after plant death. In the resistant clone 111-1 (B), foliage turned brown in an acropetal progression and most plants underwent subsequent defoliation, but buds were viable and new foliage rapidly developed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

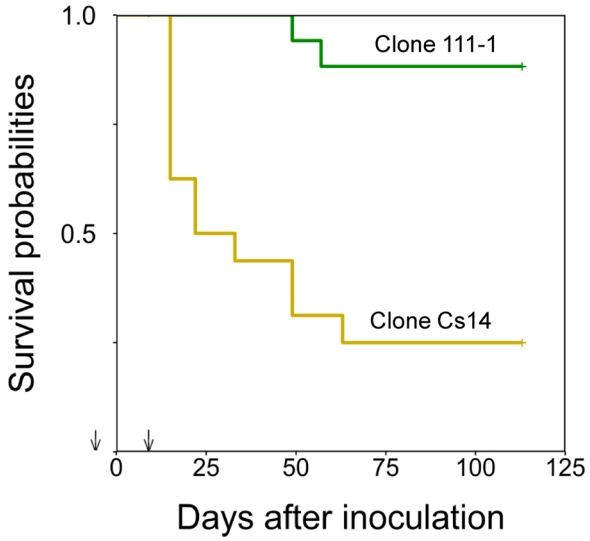

Fig. 2. Plot of survival probabilities showing differences in tree mortality of chestnut clones Cs14 (susceptible) and 111-1 (resistant) after inoculation with *Phytophthora cinnamomi* ($P < 0.001$; log rank test, n = 15 plants per clone). Arrows indicate time of plant measurements and tissue sampling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

inoculation despite that half of plants turned yellow (Fig. 1A). By contrast, in plants of the resistant clone, values of A and g_s significantly decreased (Fig. 3A and B). F_v/F_m readings significantly decreased upon inoculation regardless of clone (Fig. 3C), and SPAD values were unaltered (Fig. 3D).

3.2. Hormonal profile of leaves and roots

In the absence of infection, leaves of both clones displayed higher basal levels of hormones as compared with the roots (Figs. 4 and 1S). The resistant clone showed higher basal SA levels in roots than the susceptible clone while levels of JA, JA-Ile and ABA were similar. However, the resistant clone showed higher basal levels of JA, JA-Ile and ABA in leaves than the susceptible clone (Fig. 4). Constitutive levels of SAG plus SGE and IAA were similar in roots and leaves of both clones (Supplementary Fig. A1).

At day 9 after inoculation both clones responded by decreasing JA levels in leaves and increasing them in roots. Moreover, the resistant clone displayed a dramatic increase of ABA and JA-Ile levels in roots, suggesting an enhanced response to the infection. No other hormonal change in leaves was detected in the susceptible clone after infection and JA-Ile in leaves and roots was close to the detection limit. In the resistant clone, the levels of ABA, JA and JA-Ile in leaves were significantly reduced by infection (Fig. 4B–D). *Pc* had no significant impact on the accumulation of SA, SAG plus SGE and IAA neither in the susceptible nor in the resistant interaction (Fig. 4A and Supplementary Fig. A1).

Principal Component Analysis (PCA) based on content of SA, ABA, JA and JA-Ile in leaves and roots revealed segregation between the susceptible and resistant chestnut clones before *Pc* inoculation (Fig. 5A). PCA also showed a different hormonal profile between clones after infection. A strong impact in the roots of the resistant clone was observed according to the variation of the position of samples before and after infection (Fig. 5A).

3.3. Metabolites in leaves and roots

Before inoculation, the concentration of soluble sugars was higher in leaves of the susceptible clone than in leaves of the resistant clone (Fig. 6A), while proline was similar in both clones (Fig. 6B). Content of total polyphenolics and flavonoids were higher in leaves of the resistant clone (Fig. 6C and D). Roots had similar levels of constitutive metabolites between clones, except for total polyphenolics and condensed tannins, which were higher in the susceptible clone (Fig. 6).

After infection, leaf soluble sugars significantly increased in the resistant clone, whereas no change in root soluble sugars content was detected in none of the clones (Fig. 6A). The susceptible clone responded to *Pc* by increasing the levels of proline and condensed tannins in leaves, and the latter were reduced in roots (Fig. 6B and C). The resistant clone responded to the infection by reducing phenolics and by increasing condensed tannins in leaves (Fig. 6C), whereas in roots the opposite pattern was observed, as kind of compensation. The infection produced a reduction of flavonoids in the leaves of the resistant clone and in the roots of the susceptible clone (Fig. 6D).

Similar to hormones, the PCA based on all the secondary metabolites studied showed a clear separation between non-inoculated susceptible and resistant plantlets (Fig. 5B), indicating a different preformed metabolite profile. This separation seemed to be mainly due to a different partitioning of total polyphenolics and flavonoids between leaves and roots in each clone (Fig. 5B). *Pc* had a strong impact in the

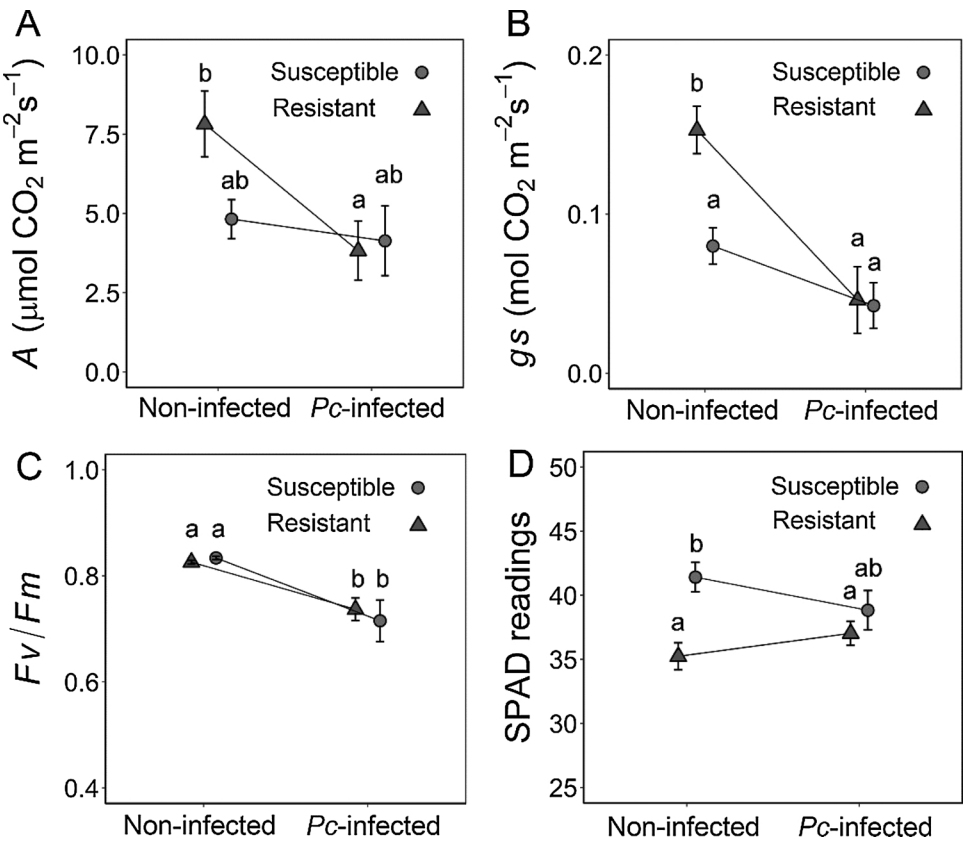

Fig. 3. Effect of experimental inoculation of 2-year-old chestnut clones with *Phytophthora cinnamomi* on *A* (A), g_s (B), *Fv/Fm* (C), and SPAD readings (D). Measurements were done 5 and 9 days before and after inoculation with *Pc*, respectively. Error bars indicate one standard error of the mean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

behaviour of the secondary metabolites taken together since both the susceptible and the resistant clones positioned closer following infection (Fig. 5B). Changes in leaf proline and root total polyphenolics were responsible for the segregation between susceptible and resistant interactions after infection (Fig. 5B), confirming what was observed in Fig. 6.

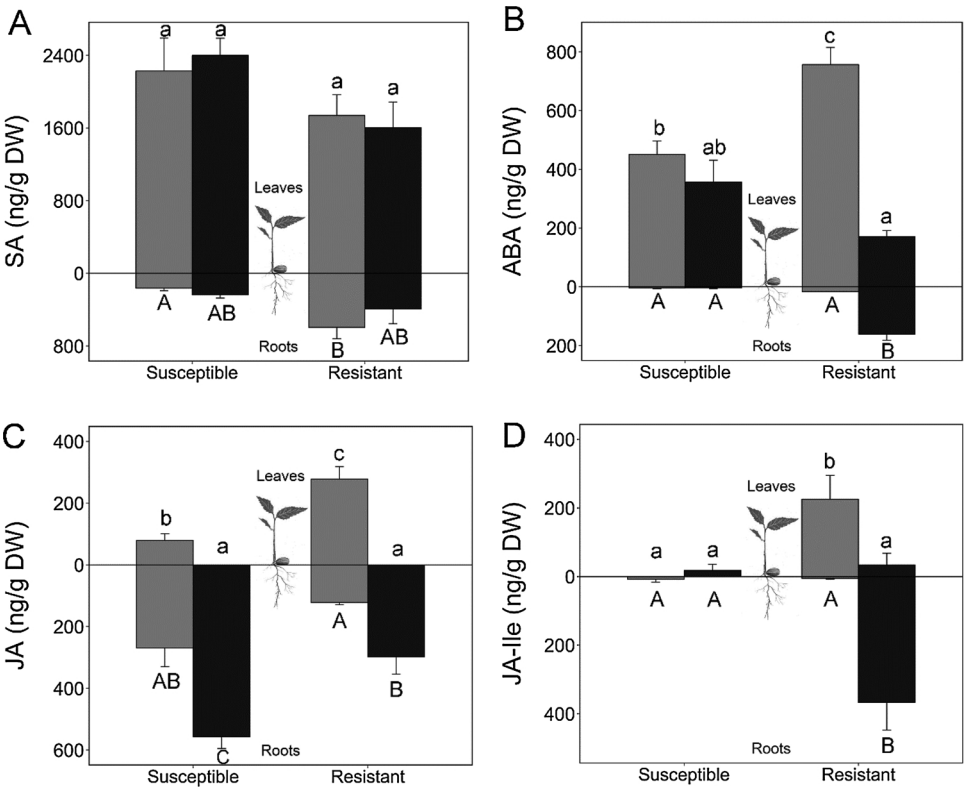

Fig. 4. Hormonal content in leaves (above the zero-line) and roots (below the zero-line) of susceptible and resistant chestnut clones 5 days before (grey bars) and 9 days after (black bars) inoculation with *Phytophthora cinnamomi* for SA (A), ABA (B), JA (C) and JA-Ile (D). Note the distinct scales.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

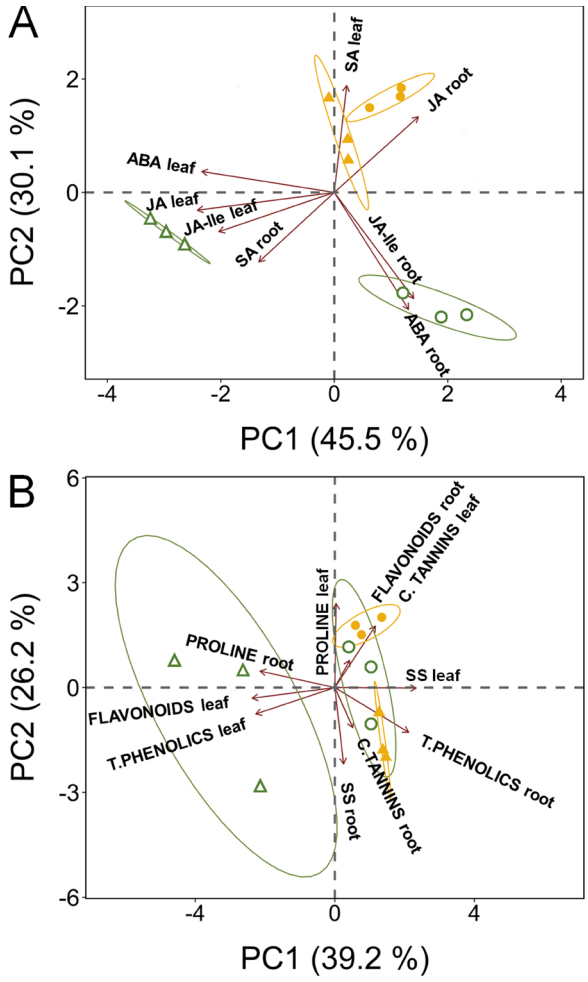

Fig. 5. PCA biplots showing the ordination of non-inoculated (triangles) and inoculated (circles) plantlets of the susceptible (orange filled symbols) and resistant (green empty symbols) clones along the two first principal components defined by (A) the content of SA, JA, JA-Ile and ABA in leaves and roots and (B) all the studied stress-related metabolites in leaves and roots. Names of variables are indicated along with their vectors, and the variance explained by each axis is shown in parenthesis. SS: soluble sugars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The challenge addressed in this work was to provide information about metabolite and hormone contents in roots and leaves of two chestnut genotypes before and after *Pc* infection. The two studied genotypes showed a contrasted defense-related hormone and metabolite profile, confirming the hypothesis that there are constitutive and *Pc*-induced differences in the hormone and metabolite contents of leaves and roots in chestnut.

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

In our study, both chestnut clones showed foliar symptoms nine days after infection but leaf physiology and mortality patterns between clones were drastically different. Gas exchange parameters (g_s and *A*) were not significantly altered by *Pc* infection in the susceptible clone but in the resistant one, where g_s and *A* decreased. This is in contrast to the decreased *gs* and *A* reported by Dinis et al. (2011) 9 days after *Pc* infection in a susceptible chestnut cultivar while no change was observed in a *Pc* resistant chestnut hybrid. Weeks after infection, Maurel et al. (2001a) showed that g_s was substantially reduced in *C. sativa* seedlings under severe and very severe *Pc* root rot. Differences could have been due to the different plant material, inoculation method (inoculation in stem wound *vs* soil infestation) and the *Pc* strain used. However, the strong reduction in g_s and *A* in the resistant clone is in agreement with the strengthened metabolic response to infection observed in *Pc* resistant chestnuts (Dinis et al., 2011; Serrazina et al., 2015; Santos et al., 2017a). Decreased F_v/F_m during both susceptible and resistant interactions reflected changes in the efficiency of PSII of infected chestnuts, which could be attributed to increased photoinhibitory damage in response to *Pc* (Corcobado et al., 2015; Camilo-Alves et al., 2017).

The general overview of the metabolic responses to *Pc* obtained by PCA analysis revealed a different constitutive metabolite and hormone fingerprinting between genotypes, and indicated a highly dynamic response of the resistant clone. This is in agreement with observations in leaf physiology. Results suggest that preformed accumulation of stress-related metabolites (ABA, JA, JA-Ile, flavonoids and total phenolics in leaves, and SA and proline in roots), and a dynamic accumulation of stress-related metabolites (JA-Ile, ABA and total phenolics in roots) are relevant for chestnuts to resist *Pc*. Differences in the hormone profile between the chestnut clones suggests that more research has to be done using more genotypes.

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

This is the first study reporting higher constitutive levels of defence-related hormones associated with resistant chestnut-*Pc* interactions. In roots, 111-1 resistant trees displayed enhanced SA levels as compared with the susceptible clone before infection, which may have a positive influence during the very early stages of pathogenic penetration and disease establishment. SA has been widely described to trigger and coordinate defence against biotrophic pathogens (de Torres-Zabala et al., 2009; Denancé et al., 2013), and is a component of the signal transduction pathway leading to defence reactions against pathogens *via* endochitinase production and H_2O_2 oxidative burst in *C. sativa* (Harfouche et al., 2008). After exogenous SA application, enhanced resistance to *Pc* due to SA accumulation in roots has been reported for *P. americana* and *Lupinus angustifolius* (García-Pineda et al., 2010; Groves et al., 2015).

In our study, JA-Ile was only constitutively detectable in leaves of the resistant clone. JAs-mediated signalling is involved in the production of antifungal metabolites in many plant species including *Castanea* spp. (Antico et al., 2012; Serrazina et al., 2015; Di et al., 2017). Whether the constitutive signalling by JA-Ile played a role in the defensive status of resistant plants is unknown, but this result suggests the use of hormones as potential stress biomarkers, which has been pointed out before but rarely applied (Kosakivska, 2008). The constitutive JA-Ile should be explored across more chestnut taxa and genotypes.

Phenolic compounds including simple phenolic acids and flavonoids are a large class of plant secondary metabolites involved in plant defence against herbivores and pathogens (Martín et al., 2008; Conrad et al., 2017; Gallardo et al., 2019), being also precursors of phytoalexins (Treutter, 2006). The constitutive total phenolics and condensed tannins contents in roots of the susceptible clone, higher than in the resistant one, were not enough to hinder *Pc* at the site of infection. This is in line with root preformed phenol assessments made in *Eucalyptus* spp. before *Pc* inoculation (Cahill and McComb, 1992; Cahill et al., 1993).

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

Unlike the susceptible clone, the resistant one showed a highly dynamic hormonal response by increasing the root JA-Ile and ABA levels following infection. ABA has recently emerged as a key player in

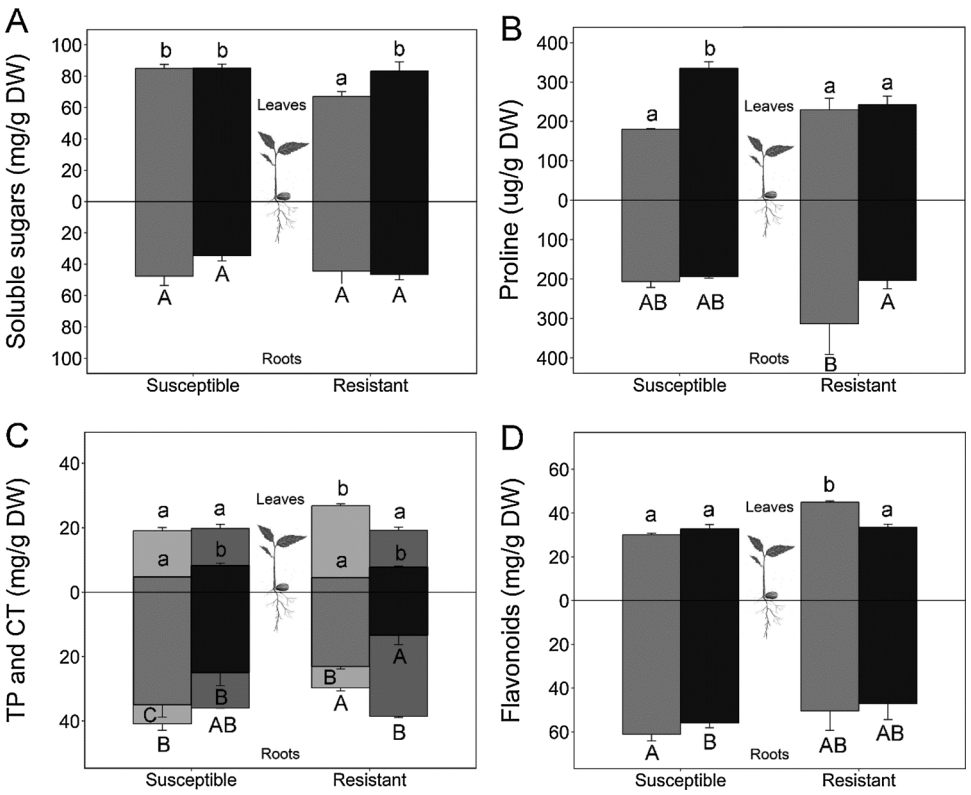

Fig. 6. Secondary metabolite content in leaves (above the zero-line) and roots (below the zero-line) of susceptible and resistant chestnut clones 5 days before (grey bars) and 9 days after (black bars) inoculation with *Phytophthora cinnamomi* for soluble sugars (A), proline (B), total phenolics (TP,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

callose deposition against necrotrophs and was linked to starch degradation and sugar mobilization in infected tissues of plants (Ton et al., 2009; Mohr and Cahill, 2007; Liu et al., 2016; Gamir et al., 2018). Because ABA modulates JAs-induced defences and acts synergistically with JAs on the expression of the MYC genes from the JAs pathway (Proietti et al., 2018), a positive synergistic effect of ABA and JA-Ile on the defence response of plants to *Pc* could be expected. This may explain why plants from the resistant clone recovered while plants from the susceptible clone started to die. Phytohormones regulate plant functioning and defence by regulating the expression of many hormone-responsive genes (Chapman and Estelle, 2009). Therefore, differences in hormonal responses between susceptible and resistant chestnut clones in our study are in line with the more abundant and higher expressed defence genes involved in response to *Pc* of the resistant *C. crenata* as compared with *C. sativa* (Serrazina et al., 2015).

Several studies have shown that defence strategies of plants are trophic dependent (van den Berg et al., 2018 and references therein). For example, a biphasic defence response has been reported to occur in *P. americana* when facing *Pc*: an initial biotrophic plant-based response followed by the enrichment of JA-mediated defence during the necrotrophic phase (van den Berg et al., 2018). Because of the enrichment of JA and JA-Ile in roots after infection, our results suggest a necrotrophic plant-based response occurring during the expression of aerial symptoms. This in turn provides evidence that at 9 days after infection *Pc* has a necrotrophic lifestyle, which is in agreement with histological observations in the *Pc*-susceptible *Quercus ilex* tree (Redondo et al., 2015).

The susceptible genotype was not able to convert increased JA levels into the active form of the hormone (JA-Ile). On the contrary, this step was strongly induced in the resistant 111-1 plants. We ignore if these differences are related to pathogen hijacking of the JA-dependent responses in the susceptible clone, but they should be the objective of further studies. JA-dependent signalling has been described to be a target for pathogen effectors that interact with JAZ repressors in order to avoid effective plant defences (Kazan and Lyons, 2014; Shen et al., 2018). *Pc* (as other *Phytophthora* species) is able to release a plethora of effectors highly efficient in manipulating and hijacking plant host defences (Hardham and Blackman, 2018).

Increases in the concentration of the amino acid proline were reported in plants both under water deficit and pathogen attack (Kaur and Asthir, 2015; Cerqueira et al., 2017). In this study, proline showed a clone- and organ-specific dynamic in response to infection, increasing in leaves in the susceptible clone and decreasing in roots in the resistant clone. In the susceptible clone, proline could have increased due to *Pc*-induced water stress, which is consistent with the wrinkling of leaves observed. Sixteen days after inoculation, larger reductions in leaf water potential in susceptible *C. sativa* (93%) plantlets than in resistant hybrid plantlets (36%) were reported (Dinis et al., 2011). In the resistant clone, decrease of proline in roots could have been occurred by stimulation of the proline-P5C cycle, although this is just a hypothesis. In *A. thaliana*, proline catabolism has been associated to pathogen defence (Cecchini et al., 2011).

The increased concentration of leaf soluble sugars in the 111-1 clone after infection was also reported in a *Pc* resistant hybrid chestnut (Dinis et al., 2011), in agreement to the often reported inducible immunity mechanism in plants known as 'high-sugar resistance' (Reimer-Michalski and Conrath, 2016). The increased concentration of root total phenolics in the 111-1 clone is in agreement with the often observed accumulation of phenolics in roots after *Pc* infection in resistant interactions with *Pc* (Osswald et al., 2014). Phenols can be polymerized into a matrix of lignin, reinforcing cell walls, or act as antimicrobial compounds (Martín et al., 2008; Osswald et al., 2014). The role of condensed tannins in the susceptible and resistant chestnut-*Pc* interactions is difficult to clarify, as they behaved similarly in the two genotypes. However, because of the negative crosstalk with total phenolics observed in leaves and roots of the resistant genotype after infection (Fig. 6C), the involvement of condensed tannins in a dynamic response of total phenolics to resist *Pc* cannot be ruled out. Results suggest that the switch of total phenolics between the roots and leaves during infection in resistant plants may be responsible for a better defensive status of trees. Considering that both families of compounds utilize the same phenolic acid precursors, the degradation of condensed tannins may constitute a dynamic supply of phenolics contributing to this

response.

Reductions of root total flavonoids observed here are in agreement with reductions of the flavonoid epicatechin in roots of susceptible *Persea americana* seedlings upon *Pc* infection (García-Pineda et al., 2010). Given the antioxidant functions that flavonoids perform, our results could indicate decreased antioxidant capacity in roots of the susceptible clone, as shown in susceptible *Eucalyptus* spp. after *Pc* infection (Dempsey et al., 2012).

5. Conclusions

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

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.153030.

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