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Title page

Title: Antifungal effect and reduction of *Ulmus minor* symptoms to *Ophiostoma novo-ulmi* by carvacrol and salicylic acid

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Key words: Dutch elm disease; phenolic compounds; tree resistance; tree phenology

Abstract: There are still no effective means to control Dutch elm disease (DED), caused by the vascular fungi *Ophiostoma ulmi* and *O. novo-ulmi*. Plant phenolics may provide a new strategy for DED control, given their known antifungal activity against pathogens and their involvement in plant defense mechanisms. The *in vitro* antifungal activity of salicylic acid, carvacrol, thymol, phenol, *o*-cresol, *m*-cresol, *p*-cresol, and 2,5-xylenol against the DED pathogens was tested. Also, the protective effect of watering *Ulmus minor* seedlings with these compounds was tested against *O. novo-ulmi*. Salicylic acid, carvacrol, and thymol showed the strongest antifungal *in vitro* activity, while carvacrol and salicylic acid provided the strongest *in vivo* protection against *O. novo-ulmi* (63 and 46% reduction of leaf wilting symptoms with respect to controls, respectively). The effect of the treatments on tree phenology was low, and a significant negative relation was observed between the number of days to bud burst and the leaf wilting symptoms after inoculation, probably determined by genetic differences among the elm tree progenies used. The treatments with salicylic acid, carvacrol and thymol induced the highest shift in phenolic metabolite profile with respect to control trees. The protective effect of carvacrol and salicylic acid is discussed in terms of their combined activity as antifungal compounds and as inductors of tree defense responses.

Abbreviations:

DED: Dutch elm disease

Dpi: days post inoculation

SA: salicylic acid

SIR: systemic induced resistance

Introduction

Most mature elms in Europe and North America have died as consequence of Dutch elm disease (DED), which is caused by the vascular fungi *Ophiostoma ulmi* (Buisman) Nanf. and the more virulent *O. novo-ulmi* Brasier (Brasier 1991). Several species of elm bark beetles, mainly in the genus *Scolytus* and *Hylurgopinus*, have been recognized as the main vectors of the disease (Webber 2004). The effective spread and complex interactions between several biotic and abiotic environmental factors make DED is extremely difficult to control.

Nowadays, there are no effective means to prevent or control DED in practical scale. Some fungicides against the DED pathogens, based on benzimidazoles (benomyl, carbendazim, and thiabendazole) and sterol biosynthesis inhibitors (Stennes 2000) are available in the market. The use of these direct control treatments is limited because the injection of fungicide requires several hours per tree and specialized personnel, and should be repeated each two years. Control of disease vectors through chemical insecticides is environmentally hazardous, often inefficient and economically impractical for large-scale treatments. As an option to control disease especially in urban trees, the induction of resistance on the tree by means of artificial inoculations with non-pathogenic microorganisms has been widely studied (e.g., Solla et al. 2003; Scheffer et al. 2008). Although promising, this biological control method is limited by the short-term effect of the protection time span (one vegetative season), and by the variable results obtained depending on the tree genotype. So far, the most successful strategy for the elm recovery has been breeding for resistance using Asian elm species as sources of resistance genes (Smalley and Guries 2000). However, the breeding of native European and American elms is proceeding slowly, mainly due to the lack of

highly resistant genotypes and the long cycles needed. Thus, there is a need to find alternative methods to slow down the progress of the disease.

An alternative strategy for DED control may consist in identifying natural molecules with anti-pathogen properties which can be transported over long distances in the tree. Certain low molecular weight plant phenolics might fulfil those requeriments, given their known chemical and biological properties in tree-pathogen interactions (Witzell and Martín 2008). Perhaps the best known of these compounds is salicylic acid (SA), the crucial role of which in the induction of systemic induced resistance (SIR) is well documented (Heil and Bostock 2002). Moreover, the monoterpene phenolics carvacrol and thymol have been reported to possess strong antimicrobial activities (Roller and Sheedhar 2002; Kordali et al. 2008). As a potential source of these and other antimicrobially active phenolics, essential oils isolated from plants are now receiving increasing interest. A very attractive reason of using essential oils or their constituents as plant protectants is their low environmental toxicity (Isman 2000).

One line of evidence indicating that exogenously applied phenolics may protect the elms against DED comes from the observation that the soil application of disinfectant products for the cattle, based on simple phenols, seems to lead to increased elm resistance to DED (Martín et al. 2008). In the current study, we hypothesize that exogenously added phenols, especially those derived from essential oils, such as carvacrol and thymol, or those involved in induction of resistance, such as SA, have the potential to reduce DED symptoms in elms. The *in vitro* effect of carvacrol, thymol and SA against the DED fungi was tested, and their antifungal efficacy in comparison to other five simple phenols, commonly used in pesticides and disinfectants (phenol, *o*-cresol, *m*-cresol, *p*-cresol, and 2,5-xylenol) tested. Also, the effect of the same phenols, applied with watering, on the resistance of young elm trees against *O. novo-ulmi* was

tested. The effects of phenols on leaf phenology, xylem radial growth, and chemical profile of the xylem were studied and discussed with respect to the resistance levels shown by the trees.

Materials and methods

In vitro experiment

The antifungal effect of the phenols SA, carvacrol, thymol, phenol, *o*-cresol, *m*-cresol, *p*-cresol, and 2,5-xylenol (all the phenols provided by Sigma-Aldrich®, Steinheim, Germany) on the DED pathogens was tested *in vitro*. Three *Ophiostoma* isolates, labelled P98 (*O. ulmi*), P114 (*O. novo-ulmi* ssp. *novo-ulmi*) and NA-PE (*O. novo-ulmi* ssp. *americana*) were used for the experiment. The isolates P98 and P114, kindly provided by C. M. Brasier and S. Kirk, are reference strains collected in 1980 in Krzeszyce and Troszyn (Poland), respectively. These strains showed an *in vitro* growth on 2% malt extract agar (MEA; 20º C) of 2.1 and 3.4 mm day^{-1}, respectively. The isolate NA-PE was collected in 2002 from an infected *U. minor* tree in Navarra (Spain) and showed an *in vitro* growth on 2% MEA (20º C) of 5.9 mm day^{-1}. The fungi were conserved at 4º C in the dark. At 3-months intervals, they were subcultured and since their *in vitro* growth rate did not significantly change with time, it was assumed that no significant loss of virulence had occurred during maintenance.

The effect of the phenols on the growth of the *Ophiostoma* isolates was evaluated in 96-well microtiter plates, following the methodology of Raposo *et al*. (1995). Conidial suspensions of the three isolates were prepared by adding 2 × 2 mm plugs from the edge of 7-day-old cultures on MEA to 50 mL of Tchernoff's liquid medium

(Tchernoff 1965) in sterile Erlenmeyer flasks, followed by shaking in darkness for 4 days at room temperature. The concentration of the conidial suspension was adjusted to 10^4 conidia per mL using a hemacytometer. Then, 100 µL of the suspension were added to each well of the microplate. Each well also received 100 µL of a dilution of the different phenols in Tchernoff's liquid medium, resulting in a final volume of 200 µL per well. Final test concentrations of phenols in the wells were 0 (control wells), 0.2, 1, 10, 50, 100, and 500 µg/mL. Four replicate wells per each phenolic concentration were included. Microplates were incubated at 20ºC, and optical densities were measured at 48 h with a microplate reader (ELx808, Bio-Tek, Vermont, USA) at 492 nm wavelength. All values were corrected for optical densities at time zero, and percent growth was calculated by dividing the corrected optical density readings of each well by the mean corrected optical density of control wells. The experiment was conducted four times per isolate in separate trials.

In vivo experiment

Plant material

The experiment included 200 *U. minor* seedlings obtanied from open-pollinated seeds collected from 10 trees at Rivas-Vaciamadrid elm stand (Madrid, Spain, 40º20'N, 3º33'W). Twenty seedlings per tree were used. The nursery-grown seedlings were 30-50 cm in height when transplanted at the Forest Breeding Centre in Puerta de Hierro (Madrid, Spain) to 30 L pots containing a sandy loam substrate. When the first treatments were applied, the ramets were 4 years old and 110-260 cm in height. Plants were placed outside under a shading mesh providing 25% of full sunlight throughout the

experiment. Pots were distributed randomly, with a spacing of 0.5 × 0.5 m, and were irrigated in spring and summer to avoid drought stress.

Treatments

The same eight phenolic compounds tested *in vitro* were used for watering the plants. The concentration of phenols used was decided on basis of an earlier study (Martín et al. 2008), in which elms treated with a mixture of phenol and cresols at 200 mg/L showed around 60% reduction of DED symptoms in comparison to control trees. With the aim to improve these results, a concentration of 400 mg/L was selected for the present experiment. Every two weeks, plants were manually watered with 1 L of the phenolic solution. Twenty seedlings per phenolic compound were used, and each lot of these 20 plants included 2 seedlings per each of the 10 mother trees used. Forty additional seedlings (including 4 seedlings per each of the 10 trees) received 1 L of water per plant, and were used as controls. The treatment period was from 15 February to 24 May 2006.

Inoculation and symptom evaluation

On 1 May 2006, a time of year when elm trees are generally very susceptible in the local area, all phenol-treated trees were inoculated with *O. novo-ulmi*. Control trees were divided into trees inoculated with *O. novo-ulmi* (+I control trees; N=20), and trees inoculated with distilled water (-I control trees; N=20). Inoculations were carried out following the procedure described in Martín et al. (2005), with spore suspensions adjusted to 10^6 conidia mL^{-1}. The *O. novo-ulmi* strain NA-PE was used for inoculations

because of its high growth rate in MEA (see the *in vitro* experiment section). Disease severity was evaluated by recording the percentage of leaf wilting in the crown of each tree at 30, 60 and 120 days post inoculation (dpi).

Leaf phenology and apical growth

The leaf phenology was studied from 1 March to 1 May during 2006. Bud development was assessed weekly using the five stages described by Santini et al. (2005): 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 leaves or more completely expanded. Twenty buds per tree were screened. A plant was considered to have reached a certain stage as soon as more than half the lateral buds had achieved that stage. For each tree these data were summarized in terms of the number of days from 1 January required to reach the stage 3. Plant height was measured on dormant trees before the treatments and at the end of the 2006 growing season, thus obtaining the measure for the seasonal apical growth.

Histology

On 1 May, 15 June and 1 August 2006, the main trunk of 10 seedlings per treatment (one per progeny) was wounded to the depth of the vascular cambium in order to mark the seasonal increment of the growth ring. Each trunk was wounded three times, one per wounding date, 5 cm above the soil surface. The first wound was made at the south, the second at the northeast and the third at the northwest side, with 120º of separation between wounds. Wounds were done longitudinally (10 × 2 mm) with a flame-sterilized

scalpel. In November 2006, the trees were sawed at the height of the wounds and trunk segments containing the wounds were placed in formaldehyde–acetic acid–70% ethanol (5:5:90, v/v/v) fixative. Transverse sections (15 µm thick) were obtained from the trunk segments using a sliding microtome, and were observed under an Olympus BX50 light microscope (Olympus Optical Company, Shibuya-ku, Tokyo, Japan). Seasonal increments of radial growth were obtained by measuring the distances from the wound surfaces to the beginning of the 2006 growth ring.

Chemical analysis

On 30 April 2006, one day before inoculation, two twig samples (2 years old and 3-cm long) were removed from each tree. Twigs were located in the upper half of the crown, (northern side, where available). The samples were immediately frozen in liquid N_2 and conserved at –80 ºC. Since the alteration of the phenolic metabolism has been frequently associated with defense responses of woody plants against fungi (Witzell and Martín 2008), a chemical analysis of the twig samples was conducted in order to trace possible changes in their phenolic profile due to the treatments with phenols.

The bark tissues of the twigs were removed, and the xylem of the outermost growth ring was separated using a razor blade. The xylem slides obtained were freeze-dried and pulverized using a ball mill. Then, 10-11 mg of wood powder were extracted with methanol as described by Witzell el al. (2003). Methanol soluble phenolics were analysed with liquid chromatography (HPLC) as described by Shrivastava et al. (2007). Chromatogram peaks were integrated by means of HSM D-7000 chromatography data station software (Merck – Hitachi, Darmstadt, Germany) at 220 nm. Standard samples of the eight phenolic compounds used for the treatments were analyzed by HPLC in

order to observe the possible accumulation of the compounds in xylem tissues. Peaks were also compared to a selection of other phenolics (an UV-spectrum library containing, e.g. catechins and common phenolics acids) but most of them could not be identified with HPLC. Peak areas were related to the sample weight in each injection to obtain comparable data. The total concentration of HPLC metabolites per sample was estimated by the sum area of all peaks in the chromatogram.

Statistical analysis

The optical densities of the microplate wells obtained from the *in vitro* experiment were compared by means of two-way analysis of variance (ANOVA) with *concentration of phenols*, and *fungal isolate* as factors. *Concentration of phenol* × *fungal isolate* interactions were considered. The concentration of each phenolic compound that inhibited fungal growth by 50% relative to control wells (EC_{50} value) was calculated. Thus, the percentage reduction in optical density of phenol-containing wells relative to control wells was regressed on $\log_{10}$ of phenol concentration, and EC_{50} values were obtained by substituting the value 50% into the regression equation. For each phenolic compound, EC_{50} values were compared by means of one-way ANOVA with *fungal isolate* as factor. Values of leaf wilting and apical growth were compared by means of three-way ANOVA with *phenol treatment*, *mother tree*, and *wounding* (i.e., trees wounded for histological study vs. non-wounded trees) as factors, and *plant height* as covariate. Values of days to bud burst, apical growth and radial increment were compared by means of two-way ANOVA with *phenol treatment*, *mother tree* as factors, and *plant height* as covariate. Comparisons between means were made by means of multiple range tests with Fisher's least significant difference (LSD) intervals. Simple

regression analyses were made between leaf phenology, apical growth, radial increment, and foliar wilting.

Results from HPLC analysis were analyzed by means of a discriminant function analysis (DFA). The chemical profile of each sample was defined by a pattern of 32 chromatogram peaks, whose normalized areas were used as input variables for the DFA, with *a priori* information about sample grouping in the data. This information was used to produce measures of within-group variance and between-group variance, and then, to define discriminant functions (DFs) that optimally separate the a priori groups. Eight DFAs were implemented, one per each phenolic compound tested. Thus each DFA included two *a priori* groups, relative to samples from trees treated with a certain phenol and relative to samples from the control trees. In order to estimate the discriminating power of the DFs, Wilks' Lambda tests were performed. The coefficients by which the original variables (peak retention times) are multiplied to obtain the DFs are called loadings. Since the numerical value of a loading of a given variable on a DF indicates how much the variable has in common with that DF, loadings were used to ascertain the most significant peaks related to the discrimination between samples. All statistical analyses were performed by means of Statgraphics Plus v5.1 software (Manugistics, MD, USA).

Results

In vitro experiment

For all phenolic compounds, the effect of the concentration of phenols on the optical density of the microplate wells was significant ($P < 0.01$). Except for *m*-cresol, the

effect of the fungal isolate on the optical density was significant for all phenolic compounds tested ($P < 0.05$). Interactions between the concentration of phenols and the fungal isolates on the optical density were also significant for all the compounds tested ($P < 0.05$), except for *m*-cresol. The most effective antifungal compounds were SA, carvacrol and thymol, with EC_{50} values below 50 µg/mL (Table 1). EC_{50} values for carvacrol, phenol, *o*-cresol, *m*-cresol, and *p*-cresol significantly varied depending on the fungal isolate trested ($P < 0.05$) (Table 1).

Table 1

In vivo experiment

Carvacrol was the most effective compound in protecting trees against *O. novo-ulmi*. At 60 and 120 dpi, the mean wilting percentages shown by trees treated with this compound was significantly lower than the wilting percentages of +I control trees. ($P < 0.05$) (Table 2). Although a reduction of symptoms was found at 30 dpi ($P < 0.10$), the protective effect of carvacrol increased with time, with a maximum of 63% reduction of symptoms at 120 dpi. The second most-effective compound was SA, which reduced wilting symptoms in comparison to +I control trees at 60 ($P < 0.10$) and 120 ($P < 0.05$) dpi, with a 54.4% reduction of symptoms at 120 dpi. Treatments with thymol and *p*-cresol reduced wilting symptoms at 30 dpi ($P < 0.05$), and phenol reduced symptoms at 60 dpi ($P < 0.10$), but their protective effect was not significant at the end of the experiment. Mean wilting values increased significantly with time ($P < 0.01$), and the mother effect was slightly significant ($P < 0.10$; data not shown). The wounding effect on wilting values was not significant ($P > 0.30$).

Table 2

The effect of the treatment on bud burst phenology was not significant ($P > 0.17$). while the effect of the mother tree was highly significant ($P < 0.001$; data not shown).

All lots of inoculated trees showed a significant lower apical growth than the -I control trees ($P < 0.01$). Both, the treatment and wounding effects on the apical growth were not significant ($P > 0.10$), while the mother tree factor showed differences at $P < 0.10$ (data not shown).

Both, the treatment and mother tree effects on radial xylem increments were significant ($P < 0.01$). Mean values of xylem growth increments along the season are shown in Table 3. If cumulative values of growth are considered, on 1 May no significant differences between treated and +I control trees were obtained, except for the slightly lower increment shown by trees treated with phenol ($P < 0.10$). On 15 June, SA-, carvacrol-, *o*-cresol- and 2,5-xylenol-treated trees, as well as –I control trees showed a higher radial increment than +I control trees ($P < 0.05$). On 1 August and at the end of the experiment, only carvacrol-treated and –I control trees grow radially more than +I control trees ($P < 0.05$). If the period of radial growth from 1 May to 15 June is considered, SA-, carvacrol-, phenol-, *o*-cresol-, and *p*-cresol- treated trees, as well as –I control showed higher increment growths than +I control trees ($P < 0.05$).

Table 3

The days to bud burst were negatively related to the radial increment at 1 May, to the total apical growth, and to leaf wilting at 60 and 120 dpi ($P < 0.05$) (Table 4). In contrast, the radial increment at 1 May was positively related to 30 dpi leaf wilting ($P < 0.05$), and the total apical growth was positively related to 60 dpi leaf wilting ($P < 0.05$).

Table 4

The exogenously applied phenolic compounds used were not detected in the xylem tissues by means of the HPLC analysis. However, all DFs were observed to be significant ($P < 0.05$) (Table 5). The most powerful discriminations were obtained when comparing profiles of control trees with profiles of trees treated with SA ($P < 0.001$), carvacrol and thymol ($P < 0.01$). Watering with SA caused the largest distance between

the centroids of control and treated trees, followed by watering with carvacrol and thymol. Peaks with the highest loading values varied depending on the treatments, but coincided when trees were watered with SA or carvacrol (peak at 14.79 min), and when trees were watered with thymol, phenol or o-cresol (peak at 14.55 min). Only samples from trees watered with 2,5-xylenol showed a higher level of total HPLC metabolites than samples from control trees ($P < 0.05$).

Table 5

Discussion

The present study shows the potential of some exogenous phenols, especially of carvacrol and SA, to reduce the symptoms of DED in young elms. Different modes of action of these phenols, working separately or in synergy could be involved in the observed reduction of DED symptoms.

First, the low-molecular weight phenolics could function as direct antifungal agents in elm tissues, limiting fungal growth. It has been proposed that SA has no direct antifungal activity (Mills & Wood 1984; Okuno et al. 1991), but also contrasting results have been found (e.g., Georgiou et al. 2000; Amborabe et al. 2002). Our results indicate a strong antifungal effect of SA towards *O. ulmi* and *O. novo-ulmi*. Carvacrol and thymol, both derived from essential oils, were also powerful inhibitors of the DED fungi. This is consistent with previous research reports showing a strong inhibitory activity of these compounds or their original essential oils against a wide variety of phytopathogenic fungal species at concentrations below 500 µg/mL (e.g., Lee et al. 2007; Kordali et al. 2008). Carvacrol, thymol and other active components of the essential oils containing them have been reported to cause alterations in the hyphal

morphology, such as cytoplasmic coagulation, vacuolations, hyphal shrivelling and protoplast leakage (Soylu et al. 2006).

A strong correspondence between the *in vitro* antifungal activity of some phenols and their *in vivo* protective effect against *O. novo-ulmi* inoculation was shown; SA and the monoterpene phenolics, carvacrol and thymol, caused the strongest *in vitro* inhibition of *Ophiostoma* isolates and significant reduction of DED symptoms after inoculation with *O. novo-ulmi*. Watering the trees with *p*-cresol also resulted in reduced symptoms, but the *in vitro* antifungal effect of this compound on the pathogen was very weak. This suggests that other mechanisms than a direct antifungal activity were involved in the enhancement of resistance in *p*-cresol treated trees.

A second mechanism explaining the reduction of DED symptoms could be the effect of the treatments on tree phenology. Tree phenology, in particularly timing of wood formation, may strongly affect the DED pathogenesis, since fungal propagules, mainly yeast-like spores, are passively transported by the sap stream. To be effective, this transport should occur by means of the wide, highly conductive, earlywood vessels. Moreover, fungal development inside xylem vessels is thought to be involved in the cavitation of the water columns (Ouellette et al. 2004, and references therein), and this process ultimately leads to foliar wilting. There is a period of maximum susceptibility of elms against DED, starting when the first earlywood wide vessels became functional and ending when a certain proportion of the latewood narrow vessels have been formed (Solla et al. 2005). A plant will show maximum symptoms only if the artificial inoculation with *O. novo-ulmi* is carried out within that period. For instance, the dispersal of *O. novo-ulmi* propagules in the sap stream could be limited if the large earlywood vessels are not yet fully functional.

In our study, the negative correlation found between the number of days to bud burst and the foliar wilting (Table 4) suggests that early flushing trees may have shown higher DED symptoms than late flushing trees because they were at the period of maximum susceptibility by the time they were challenged by the fungus. It is likely that the early flushing trees had already completed formation of their earlywood vessels by the time of plant inoculation, while some earlywood vessels of late flushing trees may have not been fully functional (Fig. 1). This possibility is indirectly supported by the significant negative relation found between the days to bud burst and the radial increment of trees at 1 May (inoculation day), and by the positive relation found between radial increment before inoculation and the 30 dpi leaf wilting. The lack of relation between the radial increment at 1 May and the foliar wilting at 60 and 120 dpi suggests that a delayed xylem formation only implied reduction of symptoms at the initial stages of the infection, and lately other factors were probably stronger determinants of tree resistance. An early bud burst was accompanied by a higher apical growth, probably as consequence of a longer period of cambial activity. The relations found between the tree phenology parameters and the foliar wilting symptoms were conditioned by the effect of the mother tree. Thus, different plant genotypes may show different symptoms depending on their earlywood development when they are inoculated at the same time. This emphasizes the importance of using clonal material, or the need of distributing equally the progenies among the different treatments if seedlings are used.

Fig. 1

After inoculation, differences in radial increments observed between treated and control plants occurred likely as a consequence of fungal inhibition by the phenols, allowing a higher growth rate of the treated plants. Thus, from 1 May to 15 June, trees treated with SA, carvacrol, phenol, and *p*-cresol, showed a higher radial increment than

untreated +I control trees, probably because these treatments protected somehow the plants against fungal development. Only in trees treated with carvacrol, the most effective antifungal compound, the total radial growth was higher than in +I control plants.

A third mechanism that could have influenced the plant resistance level is the role of phenols as activators of plant defense mechanisms. The DFA indicated that the trees treated with SA, carvacrol, thymol and *p*-cresol differed most from control trees with respect to their phenolic profiles, and these four phenolics also increased tree resistance against *O. novo-ulmi* (Tables 3 and 5). This correspondence strongly suggests that differences in phenolic profiles of xylem tissues were translated into differences in tree resistance levels. Previous research on leaf phenolics of elms also revealed qualitative differences in the phenolic metabolite profile between resistant and susceptible elms to DED (Heimler et al. 1994).

The activation of phenolic metabolism and the accumulation of certain phenolics appear commonly associated with SIR (Conrath 2006). Given the essential role of SA in the establishment of SIR in remote tissue (Heil and Bostock 2002), the highest shift in phenolic metabolism found in SA-treated trees with respect to controls could be related to the activation of SIR mechanisms. The examination of the DF loadings may reveal the importance of some chromatogram peaks for the discrimination between phenol-treated and control plants. In particular, the loadings showed that the peaks at 14.79 and 14.55 min would deserve further research with more specific techniques, since the role of these compounds in disease resistance is uncertain. The fact that the exogenously applied phenols were undetectable in xylem tissues may have been due to the limits of the method, or to the metabolization of these compounds by the plants. It has been proven that higher plants are able to metabolize exogenous aromatic hydrocarbons and

simple phenols via aromatic ring cleavage (Korte et al. 2000). The carbon atoms of these compounds, as a result of cellular metabolism, are incorporated into the molecules of endogenous metabolites as carbonic acids and amino acids.

In recent years, there has been an increasing interest to develop practical applications based on induction of resistance through exogenous chemicals (e.g., Percival 2001; Zenelli et al. 2006). The focus has been mainly on jasmonates, molecules that regulate various plant defense responses through signaling pathways that are distinct from the SA-mediated SIR pathway. Our results suggest that exogenous low molecular weight phenolics may be an alternative to increase plant resistance against pathogens, since they have the potential to act through several mechanisms, such as antimicrobial compounds, or as inducers of chemical changes that could lead to increased resistance levels. The 63% reduction of wilting symptoms obtained with carvacrol is a promising first step for a control strategy of DED based on this compound. However, the relatively low wilting symptoms showed by control plants (47.6%) indicate that these results should be interpreted with caution. The symptoms obtained with plants planted in pots are generally lower than those showed by plants growing in plots, probably due to the limited root and xylem development of the potted plants. The results obtained here will thus be validated through treatments on older elm trees, planted at wide spacing in controlled plots. Testing phenols at other doses and application methods (e.g. trunk injection) is already in progress.

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Figure legends

Fig. 1. Light microscope photographs of transverse sections of the main trunk of two control trees from two different progenies. (**a**) Early-flushing tree. (**b**) Late-flushing tree. Thirty days after inoculation with *Ophiostoma novo-ulmi*, the wilting percentages shown by (**a)** and (**b)** trees were 30 and 15 %, respectively. The earlywood formation before inoculation in 2006 (e) was determined by the distance between the end of the 2005 growth ring and the mark caused by the wound (w) made at 1 May 2006 (distance between the two dotted lines). A notably higher increment of earlywood is appreciated in (**a)** with respect to (**b**), probably as consequence of a late initiation of cambial activity in (B). t = total growth increment at the end of the season. Bar = 300 µm.

Table 1. *F* statistics and probabilities of greater *F* values (*P*) from two-way analyses of variance for the *in vitro* growth (optical density) of the strains P-98 (*Ophiostoma ulmi*), P-114 (*O. novo-ulmi* ssp. *novo-ulmi*) and NA-PE (*O. novo-ulmi* ssp. *americana*) cultivated in liquid medium containing eight phenolic compounds. Six doses (0, 0.2, 1, 10, 50, 100, and 500 µg/mL) of each phenolic compound were tested.

		Salicylic acid		Carvacrol		Thymol		Phenol		*o*-Cresol		*m*-Cresol		*p*-Cresol		2,5-Xylenol	
Factor	df[a]	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Dose	5	125.55	<0.01	103.81	<0.01	78.90	<0.01	11.21	<0.01	15.33	<0.01	7.02	<0.01	20.74	<0.01	10.54	<0.01
Isolate	2	4.33	0.02	26.16	<0.01	7.48	<0.01	10.01	<0.01	6.34	<0.01	1.52	0.22	27.44	<0.01	15.65	<0.01
Dose-isolate	10	2.83	<0.01	2.27	0.02	2.61	<0.01	4.77	<0.01	2.00	0.04	0.81	0.62	3.65	<0.01	3.09	<0.01

[a] Degrees of freedom.

Table 1. EC_{50} values (mean ± SE; µg/ml) of eight phenolic compounds tested *in vitro* against the strains P-98 (*Ophiostoma ulmi*), P-114 (*O. novo-ulmi* ssp. *novo-ulmi*) and NA-PE (*O. novo-ulmi* ssp. *americana*). For each compound, different letters indicate significant differences between strains (LSD; $P \leq 0.05$).

Compound	EC_{50} value (µg/mL)			ANOVA *P*-values[a]
	P98	P114	NA-PE	
Salicylic acid	45.1 ± 17.2 a	29.5 ± 10.9 a	21.3 ± 2.1 a	0.39
Carvacrol	3.1 ± 0.7 a	4.6 ± 0.4 a	13.3 ± 2.8 b	<0.01
Thymol	21.8 ± 10.8 a	3.9 ± 0.2 a	8.7 ± 1.4 a	0.17
Phenol	521.3 ± 72.4 b	389.0 ± 68.8 a	407.7 ± 80.1 a	<0.05
o-Cresol	>1000 b	576.3 ± 81.1 a	682.1 ± 105.0 a	<0.01
m-Cresol	646.0 ± 74.8 b	219.7 ± 37.8 a	220.2 ± 65.1 a	<0.01
p-Cresol	>1000 b	319.7 ± 30.4 a	>1000 b	<0.01
2,5-Xylenol	>1000	>1000	>1000	-

[a] Signification obtained from one-way ANOVA of EC_{50} values, considering the fungal strain as factor.

Table 2. Mean values (mean ± SE) of leaf wilting, bud-break date and apical growth of *Ulmus minor* trees treated with eight phenolic compounds, and untreated control trees. All trees were inoculated with *Ophiostoma novo-ulmi* at 1 May 2006, with the exception of –I control trees. Within each column, asterisks indicate significant differences with respect to +I control trees at $P \leq 0.10$ (*), $P \leq 0.05$ (**), and $P \leq 0.01$ (***).

Treatment	30 dpi[a] leaf wilting (%)	60 dpi leaf wilting (%)	120 dpi leaf wilting (%)	Days to bud burst	Total apical growth (cm)
Salicylic acid	17.6 ± 3.6	21.9 ± 4.8 *	25.9 ± 5.8 **	99.1 ± 10.9	19.9 ± 4.5
Carvacrol	14.3 ± 3.3 *	15.4 ± 5.2 **	17.8 ± 4.4 ***	90.6 ± 11.6	19.0 ± 4.6
Thymol	14.6 ± 2.5 **	23.9 ± 5.4	35.1 ± 7.0	95.3 ± 13.5	20.9 ± 6.0
Phenol	20.1 ± 4.9	20.9 ± 4.9 *	34.3 ± 6.2	106.8 ± 15.2	21.7 ± 4.2
o-Cresol	22.3 ± 3.7	25.2 ± 5.2	32.9 ± 6.1	113.8 ± 10.5	19.9 ± 5.7
m-Cresol	21.3 ± 4.8	26.9 ± 6.0	30.0 ± 5.8	103.3 ± 14.1	20.4 ± 7.2
p-Cresol	14.1 ± 3.7 **	27.0 ± 4.9	38.5 ± 6.3	110.4 ± 9.9	22.7 ± 4.6
2,5-Xylenol	18.6 ± 3.9	31.7 ± 6.2	39.6 ± 7.4	94.8 ± 13.6	22.8 ± 5.5
+I control	26.2 ± 4.6	34.9 ± 5.5	47.6 ± 7.4	110.7 ± 9.8	20.3 ± 5.2
-I control	0.0 ± 0.0 ***	00.0 ± 0.0 ***	00.0 ± 0.0 ***	105.1 ± 12.0	40.1 ± 7.4 ***

[a] days post-inoculation

Table 3. Xylem radial increments of the 2006 growth ring of *Ulmus minor* trees treated with eight phenolic compounds, and untreated control trees. All trees were inoculated with *Ophiostoma novo-ulmi* at 1 May 2006, with the exception of -I control trees. Within each column, asterisks indicate significant differences with respect to +I control trees at $P < 0.10$ (*), $P < 0.05$ (**) and $P < 0.01$ (***).

	Cumulative radial increment (μm)				Period to period radial increment (μm)	
Treatment	1 May	15 June	1 August	Total	1 May – 15 June	15 June – 1 August
Salicylic acid	406.7 ± 51.2	710.8 ± 306.2	745.0 ± 91.6 **	1261.5 ± 121.2	439.9 ± 100.2 **	271.2 ± 253.2
Carvacrol	317.5 ± 62.6	889.5 ± 106.9 ***	1706.25 ± 248.7 **	1865.0 ± 141.4 ***	531.5 ± 111.5 **	609.2 ± 188.8
Thymol	465.4 ± 48.2	747.2 ± 94.6	1279.5 ± 251.3	1697.1 ± 115.7 *	316.6 ± 96.4	640.6 ± 175.6
Phenol	258.5 ± 47.3 *	634.1 ± 85.5	1312.4 ± 236.5	1403.4 ± 115.1	481.5 ± 92.2 **	846.3 ± 170.8 *
o-Cresol	330.4 ± 43.3	815.3 ± 92.9 **	1130.8 ± 213.6	1437.9 ± 110.6	484.5 ± 84.6 **	381.6 ± 166.2
m-Cresol	311.1 ± 45.4	610.6 ± 83.6	1346.3 ± 275.2	1401.1 ± 107.4	276.8 ± 74.3	574.0 ± 197.2
p-Cresol	273.6 ± 55.3	692.4 ± 113.5	1355.2 ± 263.4	1114.7 ± 130.1	461.9 ± 94.9 **	383.2 ± 359.8
2,5-Xylenol	448.2 ± 48.2	835.1 ± 74.7 **	1416.3 ± 228.4	1415.7 ± 102.5	412.9 ± 69.8	492.6 ± 184.1
+I control	399.9 ± 44.0	522.2 ± 82.3	928.0 ± 243.5	1371.0 ± 109.1	210.4 ± 81.2	355.4 ± 199.0
-I control	372.5 ± 52.2	1212.2 ± 87.4 ***	1222.4 ± 354.8 **	2526.9 ± 123.5 ***	848.1 ± 95.4 ***	194.5 ± 260.1

Table 4. Correlation matrix and levels of significance among variables. Asterisks indicate significances at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***); ns = non significant.

Variable	Radial increment at 1 May	Total apical growth	30 dpi leaf wilting	60 dpi leaf wilting	120 dpi leaf wilting
Days to bud burst	- 0.22 **	- 0.28 ***	ns	- 0.20 *	- 0.20 *
Radial increment at 1 May		ns	0.21 *	ns	ns
Total apical growth			ns	0.14 *	ns
30 dpi leaf wilting				0.67 ***	0.55 ***
60 dpi leaf wilting					0.86 ***

Table 5. Results of secondary metabolite profile analysis of xylem samples of *Ulmus minor* trees treated with eight phenolic compounds if compared with untreated control trees. All samples were gathered on 30 April 2006, before inoculation with *Ophiostoma novo-ulmi*, from trees treated with different phenols. The metabolic profile was defined by 32 HPLC chromatogram peaks per sample, and a discriminant function analysis (DFA) was implemented considering the peak areas as input variables.

Treatment	DFA of treated vs. control trees				Total metabolites relative to controls[b] (mean ± SE)
	P-value	Distance between centroids of treated and control trees	Samples correctly classified (%)	Peaks with the highest DF loading (minutes[a])	
Salicylic acid	< 0.001	14.74	100	14.79	1.07 ± 0.12
Carvacrol	< 0.01	4.84	100	14.79	1.04 ± 0.12
Thymol	< 0.01	4.94	100	14.55	1.09 ± 0.15
Phenol	0.03	1.71	80	14.55	0.92 ± 0.15
o-Cresol	0.04	1.53	80	14.55	1.18 ± 0.15
m-Cresol	0.03	1.66	70	10.95	1.14 ± 0.14
p-Cresol	0.01	2.01	80	10.71	1.09 ± 0.17
2,5-Xylenol	0.05	1.48	90	17.89	1.38 ± 0.15 *

[a] Expresed as retention time in the HPLC chromatogram.
[b]Obtained by dividing the sum of all peak areas of the treated trees by the sum of all peak areas of the control trees. Asterisks indicate significant differences between mean peak areas of treated and control trees ($P < 0.05$)

Fig. 1.