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

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Abstract

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 the present work, the expression of defense-related genes to the causal agent of DED, *Ophiostoma novo-ulmi*, were analyzed in in vitro clonal plantlets from two DED-resistant and two DED-susceptible *U. minor* 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 to discriminate the susceptible from the resistant genotypes, showing higher levels of oxidative stress 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 multifactorial 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: *Ophiostoma novo-ulmi*; resistance; biocontrol; pink-pigmented yeast; *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* and *O. novo-ulmi*. 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* germplasm have become a priority to conserve and restore lost elm populations (Martín et al. 2019b). In Spain, large efforts have been invested to breed resistant *U. minor* genotypes by screening plant materials from all along the country. By now, 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 (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. 2013a), although recent research has shown that some resistant genotypes also form wide vessels (Martín et al. 2020). Beyond these differences, other key resistance traits are possibly encrypted in the genetic code of each genotype. The genetic basis of the *U. minor* response to *O. novo-ulmi* has just started to be elucidated by using classical and novel “omic” technics, such as 454-sequencing (Perdiguero et al. 2015) and RNA-sequencing (authors, 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. SA-dependent defense responses are typically triggered by biotrophic

pathogens (Pieterse et al. 2009). *O. 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). SA-dependent defenses can be activated systemically to distal parts of the plant through molecules such as methyl-SA (MeSA) or glycerol-3-phosphate (G3P), where they play a role in the activation of systemic acquired resistance (SAR) (Fu and Dong 2013). Both, 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).

Plant-microbe symbiotic associations provide plants with higher phenotypic plasticity to changing environments, including biotic and abiotic stresses (Liu et al. 2020). Enhancement of nutrient acquisition and defensive metabolism in the plant are among the main factors involved in stress tolerance mediated by symbiotic 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 (ISR) boosted by the cross-talking among different hormone pathways (Van Wees et al. 2008; Morán-Díez et al. 2012). The derived molecular signals spread towards 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. 2013b). 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). Certain elm endophytes have demonstrated biocontrol potential against DED (Martínez-Arias et al. 2021c), and activity in their hosts as growth stimulators and abiotic stress reducers (Martínez-Arias et al. 2021b). 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. 2019a; Martínez-Arias et al. 2021b). By using this in vitro technique, the present 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 that *Cystobasidium* sp. was classified as plant growth-promoting yeast (Joubert and Doty 2018) and is within the group of endophytes associated with 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*.

Material and Methods

Plant material

Four Spanish *Ulmus minor* genotypes were used for the experiment. The genotypes M-DV2.3 (Dehesa de Amaniél, 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 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. (2019a). 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 DKW basal medium (pH 5.7; Driver & 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 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. (2019a). Fungal plugs were grown on malt extract agar (MEA) 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 to remove germinated spores and centrifuged to collect the spores. Tchernoff medium was removed and substituted by sterile distilled water. The spore concentration was set at $4 \cdot 10^7$ blastospores ml⁻¹ using a hemocytometer.

A fungal endophyte identified as *Cystobasidium* sp. (deposited in the Spanish Type Culture Collection (CECT) under the reference CECT13192; Martínez-Arias et al., 2021b) 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) and was coded as P5. To obtain P5 inoculum, yeast cells were refreshed twice by growing them on yeast extract agar (YEA). 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 $4 \cdot 10^7$ 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 foreign contaminations. The experiment comprised 64 in vitro plants, i.e., 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 cutting the root system at 3 cm from the callus and maintaining it submerged in the treatment suspension for 1 min (Martín et al. 2019a). Then, plants were transferred into individual sterile glass vessels (6 cm diameter x 9.5 cm height) containing 50 g of autoclave sterilized sand as substrate, and supplemented with 10 ml of 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 (Fig. 1). The presence of new roots was visually evaluated according to the following scale: (-) absence, (+) presence, (++) high presence. Shoots and roots were immediately detached and frozen using liquid nitrogen for being stored at -80 °C until use for molecular and biochemical analyses (see below). For all the analyses, the frozen plant material was ground to a fine powder using a Mixer mill MM 400 (Retsch GmbH, Haan, Germany) set to 30 Hz for 30 sec.

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 ITS2 region of *O. novo-ulmi* and P5 rRNA using Primer3 Version 0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) (Table S1). Fungal DNA quantifications were performed with SYBR Green Master Mix (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 $2^{-\Delta Ct}$ 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 twelve defense-related genes was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in shoot tissues (Table S1). 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). 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 cDNA was synthesized from 1 µg of total RNA from each sample using RevertAid H minus Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer instructions. Quantitative RT-PCRs were performed using the SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) 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 (Ref) for *U. minor*. Gene expression was considered to be up- or down-regulated if fold-change values were ≥ 1.5 or ≤ 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), 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 reagent (F-C) according to a microplate-adapted protocol described by Ainsworth & Gillespie (2007). On the other hand, TFC was determined by the colorimetric method described in Barreira et al. (2008), adapting volumes for a microplate reader protocol. Results were expressed as mg of gallic acid and quercetin equivalents, for TPC and TFC respectively, per gram of fresh weight of sample.

MDA quantification

Lipid peroxidation was determined as an oxidative stress parameter by measuring the malondialdehyde (MDA) content on 50 mg of powdered shoot material. MDA concentration was determined according to Ortega-Villasante et al. (2005). Results were expressed as nmol of MDA per gram of fresh weight of sample.

Statistical analysis

All the dependent variables, except gene expression, were analysed using 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 one-way ANOVA, followed by Fisher's Least Significance Difference (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 1). 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 1). *O. novo-ulmi* colonization was more successful in roots (inoculation organ) than in shoots (Fig. 2). Focusing on Oph-treated plants, the resistant genotype M-DV2.3 showed the lowest pathogen presence in root tissues ($p < 0.05$; Fig. 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 ($p < 0.05$; Fig. 2A).

Furthermore, pre-inoculation with P5 markedly reduced *O. novo-ulmi* presence in M-DV2.3 shoots and in VA-AP38 roots ($p < 0.05$; Figs. 2A, 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 ($p < 0.05$; Fig. 2B; Table 1). 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 ($p < 0.05$; Fig. 2A; Table 1).

Gene expression analysis

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

The two DED-resistant genotypes showed an up-regulation of the *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 (Fig. 3).

The susceptible genotype VA-AP38 showed the highest number of up-regulated genes in response to *O. novo-ulmi* inoculation with seven out of the twelve studied genes up-regulated (58.3%; $p < 0.05$) (Fig. 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 up-regulation of defense-related genes was observed when compared to the pathogen inoculation (Fig. 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 total flavonoid content (TFC) and total phenolic content (TPC) ($p < 0.05$; Table 1). The *O. novo-ulmi* inoculation increased TPC levels in all genotypes, except in the resistant M-DV2.3 (Fig. 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 (Fig. 4).

Oxidative stress in plants after pathogen and endophyte inoculations was estimated through the level of lipid peroxidation (MDA content) in shoot tissues (Fig. 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 ($p < 0.05$; Fig. 5).

Discussion

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

The present 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 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 (Fig. 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* dispersal was observed in M-DV1 than in M-DV2.3 plants (Martínez-Arias et al. 2021a), indicating a consistent behavior of M-DV2.3 in limiting *O. novo-ulmi* spread when compared to other genotypes, possibly because this genotype develops vessels of smaller diameter (Pita et al. 2018) and length (authors, 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 1), while the abundance of P5 did not change after *O. novo-ulmi* inoculation ($p = 0.228$; Table 1). 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 (Fig. 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.

P5 attenuated plant defense responses to the pathogen

The pre-inoculation of the endophyte diminished the number of up-regulated plant defense-related genes induced by *O. novo-ulmi* (Fig. 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 the one hand, the down-regulation of

defense-related genes after *O. novo-ulmi* inoculation might indicate a mechanism to weaken the plant defense system to prevent a strong reaction against the pathogen, similarly as occurs in mutualistic interactions of microbes and plants, where SA-mediated plant defenses are inhibited or attenuated to allow these organisms to live in their tissues (Zamioudis and Pieterse 2012; Plett and Martin 2018). By down-regulating defense responses, the plant could invest resources in other processes than defense (e.g., root or shoot growth), which could promote plant tolerance to the infection (Martín et al. 2019b). This hypothesis agrees with the fact that down-regulation 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. 2019b). On the other hand, the down-regulation of defense-related genes induced by P5 inoculation suggests that attenuation of pathogen-induced stress may be also 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 down-regulation of a higher number of defense-related genes after pathogen infection than susceptible trees. However, the limited number of genes explored in this work makes it necessary to confirm this trend by analyzing a larger array of genes, e.g., 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 stress 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 (Fig. 5). The increase of oxidative stress and phenolic metabolites after *O. novo-ulmi* infection was mainly observed in susceptible genotypes, similarly as observed in a previous work (Martín et al. 2019a). Total phenolics and flavonoids accumulation induced by *O. novo-ulmi* can be related to an attempt of 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 of oxidative stress was observed in response to P5 inoculation, which evidences the non-pathogenic nature of this endophyte towards elm. Concerning the second mechanism (promotion of root growth), a positive effect of P5 on root growth stimulation was observed in P5-inoculated plants (Figure S1). 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 acetic acid (IAA) by P5 endophyte was demonstrated in a previous work (Martínez-Arias et al. 2021c) 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. 2021b), 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 the present 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. 2021c). Particularly, P5 was able to grow in 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 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 (Fig. 3). The different response of the two resistant genotypes to DED was evidenced, for example, by the down-regulation of *NRG1* in M-DV2.3, and the up-regulation of the same gene in V-AD2. The multigenic nature of elm resistance to DED have already been proposed in previous works (Townsend and Santamour 1993; Martín et al. 2019b) 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. The genes *PR4* and *CYP71A1* were found up-regulated 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, being those four genes, the only ones activated in the susceptible M-DV1 among the studied genes. Yet, the other susceptible genotype (VA-AP38) up-regulated 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 up-regulation 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 the huge transcriptional regulation, those trees were not able to stop disease progression and died. The high activation of defense pathways in this clone is clearly ineffective against the pathogen, causing a concomitant high oxidative stress. Finally, the up-regulation 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.

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 *Ulmus minor* in vitro plantlets has been previously reported (Martín et al. 2019a), 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 up-regulation of plant defenses, and lower levels of oxidative stress. Root growth promotion induced by P5 was also possibly important in the maintenance of water uptake during DED infection.

Supplementary Data

Table S1. List of genes analysed by quantitative-PCR.

Figure S1. Presence of new roots in *Ulmus minor* plantlets one week after inoculation.

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Conflict of interest

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Figure captions

Figure 1. Experimental design and scheme of sampling for the in vitro-grown elm plantlets used in the study and the subsequent measurements performed in each organ. (MDA = quantification of malondialdehyde).

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. novo-ulmi* presence (grey 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).

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 down-regulated for a genotype if fold-change values were ≥ 1.3 (purple-colored) or ≤ 0.75 (yellow-colored) respectively, besides being statistically significant (dark colors for $p < 0.05$ and light colors for $p < 0.1$; Fisher's LSD test).

Figure 4. Total flavonoid content (TFC) expressed as quercetin equivalents (**A**), and total phenolic content (TPC) expressed as gallic acid equivalents (**B**), measured in shoots of elm plantlets from DED-resistant and DED-susceptible *Ulmus minor* genotypes. Within each genotype, different letters indicate significant differences between treatments ($p < 0.05$; Fisher's LSD test; ns = non-significant differences).

Figure 5. Lipid peroxidation measured through the quantification of malondialdehyde (MDA) produced in shoots of elm plantlets from DED-resistant and DED-susceptible *Ulmus minor* genotypes. Within each genotype, different letters indicate significant differences between treatments ($p < 0.05$; Fisher's LSD test; ns = non-significant differences).

MEASUREMENTS

- Fungal quantification
- Gene expression
- Lipid peroxidation (MDA)
- Total phenolic content (TPC)
- Total flavonoid content (TPC)

- Fungal quantification

ROOT / SHOOT

SAMPLING

+1 week

Water inoculation P5 inoculation *O. novo-ulmi* inoculation

TREATMENTS

C

Oph

P5

P5+Oph

-1 week

|

|

	RESISTANT						SUSCEPTIBLE					
	M-DV2.3			V-AD2			M-DV1			VA-AP38		
	Oph	P5	P5+Oph	Oph	P5	P5+Oph	Oph	P5	P5+Oph	Oph	P5	P5+Oph
<i>PAL</i>	4.4	1.9	0.2	2.1	1.2	0.3	2.1	1.0	3.0	6.9	4.1	1.0
<i>CYP71A1</i>	1.8	0.8	0.7	4.7	1.2	1.4	5.0	0.8	1.0	3.8	1.4	0.7
<i>DIR22</i>	2.4	1.7	1.7	3.2	0.9	0.2	1.9	1.6	0.6	3.2	0.8	1.6
<i>GLP1.13</i>	1.1	0.9	1.1	1.5	1.0	1.0	0.7	1.6	0.8	3.0	1.6	2.1
<i>NRG1</i>	0.1	0.3	0.2	2.8	1.5	0.3	2.1	0.2	0.5	10.4	0.1	0.5
<i>EDS1</i>	3.9	2.7	3.5	3.1	3.7	2.6	1.5	0.7	1.1	2.0	1.7	1.2
<i>WRKY33</i>	0.8	0.7	0.2	0.5	0.5	0.3	1.3	0.7	1.2	1.3	1.2	0.8
<i>WRKY40</i>	1.2	0.3	0.3	1.0	0.8	0.9	1.6	0.6	1.9	2.4	1.8	2.1
<i>WRKY70</i>	1.4	1.0	0.3	2.7	1.8	0.6	4.7	5.0	1.9	2.4	1.2	4.2
<i>PR1</i>	1.8	1.0	0.3	1.9	1.4	0.6	4.3	0.8	1.3	4.0	1.9	0.6
<i>PR4</i>	3.5	0.2	0.5	2.9	1.4	2.0	5.0	0.6	2.9	5.9	5.6	4.2
<i>PR14</i>	0.9	0.3	0.3	8.3	3.3	7.0	3.9	0.4	2.6	13.0	1.6	3.6

Up-regulated gene

Down-regulated gene

No significant differences

A **SUSCEPTIBLE** **B**

Cnt

Oph

P5

P5 + Oph

RESISTANT**SUSCEPTIBLE**

Table 1. Results of factorial ANOVA of variables measured in *Ulmus minor* plantlets. The effect of Genotype, Treatment and their interaction, Genotype × Treatment, was studied. Red and bold numbers in *p*-value indicate statistically significant differences at *p* < 0.05. (TPC: Total Phenolic Content; TFC: Total Flavonoids Content; MDA: malondialdehyde).

Variable	Effect	Sum of squares (SS)	Degrees of freedom (df)	Mean squares (MS)	F-ratio	<i>p</i> -value
<i>O. novo-ulmi</i> 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
<i>O. novo-ulmi</i> 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
<i>O. novo-ulmi</i> 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	10300	3	3419	1.484	0.232
	Treatment	57200	3	19100	8.276	0.000
	Genotype*Treatment	51000	9	5664	2.458	0.023