Deciphering the role of the phenylpropanoid metabolism in the tolerance of pepper (*Capsicum annuum* L.) to *Verticillium dahliae* Kleb.

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Abstract

Verticillium dahliae is an economically relevant soilborne pathogen that causes vascular wilt in several crops, including pepper (*Capsicum annuum*). Fungal infection is usually visualized as a vascular browning, likely due to the onset of phenylpropanoid metabolism, which also seems to play a crucial role in the tolerance of some pepper varieties. In the current work, the potential function of distinct phenylpropanoid derivatives (suberin, lignin and phenolic compounds) in the pepper tolerance response against *V. dahliae*, was investigated. Histochemical and biochemical analyses ruled out suberin as a key player in the pepper-fungus interaction. However, changes observed in lignin composition and higher deposition of bound phenolics in infected stems seemed to contribute to the reinforcement of cell walls and the impairment of *V. dahliae* colonization. Most importantly, this is the first time that the accumulation of the hydroxycinnamic acid amide *N*-feruloyltyramine was reported in pepper stems in response to a vascular fungus. Fungitoxic activity for that hydroxycinnamate-tyramine conjugate was demonstrated as well.

Key words: *Verticillium wilt*, lignin, bound phenolics, *N*-feruloyltyramine, fungitoxic activity

1. Introduction

Verticillium dahliae Kleb. is a soil-borne pathogen that causes plant vascular wilt worldwide, limiting the yield of a wide range of economically important crops, including pepper (*Capsicum annuum* L.) [1]. The extraordinary impact of this fungus is largely due to the production of survival structures called microsclerotia, which can persist under field conditions for long periods even in the absence of a host. When the microsclerotia are close to the host plant root and the environmental conditions are favorable, they germinate causing infection [2]. Thus, the fungal hyphae usually penetrate the host through wounded roots, and spread systemically across the xylem. The growth and activity of the fungus in the vascular tissue, including the occlusion of xylem vessels by tyloses and vessel-coating materials leads to the appearance of ‘wilt’ disease symptoms [3]. Those include stunting, epinasty, wilting, foliar chlorosis, progressive necrosis, vascular browning, and leaf abscission. As long as the host plant remains alive, *V. dahliae* usually colonizes only host xylem vessels, in which the hyphae compete with plant tissues for nutrients. Only after the host dies, does it colonize senescent tissues [3].

The only known resistance genes against *V. dahliae* in Solanaceae are those of the tomato *Ve* locus [4]. In pepper, resistance to this pathogen has been observed mainly in *C. frutescens* and *C. baccatum* and, to a lesser extent, in *C. annuum* [5]. However, various reports have suggested that different degrees of resistance might exist in the widely cultivated *C. annuum*, for which various varieties with high levels of tolerance to *V. dahliae* have been described [6]. Such tolerance appears to arise primarily from a combination of distinct mechanisms that are activated in the course of the plant defence response, constraining the fungal colonization and avoiding the entire invasion of the host and subsequent plant death [7,8]. Among those, the onset of phenylpropanoid metabolism seems to play a crucial role, leading to the synthesis and accumulation of defensive structures. Indeed, the phenylpropanoid derivatives and their polymers might be among the principal compounds responsible for the darkened brown areas around xylem vessels, acting directly as

pathogen inhibitors or indirectly by contributing to the reinforcement of the plant cell wall [9,10]. Phenolic compounds have been long correlated with the resistance of plants to infective agents [11]. The phenylpropanoid pathway is usually activated as a result of the pathogen's recognition by the plant. The first key step in the biosynthesis is carried out by the enzyme phenylalanine ammonia lyase (PAL), which catalyzes the deamination of phenylalanine to *trans*-cinnamic acid. In higher plants, the hydroxycinnamic acids (HCA), such as *p*-coumaric and ferulic acids, possess broadly antimicrobial activity by disrupting the membrane integrity and decoupling the respiratory proton gradient [12,13]. Recently, also poacic acid, a derivative released from a plant diferulate, was showed to disrupt fungal cell wall biosynthesis and is consequently a potent antifungal [14]. Additionally, HCA serve as precursors for more complex phenolic polymers that may act as protective physical barriers through the strengthening of the plant cell wall structure [15]. Among those polymers, suberin and lignin have been proposed as the principal players in the cell wall modifications [16]. The term suberin is usually applied to complex heteropolymers constituted by poly-aliphatic and poly-aromatic domains. Most of the aliphatic monomers are long-chain α,ω -diacids and α -hydroxyacids, whereas the aromatic domains are polyphenolic substances mostly derived from hydroxycinnamic acid derivatives [17]. Suberized cells play a vital role affording protection against environmental aggressions and pathogens [18]. Thus, suberin is thought to accumulate on the interior cell walls of xylem vessels as a part of the plant's constitutive defense system [19,20]. Lignins are heteropolymers resulting from the oxidative coupling of, principally, three *p*-hydroxycinnamyl alcohols (monolignols): *p*-coumaryl, coniferyl and sinapyl alcohols [21]. The cross-coupling reaction of monolignol radicals, primarily with the growing polymer, produces a hydrophobic heteropolymer composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. These polyphenolic compounds are mainly deposited in tracheids, vessels, fibres of both xylem and phloem, and in the sclerenchyma [22]. Lignification of cell walls is a key event in resistance against pests in herbaceous or woody plants and resistant genotypes usually possess a

higher level of lignin [23,24]. Likewise, different stresses, such as pathogen attack, sometimes provoke a change in lignin amount and composition due to the so-called ‘wound-induced’ lignin [25]. Deposition of lignin has been hypothesized to interfere with the enzymatic hydrolysis and mechanical penetration of plant tissue by the fungus, and lignin precursors might also exert a biocidal effect on the pathogen [10,26].

As far as we know, although phenylpropanoids might constitute the first barrier, either chemical or physical, to hyphal growth, the alterations in such plant metabolism have not been widely studied for their impact on the *V. dahliae* pathogen interactions in pepper. The aim of this work was to investigate the potential role of distinct phenylpropanoid derivatives in the pepper defence response against *V. dahliae*, as well as their relationship to the vascular browning that usually accompanies the fungal infection. We describe new aspects of the events occurring at the stem xylem vessels and identify *N*-feruloyltyramine as the major component among inducible phenolic components.

2. Materials and methods

2.1. Plant material, fungal isolate, and inoculation procedure

Seeds of the cultivar Luesia (*Capsicum annuum* L. var. *annuum*), which was catalogued as tolerant to *V. dahliae* in a previous work [27], were disinfected with sodium hypochlorite (0.3% for 30 min) and grown in a substrate composed of potting soil and perlite in a 2:1 (v/v) ratio, previously sterilised at 120 °C for 45 min. The fungal isolate UDC53Vd was collected by the research group from Universidade da Coruña in northwest Spain (Galicia) from infected pepper plants during a survey carried out in summer of 1998 [28]. This isolate, with a high aggressiveness on pepper [27], was grown on PDA (potato-dextrose-agar) plates at room temperature for 20 days until inoculation. Ten 10-week-old plants were inoculated with *V. dahliae* by using a modified method of Melouk [29]. Briefly, a conidial suspension was prepared by washing each plate with sterile water and scraping the cultures with a rubber spatula. Mycelial fragments were removed by filtration through a double layer of sterile cheesecloth and the inoculum was adjusted to 10^6 conidia mL⁻¹. For inoculation, the roots were cut approximately 1 cm from the apex and suspended in the conidia for 45 min. A control group of 10 plants was treated with sterile water instead of the inoculum. The control and treated plants (after inoculation) were transplanted individually into 12 cm diameter pots with the same substrate. The experiments were repeated three times and conducted in a greenhouse at 25 °C with a 16 h light and 8 h dark photoperiod. The time course of infection was followed by visual inspection of wilt symptoms, as previously described [27]. Samples for the different analyses were taken at 29 days post-inoculation (dpi).

2.2. Histological and histochemical assays

Cross-sections with a thickness of between 100 and 300 µm were taken on the stems of control and inoculated plants at 29 dpi. Sections were firstly examined with a stereoscopic Nikon SM7 1500 microscope and pictures were taken with a Nikon DXM 1200 digital camera. The fluorescence

microscopy was carried out using a Nikon eclipse E-400 microscope fitted with a lamp of 330-380 nm and a Nikon Coolpix 990 camera.

Lignin was detected using the Wiesner test [30], as described by Pomar *et al.* [31]. Stem sections were soaked in 1% (w/v) phloroglucinol-HCl in 25:75 (v/v) HCl/ethanol for 10–15 min. The presence of lignin was visualized by its characteristic red-pink colouration.

Cell wall-bound polymers (cutin or suberin) were stained with the method described by Krishnamurthy [31] with slight modifications. Thus, stem sections were firstly incubated in ethanol 50% and then 30 min in a Sudan III solution. Cell-wall-bound polymers were observed as having a red colour. In addition, suberin was assessed by incubating the sections in 0.2 M phosphate buffer, pH 9.1 for 5 min, followed by excitation with UV light. Under these conditions, the suberin is manifested by a typical yellow fluorescence [32].

Phenolic compounds were detected with the Hoepfner-Vorsatz test [33]. Sections were treated with a solution of NO_3Na (10%), acetic acid (10%) and urea (10%) for 3 minutes and then 2 volumes of NaOH (2 N) were added. The presence of phenols normally became visible under white light as a yellow reddish coloration [34]. However, as the outcome of this test is sometimes ambiguous, the stained sections were observed under UV light. In that case, the blue fluorescence derived from the ferulic acid becomes green whereas that arising from lignin remains blue. Further evidence of phenol localization was obtained by treating the sections with ammonia and visualizing them with an UV microscope. As above, the fluorescence from phenolic acids changes to green.

2.3. Biochemical analyses

2.3.1. Lignin analyses

Total lignin content was determined in the cell walls of healthy and inoculated plants by the acetyl bromide–acetic acid method, with modifications [7]. Fourier transform infrared (FT-IR) spectra of lignifying cell walls were recorded on a Bruker Vector 22 FT-IR spectrophotometer using KBr

pellets containing 1% (w:w) finely ground samples. Aldehyde groups in lignifying cell walls were reduced with sodium borohydride, as described by [26]. Alkaline nitrobenzene oxidation of lignifying cell walls and high-pressure liquid chromatography (HPLC) analyses were performed essentially according to [7].

2.3.2. Analyses of phenolic compounds

Free-phenolic compounds were obtained from 0.5 g of tissue with 70% methanol for 30 minutes in a reflux system. After evaporating the methanol, phenolics were extracted three times with ethyl acetate. The ethyl acetate phase was evaporated under vacuum to dryness and dissolved in 1 mL of methanol. For the extraction of bound phenolics, tissue debris was subjected to alkaline hydrolysis with 4.0 M NaOH for 4 h at 37 °C. The pH was adjusted to 1.0–2.0 with HCl, and the previously bound (as esters) phenolics were extracted with diethyl ether. The ether phase was evaporated to dryness, the residue dissolved in methanol and filtered through a 0.45 µm membrane (Millex_HV, Millipore). The quantitative determination of both free and bound phenolic compounds was accomplished using Folin–Ciocalteu reagent. Ferulic acid was used as standard and results were expressed as ferulic acid equivalents (FAE) g⁻¹ FW. The identification and quantification of individual phenols were performed as described by Gayoso *et al.* [35], using a Waters Alliance Chromatographic system with a reverse-phase C18 column (SunFire™ 5 µm particle size, 150 mm x 4.6 mm I.D.).

The identification of the phenolic compound induced only in diseased plants was performed by high-performance liquid chromatography-mass spectrometry (HPLC-PDA-MS-MS). LC analysis was carried out on a Thermo Accela high-performance liquid chromatograph, equipped with a Thermo LTQ Orbitrap Discovery detector, by using the same column as above. The gradient profile was 5% A at 0 min, 75% A at 10 min, 100% A at 15 min. Each sample (10 µL) was injected and run at 30 °C with a constant flow rate of 1 mL min⁻¹. The mass spectrometry system was an LTQ-Orbitrap[®] Discovery mass spectrometer (Thermo-Fisher Scientific) equipped with an electrospray

ionization (ESI) source operating in positive-ionization mode. The ESI source conditions were as follow: source voltage -3.51 kV, heated capillary temperature 350 °C, capillary voltage -35 V and sheath gas and auxiliary gas, 50 and 10 (N₂ arbitrary units). For full-scan MS analysis, the spectra were recorded in the range of *m/z* 80 to 800 with a scan speed of 1 scan/s. The analysis was carried out with source fragmentation at 35V and the mass resolution was set at 30,000. For MS² analysis, the Normalized Collision Energy was adjusted to 35%, *q* activation to 0.25, and the scan range (*m/z*) was 50 to 350. The Orbitrap Instrument was calibrated using a calibration solution according to the manufacturer's instructions. Data acquisition and processing were performed using Xcalibur 2.0.7 software (Thermo-Fisher Scientific).

2.4. Synthesis of *N*-feruloyltyramine and assay of toxicity

Ferulic acid (8.6 g) was dissolved and stirred in ethyl acetate (500 mL) in a round bottom flask cooled by ice-water bath. 2',2',2'-trichloroethyl chloroformate (18.8 g) was added into the ferulic acid followed by slow addition of triethylamine over 20 min. The mixture was kept stirring for 1 h. The resultant white solid was filtered and washed with dry ethyl acetate (10x2 mL). Tyramine (6.7 g) was added in to the obtained filtrate and stirred for 48 h. Then the products in ethyl acetate were washed with 1 M HCl (3x150 mL), the organic phase was dried with anhydrous MgSO₄. After removal of the solid MgSO₄ by filtration, the ethyl acetate solvent was evaporated resulting a white solid. Crystallization of the products from ethyl acetate/hexenes produced 2',2',2'-trichloroethyl carbonate-protected feruloyl tyramine in 75% yield. Deprotection with zinc in acetic acid resulted in *N*-feruloyltyramine in 85% yield. NMR and MS data are consistent with published data [36].

The toxicity of *N*-feruloyltyramine against *V. dahliae* was checked in conidial suspensions adjusted to 10⁶ conidia mL⁻¹. The *N*-feruloyltyramine was diluted in methanol to the following concentrations: 0.375, 0.75, 1.5 and 3 mM. Forty µL of each dilution or pure solvent was pipetted into ring slides. The content of each slide was evaporated and 40 µL of the *V. dahliae* conidial

suspension were added. Each slide was transferred to an individual Petri dish containing moistened filter paper and incubated for 48 h at 25 °C. Conidia were considered to germinate when the germ tube length was longer than the spore diameter. Slides were observed using an optical microscope, and three replicate counts of 100 conidia were randomly chosen on each slide to score the spore germination. The mean from each count was estimated and expressed as the percentage of germinated conidia.

2.5. *Statistical analyses*

Experiments were repeated at least three times and all data are given as the mean $\pm$ SD. Data were analyzed by the Student's *t* test ($P = 0.05$) to confirm data variability and comparisons of means. All statistical analyses were performed with SPSS v.13.0.

3.1. Results and discussion

3.2. Disease development and vascular browning

Symptoms were apparent in infected pepper plants at approximately 9-10 days after inoculation and became evident at 29 dpi, primarily in the form of foliar wilting, defoliation, moderate chlorosis, stunting, and vascular browning. This darkening of the xylem vessels was clearly observed under the stereoscopic microscope in stem cross-sections of inoculated plants at 29 dpi, but was absent in control plants (Supplemental Fig. S1). The exposition of sections to UV light (λ 330-380 nm) revealed an increase in the blue autofluorescence of inoculated plants, spatially overlapping with those vascular regions with brown vessels (Supplemental Fig. S1). The browning of the xylem vessels, typical of diseases caused by vascular fungi, seems to be due to the deposition of different materials that hypothetically function as both physical barriers and toxic components [9]. The blue autofluorescence has been scarcely investigated and primarily attributed to the accumulation of different phenylpropanoid derivatives, such as free ferulic acid, cell wall esterified phenolic compounds, suberin, and lignin [37-39]. As far as we know, there is no report in Solanaceae attempting to investigate the origin of those materials deposited in vascular bundles and their association to a partial resistance against the fungal pathogen. In the following sections, several approaches were employed in order to elucidate the chemical nature of those compounds causing the vascular browning, trying to establish or discard the role of different phenylpropanoid derivatives.

3.3. The potential role of suberin

To elucidate whether suberin could be involved in the brown-blue autofluorescence, cross-sections of stems were incubated in 0.02 M pH 9 phosphate buffer and examined under UV light. The expected yellow fluorescence was observed neither in inoculated nor in control plants

(Supplemental Fig. S2). Furthermore, staining with Sudan III did not show an accumulation of suberin (red color) in inoculated plants at 29 dpi (Supplemental Fig. S2).

Suberin and lignin are deposited in response to pathogen attack as a physical barrier against fungal degradative enzymes and subsequent colonization [16]. Both lignification and suberization involve the formation of a polyphenolic matrix initially within the carbohydrate frame of cell wall [40]. However, lignin and suberin differ distinctly in their chemical compositions. The suberized cells develop a poly-aliphatic layer described as a polyester connected through primary ester bonds between the major aliphatic components. In addition, the suberin polyphenolic domain consists of a covalently cross-linked hydroxycinnamate/hydroxycinnamyl alcohol-derived matrix [17,41]. To date, various reports have demonstrated that fungal or viral attack elicits the deposition of suberin in the walls of cells in and around the site of penetration, even in *Verticillium* wilts [20,42]. However, our results did not point to suberin as an essential component of those mechanisms causing vascular browning and serving to limit the spread of infection in the pepper-*V. dahliae* interaction.

3.4. The potential role of lignin

Total lignin content, determined by the acetyl bromide-acetic acid method, did not show a significant increase in inoculated stems ($9.30 \pm 1.11 \text{ mg g}^{-1} \text{ FW}$) vs control plants ($8.33 \pm 0.99 \text{ mg g}^{-1} \text{ FW}$). The histochemical analysis of stem cross-sections with the Wiesner test, based on the incubation with phloroglucinol in acid media, revealed the existence of a centripetal gradient of lignification, in both control and inoculated stems (Supplemental Fig. S3). Lignin in healthy plants showed a pink colour, whereas in infected plants appeared as a red-brown colour. The former was characteristic of the reaction of phloroglucinol with hydroxycinnamaldehydes, which absorb in the range of 550-570 nm, whereas the latter occurs when the phloroglucinol associates with hydroxybenzaldehydes (vanillin or syringaldehyde), with maximum absorption in the range 486-503 nm [31]. After treatment with phloroglucinol, the stained cross-sections were subjected to UV light, as this reagent produces the quenching of lignin autofluorescence [43]. As predicted, the blue

fluorescence clearly decreased under UV light in both inoculated and control stems (Supplemental Fig. S3). However, such quenching did not occurred around xylem vessels, where vascular browning was highly apparent (Supplemental Fig. S3). These results suggested that lignin might be partially responsible for the browning observed after infection of pepper with *V. dahliae*, but they also pointed to the presence of other mechanisms playing a role in the impairment of fungal growth. The increase in lignification after bacterial, viral, or fungal infection has been frequently reported [44-46], although this is not the general rule, and no changes in lignin content have been observed as a result of various phytopathogenic interactions either [47,48]. The histochemical approach unveiled higher accumulation of lignin in specific areas, such as around xylem vessels. These observations suggest that the role of lignification in this particular interaction might be attributed somehow to rearrangements and qualitative modifications in infected stems, more than to large fluctuations at the quantitative level. This led us to investigate the monomer composition and cross-linking of lignin in the cell walls of both control and inoculated plants.

The lignin composition of lignifying stems was investigated with Fourier transform infrared spectra and nitrobenzene oxidation. The FT-IR spectroscopy of cell walls derived from inoculated and control plants showed clear absorption bands at 3436 cm^{-1} , 2921 cm^{-1} and 1734 cm^{-1} , attributable to hydroxyl, methyl, and non-conjugated carbonyl groups, respectively (Table 1). Some alterations were found in the band of $1600\text{--}1650\text{ cm}^{-1}$, which corresponds to a combination of the vibration in the aromatic skeleton in lignin and a C=O stretch attributable to conjugated/aromatic aldehydes, where the carbonyl oxygen atom sustains either intra- or intermolecular H-bonds, as well as in-plane bending in the C-H group [49]. In control plants this band was centred at 1624 cm^{-1} , whereas in infected plants the band showed a maximum at 1647 cm^{-1} . This shift suggests an accumulation of conjugated aldehydes. Moreover, in diseased plants, the intensity of this complex band is higher than in control plants, supporting the presence of higher levels of carbonyl groups in the plant cell wall (Table 1). Such increase of carbonyl groups might be attributed to a higher amount of

hydroxycinnamaldehydes in the lignin matrix of infected stems as well as the accumulation of *p*-hydroxybenzaldehydes, as suggested by the Wiesner test. Increments in cell wall-bound aromatic aldehydes were also described in our previous work, which reported a higher accumulation of those aldehydes in the pepper cv. Padron in response to the infection with *V. dahliae* [7]. Likewise, an increase in the quantity of aldehyde groups in inoculated cell walls was observed in both susceptible (*ve/ve*) and resistant (*Ve/Ve*) tomato lines infected with *V. dahliae* [35]. The larger number of metabolites with carbonyl groups may confer a hydrophobic character to lignin, protecting them against the action of cellulolytic enzymes [50]. In addition, the conjugated carbonyl groups of hydroxybenzaldehydes may form Schiff bases with free NH₂ groups of proteins, thus inactivating fungal enzymes [26]. Lignin with enhanced aldehyde levels conferred brown colour to the secondary xylem of pine [51]. In addition, the *in vitro* polymerization of hydroxycinnamaldehydes by peroxidase produced a brownish coloration of lignin due to the presence of carbonyl conjugated groups [52]. These reports support the assumption that the qualitative changes detected in the lignin composition in our work might be partially responsible for the browning observed in pepper vascular tissue after the fungal infection.

Nitrobenzene oxidation solubilizes both the condensed and non-condensed lignin fractions present in secondary cell walls forming *p*-hydroxybenzaldehyde from *p*-hydroxyphenyl (H), vanillin from guaiacyl (G), and syringaldehyde from syringyl (S) lignin units [53,54]. Table 2 shows the relative monomeric composition of cell walls of infected and healthy stems, calculated after nitrobenzene oxidation. Control stems showed an H:G:S proportion of 1:66:33, whereas stem lignin from diseased plants contained greater amounts of *H* units, with an H:G:S ratio of 3:66:31 (Table 2). Therefore, stem lignin from infected plants were putatively more cross-linked as they exhibited higher contents of H units derived from *p*-coumaryl alcohol. This result is in accordance with those obtained by Pomar *et al.* [7], who detected higher amounts of *p*-hydroxyphenyl units in pepper cv. Padron inoculated with *V. dahliae*. Similarly, Gayoso *et al.* [35] found an increased proportion of H

groups in susceptible and resistant tomato lines after infection with the same fungus. Differences in monolignol composition between the lignin of healthy plants and resistant-related lignin of infected plants have been described in a number of host/pathogen interactions [25]. In our work, although no changes occurred in the amount of lignin deposited in infected pepper stems, the modifications in lignin compaction, due to divergent crosslinking properties of monomers, might be sufficient to restrict *V. dahliae* hyphal growth to the xylem apoplastic cavity.

3.5. *The potential role of phenolic compounds*

The assay of stem cross-sections with the Hoepfner-Vorsatz test, followed by an alkaline incubation with sodium hydroxide and visualization under UV light, revealed a clear turn into green in the fluorescence of xylem vessels from inoculated plants, while those from healthy plants stayed blue (Fig. 1 A, B). Such change was also observed when pepper stem sections were incubated in ammonia (Fig 1 C, D). This accumulation of phenolic compounds in response to the pathogenic invasion could be responsible for the remaining blue autofluorescence observed above after the treatment with phloroglucinol (Supplemental Fig. S3). In order to investigate whether the phenolic metabolism was also quantitatively modified during the *Verticillium* wilt of pepper, changes in total free and bound phenolics were quantified in pepper stems at 29 dpi. A significant increase in both types of phenols was recorded in infected stems in relation to control plants, being more evident for the free fraction (Table 3). Phenolic compounds are the primary downstream products of the phenylpropanoid pathway and increments in their concentration upon a challenge by pathogens have been described in a great variety of studies dealing with different pathogens, including *V. dahliae* [10,35,55]. In pepper, phenol content was much higher in chili cultivars resistant to Fusarium wilt than in susceptible ones [56]. Similarly, Pomar *et al.* [7] reported a notable increase in free and bound phenolics in the partially resistant pepper cv. Padron after infection with *V.*

dahliae. As in our case, those phenols may act directly by inhibiting the growth of the pathogen or indirectly by sealing the plant cell walls and hence delaying the fungal colonization.

The chemical nature of both free and bound phenols was further analysed by using reverse-phase HPLC. The chromatographic analysis of the bound phenols indicated differences between healthy and diseased plants. Thus, the levels of caffeic acid and vanillin notably increased in infected plants, whereas other phenols, such as ferulic acid, exhibited a more modest raise (Table 4). The increment displayed by the vanillin confirmed the FT-IR analysis, which showed a greater area for the 1650 cm^{-1} band (Table 1), attributed to the vibration of carbonyl conjugated groups, such as those in *p*-hydroxybenzaldehydes like vanillin, and the red-brown staining observed after Wiesner staining (Supplemental Fig. S3). During infection and colonization, the speed of lignification and phenolic accumulation may be important for successful plant defence [57]. Although lignification of stem cell walls might require longer times to produce an effective physical barrier against fungal attack, the strengthening of the cell wall through the accumulation of *p*-hydroxybenzaldehyde and esters of hydroxycinnamic acids, such as ferulic acid and its dehydrodimers, followed by crosslinking with cell wall polysaccharides could be a more rapidly inducible mechanism upon pathogen infection. The tolerance observed in pepper plants, which is displayed as a vascular browning, could be partially explained by changes in lignin monomers and deposition of bound phenolics, which are not compiled with enough celerity and to the concentration required to provide resistance.

The chromatograms obtained with the free-phenolic fraction revealed the presence, only in infected plants, of a phenolic compound with retention time at 62.5 minutes. The absence of this peak in healthy stems suggests that its synthesis might be triggered by the infection of pepper plants with the fungal pathogen. The absorption spectrum of this compound (318,293 sh) showed similarities with those observed for hydroxycinnamic acids, such as ferulic, caffeic or *p*-coumaric acids, suggesting that it might be a derivative of those phenylpropanoids. The nature of this phenolic compound was investigated in detail by using LC-MS analyses. A pseudo molecular ion $[\text{M}+\text{H}]^+$ at

m/z 314.1387 was observed. The fragmentation of $[M+H]^+$ preferentially led to the cleavage of the amide bond to eliminate the tyramine moiety (137 Da) and produced a base ion at m/z 177, which could be ascribed to a feruloyl group. Further fragmentation was due to the consecutive loss of CH_3OH (32) and CO (28) corresponding to m/z 145 and 117, respectively (Fig. 2). All of these data suggested that the structure of that inducible phenol corresponded to a hydroxycinnamic acid amide and, in particular, to *N*-feruloyltyramine (presumably as its *trans*-isomer). This interpretation was confirmed later by comparisons with the chemically synthesized standard for *N*-feruloyltyramine. Thus, the exact mass data, MS and MS/MS fragmentations coincided with the predicted ones (Table 5).

Hydroxycinnamic acid amides (HCAAs) are formed by the condensation of hydroxycinnamic acids, such as ferulic (4-hydroxy-3-methoxycinnamic acid), with different amines, in a reaction catalyzed by the enzyme hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyl-transferase (THT) [58]. These low molecular-weight phenolic compounds have been described in a wide range of plant families, associated with various developmental processes [59-61]. HCAAs also play crucial roles in plant defence and their synthesis is induced in response to biotic and abiotic stresses [62]. They have been particularly reported in Solanaceae, where they accumulated upon pathogen infection playing two major roles: acting directly as antimicrobial agents or incorporating into the plant cell wall in order to strengthen it against microbial degradation [63-64]. *N*-feruloyltyramine is known to be accumulated in tobacco after infection with TMV [65], in potato after the treatment with an elicitor from *Phytophthora infestans* [66], in onion against *Botrytis allii* [67] and tomato in response to *Pseudomonas syringae* pv. *tomato* [68]. Also in tobacco, *N*-feruloyltyramine was found at higher levels in the lignins of CCR-deficient transgenics [69]. In pepper, increased levels of *N*-feruloyltyramine were detected in plants infected with the bacterial pathogen *Xanthomonas campestris* [70]. HCAAs derived from tyramine are likely synthesized in the cytosol and transported into the cell wall, where they undergo peroxidative polymerization mostly upon

wounding or pathogen attack. The incorporation of hydroxycinnamate conjugates into the cell wall guarantees a less digestible and permeable but highly fortified cell wall, which ensures exclusion of the pathogen from accessing plant nutrients and water [63,71]. Aside from cell wall fortification, the free form of those HCAA may have additional functions as massive production normally exceeds the rate of incorporation into the cell wall [62]. Indeed, the *N*-feruloyltyramine accumulated in pepper plants has been reported to possess antibacterial activity *in vitro* [70]. Similarly, various HCAAs derived from tyramine reported in tomato infected with the bacteria *P. syringae* act as antibacterial and antioxidant compounds [64].

To the best of our knowledge, this is the first time that the accumulation of *N*-feruloyltyramine is detected in pepper stems in response to a vascular fungus. Interestingly, the host-pathogen interaction assessed in the present work does not constitute an incompatible interaction, in which HCAAs have been primarily described [62]. McLusky *et al.* [67] reported that the blue autofluorescence observed in the cell walls of onion epidermal cells after attempted penetration by *Botrytis allii* was fundamentally caused by the accumulation of *N*-feruloyltyramine and other HCA-tyramine derivatives. In our work, the inducible phenol identified as *N*-feruloyltyramine in the chromatograms was not directly detected in the bound phenolics fraction, probably due to the extraction method used. However, its specific deposition around xylem vessels after pathogen attack might greatly contribute to the fluorescence observed in the cell wall of vascular bundles. Accordingly, the role of this amide in our particular interaction does not appear to be exclusively related to the cell wall fortification but also to an activity as a fungitoxic agent. The antimicrobial properties of phenolic compounds in general and hydroxycinnamic acids in particular have been broadly reported [12,13,72]. Likewise, *N*-feruloyltyramine isolated from infected pepper and onion displayed antibacterial and antifungal activity, respectively [70,73]. Bearing this in mind, the antifungal activity of chemically synthesized *N*-feruloyltyramine was tested against *V. dahliae* conidia. For that purpose, the inhibition of spore germination was microscopically assessed by

using different concentrations of the phenolic compound. The lowest concentrations (0.375 mM) did not have any effect on the germ tube growth (Fig. 3). However, higher amounts of *N*-feruloyltyramine (from 0.75 mM onwards) caused a remarkable reduction in the capacity of conidia to germinate, reaching an inhibition of up to 95% at the highest concentration (3 mM) (Fig. 3). The role of *N*-feruloyltyramine as an antimicrobial agent was demonstrated previously against the bacteria *Xanthomonas campestris* pv. *campestris* and the fungus *Fusarium culmorum*, also in a dose-dependent manner [70,73]. However, this plant amide has no effect against conidia and germ tubes of *Botrytis allii* and *Botrytis cinerea* [67]. In our work, *N*-feruloyltyramine seems to be effective by directly controlling the causal agent of *Verticillium* wilt disease in pepper, although additional functions should not rule out. Indeed, HCAA accumulation has been recently found to induce intermediary signals like salicylic acid, which mediate in the activation of certain plant defence responses, such as the synthesis of pathogenesis-related (PR) proteins [68].

4. Conclusions

Taking into account the results obtained in the different approaches performed in the current work, we contend that the tolerance of pepper to *V. dahliae* arises from various mechanisms involving phenylpropanoid metabolism, which likely underscore the vascular browning and the consequent autofluorescence. Both the reinforcement of the cell wall and the bioactive effect of some phenylpropanoid derivatives seem to play a role in the impairment of *V. dahliae* colonization. The achievement of a more compact cell wall, which acts as an initial physical barrier, was primarily attained through changes in lignin composition and deposition of cell-wall-bound phenolics, whereas fungitoxic activity relies on hydroxycinnamate-tyramine conjugates, which were mainly accumulated as soluble phenolics only in infected stems. The lack of total resistance could be explained as a matter of time and speed in the activation of the phenylpropanoid pathway leading to the synthesis of the necessary plant defence-related metabolites.

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Table 1 Fourier transform infrared spectroscopy analysis

Functional group or bond	Peak wavenumber ¹ (cm ⁻¹)	Control		Inoculated	
		Wavenumber (cm ⁻¹)	Intensity	Wavenumber (cm ⁻¹)	Intensity
-OH-	3436	3356	98.46	3354	93.26
-CH ₂ -	2921	2930	16.63	2931	12.97
-CO- (Non conjugated)	1734	1735	11.16	1734	2.45
-CO- (conjugated)	1650				
Aromatic skeleton	1600	1636	59	1651	99.74

¹ Peak wavenumber according to Faix [74].

Table 2 Changes in lignin composition after inoculation with *V. dahliae*

	<i>p</i> -Hydroxybenzaldehyde (H) (μg mg ⁻¹ FW)	Vanillin (G) (μg mg ⁻¹ FW)	Syringaldehyde (S) (μg mg ⁻¹ FW)
Control	7.78 ± 0.56	679.4 ± 0.51	339.5 ± 0.13
Inoculated	20.68 ± 0.44	571.6 ± 1.41	272.0 ± 1.84

Table 3 Free and bound phenolic content in control and infected pepper stems

	Phenols content (mg FAE g ⁻¹ FW)	
	Control	Inoculated
Free phenols	1.731 ± 0.323	3.342 ± 0.635
Bound phenols	0.166 ± 0.025	0.244 ± 0.027

Table 4 Changes in individual phenols ($\mu\text{g g}^{-1}$ FW) in the bound fraction after inoculation with *V. dahliae*

	Retention time (minutes)	Control	Inoculated
Protocatechuic acid	10.0	0.29	0.16
Caffeic acid	20.5	0.97	1.23
Vanillin	31.0	1.80	2.41
<i>p</i> -Coumaric acid	32.5	4.21	3.92
Syringaldehyde	33.9	0.28	0.30
Ferulic acid	37.1	0.24	0.26

Table 5 Chromatographic behavior and mass spectral fragmentation of the induced compound and feruloyltyramine

Peak (retention time)	Mass characteristics			
	Exact mass	Mass error	MS	MS ²
Theoretical feruloyltyramine	314.13868	-	-	-
Induced compound (16.37 min)	314.13876	0.16 ppm	314, 177, 145	314 → 177, 145, 117
Feruloyltyramine (16.40 min)	314.13849	0.24 ppm	314, 177, 145	314 → 177, 145, 117

Figure captions:

Figure 1. Stem cross-sections of control plants (A, C) and infected plants (B,D) observed under UV light, after Hoepfner-Vorsatz test (A,B) and after incubation with ammonia (C, D). The bar represents 50 μm .

Figure 2. Fragmentation pathways proposed for N-*trans*-feruloyl tyramine.

Figure 3. Assay of N-feruloyltyramine toxicity on the germination of *V. dahliae* conidia