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Effect of grafting on phenology, susceptibility to *Phytophthora cinnamomi* and hormone profile of chestnut

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ABSTRACT

Ink disease caused by the root-rot pathogen *P. cinnamomi* (*Pc*) threatens European sweet chestnut (*Castanea sativa* Mill.) orchards, and growers increasingly graft susceptible *C. sativa* traditional varieties on *Pc*-resistant hybrid commercial rootstocks. The influence of the scion, the rootstock, and grafting *per se* on the vegetative budburst, growth, susceptibility to *Pc* and defence-related hormone profile of *Castanea spp.* are unknown. In a greenhouse experiment, these effects were evaluated by reciprocally grafting two *Pc* resistant *C. crenata* x *C. sativa* clones and two *Pc* susceptible *C. sativa* clones. Resistance to *Pc* and the hormone content of leaves and roots were rootstock-dependent, and survival rates of susceptible chestnuts strongly increased when grafted on resistant rootstocks. The scion had no effect on the resistance to *Pc* and the hormone profile of leaves and roots of grafted trees, but influenced vegetative budburst and primary growth. Grafting *per se* increased susceptibility to *Pc* and altered the defence-related phytohormone content of trees, especially in resistant rootstocks, but did not influence budburst and growth of trees. Grafting-induced alteration of the constitutive defense-related hormone profile could explain the increased susceptibility of resistant rootstocks to *Pc*. Nine days after infection, a dynamic hormonal response consisting of decreased jasmonates (JA and JA-Ile) in leaves and increased ABA and JA-Ile in roots was observed in resistant chestnuts. This is the first study addressing the role of grafting in modulating resistance to the soil-borne pathogen *Pc* in chestnut trees.

1. Introduction

Grafting is an old horticultural technique in which two plant tissues fuse together, establish a vascular continuity and raise a new composite organism that functions as a whole (Mudge et al., 2009). After grafting, the upper part or scion of a plant grows on the root system or rootstock of another plant. Self-grafted plants use to be fully compatible while the success of grafting different plants (heterografts) diminishes if their phylogenetic distance increases (Mudge et al., 2009). Agricultural applications of grafting include vegetative propagation of cultivars, control of plant size or the use of resistant rootstocks to protect susceptible cultivars from soil-borne plant pathogens (Mudge et al., 2009; Warschefsky et al., 2015; Lazare et al., 2021).

One of the most intriguing aspects of grafting is that the physiological state of a scion is modulated by the rootstock (Warschefsky et al., 2015; Lazare et al., 2021). Rootstocks control the growth, size and morphology of the grafted scion (Sorce et al., 2002; Hooijdonk et al., 2011; Tworkoski and Fazio, 2016), determine aboveground resistance to foliar bacterial and fungal diseases (Russo et al., 2007; Jensen et al., 2012; Chitarra et al., 2017; Flores-León et al., 2021) and modulate tolerance to drought (Camisón et al., 2021; López-Hinojosa et al., 2021). Conversely, the physiological state of a rootstock can also be influenced by the grafted scion (Gautier et al., 2020; Camisón et al., 2021). However, little is known about how the scion influences the tolerance of rootstocks to pathogens as most of studies are addressed from the point of view of the rootstock influencing the scion (Warschefsky et al., 2015; Wang et al., 2017; Gautier et al., 2020). A study in eggplant showed that a susceptible scion increases the susceptibility of a resistant rootstock to

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the soil-borne bacteria *Ralstonia solanacearum* (Huang et al., 2019).

Mobile endogenous factors including mRNA and phytohormones are exchanged between the rootstock and the scion across the graft union thus allowing for long distance communication between both partners (Aloni et al., 2010; Li et al., 2016; Xia et al., 2018). Phytohormones are small signalling molecules that regulate gene expression patterns and coordinate development and defence reactions of plants to abiotic and biotic stress (Kalantidis 2004; Martín et al., 2010, 2012; Guan et al., 2012; Wang et al., 2017). The deployment of a fine-tuned immune response after pathogen attack depends on the maintenance of the plant hormonal homeostasis by means of the crosstalk between hormone signalling pathways (Vos et al., 2013).

Grafting involves wounding a plant and this action *per se* can disrupt its hormonal balance (Wang et al., 2017). In the same way as pathogens alter the hormonal profile of infected trees during attack (de Torres Zabala et al., 2009; Pozo et al., 2015; Camisón et al., 2019a), mechanical damage caused by grafting could also be expected to alter the hormonal profile of trees (Gainza et al., 2015). The effects of grafting on the physiology of a tree may also depend on the graft union incompatibility and wound sealing. It is ignored if the injury caused by grafting may influence the content of defense-related hormones in trees, and if this hypothetical shift translates into changes in the phenology, growth and resistance to pathogens of rootstocks. No studies have addressed how grafting affects resistance of rootstocks to soil-borne pathogens in trees.

Castanea sativa Mill. (Sweet chestnut, *Fagaceae*) is an important tree species in Europe for its edible nuts, and is usually cultivated by grafting profitable *C. sativa* cultivars onto hybrid rootstocks resistant to ink disease (Fernández-Lorenzo and Crecente-Campo, 2010; Alessandri et al., 2022). Ink disease in chestnuts is mainly caused by *Phytophthora cinnamomi* Rands. (*Pc*), an invasive soil-borne pathogen widespread worldwide (Scott et al., 2019). The main strategy to control ink disease in Europe consists of using resistant rootstocks obtained after crossing *C. sativa* with Asiatic *C. crenata* and *C. mollissima* species (Alcaide et al., 2022; Fernandes et al., 2022). At the time grafted chestnuts are planted in orchards, the graft union is not always sealed. Alternatively, grafting is performed in the following months after plantation, in early spring or autumn, coinciding with favourable conditions of *Pc*-infection. In this context, information about the effects of grafting, the scion and the rootstock on the phenology, growth, resistance to *Pc* and hormone content of chestnut is lacking. The objective of this work was to test in chestnut the hypotheses that (i) grafting and (ii) the resistance of the scion to *Pc* influence vegetative budburst, growth, resistance to *Pc* and the hormone profile of the rootstock. A reciprocal grafting experiment using *Castanea* spp. genotypes with contrasted hormone content and susceptibility to *Pc* was performed.

2. Materials and methods

2.1. Plant material

Four *Castanea* spp. genotypes of contrasted susceptibility to *Pc* were used. The susceptible *C. sativa* clones 'Cs12' and 'Cs14' native to the North-Atlantic coast of Spain (Galicia) were selected because grafted well and were characterized in previous studies (Camisón et al., 2019a; Alcaide et al., 2020). The resistant *C. sativa* × *C. crenata* hybrid commercial clones '111-1' and 'PO11' were selected because they showed a high degree of compatibility with Iberian traditional varieties of *C. sativa* (Cuenca et al., 2018) and are widely used as rootstocks in Spain (Camisón et al., 2019a; Alcaide et al., 2020). In August 2015, the four genotypes were propagated *in vitro* according to Vidal et al. (2015) and grown in a greenhouse in 2-L pots with a mixture of peat, vermiculite and perlite (1:1:1). In August 2016, plantlets from each clone were grafted in the main stem by 'green grafting' (Cuenca et al., 2018) in a specialized tree nursery (Grupo TRAGSA-SEPI, Maceda, Spain). Besides non-grafted controls of each clone, twelve scion/rootstock combinations were produced, of which four were self-grafts (one per clone, Cs12/Cs12, Cs14/Cs14, 111-1/111-1 and PO11/PO11 combinations) and eight were heterografts from scions and rootstocks of different susceptibility to *Pc* (Cs12/111-1, Cs12/PO11, Cs14/111-1, Cs14/PO11, 111-1/Cs12, 111-1/Cs14, PO11/Cs12, PO11/Cs14). Non-grafted controls of each clone were also produced. Because of differences in stem diameter between plants (stems should be equal in diameter to ensure fusion of the phloem), grafting heights varied from 23.3 ± 7.9 cm in susceptible rootstocks to 42.2 ± 18.2 cm in resistant rootstocks (Table 1). In October 2016, the plant material was placed in the greenhouse at the Faculty of Forestry of Plasencia, western Spain (40°02N, 6°05W; 374 m a.s.l.), fertilized with Osmocote Pro 3-4M (Osmocote® Pro) at 4 g L^{-1} and grown under optimal watering conditions.

2.2. Experimental design

In January 2017, 9 to 18 plants (mean = 13.5) from each non-grafted clone and scion/rootstock combination (total 212 plants) were arranged in a complete randomized block design of four blocks. The plant material was merged into six groups of plants according to the susceptibility of the scion and the rootstock to *Pc* (see Table 1 and Fig. 1): S and R (susceptible and resistant non-grafted controls), S/S and R/R (susceptible and resistant self-grafts), and R/S and S/R (susceptible and resistant heterografts). The different clones were used as replicates. The experiment was performed in a greenhouse at the Faculty of Forestry of Plasencia, Spain.

To test if grafting influences the vegetative budburst, growth, resistance to *Pc* and hormone profile of chestnut plants (first hypothesis), the self-grafted plant material (S/S, R/R) was compared with non-grafted plant material (S and R controls, Fig. 1). To test if the scion has an effect on the resistance to *Pc* and hormone profile of the rootstock (second hypothesis), S/S and R/R self-grafts were compared with R/S and S/R heterografts (Fig. 1).

2.3. Vegetative budburst and plant growth assessment

Vegetative budburst, primary growth and secondary growth were assessed in all trees of the experiment. Bud development was assessed in April 2017 as follows (Solla et al., 2015): 1 = dormant buds; 2 = swollen buds, but scales closed; 3 = bud scales open and extremities of the first leaf visible at the apex of the buds; 4 = extremities of all leaves out; and 5 = two or more leaves completely expanded. Primary growth of plants was measured in July 2017, before inoculation, from the graft union to the tip of the scion. Secondary growth was obtained before inoculation by differences of stem diameters in January 2017 and July 2017. Stem diameters were obtained by the average of two measurements made orthogonally ca. 5 cm from the ground level, where a white stripe in January was painted. Diameters in July were measured at the stripes.

2.4. Plant resistance to Pc inoculations

In July 6 2017, when the trees were two years old and 108.9 ± 35.8 cm in height (Table 1), all the plant material was inoculated with *Pc* through the soil infestation method. An aggressive single A2 strain (coded Ps-1683) isolated from a diseased *C. sativa* tree in Galicia (43°18′32″N 8°13′57″W, northern Spain) was used. The strain was proven to be highly virulent in *C. sativa* (Camisón et al., 2019a; Alcaide et al., 2020). The inoculum was prepared according to Jung et al. (1996) and incubated for 5 weeks in 1-L Erlenmeyer flasks. Soil infestation was done by mixing 12 ml of the inoculum with the first 3 cm of soil for each plant (Camisón et al., 2019b). After inoculation, the substrate was moistened by slight watering and the day after, plants were flooded for 48 h in chlorine-free water to encourage the production, releasing and dispersal of zoospores. Plant mortality was assessed weekly for four months. At the end of the experiment, in October 2017, *Pc* was successfully re-isolated from inoculated roots.

Table 1
Sample size (n), grafting height, tree height, bud development, growth and mortality after inoculation with *Phytophthora cinnamomi* of the six *Castanea* spp. scion/rootstock combinations used in this study. Combinations are formed according to the inherent resistance of the scion and the rootstock to *Pc*. Accumulated mortality in each group at the end of the experiment (four months after inoculation) is shown. Values are means ± standard deviation of the mean. Different letters indicate significant differences between groups ($P < 0.05$; Tukey's HSD test).

Rootstock	Group[1]	n	Grafting height (cm)	Tree height before inoculation (cm)	Vegetative budburst	Primary growth (cm)	Secondary growth (%)	Mortality after inoculation (%)
Susceptible	S	31	-	97.4 ± 33.8	2.6 ± 1.6a	-	39.6 ± 32.0a	83.8
	S/S	28	23.3 ± 7.9	99.2 ± 33.9	3.1 ± 1.1a	68.2 ± 33.1a	49.0 ± 35.7ab	85.7
	R/S	51	24.0 ± 7.9	95.3 ± 35.1	4.7 ± 0.5b	67.1 ± 31.5a	68.4 ± 46.2b	98.0
Resistant	R	33	-	114.8 ± 36.6	3.2 ± 1.6a	-	64.8 ± 56.7ab	18.7
	R/R	26	37.6 ± 14.3	125.4 ± 31.8	3.2 ± 1.1a	82.5 ± 28.2a	74.6 ± 41.6b	42.3
	S/R	43	42.2 ± 18.2	125.5 ± 30.5	2.9 ± 1.4a	65.4 ± 33.8a	73.0 ± 39.9b	33.3

[1] S and R: susceptible and resistant non-grafted controls; S/S and R/R: susceptible and resistant self-grafts; R/S: resistant scion onto susceptible rootstock; S/R: susceptible scion onto resistant rootstock.

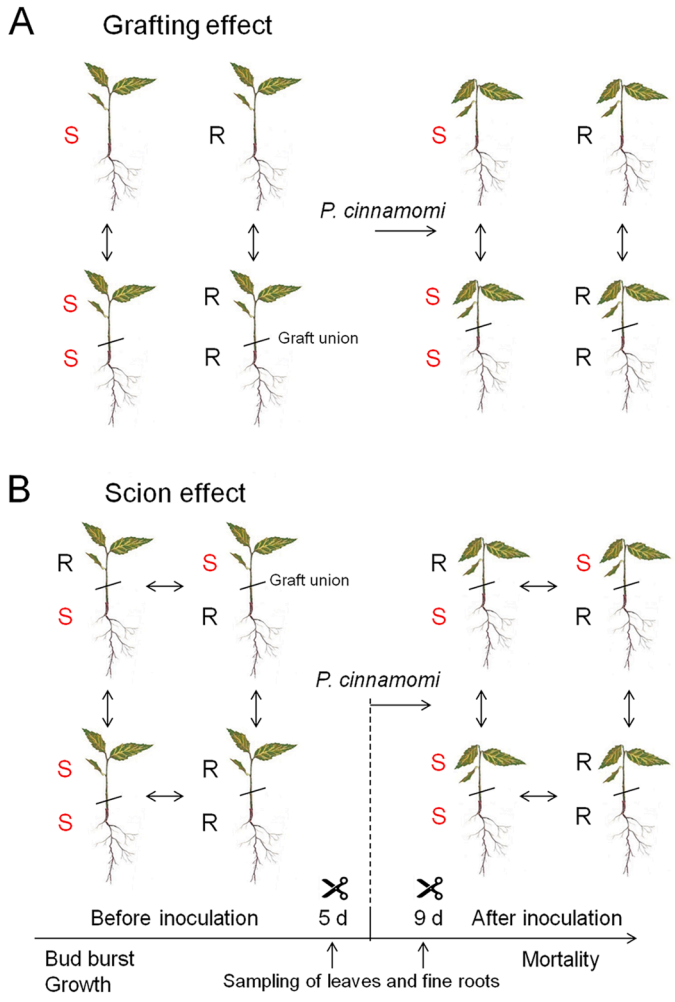

Fig. 1. Experimental design to test if grafting (**A**) and scion (**B**) have an effect on the budburst, growth, resistance to *Phytophthora cinnamomi* (*Pc*) and the hormone profile of *Castanea* spp. grafted trees. Two clones resistant (R) to *Pc* and two clones susceptible (S) to *Pc* were used. Sampling points are indicated with scissors in days (d) before or after inoculation.

2.5. Hormone profile assessment

Fifteen trees from each of the six groups of plants described in Table 1 were sampled for hormone content determination. The hormone and secondary metabolite profiling of 111-1 and Cs14 clones in response to *Pc* was described in a previous study (Camisón et al., 2019a), thus hormone assessment was performed in the groups of plants including this material only. Sampling was performed twice aboveground and belowground, 5 days before inoculation (July 1 2017) and 9 days after inoculation (July 10 2017). Aboveground sampling was done by collecting the apex from one fully-developed leaf at the top of the stem. Belowground sampling consisted of carefully excising five outermost fine root segments from the root ball of each plant. After collection, samples were immediately frozen in liquid N and samples from five different plants within each group were pooled together to get a sample size of three biological replicates per group of plants. Samples were kept at -80 °C until freeze drying with a FreeZone 6 Liter Benchtop (Labconco, Kansas City, USA). Samples were then ground in a ball mill (Mixer Mill MM 400, Retsch, Germany) and passed through a 0.42 mm screen.

Four plant hormones related to signalling of plant defense against pathogens, salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), and its conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) were determined. Hormones were extracted from lyophilized powdered plant tissue. 1 ml 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 at 4°C for 30 min to allow for samples to rehydrate. After mixing in a mixer mill with glass beds (30 Hz, 3 min.), samples were centrifuged (13.000 rpm, 30 min, 4°C) 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 the same as in Gamir et al. (2012).

2.6. Data analysis

To assess the effect of grafting and the scion on the phenology and growth of plants, linear mixed models (LMM) were fitted considering 'vegetative budburst', 'primary growth' and 'secondary growth' values as dependent variables. To assess the effect of grafting and the scion on the resistance to *Pc* of plants, Survival Time Analysis was used (Solla et al., 2011). The Kaplan–Meier estimator was used to obtain plant survival probabilities over time and statistical differences between survival curves were tested with the log-rank test. To estimate the effects of predictors and continuous covariates on survival probabilities of plants, Cox proportional hazards models were fitted to the survival data. In the

models used to analyze the effect of grafting, the variables 'grafting' (with levels 'yes' and 'no'), 'rootstock resistance to *Pc*' (with levels 'R' and 'S', i.e. resistant and susceptible, respectively) and their interaction were considered, while in models to analyze the effect of the scion, the variables considered were 'scion resistance to *Pc*' (with levels 'R' and 'S'), 'rootstock resistance to *Pc*' and their interaction. In all the above models, all mentioned variables were fixed factors, 'block' was a random factor, and 'plant height' was a covariate.

To assess the effect of grafting and the scion on the hormone profile of chestnuts before and after challenging plants with *Pc*, LMM were used. In these models, 'SA', 'ABA', 'JA' and 'JA-Ile' hormones were the response variables, the effects were the same as described above, and the fixed factor 'inoculation with *Pc*' (hereafter '*Pc*' with levels 'yes' or 'no') was included. Models were run separately for data of leaves and fine roots. Differences between means of variables in the study were tested with Tukey's HSD tests with the Benjamini-Hochberg *P*-value correction to control for the false discovery rate. To identify variation patterns in the hormone profile of groups of plants, Principal Component Analysis (PCA) of hormones in leaves and fine roots, before and after *Pc* inoculation was applied. Statistical analyses were performed in R software environment version 3.4.2 (R Foundation for Statistical Computing).

3. Results

3.1. Budburst, growth and mortality in response to Pc of chestnut grafts

Grafting had no effect on budburst and secondary growth of plants (Tables 1 and 2). Cox models indicated that grafting significantly increased tree mortality due to *Pc* (Tables 1 and 2; Fig. 2A) and this effect was stronger in resistant rootstocks than in susceptible rootstocks (significant 'grafting' × 'rootstock resistance to *Pc*' interaction, Table 2; Fig. 2A). As compared to non-grafted controls, mortality at the end of the experiment increased by 2 and 125 % in susceptible and resistant self-grafted rootstocks, respectively. According to a log rank test, survival probabilities over time of R/R trees were marginally lower than in R trees ($P = 0.069$, Fig. 2A).

Vegetative budburst and primary growth were determined by the scion and the 'scion resistance to *Pc*' × 'rootstock resistance to *Pc*' interaction (Table 3). Resistant scions flushed earlier and grew more in height than susceptible scions (4.2 *vs* 3.0 budburst values and 72 *vs* 66 cm, respectively), and differences increased when a susceptible rootstock instead of a resistant rootstock was used (4.7 *vs* 3.1 and 3.2 *vs* 2.9 values for budburst, and 82 *vs* 65 and 68 *vs* 67 cm for height growth, respectively) (significant 'scion resistance to *Pc*' × 'rootstock resistance to *Pc*' interaction, Table 3). No effect of the scion and rootstock on secondary growth was observed (Table 3). Mortality due to *Pc* of chestnut heterografts was not affected by the resistance of the scion but by the resistance of the rootstock (Fig. 2B). R/R and S/R trees had significantly higher survival probabilities over time than S/S and R/S trees (Fig. 2B).

3.2. Hormone profiling of chestnut grafts

Grafting affected the content of SA and JA-Ile in chestnut trees (Table 4). Non-inoculated R/R and S/S trees showed reduced contents of leaf SA, although this effect was significant only in R/R trees (significant 'grafting' x 'rootstock resistance' interaction, Fig. 3A, Table 4). Prior to inoculation, R/R trees showed increased JA-Ile levels in roots (Fig. 3H) (significant 'grafting' x 'rootstock resistance' interaction, Table 4). After inoculation with *Pc*, leaf SA content increased only in grafts (significant 'grafting' x '*Pc*' interaction, Table 4; Fig. 3A).

The most relevant effects when assessing the reciprocal influence of scion *vs* rootstock in the hormone content of chestnut grafts were 'rootstock resistance' and '*Pc*', but not the 'scion resistance' (Table 5). The rootstock determined the SA, ABA and JA-Ile contents in leaves and the ABA and JA-Ile contents in fine roots (Table 5). Before inoculation, leaf JA-Ile levels were highest in scions grafted onto resistant rootstocks and lowest in scions grafted onto susceptible rootstocks (Fig. 3G). After inoculation, the increase in the leaf SA levels in grafts was higher in plants with a resistant rootstock than in plants with a susceptible rootstock (significant 'rootstock resistance' × '*Pc*' effect, Fig. 3A), and the 'scion resistance' did not change the root hormone profile in response to *Pc*. There were significant effects of the 'rootstock resistance', as the levels of root ABA and JA-Ile after infection were high and low in grafts with resistant and susceptible rootstocks, respectively (Fig. 3D, H).

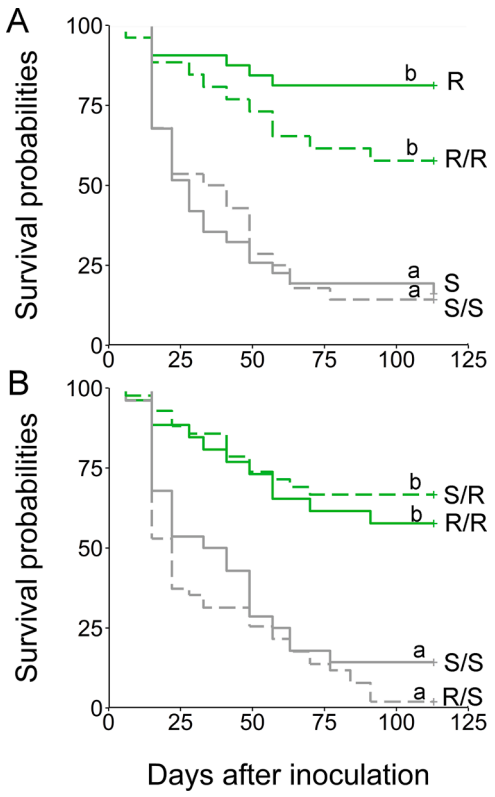

Fig. 2. Kaplan-Meier survival curves showing survival probabilities after inoculation with *Phytophthora cinnamomi* of susceptible (S) and resistant (R) non-grafted controls, self-grafts (S/S and R/R) (**A**) and heterografts (R/S and S/R) (**B**) of *Castanea* spp. trees. Different letters indicate significant differences between survival curves ($P < 0.05$; log rank test).

Table 2
Results of models used to analyze the effect of grafting on the vegetative budburst, growth and mortality after inoculation with *Pc* of *Castanea* spp. grafted trees. Degrees of freedom (df) and *F*-ratios for the fixed factors are shown. Significant *P*-values are indicated in bold.

		Vegetative budburst		Primary growth		Secondary growth		Mortality	
Fixed factors	df	*F*	*P*	*F*	*P*	*F*	*P*	X^2	*P*
Grafting (G)	1	3.06	0.082	164.6	**<0.001**	0.5	0.458	8.8	**0.009**
Rootstock resistance to *Pc* (R_{Pc})	1	0.3	0.629	0.8	0.935	1.2	0.378	13.6	**<0.001**
G × R_{Pc}	1	0.9	0.331	0.5	0.463	0.2	0.619	5.7	**0.046**
Covariate									
Plant height	1	20.8	**<0.001**	111.6	**<0.001**	16.9	**<0.001**	18.5	**<0.001**

Table 3
Results of models used to analyze the reciprocal effect of scion *vs* rootstock on the vegetative budburst, growth and mortality after inoculation with *Pc* of *Castanea* spp. grafted trees. Degrees of freedom (df) and *F*-ratios for the fixed factors are shown. Significant *P*-values are indicated in bold.

		Vegetative budburst		Primary growth		Secondary growth		Mortality	
Fixed factors	df	*F*	*P*	*F*	*P*	*F*	*P*	X^2	*P*
Scion resistance to *Pc* (S_{Pc})	1	27.3	**<0.001**	5.8	**0.017**	2.0	0.153	0.9	0.235
Rootstock resistance to *Pc* (R_{Pc})	1	1.2	0.381	7.1	0.083	0.1	0.796	30.7	**<0.001**
$S_{Pc} \times R_{Pc}$	1	16.6	**<0.001**	4.1	**0.042**	3.0	0.084	1.1	0.123
Covariate									
Plant height	1	1.8	0.173	129.6	**<0.001**	19.0	**<0.001**	**20.2**	**<0.001**

Table 4
Results of models used to analyze the effect of grafting on the hormone content in leaves and fine roots of *Castanea* spp. grafted trees before and after inoculation with *Phytophthora cinnamomi*. Significant *P*-values are indicated in bold.

			SA		ABA		JA		JA-Ile	
Organ	**Effect**	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Leaves	Grafting (G)	1	74.0	**<0.001**	0.1	0.704	0.0	1.000	0.0	0.982
	Rootstock resistance to *Pc* (R_{Pc})	1	6.0	**<0.001**	0.0	0.997	0.0	1.000	14.7	**<0.001**
	Inoculation with *Pc* (*Pc*)	1	0.1	0.661	15.2	**<0.01**	76.3	**<0.001**	138.5	**<0.001**
	$G \times R_{Pc}$	1	32.5	**<0.001**	2.3	0.142	2.4	0.140	0.8	0.350
	$G \times Pc$	1	37.6	**<0.001**	0.6	0.423	0.0	0.991	21.1	**<0.001**
	$R_{Pc} \times Pc$	1	0.4	0.496	0.5	0.473	0.0	0.985	84.2	**<0.001**
	$R_{Pc} \times G \times Pc$	1	5.2	**<0.05**	8.4	**<0.05**	2.3	0.143	4.2	**<0.05**
Fine roots	Grafting (G)	1	74.0	**<0.001**	1.1	0.288	1.0	0.315	27.3	**<0.001**
	Rootstock resistance to *Pc* (R_{Pc})	1	6.0	**<0.05**	7.5	**<0.01**	17.5	**<0.001**	10.7	**<0.01**
	Inoculation with *Pc* (*Pc*)	1	0.1	0.661	6.2	**<0.05**	21.8	**<0.001**	1.7	0.189
	$G \times R_{Pc}$	1	32.5	**<0.001**	6.1	**<0.05**	0.4	0.511	4.8	**<0.05**
	$G \times Pc$	1	37.7	**<0.001**	1.3	0.240	0.7	0.389	0.3	0.568
	$R_{Pc} \times Pc$	1	0.4	0.496	0.3	0.547	0.9	0.355	8.2	**<0.01**
	$R_{Pc} \times G \times Pc$	1	5.2	**<0.05**	6.3	**<0.05**	1.0	0.326	11.4	**<0.001**

Regardless of the rootstock, the levels of JA and JA-Ile in leaves dropped almost to zero after infection (significant '*Pc*' effect, Table 5, Fig. 3E, G). Hormonal changes induced by grafting and *Pc*-infection are summarized in Fig. 4.

3.3. PCA overview of the constitutive and Pc-induced hormonal profiles of chestnuts

The impact of grafting on the hormonal profile of trees was dependant on the resistance of the rootstock and the organ (Fig. 5A, B). Susceptible non-grafted and grafted trees (S and S/S) segregated in leaves due to differences in SA and ABA contents (Fig. 5A). Resistant non-grafted and grafted trees (R and R/R) segregated in roots due to differences in JA and JA-Ile contents (Fig. 5B). PCA also revealed segregation between trees attributable to the resistance of rootstocks to *Pc*: contents of JA and JA-Ile in leaves and SA and ABA in roots were responsible for segregation between susceptible and resistant rootstocks (Fig. 5A and B). After *Pc* inoculation, segregation between susceptible and resistant groups of trees was only observed for fine roots (Fig. 5C and D), associated to variations in the JA-Ile and ABA content (Fig. 5D).

4. Discussion

4.1. The scion determines vegetative budburst and primary growth, but does not influence the susceptibility to Pc and hormone profile of grafted chestnuts

The moment vegetative budburst occurs and its modulation by grafting practices is relevant for horticulture, especially in orchards under continental climate because of the high sensitivity of chestnuts to late spring frosts. Because of their Asian germplasm, resistant hybrid chestnuts show an earlier budburst, flowering and fruiting phenology than native *C. sativa* trees (Serdar et al., 2011; Larue et al., 2021). Vegetative budburst was affected here by the scion and its interaction with the rootstock, and R scions grafted on S rootstocks had the earliest budbreak. In grafted nut trees, budburst phenology is known to be a complex trait which often depends on specific scion-rootstock combinations (Pica et al., 2021; Vahdati et al., 2021). Results suggest that the use of *Pc* resistant hybrid rootstocks does not increase exposure of susceptible *C. sativa* scions to late frosts.

Very little is known about size-controlling processes in grafted *Castanea* spp. trees. Literature pinpoints the major control of tree height by rootstocks, but scions are also known to determine tree canopy height and habit (Sorce et al., 2002; Warschefsky et al., 2015; Tworkoski and Fazio, 2016). In our experiment, primary growth of grafts was influenced by the scion and not by the rootstock. Similar results were found by Tworkoski and Fazio (2016) in a greenhouse experiment with well-known size-controlling apple rootstocks. However, it has been shown that size-controlling processes of rootstocks take time to occur and are influenced by growing conditions affecting root development, different in the greenhouse and in the field, and dependent on the size of the pot (Tworkoski and Fazio, 2016),

Grafting resistant scions on susceptible rootstocks did not increase the resistance of plants to *Pc* and grafting susceptible scions onto resistant rootstocks did not increase the susceptibility of plants to *Pc*. During the interaction of trees with the pathogen, the genetic background of the rootstock prevailed over possible above-ground effects derived from the scion. This result contrasts with the study by Huang et al. (2019) in eggplant, where a susceptible scion increased susceptibility of a resistant rootstock to *Ralstonia solanacearum*. Our findings demonstrate that resistance to ink disease in grafted chestnuts requires resistance only in the rootstock, reasonably because *Pc* recognition and defense reactions occur belowground (Redondo et al., 2015; Camisón et al., 2019a).

Defense-related phytohormones regulate plant responses to pathogens by modulating the expression patterns of transcription factors and genes encoding for e.g. phytoalexins and pathogenesis-related (PR) proteins. In this work, the scion had no significant effect on the constitutive and *Pc*-induced leaf and root hormone profile of trees which was rather rootstock-dependent. This may explain the lack of effect of the 'scion resistance' on the survival of grafted trees when challenged with

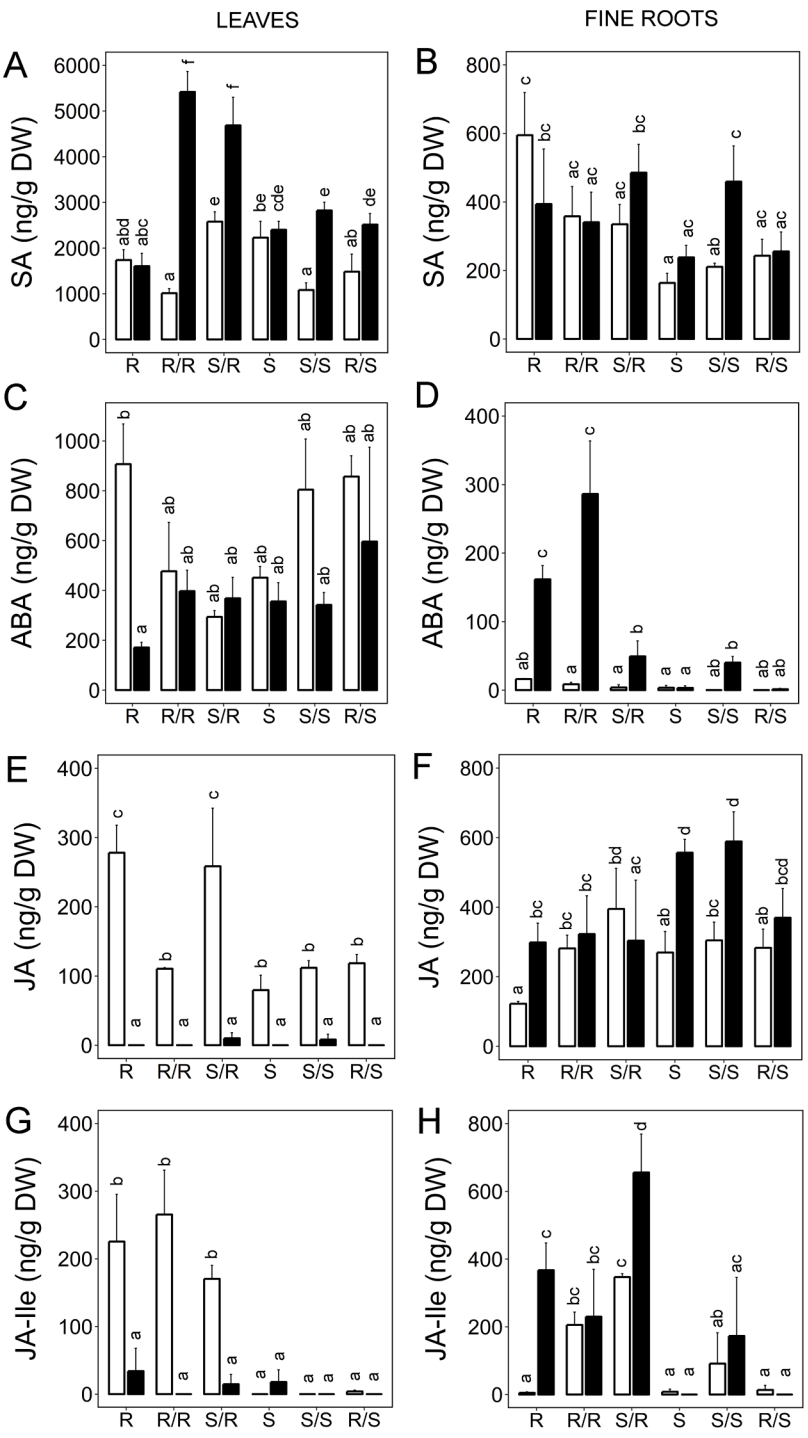

Fig. 3. Salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) contents in leaves (**A, C, E, G**) and fine roots (**B, D, F, H**) of *Castanea* spp. trees before (white bars) and after inoculation with *Phytophthora cinnamomi* (*Pc*) (black bars). Trees included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

Pc. In a previous work (Camisón et al., 2019a), we pointed out JA-Ile as a potential biomarker for *Pc* resistance in chestnut due to its constitutive presence in leaves of the 111-1 clone. In the present work, the use of reciprocal grafts between susceptible and resistant chestnuts demonstrated that over accumulation of JA-Ile in leaves of the clone 111-1 was a graft-transmissible trait dependent on the root system.

4.2. Grafting does not affect vegetative budburst and growth, but alters the susceptibility to Pc and the hormone profile of grafted chestnuts

In the last decades, there has been a significant advance in rootstock breeding and development of tree nut crops including chestnuts (Vahdati et al., 2021) calling to investigate how grafting *per se* influences the physiology of rootstocks. We found no significant effect of grafting on vegetative budbreak and growth of chestnuts. However, in a previous study we observed a delay in budburst of *C. sativa* due to grafting (Camisón et al., 2021).

To the best of our knowledge, this is the first report of increased susceptibility to a soil-borne pathogen in a tree species due to the fact of being grafted. The graft unions of the chestnut grafts used in this study were not completely healed at the time of inoculation (Fig. S1), and therefore incomplete wound healing and vascular discontinuity likely played a role. This would explain observations of survival failure in orchards of chestnut trees recently grafted. The incomplete wound

Table 5
Results of models used to analyze the reciprocal effect of scion *vs* rootstock on the hormone content in leaves and fine roots of *Castanea* spp. grafted trees before and after inoculation with *Phytophthora cinnamomi*. Significant *P*-values are indicated in bold.

			SA		ABA		JA		JA-Ile	
Organ	**Effect**	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Leaves	Scion resistance to *Pc* (S_{Pc})	1	0.6	0.446	1.0	0.313	3.4	0.083	1.3	0.258
	Rootstock resistance to *Pc* (R_{Pc})	1	37.1	**<0.001**	4.5	**<0.05**	2.6	0.122	40.3	**<0.001**
	Inoculation with *Pc* (*Pc*)	1	95.1	**<0.001**	2.1	0.162	45.3	**<0.001**	36.6	**<0.001**
	$S_{Pc} \times R_{Pc}$	1	0.9	0.343	0.0	0.851	3.3	0.087	1.2	0.279
	$S_{Pc} \times$ *Pc*	1	2.7	0.113	0.0	0.926	2.0	0.171	2.5	0.131
	$R_{Pc} \times$ *Pc*	1	15.4	**<0.01**	2.0	0.168	2.5	0.132	35.2	0.588
	$S_{Pc} \times R_{Pc} \times$ *Pc*	1	10.0	**<0.01**	0.5	0.484	3.1	0.095	2.3	0.143
Fine roots	Scion resistance to *Pc* (S_{Pc})	1	2.0	0.172	0.25	0.615	1.4	0.246	1.0	0.296
	Rootstock resistance to *Pc* (R_{Pc})	1	2.9	0.106	35.4	**<0.001**	0.7	0.394	29.5	**<0.001**
	Inoculation with *Pc* (*Pc*)	1	3.6	0.072	40.8	**<0.001**	1.3	0.266	0.0	0.910
	$S_{Pc} \times R_{Pc}$	1	0.5	0.811	19.2	**<0.001**	0.2	0.606	8.4	**<0.01**
	$S_{Pc} \times$ *Pc*	1	3.8	0.066	3.2	0.072	0.0	0.816	0.1	0.711
	$R_{Pc} \times$ *Pc*	1	0.3	0.541	3.2	0.222	2.2	0.150	3.8	**<0.05**
	$S_{Pc} \times R_{Pc} \times$ *Pc*	1	0.1	0.744	12.6	**<0.001**	1.4	0.252	1.9	0.163

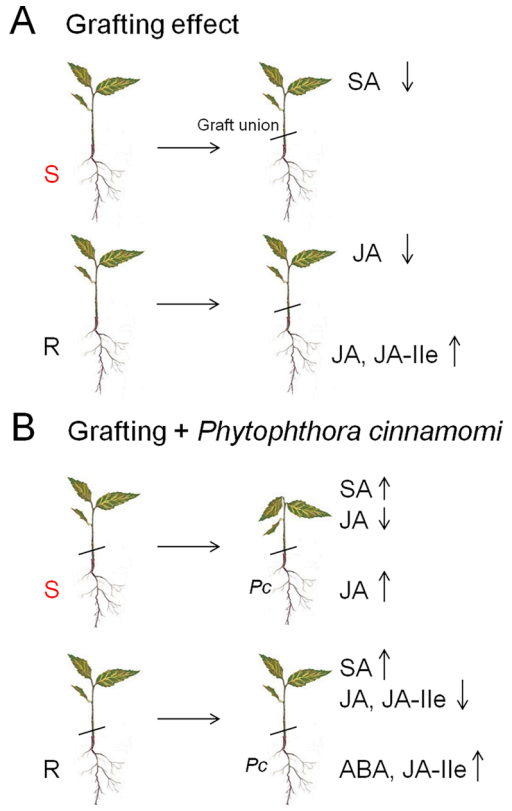

Fig. 4. Summary of the hormonal changes induced by grafting and infection with *Phytophthora cinnamomi* (*Pc*) in leaves and fine roots of susceptible (S) and resistant (R) chestnut grafts. Changes in the constitutive content of hormones relative to non-grafted controls are depicted in (**A**), while the hormone response to infection observed in grafts is depicted in (**B**). The effect of grafting was evaluated eleven months after grafting, and the effect of *Pc* in grafted trees was evaluated nine days after inoculation. Trends of increasing or decreasing hormone contents in leaves and fine roots are indicated by up and down arrows, respectively.

healing in the studied plants could induce biochemical changes (e.g. phenolic content) during the healing process (Irisarri et al., 2015; Gainza et al., 2015) which could affect the defense responses of chestnuts to *Pc*. Also, vascular discontinuity can hamper transport of water, nutrients, metabolites and signals throughout the graft (Sorce et al., 2002; Kalantidis, 2004; Martínez-Ballesta et al., 2010). This result might suggest that, in *Pc*-infected chestnut orchards, susceptibility to *Pc* of grafted resistant rootstocks may increase during the following months after grafting.

This study provides evidence for the capacity of grafting to modify the constitutive and *Pc*-induced defense-related hormone profile of chestnuts. Grafting-induced alterations of the fine-tuned hormonal balance might have affected susceptibility to *Pc* of chestnut clones. Alteration of the constitutive hormonal profile by grafting was stronger in roots of resistant trees, which could explain the higher impact of grafting on survival of resistant chestnuts. Jasmonates (JA and JA-Ile) are induced in plants in response to wounding (Pieterse et al., 2012; Wasternack and Hause, 2013), and we observed a relevant accumulation of JA-Ile in fine roots of resistant grafts before inoculation. This is consistent with the assumption that grafting induces a wounding effect. A recent study (Santolamazza-Carbone et al., 2021) reported the effect of grafting on the root ectomycorrhizal (ECM) fungal community of the resistant chestnut clone 111-1. Changes in the ECM colonization rate and the ECM species composition occurred in the field in grafted clone 111-1. Changes in the microbiome of trees are known to influence the mortality of trees if soils are infested with *Pc* (Branzanti et al., 1999; Corcobado et al., 2015; Ruiz Gómez et al., 2019).

In our study, inoculation with *Pc* triggered accumulation of SA in leaves of grafted chestnuts. This resembles Systemic Acquired Resistance (SAR), a 'whole plant' resistance phenotype occurring following a localized exposure to a pathogen characterized by the accumulation of SA in remote tissues (Reimer-Michalski and Conrath, 2016). While the development of SAR correlates to the up-regulation of PR proteins (Fu and Dong, 2013), it is unknown if the induction of leaf SA by *Pc* plays a role in chestnut defence against the pathogen, which deserves further attention. Serrazina et al. (2015) reported differentially expressed genes related to SAR in roots of both *C. sativa* and *C. crenata* within seven days after inoculation with *Pc*.

5. Conclusions

This is the first study addressing the effects of grafting on the hormone profile and resistance to the soil-borne pathogen *Pc* in chestnut. Grafting *per se* increased susceptibility to *Pc* and altered the hormone profile of resistant chestnuts. Alteration of the constitutive hormonal profile by grafting could be mediated by a wounding effect, as grafted resistant rootstocks showed increased root JA-Ile levels before infection. Coupled with other factors, alteration of the constitutive hormonal balance could underlie the increased susceptibility of grafted chestnuts to *Pc*. Likely, the grafting effect vary over time and disappear after complete graft union healing. The scion had no effect on the resistance to *Pc* of rootstocks and on the hormone profile of roots of grafted chestnut trees, and thus resistance to *Pc* in chestnut is fully dependant on

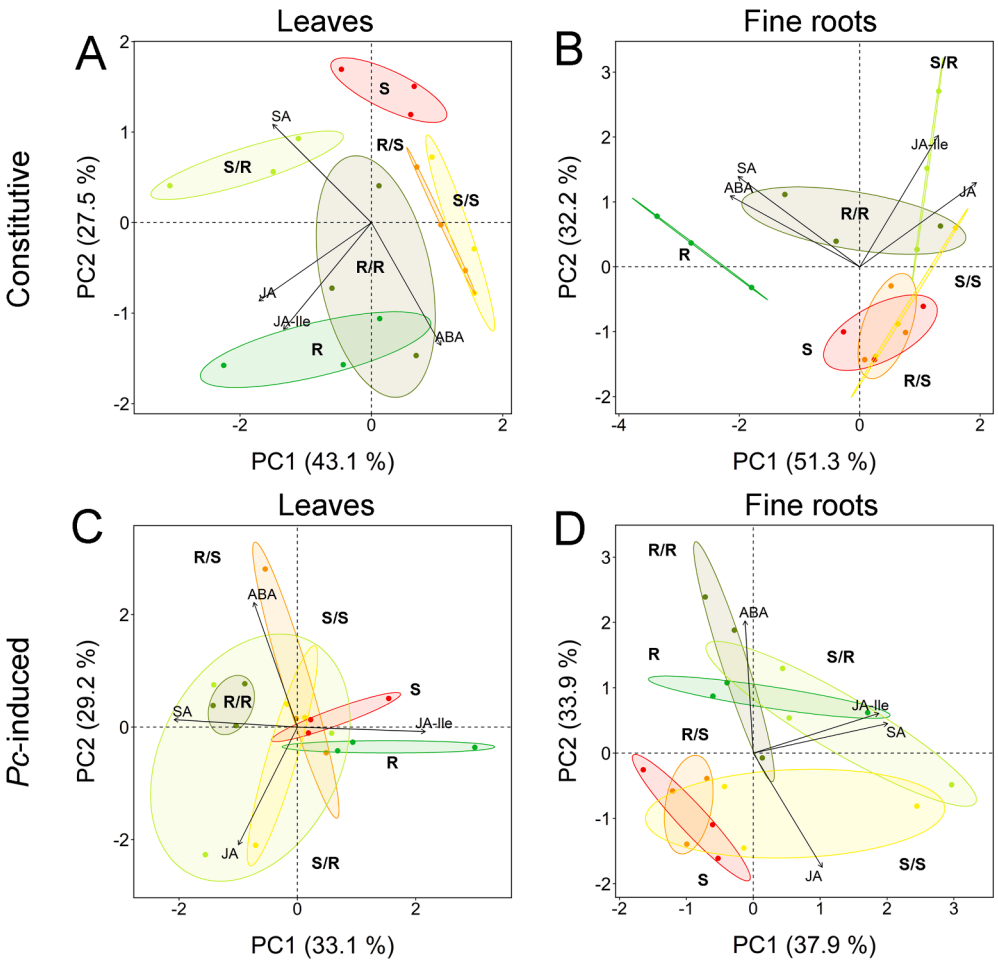

Fig. 5. PCA biplots showing the ordination of susceptible (S) and resistant (R) non-grafted controls, self-grafts (S/S and R/R) and heterografts (R/S and S/R) of *Castanea* spp. trees in the two first principal components defined by the (**A**) constitutive hormone content in leaves, (**B**) constitutive hormonal content in fine roots, (**C**) *Phytophthora cinnamomi*-induced hormone content in leaves, and (**D**) *Phytophthora cinnamomi*-induced hormone content in fine roots. Explained variance by each axis is shown in parenthesis. SA: salicylic acid; ABA: abscisic acid; JA: jasmonic acid; JA-Ile: jasmonic acid-isoleucine.

the rootstock. Nine days after inoculation, main hormonal changes associated to *Pc* infection in grafted resistant chestnuts consisted of increased levels of SA and decreased levels of JA in leaves, and increased levels of ABA and JA-Ile in roots.

CRediT authorship contribution statement

A. Solla and M. Á. Martín conceived and designed the study; A. Camisón performed the experiment, sampling and greenhouse measurements; P. Sánchez-Bel and V. Flors helped A. Camisón with the hormone analysis; M. Á. Martín, E. Cubera and A. Solla provided funding; and A. Camisón and A. Solla wrote the draft of the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111789.

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