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Research paper

No priming, just fighting—endophytic yeast attenuates the defense response and the stress induced by Dutch elm disease in *Ulmus minor* Mill.

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One century after the first report of Dutch elm disease (DED), there is still no practical solution for this problem threatening European and American elms (*Ulmus* spp.). The long breeding cycles needed to select resistant genotypes and the lack of efficient treatments keep disease incidence at high levels. In this work, the expression of defense-related genes to the causal agent of DED, *Ophiostoma novo-ulmi* Brasier, was analyzed in in vitro clonal plantlets from two DED-resistant and two DED-susceptible *Ulmus minor* Mill. trees. In addition, the effect of the inoculation of an endophytic pink-pigmented yeast (*Cystobasidium* sp.) on the plant's defense system was tested both individually and in combination with *O. novo-ulmi*. The multifactorial nature of the resistance to DED was confirmed, as no common molecular response was found in the two resistant genotypes. However, the in vitro experimental system allowed discrimination of the susceptible from the resistant genotypes, showing higher levels of oxidative damage and phenolic compounds in the susceptible genotypes after pathogen inoculation. Inoculation of the endophyte before *O. novo-ulmi* attenuated the plant molecular response induced by the pathogen and moderated oxidative stress levels. Niche competition, endophyte–pathogen antagonism and molecular crosstalk between the host and the endophyte are discussed as possible mechanisms of stress reduction. In sum, our results confirm the complex and heterogeneous nature of DED resistance mechanisms and highlight the possibility of using certain endophytic yeasts as biological tools to improve tree resilience against biotic stress.

Keywords: biocontrol, *O. novo-ulmi*, pink-pigmented yeast, resistance, *Ulmus*.

Introduction

One hundred years ago, the Dutch phytopathologist Bea Schwarz, led by Professor Johanna Westerdijk (Boonekamp et al. 2019), described for the first time the Dutch elm disease (DED) (Schwarz 1922). Since then, European elm populations have suffered a massive reduction due to the constant threat of this vascular wilt disease caused by the ascomycete fungi *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier. Bark beetles are responsible for transmitting fungal spores into healthy elm trees, where they germinate and spread into the xylem vessels inducing their blockage and embolism. Thus, water transport is critically hindered resulting in foliage wilting and tree death (Brasier 1991). Location, characterization and propagation of pure *Ulmus minor* Mill. germplasm have become a priority to conserve and restore lost elm populations (Martín, Sobrino-Plata et al. 2019*b*). In Spain, large efforts have been invested to breed resistant *U. minor* genotypes by screening plant materials from all across the country. To date, seven *U. minor* genotypes have been registered as resistant base materials for forest use (Martín et al. 2015). These resistant

genotypes are also valuable materials for elucidating the basis of *U. minor* resistance to DED (Li et al. 2016, Pita et al. 2018).

Chemical and anatomical factors partially explain DED resistance. For instance, the accumulation of lignin, suberin and mansonones in response to *O. novo-ulmi* infection is usually higher in DED-resistant than in DED-susceptible genotypes (Overeem and Elgersma 1970, Jeng et al. 1983, Duchesne et al. 1986, Martin et al. 2005, Martín et al. 2008), as well as the constitutive proportion of cellulose and hemicellulose in xylem tissues (Li et al. 2016). In contrast, DED-susceptible genotypes tend to possess wider xylem vessels and a higher proportion of large vessels than resistant ones, enabling fungal dispersal throughout the plant (Solla and Gil 2002, Martín et al. 2013*a*), although recent research has shown that some resistant genotypes also form wide vessels (Martín et al. 2021). Beyond these differences, other key resistance traits are also encrypted in the genetic code of each genotype in an as yet unknown manner. The genetic basis of the *U. minor* response to *O. novo-ulmi* has just begun to be elucidated by using classical and novel 'omic' technics, such as 454 sequencing (Perdiguero et al. 2015) and RNA sequencing (Chano et al., unpublished results). A recent work by Perdiguero et al. (2018) described the molecular responses activated over time upon *O. novo-ulmi* infection in a highly DED-susceptible clone. The results pointed to defense mechanisms that are regulated by the salicylic acid (SA) pathway. Salicylic acid-dependent defense responses are typically triggered by biotrophic pathogens (Pieterse et al. 2009). *Ophiostoma novo-ulmi* is considered a hemi-biotrophic pathogen, because it has an initial biotrophic phase during the vascular colonization of the xylem, followed by a necrotrophic phase during more advanced disease stages (Martín et al. 2012, Sherif et al. 2017). Salicylic acid-dependent defenses can be activated systemically to distal parts of the plant through molecules such as methyl-SA or glycerol-3-phosphate, where they play a role in the activation of systemic acquired resistance (Fu and Dong 2013). Local and systemic defense activation is associated with the accumulation of pathogenesis-related (PR) proteins, some of which possess antimicrobial activities against a broad range of pathogens (Bari and Jones 2009).

Beneficial microbes provide plants with higher phenotypic plasticity to change environments, including biotic and abiotic stresses (Liu et al. 2020). Enhancement of nutrient acquisition and defensive metabolism in the plant is among the main factors involved in stress tolerance mediated by beneficial microbes (Vandenkoornhuyse et al. 2001, Gehring et al. 2017). In this regard, plants can recognize microbial stimuli and microbial-associated molecules by certain transmembrane receptors. Recognition translates into the activation of an induced systemic resistance boosted by the cross-talking among different hormone pathways (Van Wees et al. 2008, Morán-Diez et al. 2012). The derived molecular signals spread toward distal parts inducing a 'primed' state in the plant and preparing its immune system to better combat subsequent pathogen attacks. The priming effect is characterized by a faster and stronger activation of defenses upon infection, resulting in an enhanced resistance level without a constant activation of the defense pathways (Martínez-Medina et al. 2016). Fungal endophytes are among the wide variety of microorganisms inducing a priming effect. For example, *Trichoderma* spp. and *Piriformospora indica* have shown the ability to reduce pathogen incidence in tomato and barley, respectively (Waller et al. 2008, Martínez-Medina et al. 2013, Pescador et al. 2022).

Apart from the molecular mechanisms induced in the plant by fungal endophytes, increased resistance to pathogens can be also exerted by direct inhibition of pathogen growth in plant tissues (Terhonen et al. 2019). Fungal endophytes of forest trees are receiving growing interest as biocontrol agents against a wide range of pathogens (Witzell et al. 2014, Romeralo et al. 2015, Rabiey et al. 2019). Concerning elms, DED-resistant plant material has been used to assess elm microbiome composition and disentangle differences in fungal communities between resistant and susceptible genotypes (Martín et al. 2013*b*). Recently, an association has been reported between the abundance of certain members of the *U. minor* core mycobiome and the degree of host resistance to DED (Macaya-Sanz et al. 2020). Several endophytic fungi have been isolated from mature, DED-resistant trees with the aim of finding those with potential implication in host resistance to DED. Among these isolated endophytes, a pink-pigmented yeast of the genus *Cystobasidium* was characterized and classified as a plant growth-promoting yeast (Joubert and Doty 2018). This isolate mitigated abiotic stress in the host plants (Martínez-Arias et al. 2021).

Furthermore, novel and low-cost screening techniques with early developed in vitro elm plants have been developed to shorten the long breeding cycles and the large experimental areas required with the current elm breeding methods (Martín et al. 2019*a*, Martínez-Arias et al. 2021*b*). By using this in vitro technique, this work aimed to evaluate: (i) the early defense response of resistant and susceptible elm genotypes to *O. novo-ulmi* and (ii) how this response is produced in elms inoculated with a core endophyte (*Cystobasidium* sp.) before exposure to *O. novo-ulmi*. It was hypothesized that a different molecular response would be detected according to the DED-resistance level of the genotype during early developmental stages. Moreover, given the potential of *Cystobasidium* sp. as plant growth-promoting yeast and its classification within a group of endophytes associated with DED-resistant elm genotypes (Macaya-Sanz et al. 2020), it was also postulated that its presence in elm tissues could trigger enhanced defense responses against *O. novo-ulmi*.

Materials and methods

Plant material

Four Spanish *U. minor* genotypes were used for the experiment. The genotypes M-DV2.3 (Dehesa de Amaniel, Madrid) and V-AD2 (Ademúz, Valencia) were selected as representatives of resistant genotypes (i.e., <10% of the crown showing foliar wilting 60 days after inoculation with *O. novo-ulmi*), while the genotypes M-DV1 (Dehesa de la Villa, Madrid) and VA-AP38 (Arrabal del Portillo, Valladolid) were selected for being highly susceptible (i.e., >80% of leaf wilting) according to the previous susceptibility tests performed by the Spanish elm breeding program (Martín et al. 2015). VA-AP38 belongs to the Atinian clone (*U. procera*) a highly DED-susceptible genotype spread mostly throughout Spain and England since Roman times (Gil et al. 2004).

In vitro plant production was performed as described by Martín et al. (2019*a*). First, buds from adult trees were sterilized with 70% ethanol for 3 min followed by 10 min incubation in 1.5% sodium hypochlorite, and then rinsed three times in distilled sterilized water. For the stimulation of aerial organs, buds were cultured in Driver and Kuniyaki Walnut (DKW) basal medium (pH 5.7; Driver and Kuniyuki 1984) gelled with 8 g l^{-1} agar and supplemented with 2.5 μM benzyl-6-amino purine. Then, aerial explants were transferred to an in vitro pot with DKW–Agar medium supplemented with 1.3 μM indole-3-butyric acid to promote the differentiation of root tissue. Cultures were kept in a growth chamber at 25 °C with 16 h photoperiod using fluorescent white light. Once developed, individual plants were transferred to the experimental system and assigned to the different treatments (see below).

Inoculum preparation

Ophiostoma novo-ulmi inoculum was produced by using the SOM-1 isolate, identified as *O. novo-ulmi* ssp. *americana* by Martín et al. (2019*a*). Fungal plugs were grown on malt extract agar for 7 days. Then, mycelial fragments from the colony edge were grown in Erlenmeyer flasks with Tchernoff's liquid medium (Tchernoff 1965) at 22 °C in the dark under constant shaking to induce sporulation. Three days later, the liquid suspension was filtered and then centrifuged to collect the spores. Tchernoff medium was removed and substituted by sterile distilled water. The spore concentration was set at 4×10^7 blastospores ml^{-1} using a hemocytometer.

A fungal endophyte identified as *Cystobasidium* sp. (deposited in the Spanish Type Culture Collection (CECT by its Spanish acronym) under the reference CECT13192; Martínez-Arias et al. 2021*b*) was selected for this study. This yeast was isolated from twigs of a DED-resistant *U. minor* genotype growing in a conservation plot at 'Puerta de Hierro' Forest Breeding Center (Madrid, Spain; 40° 27′ 24″ N; 3° 45′ 0″ W; 595 m a.s.l.) and was coded as P5. To obtain P5 inoculum, yeast cells were refreshed twice by growing them on yeast extract agar. Then, the cells were dragged from the agar by using a sterile spatula and suspended in sterile distilled water. The final concentration was adjusted to 4×10^7 cells ml^{-1} using a hemocytometer.

Experimental design and sampling

Plants from in vitro propagation were assigned to four different treatments and transferred individually to sterile glass culture vessels. In vitro and sterile conditions were maintained during the whole experiment to avoid contaminations. The experiment comprised 64 in vitro plants, that is, 16 plants per genotype and 4 biological replicates per genotype and treatment. The four treatments were (i) control plants inoculated with sterile distilled water (C treatment), (ii) plants inoculated with *O. novo-ulmi* spore suspension (Oph treatment), (iii) plants inoculated with P5 endophyte cell suspension (P5 treatment) and (iv) plants pre-inoculated with P5 a week before *O. novo-ulmi* inoculation (P5 + Oph treatment). Inoculations were performed by submerging the root system in the treatment suspension and then cutting and removing roots at 3 cm from the callus to encourage new root formation. Roots were submerged in the treatment suspension for 1 min (Martín et al. 2019*a*). Then, plants were transferred into individual sterile glass vessels (6 cm diameter × 9.5 cm height) containing 50 g of autoclave sterilized sand as substrate and supplemented with 10 ml of Murashige and Skoog (MS) nutritive medium (Murashige and Skoog 1962). Plants were grown in a chamber at 25/20 °C day/night temperatures, with a 16 h photoperiod and relative air humidity of 40%.

One week after inoculation, the four biological replicates per genotype and treatment were extracted from the culture vessel (Figure 1). The presence of new roots was visually evaluated according to the following scale: (−) absence, (+) presence of new roots shorter than 1 cm and (++) proliferation of new roots longer than 1 cm. Shoots and roots were immediately detached and frozen using liquid nitrogen and stored at −80 °C until use for molecular and biochemical analyses. For all the analyses, the frozen plant material was ground to a fine powder using a Mixer mill MM 400 with frozen adapters for 1.5–2.0 ml vials (Retsch GmbH, Haan, Germany) and setting the milling to 30 Hz for 30 s.

In planta fungal detection

Fungal colonization ability from roots to shoot tissues was evaluated. DNA was extracted from 50 mg of the root or shoot powder material using the DNeasy plant kit (Qiagen, Hilden, Germany). Specific primer sequences were designed within the internal transcribed spacer 2 (ITS2) region of *O. novo-ulmi* and P5 ribosomal RNA (rRNA) using Primer3 Version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (see Table S1 available as Supplementary data at *Tree Physiology* Online). Fungal DNA quantifications were performed with SYBR Green Master Mix

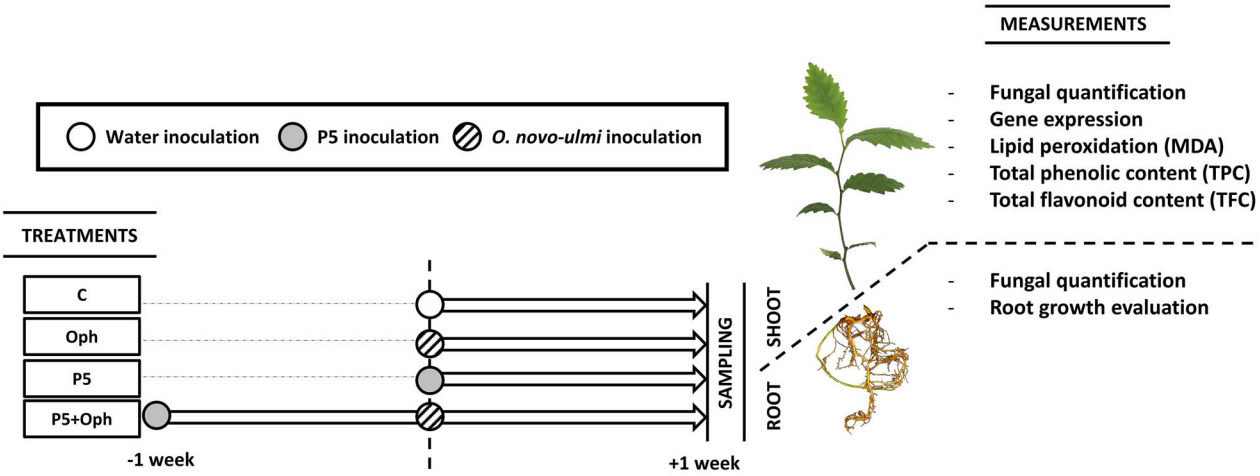

Figure 1. Experimental design and scheme of sampling for the in vitro–grown elm (*Ulmus minor*) plantlets used in the study and the subsequent measurements performed in each organ. Elm plantlets were inoculated with (i) sterile water (C = control), (ii) *Ophiostoma novo-ulmi* (Oph), (iii) P5 endophyte (P5) and (iv) P5 endophyte followed by *O. novo-ulmi* (P5 + Oph). MDA, quantification of malondialdehyde.

(Thermo Fisher Scientific, Waltham, MA, USA) in a ViiA™ 7 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with a standard amplification protocol. Fungal colonization was determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008) by subtracting the raw threshold cycle (Ct) values of *O. novo-ulmi* or P5 ITS2 from those of *U. minor* 18S-rRNA. The amplification results were expressed as *O. novo-ulmi* and P5 presence in each sample relative to average presence of both organisms in roots of Oph- and P5-inoculated M-DV2.3 plantlets, respectively.

Gene expression analysis

The expression of 12 defense-related genes was analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in shoot tissues (Table S1 available as Supplementary data at *Tree Physiology* Online). These genes were selected on the basis of the annotation results from a *U. minor* transcriptome analysis previously performed in our group (Perdiguero et al. 2015) and of their putative role in plant defense against fungal pathogens according to previous studies in model plants (see Table 1). Approximately 100 mg of shoot material was used for RNA extraction using the plant RNA isolation kit Spectrum Plant total RNA Kit (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The obtained RNA was treated with DNAse (Thermo Fisher Scientific, Waltham, MA, USA). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA from each sample using RevertAid H minus Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer instructions. Quantitative RT-PCRs were performed using the SYBR Green Master Mix (Thermo Fisher Scientific) in a ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific) with a standard amplification protocol. Three technical replicates were processed for each biological replicate. Relative quantification of specific mRNA levels was performed using the comparative method of Livak and Schmittgen (2001). Expression values were normalized using the housekeeping gene 18S-rRNA from *U. minor*. Gene expression was considered to be upregulated or downregulated if fold-change values were $\geq$1.5 or $\leq$0.7, respectively, besides being significantly different from control plants (see Statistical analysis section).

Total phenolic and flavonoid contents

For the quantification of total phenolic and flavonoid contents (TPC and TFC, respectively), around 20 mg of powdered shoot material was extracted in 1 ml of 95% methanol under constant shaking in a Precellys Evolution mixer mill (Bertin Instruments, Montigny-le-Bretonneux, France) and then incubated at room temperature for 48 h in the dark. After this time, samples were centrifuged, and supernatants were recovered. TPC was quantified using the Folin–Ciocalteu (F–C) reagent according to a microplate-adapted protocol described by Ainsworth and Gillespie (2007). Briefly, 100 μl of supernatant was mixed with 200 μl of 10% (v/v) F–C reagent and 800 μl of 700 mM Na_2CO_3 and then incubated for 2 h at room temperature in the dark. The same procedure was done with blank (95% methanol) and standards (0.025–1 mM gallic acid). Two hundred microliters of each mix were transferred to a 96-well microplate reader, and absorbance was measured at 765 nm in a microplate reader. On the other hand, TFC was determined by the colorimetric method described in Barreira et al. (2008), adapting volumes for a microplate reader protocol. In a 96-well microplate, a volume of 177.5 μl of each sample, blank and standards (0.015–0.75 mg ml^{-1} of quercetin) was transferred in an independent well. Every well was supplemented with 7.5 μl of 5% $NaNO_2$ and incubated for 6 min. Then, 15 μl of 10% $AlCl_3$ were added in each well, and after 5 min of

Table 1. List of genes analyzed by quantitative-PCR. Isotig numbers from *U. minor* transcriptome (Perdiguero et al. 2015), a brief description of their annotation and their putative role in plant defense are specified.

ID	isotig (*U. minor*)	Seq. description	Role in plant defense
CYP71A1	isotig08772	Cytochrome p450 71a1-like	This gene encodes a Cytochrome P450 monooxygenase involved in the biosynthesis of secondary metabolites in avocado and rice (Bozak et al. 1992, Lu et al. 2018). It was selected for its presumed connection with the biosynthesis of phenylpropanoids
DIR22	isotig10677	Dirigent protein 22-like	Dirigent proteins are described in different plant species in response to pathogen attack and have important roles in secondary metabolism, including lignan and lignin formation. The overexpression of DIR22 in soybean enhances plant resistance to *Phytophthora sojae* (Li et al. 2017)
EDS1	isotig02962	Enhanced disease susceptibility 1	This gene is a key immune regulator. In *Arabidopsis thaliana*, EDS1 interacts with the protein Phytoalexin Deficient 4 (PAD4) and this complex is involved in effector triggered immunity and SA accumulation in response to pathogens (Rietz et al. 2011).
GLP1.13	isotig14915	Germin-like protein subfamily 1 member 13	GLPs are involved in response to different abiotic and biotic stresses in several plant species, and their localization in plant cell walls suggests a relevant role in primary layers of plant defense (Breen and Bellgard 2010)
NRG1	isotig13421	RPW8-CNL, NRG1 (N requirement gene 1)	In *Nicotiana benthamiana*, this gene encodes an intracellular nucleotide binding/leucine-rich repeat receptor that together with EDS1 promotes host cell death and limits pathogen spread (Qi et al. 2018)
PAL	isotig14143	Phenylalanine ammonia lyase	*PAL* is involved in SA synthesis, and its upregulation has been described in several incompatible plant–microbe interactions, including the *Ophiostoma–Ulmus* pathosystem (Mauch-Mani and Slusarenko 1996, Umesha 2006, Aoun et al. 2010)
PR1	isotig16547	Pathogen-related protein 1	*PR1* is a marker gene for SA-mediated defense activation. This gene is rapidly induced in the presence of pathogens. It seems to have antimicrobial activity but its mode of action is not well known (Breen et al. 2017)
PR4	isotig04787	Wound-induced protein win2	Chitinases type I and II. These proteins degrade fungal cell wall chitins. *PR4* was selected because its upregulation has been previously described in *Ulmus* spp. in response to *O. novo-ulmi* (Aoun et al. 2010, Sherif et al. 2016, Perdiguero et al. 2018)
PR14	isotig10737	Non-specific lipid-transfer protein 1-like	The lipid transfer proteins transfer phospholipids between two membranes. The antimicrobial activity of these proteins consists of forming a pore in the membrane of the pathogen that allows intracellular loss of ions and provokes its death (Sels et al. 2008)
WRK33	isotig11160	Probable wrky transcription factor (TF) 33-like	TF of the WRKY family. In *A. thaliana* this TF positively regulates JA-mediated defense pathway and inhibits SA signaling. WRKY33 is directly involved in the activation of phytoalexins biosynthetic genes (Mao et al. 2011, Birkenbihl et al. 2012)
WRK40	isotig20762	Probable wrky TF 40-like	TF of the WRKY family. In *A. thaliana*, the expression of *WRKY40* is induced by SA and seems to have a role in post-invasion defense responses in a EDS1-independent manner (Schön et al. 2013)
WRK70	isotig03773	Probable wrky TF 70-like	TF of the WRKY family. This TF is activated by SA and repressed by JA. *WRKY70* regulates the activation of *PR* genes and other SA-responsive genes, while suppresses JA signaling (Li et al. 2004)

incubation, 50 μl of 1 M NaOH were also added, incubating the microplate for 15 min more. All the incubations were performed at room temperature. The absorbance was measured at 510 nm in a microplate reader. Results were expressed as milligrams of gallic acid and quercetin equivalents, for TPC and TFC, respectively, per gram of fresh weight of sample.

Malondialdehyde quantification

Lipid peroxidation was determined as an oxidative damage parameter by measuring the malondialdehyde (MDA) content on 50 mg of powdered shoot material. Malondialdehyde concentration was determined according to Ortega-Villasante et al. (2005). Plant material was homogenized with 1 ml of trichloroacetic acid (TCA)–thiobarbituric acid (TBA)–HCl reagent [15% (w/v) TCA, 0.37% (w/v) 2-TBA, 0.25 M HCl and 0.01% butylated hydroxytoluene]. Then, samples were incubated at 90 °C for 30 min and subsequently centrifuged. Supernatants were collected and absorbance was measured in a spectrophotometer at 535 and 600 nm, the last one to correct the nonspecific turbidity. Results were expressed as

nanomole of MDA per gram of fresh weight of sample using the extinction coefficient of the resulting chromophore from the reaction between MDA and TBA: 1.56×10^5 M cm^{-1}.

Statistical analysis

All the dependent variables, except gene expression, were analyzed using the two-way analysis of variance (ANOVA), with genotype and treatment and their interaction as between-subject factors, followed by Fisher's least significance difference (LSD) post-hoc tests to differentiate means when ANOVA showed significant effects ($P < 0.05$). Fold change values of gene expression analyses were compared between treatments within each genotype using the one-way ANOVA, followed by Fisher's LSD post-hoc test to differentiate means ($P < 0.05$ and $P < 0.1$). When needed, data were log- or arcsine-transformed prior to analysis to comply with normality and homoscedasticity assumptions. All the analyses were run using STATISTICA version 8.0 (StatSoft, Tulsa, OK, USA).

Results

Ophiostoma novo-ulmi detection in planta

Different patterns of *O. novo-ulmi* presence were observed among genotypes in root and shoot tissues ($P < 0.01$; Table 2). In the whole plant, *O. novo-ulmi* abundance was lower in plants pre-inoculated with P5 endophyte than in plants not inoculated with P5 ($P < 0.01$; Table 2). *Ophiostoma novo-ulmi* colonization was more successful in roots (inoculation organ) than in shoots (Figure 2). Focusing on Oph-treated plants, the resistant genotype M-DV2.3 showed the lowest pathogen presence in root tissues ($P < 0.05$; Figure 2B). Conversely, in shoot tissues, the genotypes M-DV2.3 and M-DV1 showed higher *O. novo-ulmi* presence than V-AD2 and VA-AP38 ($P < 0.05$; Figure 2A). Furthermore, pre-inoculation with P5 markedly reduced *O. novo-ulmi* presence in M-DV2.3 shoots and in VA-AP38 roots ($P < 0.05$; Figure 2A and B).

Endophyte detection in planta

The colonization of the P5 yeast was rather similar in shoots of all elm genotypes; however, in M-DV1 roots, the presence of this endophyte was 3.0-fold higher than in the rest ($P < 0.05$; Figure 2B, Table 2). When plants were inoculated with both the endophyte and the pathogen, P5 presence did not diminish or even increased in comparison with plants inoculated with P5 only ($P < 0.05$; Figure 2A, Table 2).

Gene expression analysis

The expression of 12 genes related to plant defense responses was evaluated in the shoot tissue. Each of the four elm genotypes evaluated showed a unique gene upregulation pattern induced by *O. novo-ulmi* inoculation (Figure 3). Yet, in all genotypes the pre-inoculation with P5 endophyte lowered the number of upregulated genes after *O. novo-ulmi* inoculation. For instance, in VA-AP38, the *O. novo-ulmi* inoculation upregulated seven genes ($P < 0.05$), but when this genotype was pre-inoculated with P5 only one gene was upregulated ($P < 0.05$) (Figure 3). Interestingly, the pre-inoculation with P5 increased the number of downregulated genes after *O. novo-ulmi* inoculation in the two resistant genotypes (Figure 3).

The two DED-resistant genotypes showed an upregulation of the *enhanced disease susceptibility* (*EDS1*) gene after *O. novo-ulmi*, P5 endophyte and their combined inoculation, while the expression of this gene did not change in the two susceptible genotypes after the same treatments (Figure 3).

The susceptible genotype VA-AP38 showed the highest number of upregulated genes in response to *O. novo-ulmi* inoculation with 7 of the 12 studied genes upregulated (58.3%; $P < 0.05$) (Figure 3). This percentage was only 16% in M-DV2.3 and 33.3% in both V-AD2 and M-DV1 genotypes.

The sole inoculation of P5 endophyte induced different responses in each genotype, but in general, a reduced upregulation of defense-related genes was observed when compared with the pathogen inoculation (Figure 3).

Biochemical analyses

The level of phenolic metabolites (flavonoids and total phenolics) was measured in shoot tissue to evaluate the plant chemical response after inoculations. Both the elm genotype and the treatment (control, endophyte, pathogen and endophyte + pathogen inoculations) performed significant effects on TFC and TPC ($P < 0.05$; Table 2). The *O. novo-ulmi* inoculation increased TPC levels in all genotypes, except in the resistant M-DV2.3 (Figure 4). The endophyte inoculation alone did not alter TFC or TPC levels. However, the pre-inoculation of the endophyte attenuated the accumulation of phenolic metabolites in response to *O. novo-ulmi* inoculation (Figure 4).

Oxidative damage in plants after pathogen and endophyte inoculations was estimated through the level of lipid peroxidation (MDA content) in shoot tissues (Figure 5). Lipid peroxidation in DED-resistant genotypes was not altered by pathogen or endophyte inoculation. Meanwhile, the pre-inoculation with the endophyte diminished the redox imbalance induced by the pathogen in DED-susceptible genotypes ($P < 0.05$; Figure 5).

Discussion

P5 endophytic yeast hindered colonization of elm plantlets by O. novo-ulmi

This work reveals intraspecific trends in *U. minor* responses to plant colonization by the DED pathogen *O. novo-ulmi* and by an endophytic yeast of the genus *Cystobasidium* (coded as P5). We found that plant colonization by the pathogen was dependent on the host genotype, with no straightforward relation with the

Table 2. Results of factorial ANOVA of variables measured in *Ulmus minor* plantlets. The effect of genotype, treatment and their interaction, genotype × treatment, was studied. Bold numbers in *P*-value indicate statistically significant differences at $P < 0.05$.

Variable	Effect	Sum of squares	Degrees of freedom	Mean squares	F-ratio	*P*-value
O. novo-ulmi presence in shoots	Genotype	0.214	3	0.071	7.813	**0.001**
	Treatment	0.024	1	0.024	2.608	0.122
	Genotype × treatment	0.037	3	0.012	1.344	0.288
O. novo-ulmi presence in roots	Genotype	69.87	3	23.29	8.966	**0.001**
	Treatment	22.54	1	22.54	8.679	**0.009**
	Genotype × treatment	22.23	3	7.41	2.853	0.066
O. novo-ulmi presence in whole plant	Genotype	75.60	3	25.20	10.540	**0.000**
	Treatment	24.45	1	24.45	10.225	**0.005**
	Genotype × treatment	23.42	3	7.81	3.265	**0.046**
P5 presence in shoots	Genotype	0.003	3	0.001	0.842	0.485
	Treatment	0.01	1	0.01	7.351	**0.012**
	Genotype × treatment	0.009	3	0.003	2.296	0.104
P5 presence in roots	Genotype	16.12	3	5.373	7.005	**0.002**
	Treatment	1.29	1	1.285	1.676	0.209
	Genotype × treatment	3.45	3	1.149	1.498	0.243
P5 presence in whole plant	Genotype	16.53	3	5.510	7.088	**0.002**
	Treatment	1.20	1	1.195	1.538	0.228
	Genotype × treatment	3.04	3	1.015	1.305	0.298
TPC	Genotype	4.194	3	1.398	6.68	**0.001**
	Treatment	8.091	3	2.697	12.89	**0.000**
	Genotype × treatment	2.033	9	0.226	1.08	0.398
TFC	Genotype	2.194	3	0.731	3.232	**0.033**
	Treatment	4.918	3	1.639	7.246	**0.001**
	Genotype × treatment	1.075	9	0.119	0.528	0.845
MDA level	Genotype	10,300	3	3419	1.484	0.232
	Treatment	57,200	3	19,100	8.276	**0.000**
	Genotype × treatment	51,000	9	5664	2.458	**0.023**

Abbreviations: TPC, total phenolic content; TFC, total flavonoids content; MDA, malondialdehyde.

DED resistance level of the host. One genotype stood out for its lower *O. novo-ulmi* abundance in root tissue: the DED-resistant M-DV2.3 (Figure 2). The reduced pathogen proliferation in this genotype may be indicative of and partly explain its high DED-resistance level. This result supports a recent work where higher *O. novo-ulmi* invasion was observed in M-DV1 than in M-DV2.3 plants (Martínez-Arias et al. 2021*a*), indicating a consistent behavior of M-DV2.3 in limiting *O. novo-ulmi* spread within the plant when compared with other genotypes, possibly because this genotype develops vessels of smaller diameter (Pita et al. 2018) and length (Chano et al., unpublished results). In turn, colonization of plants by P5 was rather similar in all elm genotypes, with the exception of the susceptible M-DV1, whose roots showed higher presence of P5. Interestingly, P5 pre-inoculation reduced the abundance of *O. novo-ulmi* in the whole plant ($P = 0.005$; Table 2), while the abundance of P5 did not change after *O. novo-ulmi* inoculation ($P = 0.228$; Table 2). This result shows that the pathogen was not able to displace the endophyte from plant tissues during the experiment. The lower pathogen presence in plants already colonized by the endophyte could directly reduce the impact of the pathogen on plant physiology and metabolism. However, the effect of the endophyte-mediated lowering of pathogen presence also varied with the plant genotype (Figure 2), revealing the complexity of host–endophyte–pathogen interactions and that the functions of endophytes on plants cannot always be generalized at the host species level (Kogel et al. 2006, Gundel et al. 2012, David et al. 2016).

P5 attenuated plant defense responses to the pathogen

The pre-inoculation of the endophyte diminished the number of upregulated plant defense-related genes induced by *O. novo-ulmi* (Figure 3). The lower presence of pathogen cells in plant tissues colonized by the endophyte probably attenuated the intensity of plant responses against the pathogen. In addition, the pre-inoculation of the endophyte increased the number of down-regulated genes in response to the pathogen in the resistant genotypes. This endophyte–host interaction can be interpreted in different ways. On one hand, the downregulation of defense-related genes after *O. novo-ulmi* inoculation might indicate a mechanism to evade the plant defense system to prevent a strong reaction against the endophytes, where SA-mediated plant defenses are inhibited or attenuated to allow these organisms to live in their tissues (Zamioudis and Pieterse 2012,

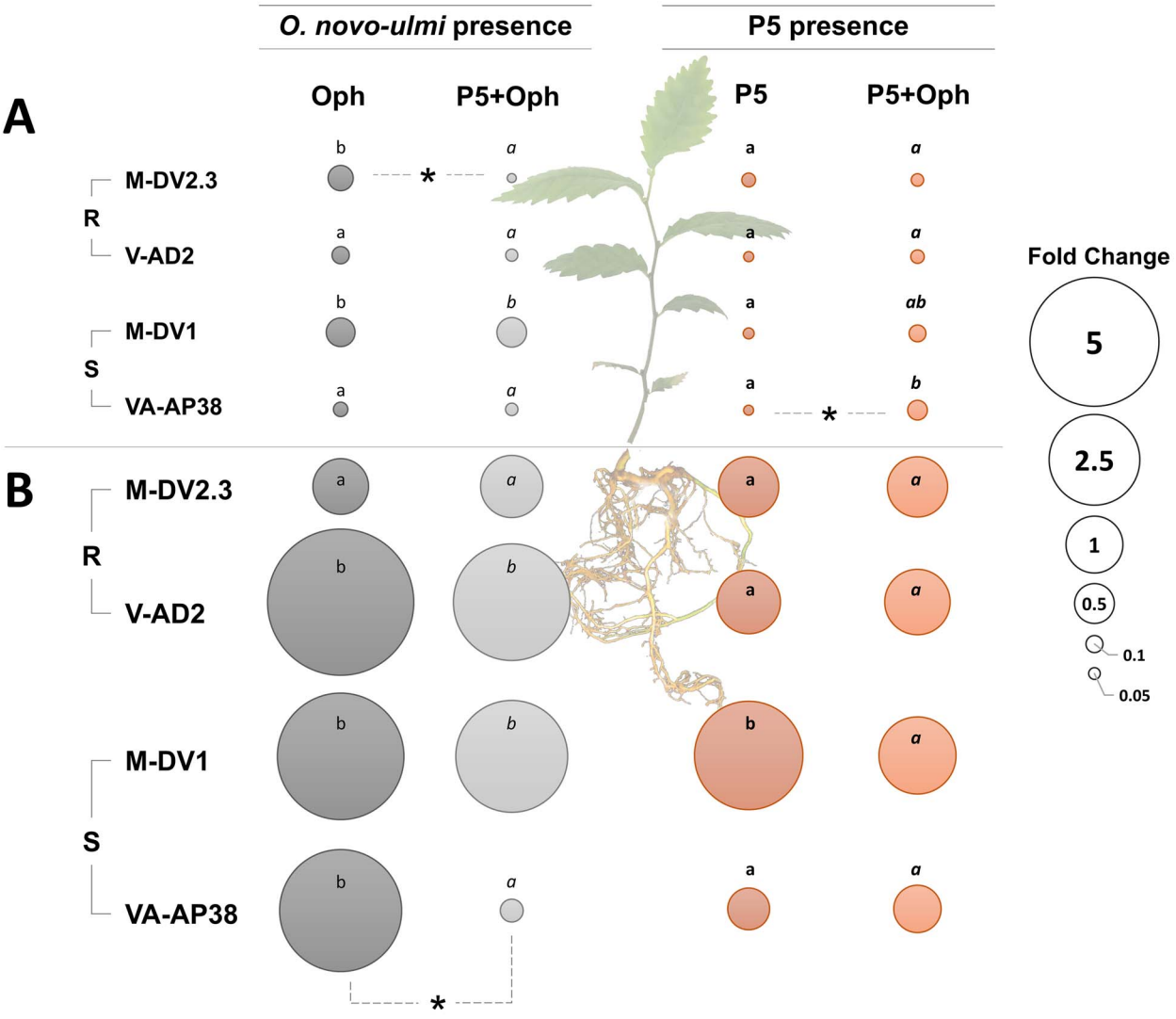

Figure 2. Relative presence of *Ophiostoma novo-ulmi* and P5 endophyte in shoots (A) and roots (B) of resistant (R) and susceptible (S) *Ulmus minor* genotypes. Results are shown as circles representing the average fold change of fungal presence relative to roots of Oph- or P5-treated plantlets from the resistant genotype M-DV2.3. Within each treatment, different letters indicate significant differences between genotypes, with independence for each plant organ ($P < 0.05$; Fisher's LSD test). Differences in *O. novoulmi* presence (gray spheres) are represented by normal letters in Oph treatment and by italic letters in P5 + Oph treatment. Regarding P5 presence (pink spheres), bold letters correspond to P5 treatment and bold italic letters correspond to P5 + Oph treatment. In each genotype, significant pairwise comparisons in Oph vs P5 + Oph or in P5 vs P5 + Oph are indicated with asterisks ($P < 0.05$; Fisher's LSD test). C, control; Oph, *O. novo-ulmi* inoculation; P5, P5 endophyte inoculation and P5 + Oph, P5 endophyte inoculation followed by *O. novo-ulmi* inoculation.

Plett and Martin 2018). Moreover, by downregulating defense responses, the plant could invest resources in processes other than defense (e.g., root or shoot growth). This hypothesis agrees with the fact that downregulation of defense-related gene expression after *O. novo-ulmi* inoculation was observed in the resistant genotypes only, which are able to continue growing after infection (Martín et al. 2019*b*). On the other hand, the downregulation of defense-related genes induced by P5 inoculation suggests that attenuation of pathogen-induced stress may be regulated at molecular level, particularly in the resistant genotypes. It should be acknowledged that a previous metabarcoding study with adult elm trees concluded that operational taxonomic units within the class Cystobasidiomycetes (which includes the P5 isolate) are more abundant in resistant than in susceptible elm genotypes under field conditions (Macaya-Sanz et al. 2020). Although this trend was not confirmed under the very different in vitro experimental conditions of the present work, our results suggest that resistant genotypes established a closer molecular interaction with the endophytic yeast than susceptible genotypes, leading to downregulation of a higher number of defense-related genes after pathogen infection than susceptible trees. However, the limited number of

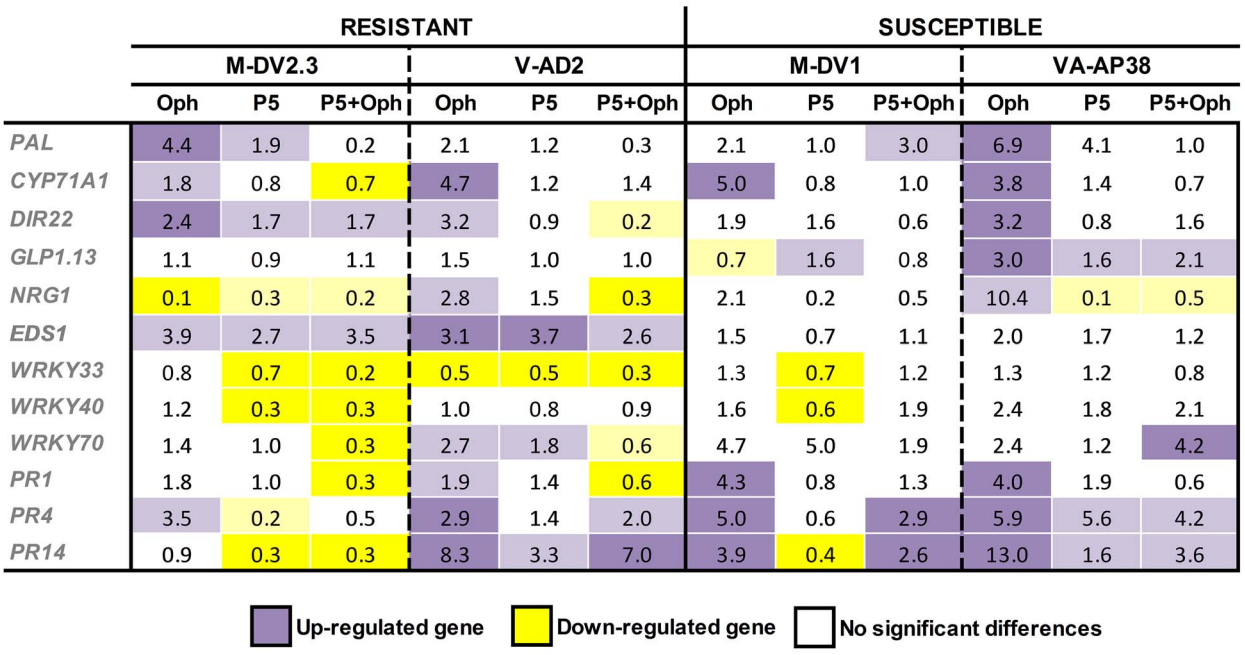

Figure 3. Transcriptional qRT-PCR profile of selected genes in shoots of resistant and susceptible *Ulmus minor* genotypes. Values are means of four independent biological replicates. Relative expression was normalized to the *U. minor* reference gene 18S-rRNA. Genes were considered to be significantly up- or downregulated for a genotype if fold-change values were $\geq$1.5 (purple-colored) or $\leq$0.7 (yellow-colored), respectively, besides being statistically significant (dark colors for $P < 0.05$ and light colors for $P < 0.1$; Fisher's LSD test). C, control; Oph, *O. novo-ulmi* inoculation; P5, P5 endophyte inoculation and P5 + Oph, P5 endophyte inoculation followed by *O. novo-ulmi* inoculation.

genes explored in this work makes it necessary to confirm this trend by analyzing a larger array of genes, for example, through high-throughput sequencing techniques (e.g., RNAseq).

P5 mitigated the pathogen-induced stress in DED-susceptible clones

Besides the observed host–endophyte–pathogen interaction in defense gene regulation, other plausible mechanisms by which P5 endophyte attenuated the stress caused by *O. novo-ulmi* in elm plants are (i) ability to control the levels of oxidative damage induced by the pathogen, (ii) promotion of root growth and (iii) direct antagonism and/or niche competition against *O. novo-ulmi*. Regarding the first mechanism, P5 pre-inoculation lowered the levels of lipid peroxidation (MDA content) in susceptible trees after pathogen infection (Figure 5). The increase in oxidative damage and phenolic metabolites (mostly flavonoids) after *O. novo-ulmi* infection was mainly observed in susceptible genotypes, similar to previous work (Martín et al. 2019*a*). Total phenolic and flavonoid accumulation induced by *O. novo-ulmi* can be related to an attempt by the plant to limit pathogen spread (Ouellette and Rioux 1992, Witzell and Martín 2008) and to counterbalance the oxidative damage derived from the incompatible interaction between the plant and the pathogen (Shalaby and Horwitz 2015). In this sense, no increase in oxidative damage was observed in response to P5 inoculation, which evidences the nonpathogenic nature of this endophyte toward elm. Concerning the second mechanism (promotion of root growth), a positive effect of P5 on root growth stimulation was observed in P5-inoculated plants (see Figure S1 available as Supplementary data at *Tree Physiology* Online). Higher formation of fine roots may help the plant-sustaining water uptake and hydraulic functioning during pathogen invasion of xylem conduits. The production of the auxin indole-3-acetic acid by P5 endophyte was demonstrated in previous work (Martínez-Arias et al. 2021*c*) and may contribute to the stimulation of root formation in host plants (Harman 2011, Sukumar et al. 2013). In other work, P5-inoculated elm plantlets showed higher root development accompanied with higher survival rates against abiotic stress than non-inoculated plants (Martínez-Arias et al. 2021*b*), evidencing the role of this yeast in counterbalancing plant stressful situations. Although the third mechanism (direct antagonism and/or niche competition against *O. novo-ulmi*) was not directly evaluated in this work, previous analyses demonstrated that liquid filtrates from P5 reduced *O. novo-ulmi* growth and that P5 partly overlapped with *O. novo-ulmi* in the metabolization of different carbon sources (Martínez-Arias et al. 2021*c*). Particularly, P5 was able to grow in the presence of defensive molecules (flavonoids and other phenolic compounds) produced either by the host or the pathogen, possibly helping the yeast to compete with the pathogen within plant tissues. Furthermore, other plant-growth promoting yeasts similar to P5 have been also

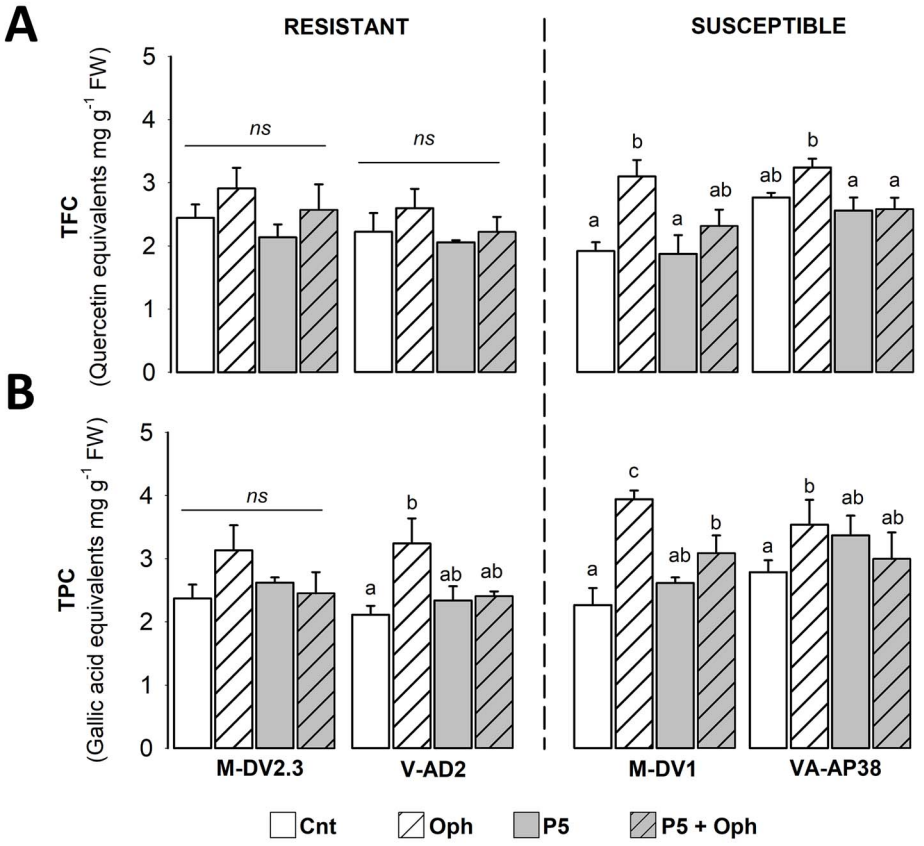

Figure 4. TFC expressed as quercetin equivalents (A), and TPC expressed as gallic acid equivalents (B), measured in shoots of elm plantlets from DED-resistant and DED-susceptible *U. minor* genotypes. Within each genotype, different letters indicate significant differences between treatments ($P < 0.05$; Fisher's LSD test; ns = nonsignificant differences). C, control; Oph, *O. novo-ulmi* inoculation; P5, P5 endophyte inoculation and P5 + Oph, P5 endophyte inoculation followed by *O. novo-ulmi* inoculation.

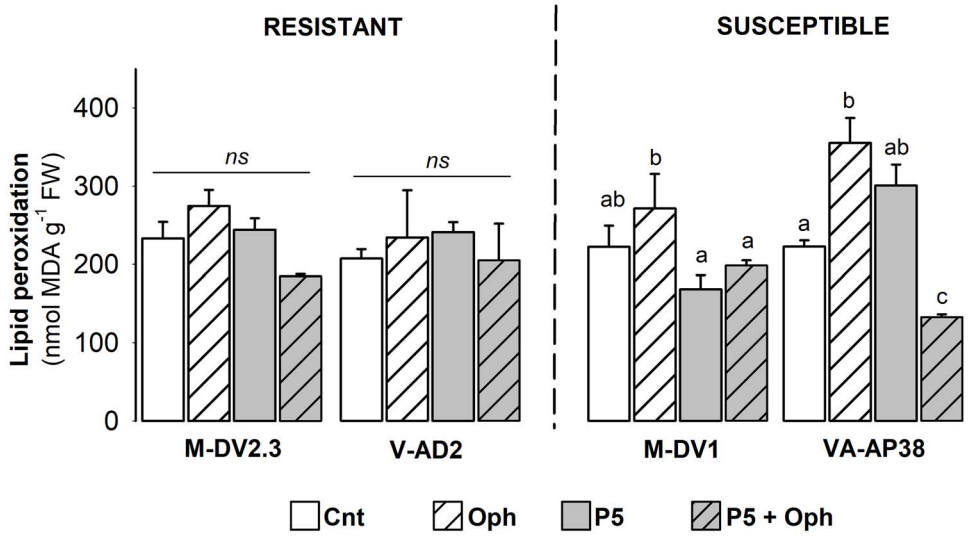

Figure 5. Lipid peroxidation measured through the quantification of malondialdehyde produced in shoots of elm plantlets from DED-resistant and DED-susceptible *U. minor* genotypes. Within each genotype, different letters indicate significant differences between treatments ($P < 0.05$; Fisher's LSD test; ns = nonsignificant differences). C, control; Oph, *O. novo-ulmi* inoculation; P5, P5 endophyte inoculation and P5 + Oph, P5 endophyte inoculation followed by *O. novo-ulmi* inoculation.

described as beneficial symbionts, not only by promoting plant growth but also by acting as inhibitors of phytopathogens (El-Tarabily 2004, Ignatova et al. 2015), enhancing plant defense or performing a direct antagonism to pathogens (Calvente et al. 2001, Kalogiannis et al. 2006, Akhtyamova and Sattarova 2012, Gava et al. 2018).

Different molecular responses evidence the multifactorial nature of DED resistance

Our results further confirm the complexity and heterogeneity of elm defense mechanisms against DED among different genotypes. In response to *O. novo-ulmi* inoculation, the two DED-susceptible genotypes and the resistant V-AD2 shared a rather similar pattern of gene regulation, while the resistant M-DV2.3 showed a distinct response (Figure 3). The different response of the two resistant genotypes to DED was evidenced, for example, by the downregulation of *N requirement gene 1* (*NRG1*) in M-DV2.3, and the upregulation of the same gene in V-AD2. The multigenic nature of elm resistance to DED has already been proposed in the previous works (Townsend and Santamour 1993, Martín et al. 2019*b*) and could have important implications for elm breeding. Thus, by crossing genotypes with different and ideally complementary defense mechanisms, it might be possible to enhance disease resistance in the offspring. *Pathogenesis-related 4* (*PR4*) and *cytochrome P450 71A1* (*CYP71A1*) were found upregulated in the four elm genotypes after pathogen infection, in agreement with previous works describing elm responses to *O. novo-ulmi* (Aoun et al. 2010, Sherif et al. 2016, Perdiguero et al. 2018). Besides these two genes, *PR1* and *PR14* were also induced by the pathogen in both DED-susceptible genotypes, and those four genes were the only ones activated in the susceptible M-DV1 among the studied genes. Yet, the other susceptible genotype (VA-AP38) upregulated eight (i.e., 66.6%) of the analyzed genes. This genotype (representative of the so-called English elm) showed the highest expression values of all genotypes in response to pathogen inoculation. In a previous study using field-grown VA-AP38 trees, *O. novo-ulmi* inoculation stimulated the upregulation of a large number of genes related to several metabolic pathways and biological processes, leading a tradeoff between expression of growth and defense genes (Perdiguero et al. 2018). Despite this huge transcriptional activation of genes, those trees were not able to stop disease progression and died, revealing the need of a well-regulated immune response to deal with the disease. In this work, the high activation of defense pathways in this clone is clearly ineffective against the pathogen, causing a concomitant high-oxidative damage. Finally, the upregulation of *EDS1* gene after pathogen, endophyte and their combined inoculation in resistant trees but not in susceptible ones deserves further research to clarify its possible involvement in DED resistance. *EDS1* in *U. minor* might be related to ROS and SA homeostasis as occurs in other plant species such as *A. thaliana*, *Nicotiana benthamiana* or poplar (Catinot et al. 2008, Rietz et al. 2011, Bernacki et al. 2018). The upregulation of this gene could have a role in the correct activation of SA-dependent pathways in response to both beneficial and pathogenic microbes.

Conclusions

Numerous screening trials to find DED-resistant elm material have been performed during the decades of the Spanish Elm Breeding Program activity. As the evaluation of adult trees is very space- and time-consuming, the development of early detection tools in which these limiting factors disappear is a key step to progress in elm restoration. The potential of early screening methods using *U. minor* in vitro plantlets has been previously reported (Martín et al. 2019*a*), showing some distinctive traits associated with DED resistance. Biochemical parameters, such as MDA and chlorophyll contents, and biometric parameters, such as shoot growth, were useful to distinguish between resistant and susceptible elm plantlets. Our study provides further evidence of the usefulness of the in vitro system for the early detection of DED-resistant genotypes, but also to investigate plant–endophyte symbioses. The MDA level was confirmed as a key parameter associated with DED-susceptibility. Similarly, an accumulation of plant phenolics in response to *O. novo-ulmi* was identified as a general response to the pathogen, especially in susceptible trees. The pre-inoculation of the P5 endophyte displayed ameliorative effects against *O. novo-ulmi*, evidenced by lower pathogen abundance, reduced upregulation of plant defenses and lower levels of oxidative damage. Root growth promotion induced by P5 was also possibly important in the maintenance of water uptake during DED infection. This work is one of the first addressing defense gene regulation in *U. minor* after inoculation with beneficial and pathogenic fungi. Recently published (Islam et al. 2022) and ongoing works (Chano et al., unpublished results) about the transcriptomic response of different *Ulmus* species to *O. novo-ulmi* will increase the set of genes potentially involved in *U. minor* defense mechanisms.

Supplementary data

Supplementary data for this article are available at Tree Physiology Online.

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Conflict of interest

None declared.

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