New Phytologist

Research

Changes in stem lignins (monomer composition and crosslinking) and peroxidase are related with the maintenance of leaf photosynthetic integrity during *Verticillium* wilt in *Capsicum annuum*

Federico Pomar[1], Marta Novo[2], María A. Bernal[2], Fuencisla Merino[2] and A. Ros Barceló[3]

[1]Centro de Investigaciones Agrarias de Mabegondo, Apartado 10, E–15080 La Coruña; [2]Department of Plant Biology (Plant Physiology), University of La Coruña, E–15071 La Coruña; [3]Department of Plant Biology (Plant Physiology), University of Murcia, E–30100 Murcia, Spain

Author for correspondence:
Alfonso Ros Barceló
Tel: +34 968 364945
Fax: +34 968 363963
Email: rosbarce@um.es

Received: *2 November 2003*
Accepted: *26 February 2004*

doi: 10.1111/j.1469-8137.2004.01092.x

Summary

• *Verticillium dahliae* is a soilborne pathogen that causes vascular wilt in pepper (*Capsicum annuum* var. *annuum*). Here we study to what extent changes in the lignification response of peppers condition tolerance of wilt.

• For this, the quantum yield (Φ_{PSII}), the linear *e*lectron *t*ransport *r*ate (ETR), and the lignification response (monomer composition and crosslinking) were studied in three *C. annuum* cultivars differing in degree of tolerance.

• The results showed that in tolerant cultivars (Padrón and Yolo Wonder), both Φ_{PSII} and ETR showed significantly higher levels at saturating photosynthetically active radiation values. This was not, however, the case for cv. Luesia, which showed a significant decrease in Φ_{PSII}, ETR and nonphotochemical quenching values, suggesting that photochemical processes are strongly damaged in this cultivar as a consequence of the disease. The analysis of stem lignins in tolerant cultivars revealed that they were mainly composed of *p*-hydroxyphenyl units, while levels of β-*O*-4-linked coniferyl and sinapyl alcohols were significantly lower.

• It is concluded that through the observed changes in stem lignins (monomer composition and crosslinking) peppers retard, since they maintain leaf photosynthetic integrity, but do not stop (since wilt symptoms are not avoided) *V. dahliae* fungal hyphae penetration.

Key words: *Capsicum annuum*, leaf photosynthetic integrity, lignins, nonphotochemical quenching, peroxidase, quantum yield, *Verticillium dahliae*.

 163: 111–123

Introduction

Verticillium dahliae is a soilborne pathogen that causes vascular wilt in over 160 agronomically important plant species worldwide, including pepper, tomato, cotton, alfalfa, cucurbits, eggplant, mint, potato, strawberry and sunflower (Kawchuk *et al.*, 2001). Few other plant-pathogenic fungi exhibit the extraordinary virulence of *V. dahliae*, and new *Verticillium* wilts are continually emerging in vegetable crops previously considered as nonhosts, such as lettuce (Sadras *et al.*, 2000). *Verticillium dahliae* penetrates the host through wounded roots, and spreads systemically through 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 (Robb *et al.*, 1991), leads to the appearance of 'wilt' disease symptoms (Goicoechea *et al.*, 2000). These symptoms 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 – the hyphae competing with plant tissues for nutrients – and only after the host dies does it colonize senescent tissues (Goicoechea *et al.*, 2000). *Verticillium* wilt causes severe economic losses, and in many crops, such as pepper (*Capsicum annuum* var. *annuum*), this disease is managed with a combination of crop

rotation, chemical and biological methods, but results obtained are far from being satisfactory. Although some *Capsicum* species, such as *C. chinense* and *C. baccatum*, are resistant to *Verticillium* wilt, there are no *C. annuum* cultivars resistant to *V. dahliae*. This led us to investigate the nature of the response of the compatible species, *C. annuum*, to *V. dahliae*. Understanding the whole physiology, recognition, signal transduction, and tolerance mechanisms of *C. annuum* against *V. dahliae* is not only of great scientific interest but is also important for controlling this worldwide plant disease.

Disease resistance in plants is often conditioned by 'gene-for-gene' interactions. Thus, when a pathogen has an avirulence (*avr*) gene and the host plant has its corresponding resistance (*R*) gene, the host plant rapidly deploys defense responses (Dang & Jones, 2001). If a host plant is resistant, the interaction is called incompatible. If a host plant is susceptible, the interaction is called compatible. Differences between resistant and susceptible host plants are mainly related to the rate at which they express structural and chemical defenses (Dang & Jones, 2001), the signal transduction mechanisms involved in compatible and incompatible interactions being largely shared (Tao *et al.*, 2003).

Owing to the lack of resistant cultivars, resistance genes have not been identified for the *C. annuum*–*V. dahliae* interaction. In alfalfa (lucerne), where resistant cultivars have been described (Gao *et al.*, 2000), an antifungal peptide has been identified, isolated and cloned in potato, which confers resistance to *V. dahliae* under field conditions (Gao *et al.*, 2000). In tomato, *V. dahliae* resistance (*R*) genes encode a class of cell-surface glycoproteins with signals for receptor-mediated endocytosis and leucine zipper or Pro-Glu-Ser-Thr (PEST) sequences (Kawchuk *et al.*, 2001). In this Solanaceae, elemental sulfur (S) appears as a phytoalexin linked with defense against *V. dahliae* (Williams *et al.*, 2002). In fact, S accumulates in vascular tissues and substantial local accumulations have been detected in xylem parenchyma cells, vessel walls, vascular gels and tyloses (Williams *et al.*, 2002). This is not surprising since *V. dahliae* penetrates the host through the roots and spreads systemically through the lignified xylem; the lignified secondary cell wall constitutes the first structural barrier of defense.

Lignins are three-dimensional phenolic heteropolymers covalently associated with polysaccharides in plant cell walls (Lewis *et al.*, 1999). They are mainly localized in the impermeable water transport conduits of the xylem and other supporting tissues, such as phloem fibers, and result from the oxidative polymerization of three *p*-hydroxycinnamyl (*p*-coumaryl, coniferyl and sinapyl) alcohols in a reaction that can be mediated by both peroxidases and laccases (Ros Barceló, 1997). The process of sealing plant cell walls through lignin deposition provides mechanical strength to the stems, protecting cellulose fibers from chemical and biological degradation (Grabber *et al.*, 1998) in the face of fungal attack. Bearing this role for lignins in mind, and since lignification mainly occurs in the secondary xylem cell wall, which is in potential contact with, and probably involved in the defense against, *V. dahliae*, it may be expected that xylem lignification acts as the primary physical barrier against *V. dahliae* (Smit & Dubery, 1997), and that through changes in monomer composition and crosslinking, the plant may develop effective mechanisms to restrict the fungal growth in the xylem, avoiding as much as possible damage of photosynthetic tissues. However, lignin is not the sole structural barrier developed by plants to restrict the fungal growth in the xylem, since the 'coating material' deposited in tomato vessels after *Verticillium albo-atrum* infection has been characterized as suberin (Robb *et al.*, 1991).

The aim of the present work was to study the lignification (monomer composition and crosslinking) and peroxidase response of three *C. annuum* (var. *annuum*) cultivars differing in the degree of tolerance against *V. dahliae*, and the relation of these responses with the protection of photosynthetic tissues. Knowledge of the tolerance mechanism of *C. annuum* cultivars against *V. dahliae* will contribute to understand the basis of the devastating effects of *Verticillium* wilts.

Materials and Methods

Plant material, fungus, inoculation and sampling procedure

Seeds of *C. annuum* L. (var. *annuum*) cvs Padrón, Yolo Wonder and Luesia were soaked overnight in tap-water before being sown in sterilized vermiculite. The seedlings were grown at 25°C under a 16-h photoperiod. *Verticillium dahliae* Kleb. (isolate VDL), kindly provided by C. Palazón (SIA, Zaragoza, Spain), was cultivated in the dark on potato dextrose agar medium at 25°C. A water–agar medium was periodically alternated with the potato dextrose agar medium to maintain fungal virulence. A conidial suspension was leached from the plate-grown mycelium by adding sterile water and gently stirring. For inoculation, the roots of 24-d-old plants were cut approx. 0.5 cm from the apex and immersed in a *V. dahliae* conidial suspension of 10^6 conidia ml^{-1}. Roots of control (healthy) plants were immersed in sterilized water. After inoculation, plants were grown as described above in a vermiculite–humus mixture. Samples of leaves, stems and roots were taken 12 h, and 1, 2, 7, 14, 21 and 28 d post-inoculation (dpi).

Time-course of *V. dahliae* colonization

The time-course of hyphal colonization was followed in the roots and stems of inoculated cv. Padrón. This was done at three times (12, 24 and 48 h) post-inoculation. Using a razor blade, 1.0-cm long NaClO surface-sterilized sections were cut from the roots (top and bottom) and the stems (divided into four quarters), and cultured for 7 d in potato dextrose agar supplemented with penicillin and streptomycin (100 mg l^{-1} each) in the dark at 25°C. Colonization was determined by the development of fungal hyphae in roots and stem sections after culture.

Leaf surface

Total leaf surface was determined from digital photographs using a Nikon DXM 1200 camera, and image processing using the ANALYSIS program (Soft Imaging System, Münster, Germany).

Chlorophyll fluorescence analysis

Modulated chlorophyll fluorescence was measured in dark-adapted leaves with a PAM-210 pulse chlorophyll fluorometer (Walz, Effeltrich, Germany). The quantum yield of photosystem II photochemistry (Φ_{PSII}) was calculated empirically as the fluorescence parameter $(F'_m - F_t)/F'_m$ (Genty *et al.*, 1989), and the maximum quantum yield of photosystem II (F_v/F_m) as $(F_m - F_o)/F_m$ (Maxwell & Johnson, 2000). The linear electron transport rate (ETR) (i.e. the overall photosynthetic capacity *in vivo*) was calculated using the equation

$$ETR = 0.5 \times PFDa \times \Phi_{PSII}$$

(Maxwell & Johnson, 2000), where PFDa is the absorbed light expressed as μmol quanta m^{-2} s^{-1}. Nonphotochemical quenching (NPQ) was calculated as a Stern–Vollmer type quenching (Bilger & Björkman, 1990). The minimal 'dark' fluorescence level following illumination (F'_o) was measured in the presence of a background far-red light to favor rapid oxidation of intersystems electron carriers.

Histochemical stains

The histochemical localization of peroxidase activity was performed by incubating hypocotyl sections (250–500 μm thick) for 20 min at 25°C in a staining solution composed of either 500 μM 3,3′,5,5′-tetramethylbenzidine (TMB) or 50 μM syringaldazine, in 50 mM Tris-acetate buffer (pH 5.0), using the proper capability of xylem tissues to produce H_2O_2 (Ros Barceló *et al.*, 2000). Inhibitors (ferulic acid, tropolone, sodium azide, potassium ferrocyanide and potassium ferricyanyde) used at 1.0 mM were added 20 min before the histochemical reaction was started by the addition of the substrate. Lignins were detected using the Wiesner test, as described (Pomar *et al.*, 2002).

Protein extraction, assay of peroxidase activity and isoelectric focusing (IEF)

Samples 1.0 g f. wt were homogenized in 2.0 ml of 50 mM Tris-HCl, 1.0 M KCl buffer (pH 7.5) containing 0.05 g polyvinylpolypyrrolidone (PVPP) and 2.0 mg sodium bisulphite at 4°C. The homogenate was kept in the dark at 4°C for 1 h, filtered through four nylon layers, and centrifuged at 27 000 **g** for 30 min. The supernatants were dialysed overnight at 4°C against 50 mM Tris-HCl buffer (pH 7.5) and stored at −20°C until use. Peroxidase activity was determined at 25°C with 4-methoxy-α-naphthol and coniferyl aldehyde, as described elsewhere (Ros Barceló & Pomar, 2001).

Isoelectric focusing was performed on a Pharmacia Multiphor II system using Ampholine PAGplate (pH = 3.5–9.5) polyacrylamide gels according to the manufacturer (Amersham Biosciences, Barcelona, Spain). An amount of protein equivalent to 50 mg f. wt per lane was deposited. Peroxidase activity was revealed by incubating the gels at 30°C in 50 mM Tris-acetate buffer (pH 5.0) containing 1.0 mM 4-methoxy-α-naphthol and 0.5 mM H_2O_2.

Extraction and determination of free and bound phenolics

Free phenolics were extracted as described by Díaz *et al.* (1997). For the extraction of bound phenolics, tissue debris from the extraction were subjected to alkaline hydrolysis in 4.0 ml 4.0 M NaOH for 4 h at 37°C. After adjusting the pH to 1.0–2.0 with HCl, phenolics were extracted with diethyl ether. The ether phase was then evaporated to dryness, the residue dissolved in methanol and filtered through a 0.45 μm membrane. The phenolic content was determined using the Folin–Ciocalteau reagent according to Díaz *et al.* (1997). Ferulic acid was used as standard. Determination of individual phenolics by high-performance liquid chromatography (HPLC) was performed as described by Díaz *et al.* (1997). Identification and quantification of phenolics was performed using the corresponding standards.

Isolation of cell walls, determination of total lignin content, Fourier transform infrared (FT-IR) spectra, alkaline nitrobenzene oxidation and thioacidolysis analyses

Cell walls from inoculated and uninoculated plants were prepared by a Triton X-100 washing procedure as described by Pomar *et al.* (2002). Total lignin content was determined by the acetyl bromide–acetic acid method with modifications (Díaz *et al.*, 1997). Fourier transform infrared 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 Grabber *et al.* (1998).

Alkaline nitrobenzene oxidation of lignifying cell walls and HPLC analyses were performed essentially as described by Pomar *et al.* (2002). Quantification of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde was performed at 280 nm using the corresponding standards. Thioacidolysis of lignifying cell walls and gas chromatography–mass spectrometry (GC-MS) analyses were performed as described by Pomar *et al.* (2002).

Chemicals

Ferulic acid, 3,3′,5,5′-tetramethylbenzidine (TMB)-HCl, tropolone, potassium ferrocyanide, potassium ferricyanide,

tyramine and syringaldazine were purchased from Sigma Chemical Co. (Madrid, Spain). 4-Methoxy-α-naphthol was from Aldrich Chemie (Madrid, Spain). Sodium azide and H_2O_2 were from Merck (Barcelona, Spain). The remaining the chemicals were obtained from various suppliers and were of the highest purity available.

Statistical analyses

Assays involved eight plants per treatment and were performed at least three times. Statistical analysis of parameters between healthy and diseased plants were performed using the Student *t*-test, at the $P = 0.05$ level of significance.

Results

Biomass and morphology

The pepper cvs Padrón, Yolo Wonder and Luesia are known to show varying degrees of tolerance to *V. dahliae* in the glasshouse. Cultivars Padrón and Yolo Wonder are more tolerant than cv. Luesia, a tolerance that is easily monitored from the reduction in f. wt, which was 69% in both cvs Padrón and Yolo Wonder and 81% in cv. Luesia (Fig. 1a). To monitor alterations in plant development as a consequence of the spread of infection, fungal penetration (hyphal colonization) and total leaf surface (Fig. 1b–d) were determined in healthy and diseased plants. After 12 h of inoculation, hyphal colonization only affected the bottom half of the root. After 24 h, hyphal colonization had reached the bottom quarter of the stem, while 48 h after inoculation, colonization of the stem was total. At 48 h, no significant differences were found between healthy and diseased plants for total leaf surface (Fig. 1B), and only 7 d post-inoculation (7 dpi) was a significant reduction in the total leaf surface detected (Fig. 1c). That is, although acropetal hyphal colonization of the stem was complete after 48 h, no significant changes in total leaf surface could be observed until 7 dpi. These changes were especially evident at 28 dpi (Fig. 1d), and indicate the strong alteration of pepper development induced by the disease.

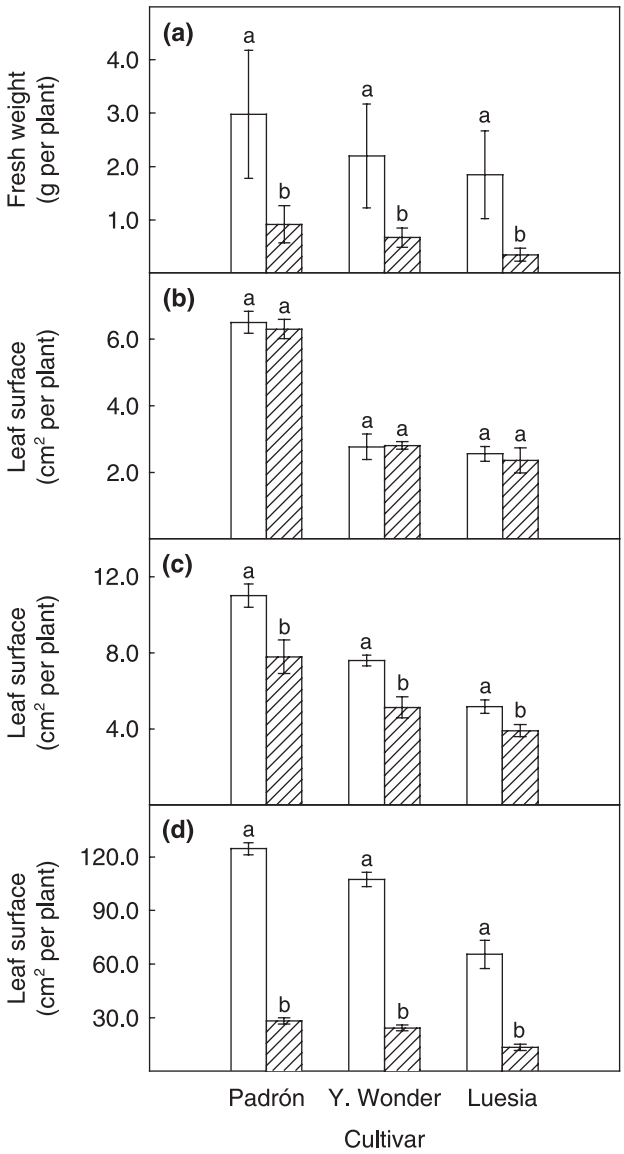

Fig. 1 (a) Effect of *Verticillium dahliae* (hatched bars) on the total fresh weight of different pepper lines 28 d post-inoculation. (b–d) Effect of *V. dahliae* (hatched bars) on the total leaf surface of different pepper lines at 48 h (b), 7 d (c) and 28 d (d) post-inoculation. Values followed by the same letter are not significantly different from controls (open bars) at $P = 0.05$.

Leaf photosynthesis

Despite the strong reduction in leaf surface observed at 7 dpi for cvs. Padrón and Yolo Wonder (Fig. 1C), both Φ_{PSII} (Fig. 2a,b) and ETR (Fig. 2d,e) showed a significant increase at saturating photosynthetically active radiation (PAR) values (500–800 μmol quanta m^{-2} s^{-1}). This increase was not observed at limiting PAR values (< 250 μmol quanta m^{-2} s^{-1}, Fig. 2d,e). Unlike cvs Padrón and Yolo Wonder, cv. Luesia showed a significant decrease in both leaf Φ_{PSII} (from 0.32 ± 0.02 to 0.27 ± 0.01, Fig. 2c) and leaf ETR (from 79.9 ± 3.40 to 67.6 ± 2.60, Fig. 2f) at saturating PAR values (600 μmol quanta m^{-2} s^{-1}), suggesting that photochemical processes are strongly damaged in this cultivar as a consequence of disease.

Similar results were obtained for cvs Padrón and Yolo Wonder at 28 dpi. At this time, when stunting and epinasty were clearly visible, only a weak stimulation of Φ_{PSII} (Fig. 2g,h) and ETR (Fig. 2j,k) was observed at saturating PAR values. At 28 dpi, *V. dahliae*-infected cv. Padrón maintained maximum quantum yields (F_v/F_m) of photosystem II (0.78 ± 0.01 in healthy vs 0.78 ± 0.02 in diseased plants) and high NPQ values (1.06 ± 0.21 in healthy vs 1.29 ± 0.28 in diseased plants). In the case of cv. Luesia, the reduction in Φ_{PSII} (Fig. 2i) and

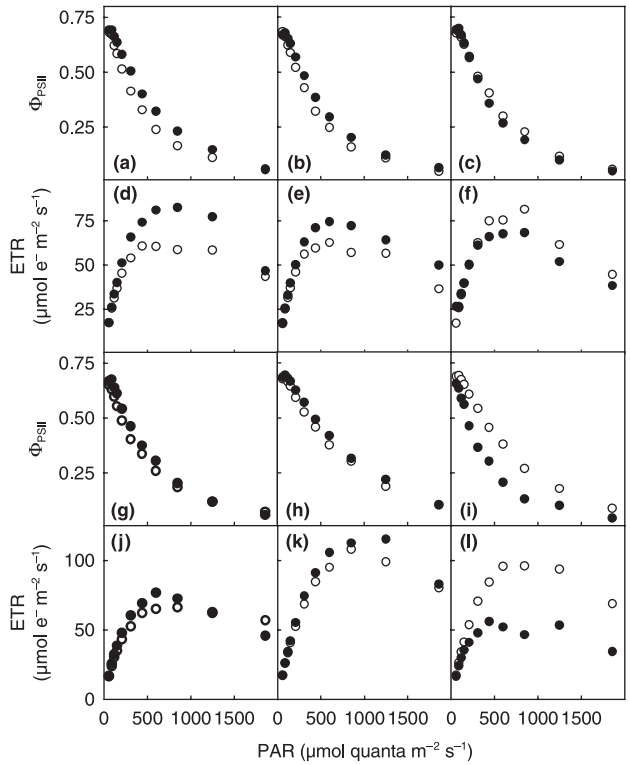

Fig. 2 Effect of *Verticillium dahliae* inoculation on the leaf quantum yield of photosystem II (Φ_{PSII}) and the leaf linear electron transport rate (ETR) of cvs Padrón (a,d,g,j), Yolo Wonder (b,e,h,k), and Luesia (c,f,i,l) pepper lines at varying photosynthetically active radiation (PAR) values. Data were taken 7 d (a–f) and 28 d (g–l) post-inoculation for controls (open circles) and inoculated (closed) plants. Error bars are SD but are hidden by symbols.

ETR (Fig. 2l) at 28 dpi was also associated with a decrease in NPQ at saturating PAR values (1.70 ± 0.18 in healthy vs 1.38 ± 0.12 in diseased plants).

Phenolic compounds

To the best of our knowledge, alterations in phenolic metabolism have not been reported during *Verticillium* wilt in peppers, although they could constitute, together with lignins and suberin, the first barrier, either chemical or physical, to hyphal growth. This may be especially relevant for the cv. Padrón, which maintains high Φ_{PSII} and ETR values (Fig. 2) regardless of the extent of *Verticillium* hyphal colonization. For this reason, phenyl-propanoid compounds (C_6C_3) and their relatives, C_6C_1, phenolic compounds, were analysed in healthy and diseased plants.

That phenolic metabolism was altered during the *Verticillium* wilt of pepper may be easily seen from the changes in total free phenolics that occurred in pepper stems from 14 dpi onwards. At 28 dpi, differences were maximal and increases in total free phenolics were greater in cv. Padrón stems than in cvs Yolo Wonder and Luesia (Fig. 3a). However, the levels of free chlorogenic acid and vanillin showed no significant changes in diseased plants (Fig. 3b). Changes therefore mainly concerned a plethora of phenolics, which remained unidentified by HPLC.

Phenolics present in plant cells may be found in either a free or a bound state. The latter group includes phenolics bound to oligosaccharides and polysaccharides by ether or ester bonds, which are easily hydrolysable in an alkaline medium. When we analysed this fraction, differences between healthy and diseased cv. Padrón plants were significant from 14 dpi onwards. At 21 dpi, levels of ferulic acid (a C_6C_3 compound)

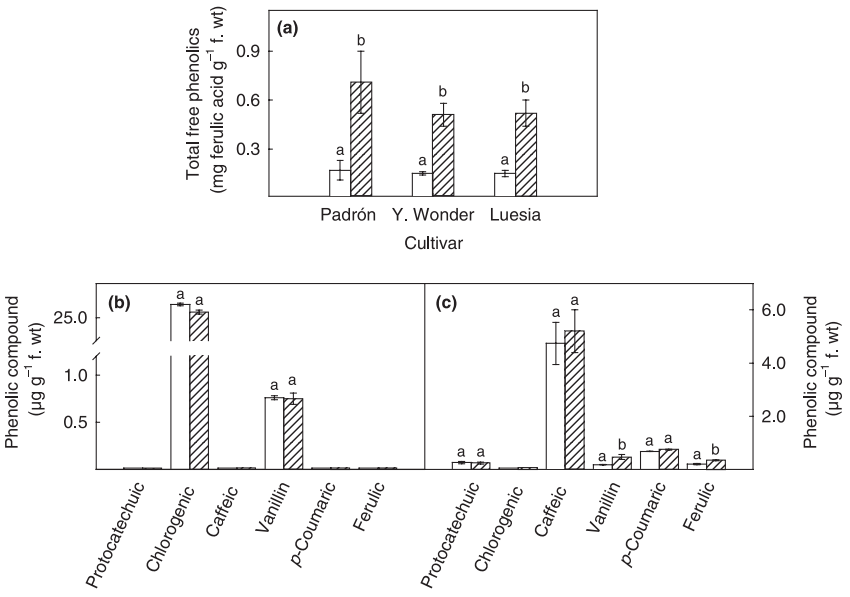

Fig. 3 (a) Effect of *Verticillium dahliae* (hatched bars) on stem total free phenolic content of different pepper lines at 21 d post-inoculation (dpi). (b,c) Effect of *V. dahliae* (hatched bars) on the level of individual phenolics in free (b) and bound (c) fractions of the cv. Padrón at 21 dpi. Values followed by the same letter are not significantly different from controls (open bars) at $P = 0.05$.

Table 1 Effect of *Verticillium dahliae* on stem lignin content of different pepper lines at 28 d post-inoculation

	Lignin content (mg g^{-1} f. wt)	
Cultivar	Control	Inoculated
Padrón	2.95 ± 0.24^a	4.03 ± 0.37^b
Yolo Wonder	2.19 ± 0.25^a	3.23 ± 0.07^b
Luesia	2.31 ± 0.09^a	3.03 ± 0.50^b

Values followed by the same superscript letter are not significantly different from controls at $P = 0.05$.

and vanillin (a C_6C_1 compound) increased significantly (Fig. 3c), while other phenolics, such as caffeic acid, did not increase in response to disease. Tyramine, a chemical marker of suberized tissues (Bernards, 2002), was not present in this fraction. Ferulic acid and vanillin are two unusual phenolic moieties which are also found in structural lignins (Boudet, 1998) and which may seal plant cell walls in response to fungal colonization. For this reason, changes in the total lignin content, lignin monomer composition and lignin crosslinking was studied in pepper stems in response to *V. dahliae* inoculation.

Lignin content, monomer composition and cross-linking

Inoculation of the three pepper lines with *V. dahliae* produced an increase in the total stem lignin content, which became significant from 14 dpi onwards. At 28 dpi, the three pepper lines respond to *V. dahliae* inoculation with a similar increase in stem lignins, which was about 30–40% (Table 1), although the degree of lignification reached in diseased plants was higher in the cv. Padrón.

In order to ascertain the nature of cv. Padrón stem lignins, cell walls isolated from lignifying stems of both healthy and diseased plants were subjected to nitrobenzene oxidation and thioacidolysis. The analysis of nitrobenzene oxidation products (Table 2) revealed that at 21 dpi, lignin content (p-Hydroxyphenyl + Guaiacyl + Syringyl (H + G + S) was 1.15-fold higher in diseased cv. Padrón plants than in controls. Stem lignins from healthy plants were mainly composed of guaiacyl/syringyl units in a 6 : 60 : 34 (H : G : S) ratio (Table 2), while stem lignins from diseased plants contained higher amounts of *p*-hydroxyphenyl units, the H : G : S ratio being 55 : 34 : 11.

In order to ascertain the nature of the phenolic moieties contained in the lineal β-*O*-4 lignin fraction, cv. Padrón stem cell walls isolated from healthy and diseased plants were subjected to thioacidolysis, and the recovered thioethylated monomers were analysed by GC-MS (Table 3). Owing to partial overlapping in the chromatograms of the peaks corresponding to *p*-hydroxycinnamyl aldehydes (Table 3, peaks 9 and 11) with peaks corresponding to *p*-hydroxycinnamyl alcohols (Table 3, peaks 8 and 10), when the total ionic current (TIC) was monitored, *p*-hydroxycinnamyl aldehydes were quantified from the chromatograms obtained for the ions of *m/z 418* (M^+, for the thioethylated monomer of coniferyl aldehyde) and *m/z* 448 (M^+, for the thioethylated monomer of sinapyl aldehyde). In these chromatograms (Fig. 4), the peaks corresponding to the thioethylated products of *p*-hydroxycinnamyl aldehydes (Fig. 4, peaks 2), and identified as such by their mass spectrum (Lapierre *et al.*, 1995; Pomar *et al.*, 2002), were clearly resolved from the two peaks (Fig. 4, peaks 1) belonging to the *erythro*- and *threo*-isomers corresponding to each *p*-hydroxycinnamyl alcohol.

The main products revealed by thioacidolysis in healthy and diseased cv. Padrón stem lignins (Table 3) were the thioethylated (*erythro*- and *threo*-isomers) monomers arising from aryl-glycerol-β-aryl ether structures derived from coniferyl (peak 8) and sinapyl (peak 10) alcohols. In addition to these thioethylated monomers arising from the cleavage of the lineal β-*O*-4 lignin fraction, thioacidolysis also revealed significant amounts of stilbene structures ($C_6C_2C_6$) derived from the β-β crosscoupling of coniferyl alcohol (peak 12), and the presence of trace amounts of C_6C_2 enol ether structures derived from coniferyl alcohol (peak 4). Thioacidolysis of both healthy and diseased cv. Padrón stem lignins also revealed the presence of the 4-*O*-linked coniferyl alcohol end group (peak 2), the 4-*O*-linked vanillin (3-methoxy-4-hydroxy-benzaldehyde, peak 3) end group, the 4-*O*-linked syringaldehyde (3,5-methoxy-4-hydroxy-benzaldehyde, peak 3) end group, and the thioethylated monomers, which arise from both the 4-*O*-linked coniferyl aldehyde end group (peak 9) and the 4-*O*-linked sinapyl aldehyde end group (peak 11).

The results obtained by thioacidolysis (Table 3) also revealed two additional striking features. First, levels of β-*O*-

Table 2 Effect of *Verticillium dahliae* on lignin monomer composition, as revealed by analysis of the nitrobenzene oxidation products of cv. Padrón stem lignins at 21 d post-inoculation

Nitrobenzene oxidation product	Control	Inoculated
p-Hydroxybenzaldehyde (H) (μg mg^{-1} CW)	0.22 ± 0.09^a	2.45 ± 0.20^b
Vanillin (G) (μg mg^{-1} CW)	2.32 ± 0.15^a	1.50 ± 0.09^b
Syringaldehyde (S) (μg mg^{-1} CW)	1.30 ± 0.05^a	0.48 ± 0.03^b
H + G + S (μg mg^{-1} CW)	3.84 ± 0.11^a	4.43 ± 0.22^b
H : G : S ratio	6 : 60 : 34	55 : 34 : 11

Values followed by the same superscript letter are not significantly different from controls at $P = 0.05$. CW, cell walls.

Table 3 Monomeric degradation products obtained by thioacidolysis of stem lignins from controls and *Verticillium dahliae*-inoculated pepper (cv. Padrón) plants at 21 d post-inoculation, and assignment of the original fragment in lignins

Peak (R_t, min)	TIC ($\times 10^8$) (area, %)		Original fragment in lignins
	Control	Inoculated	
1 (18.34)	0.08 (100)[a]	0.13 (160)[b]	*O*-4-linked vanillic acid end group
2 (24.37)	0.40 (100)[a]	0.30 (75)[b]	*O*-4-linked coniferyl alcohol end group
3 (24.49)	0.38 (100)[a]	0.40 (105)[b]	*O*-4-linked vanillin (3-methoxy-4-hydroxy-benzaldehyde) end group
4 (26.37)	< 0.05 (Tr)	< 0.05 (Tr)	C_6C_2 enol ether structure (G–CH=CHOAr)
5 (26.73)	ND	< 0.05 (Tr)	*O*-4-linked syringaldehyde (3,5-dimethoxy-4-hydroxy-benzaldehyde) end group
6 (27.67)	1.40 (100)[a]	0.90 (64)[b]	*O*-4-linked coniferyl alcohol end group
7 (28.59)	< 0.05 (Tr)	< 0.05 (Tr)	C_6C_2 enol ether structure (S–CH=CHOAr)
8 (32.53)	23.40 (100)[a]	12.05 (51)[b]	β-*O*-4-linked coniferyl alcohol
9 (32.60)	0.85 (100)[a]	0.37 (43)[b]	*O*-4-linked coniferyl aldehyde end group
10 (33.89)	12.20 (100)[a]	4.45 (36)[b]	β-*O*-4-linked sinapyl alcohol
11 (34.03)	0.68 (100)[a]	0.19 (28)[b]	*O*-4-linked sinapyl aldehyde end group
12 (34.74)	1.42 (100)[a]	0.65 (59)[b]	Stilbene structure (G–CH=CH-G)

TIC, total ionic current; G, guaiacyl; S, syringyl; Tr, trace; ND, not detectable; Rt, retention time. Values followed by the same superscript letter are not significantly different from controls at $P = 0.05$.

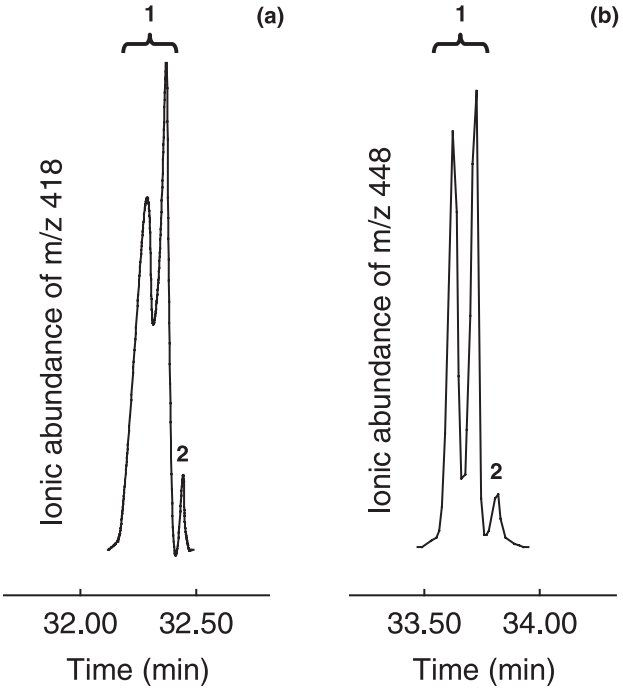

Fig. 4 Chromatographic profiles obtained for the ions of *m/z* 418 and 448, illustrating the presence of both the *erythro*- and *threo*-isomers (peak 1) of the thioethylated monomers arising from β-*O*-4 coniferyl alcohol (a) and β-*O*-4 sinapyl alcohol (b), and their corresponding *O*-4-linked aldehydes (peak 2), which were identified as such by their mass spectra (Pomar *et al*., 2002). Monomeric degradation products were obtained by thioacidolysis of stem cell walls from *Verticillium dahliae*-inoculated pepper (cv. Padrón) plants at 21 dpi.

4-linked thioethylated monomers were significantly lower in diseased plants than in controls. The total ionic current associated with both coniferyl and sinapyl alcohols was reduced from 35.60×10^8 to 16.50×10^8 (i.e. by 54%, in diseased plants). That is, diseased plants showed a 54% reduction in the amount of lineal β-*O*-4 lignins, despite the fact that total lignin quantities increased (Tables 1 and 2). Furthermore, *O*-4-linked terminal units were reduced from 3.79×10^8 to 2.29×10^8, which indicates that the polymerization degree had increased 1.65-fold. Second, results obtained by thioacidolysis (Table 3) also suggest that the levels of the *p*-hydroxybenzaldehydes, vanillin (peak 3) and syringaldehyde (peak 5) in this lineal β-*O*-4 lignin fraction increased in diseased plants.

That the level of total conjugated carbonyl groups in cell walls increased in diseased cv. Padrón was confirmed by FT-IR spectroscopy analyses. The FT-IR spectroscopy of lignifying cv. Padrón stem cell walls revealed an absorption band at 1650 cm^{-1} (Table 4), the intensity of which decreased by 21% after sodium borohydride reduction. This band is clearly attributable to the C=O stretching vibration of conjugated/aromatic aldehydes, where the carbonyl oxygen atom sustains either intramolecular or intermolecular H-bonds. Conclusive evidence for this assignation was obtained when exploring the changes in the intensity of the bands due to the O–H (3436 cm^{-1}) and C–H (2921 cm^{-1}) stretching vibrations, which increased by 7.2% and 50%, respectively, after borohydride reduction. These data agree with the fact that borohydride reduces cell wall aromatic carbonyl (C=O) groups to alcohols (HO–C–H). Diseased cv. Padrón showed increased levels of conjugated/aromatic aldehydes in cell walls since the borohydride-reducible area of the peak at 1650 cm^{-1} increased 6.6-fold (Table 4).

Cell wall bound (*in muro*) *p*-hydroxybenzaldehydes (Table 3, peaks 3 and 5) probably originated from *p*-hydroxycinnamylaldehydes (Table 3, peaks 9 and 11), by means of a spontaneous retro-aldol reaction which occurs at neutral pH values (Ralph *et al*., 1997). Since *p*-hydroxycinnamylaldehydes are incorporated in growing lignins by means of a reaction catalysed

Table 4 Fourier transform infrared spectroscopy analyses of lignifying cell walls from controls and *Verticillium dahliae*-inoculated pepper (cv. Padrón) plants at 21 d post-inoculation, and assignment of peak wave number to functional groups in native and $NaBH_4$-reduced cell walls

		Peak area (relative units)			
		Control	Inoculated		
Functional group	Peak wave number	Native $NaBH_4$-reduced (Δ)	Native $NaBH_4$-reduced (Δ)	Native	$NaBH_4$-reduced (Δ)
–OH-	3436 cm^{-1}	86.59	92.88 (+6.29)	ND	ND
$–CH_2$-	2921 cm^{-1}	14.50	21.78 (+7.28)	ND	ND
–CO- (nonconjugated)	1734 cm^{-1}	14.86	12.69 (–2.17)	2.40	7.37 (+4.97)
–CO- (conjugated)	1650 cm^{-1}	42.15	33.26 (–8.89)	80.11	21.33 (–58.78)

Δ, increase/decrease in peak area after $NaBH_4$ reduction. ND, not determined.

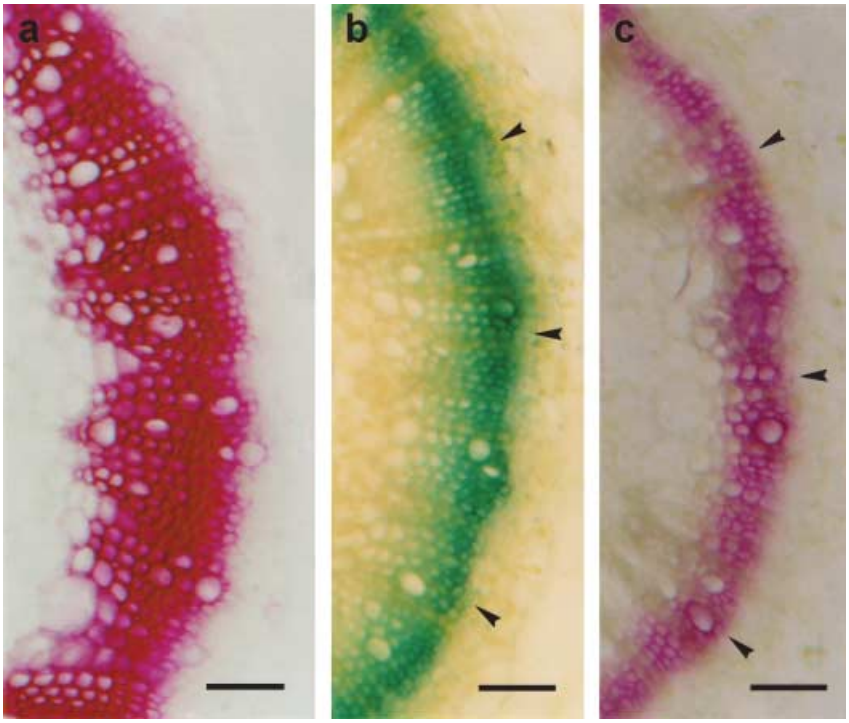

Fig. 5 Histochemical localization of *p*-hydroxycinnamylaldehydes with the phloroglucinol (Wiesner test) reagent (a) and peroxidase activities with the 3,3′,5,5′-tetramethylbenzidine (TMB) (b) and syringaldazine (c) reagent in the lignifying xylem from cv. Padrón stems. Arrowheads indicate the localization of the staining in young (differentiating) xylem elements. Bar, 50 μm.

by peroxidase (EC 1.11.1.7) (Kim *et al.*, 2000; Ros Barceló & Pomar, 2001), the next step was to study the changes in peroxidase during the *Verticillium* wilt.

Peroxidase

That peroxidase is located in the same tissues as *p*-hydroxycinnamylaldehydes and is therefore a suitable candidate for driving their cross-linking in xylem cell walls can be deduced from histochemical probes. In cv. Padrón stems, *p*-hydroxycinnamylaldehyde-containing lignins are exclusively located in xylem vessels, as revealed by the Wiesner test (Fig. 5a). Also in cv. Padrón stems, peroxidase activity is exclusively located in pro-lignifying (outermost) xylem cell layers, as revealed with 3,3′,5,5′-tetramethyl benzidine (TMB, Fig. 5b, arrowheads) or syringaldazine (Fig. 5c, arrowheads).

Additional proof for the peroxidase nature of the TMB staining of the pepper lignifying xylem was obtained from competitive inhibitor-dissected histochemistry. For this study, ferulic acid and ferrocyanide were used as competitive inhibitors of peroxidase (Ros Barceló *et al.*, 2000), and tropolone was further used to discriminate peroxidase from polyphenoloxidases activities which may also interfere with these histochemical probes (López-Serrano & Ros Barceló, 2000). Xylem staining by TMB (Fig. 6a) was totally inhibited by ferulic acid (Fig. 6b), but not by tropolone (Fig. 6c), suggesting that staining was caused by peroxidase, unlike that observed in cortical parenchyma cells, which was inhibited by tropolone.

Further evidence for the peroxidase nature of TMB staining was obtained by using the competitive inhibitor of peroxidase, ferrocyanide (Ros Barceló *et al.*, 2000), which inhibits the staining (Fig. 6d). As a positive control for ferrocyanide,

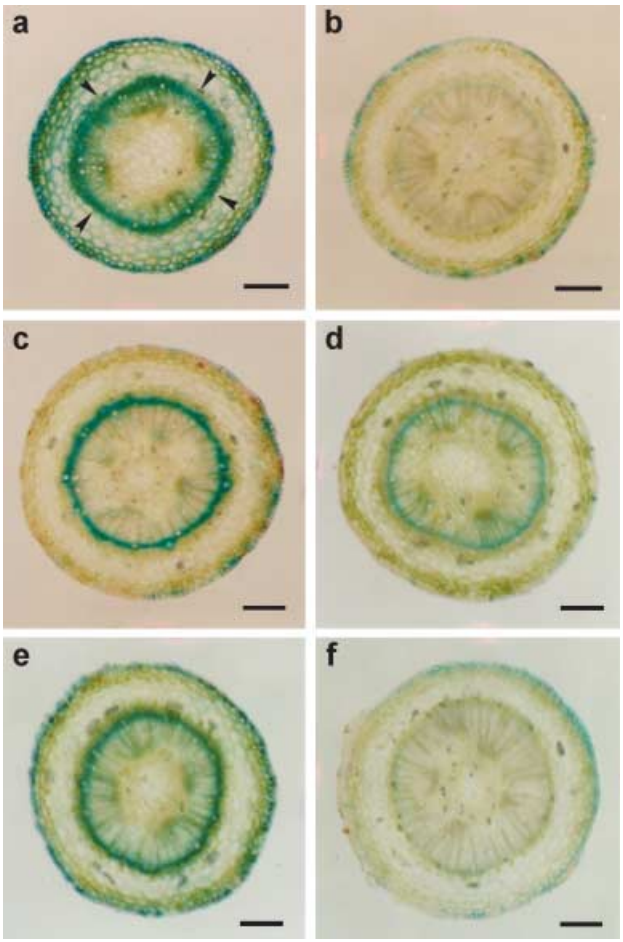

Fig. 6 Effect of inhibitors (tested at 1.0 mM) on the 3,3′,5,5′-tetramethylbenzidine (TMB) stain of the lignifying xylem from cv. Padrón stems. (a) Control section stained with the TMB reagent. (b–f) Sections stained with the TMB reagent preincubated for 20 min with (b) ferulic acid (c) tropolone (d) potassium ferrocyanide (e) potassium ferricyanide and (e) sodium azide. Arrowheads indicate the localization of the staining in young (differentiating) xylem elements. Bar, 250 μm.

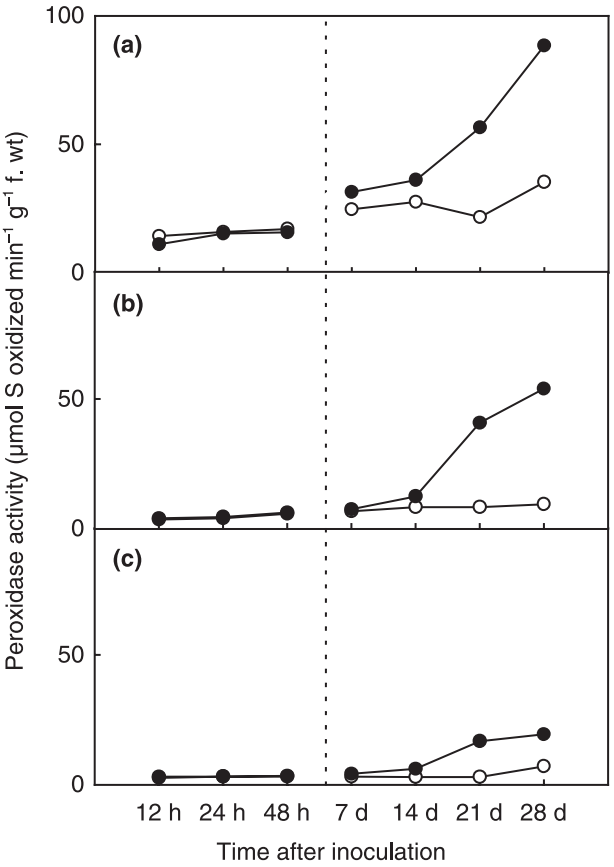

Fig. 7 Time course of changes in total peroxidase activity assayed with coniferyl aldehyde in control (open circles) and *Verticillium dahliae*-inoculated (closed circles) cv. Padrón seedlings. Peroxidase activity was measured in roots (a), stems (b), and leaves (c) of control and inoculated plants. Error bars are SD but are hidden by symbols.

we used ferricyanide. This compound, which is not a peroxidase substrate, had no effect on the TMB stain of the lignifying xylem (Fig. 6e). The inhibition obtained with sodium azide (Fig. 6f), also considered as an H_2O_2 scavenger (Ros Barceló, 1998), supports the metallo-protein nature of the enzyme. Similar sensitivity to inhibitors was obtained for the histochemical localization of peroxidase using syringaldazine in healthy plants and using TMB and syringaldazine in diseased cv. Padrón at 21 dpi (data not shown).

Verticillium dahliae inoculation of the three pepper lines led to an increase in stem peroxidase activity, which became significant from 7 dpi onwards (Fig. 7). At 21 dpi, all the three pepper lines showed a similar increase in stem peroxidase, which was two- to three-fold in roots, and 10- to 12-fold in stems (data not shown). Figure 7 shows the time-course changes in peroxidase activity measured with coniferylaldehyde in roots (Fig. 7a), stems (Fig. 7b) and leaves (Fig. 7c) of cv. Padrón. Similar results were obtained for the other cultivars and with the other substrate used to measure peroxidase activity. The strong increases in peroxidase activity in diseased cv. Padrón plants (Fig. 7) were not accompanied by drastic changes in the peroxidase isoenzyme pattern. In fact, only slight but significant changes in a strong basic peroxidase, which increased its activity in roots and stems (Fig. 8, lanes Ri and Si, arrowheads), and in a weakly acid peroxidase, which decreased its activity in leaves (Fig. 8, lane Lc, arrow), were observed.

Discussion

Verticillium dahliae is a soilborne pathogen that causes vascular wilt in pepper. No *C. annuum* cultivars are resistant to *Verticillium* wilts. However, in the glasshouse, some pepper cultivars, such as Padrón, are apparently more tolerant than others, such as Luesia, as can be quantitatively confirmed by changes in f. wt (Fig. 1a) and total leaf surface (Fig. 1c,d).

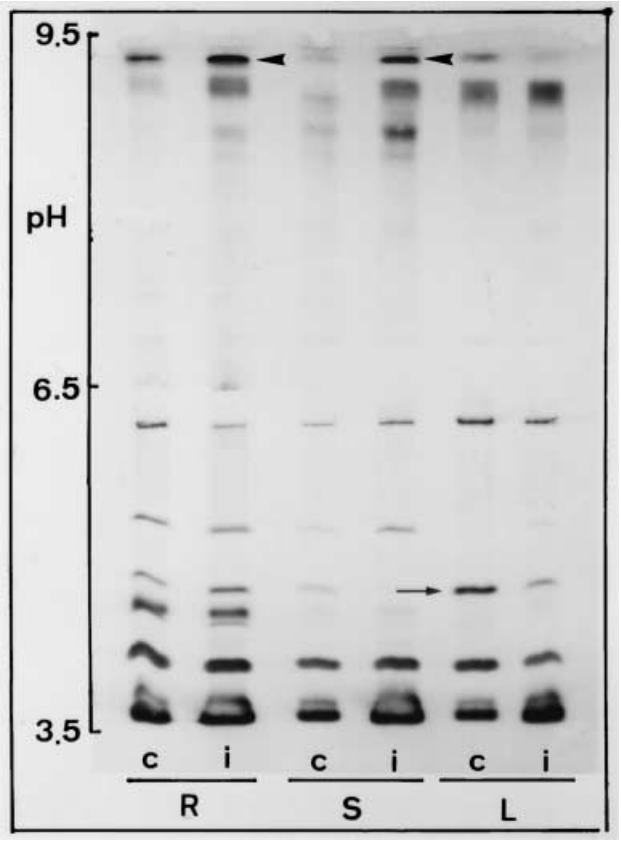

Fig. 8 Peroxidase isoenzyme patterns of control and *Verticillium dahliae*-inoculated cv. Padrón seedlings at 28 d post-inoculation obtained by isoelectric focusing in pH 3.5–9.5 gradients. Peroxidase isoenzyme patterns were analysed in roots (R), stems (S), and leaves (L) of control (c) and inoculated (i) plants, and stained for activity with 4-methoxy-α-naphthol in the presence of H_2O_2.

Despite the strong reduction in the leaf surface observed at 7 dpi in cvs. Padrón and Yolo Wonder (Fig. 1C), both Φ_{PSII} and ETR showed a significant increase at saturating PAR values (Fig. 2a,b,d,e). This increase, which was also observed during later stages (Fig. 2g,h,j,k), was, however, not observed at limiting PAR values, and may be caused by the creation of a novel sink for photosynthates by the fungal hyphae. Unlike cvs Padrón and Yolo Wonder, cv. Luesia showed a significant decrease in both leaf Φ_{PSII} and ETR values (Fig. 2c,f,i,l), especially at later stages, suggesting that photochemical processes are strongly damaged in this cultivar as a consequence of disease.

During the time when the plants were monitored, cv. Padrón maintained maximum quantum yields of photosystem II and high NPQ values, which are indicative of a high capacity to dissipate light energy safely (Müller *et al.*, 2001), and of the cultivar's ability to develop strategies for avoiding/reducing the damage provoked by fungal colonization. In the case of cv. Luesia, the reduction in Φ_{PSII} and ETR at 28 dpi (Fig. 2i,l) was also associated with a decrease in NPQ at saturating PAR values. Since the maintenance of high NPQ values under stress situations has been associated with a high capacity to dissipate light energy safely (Veljovic-Jovanovic *et al.*, 2001), the nonmaintenance of a high NPQ capacity in cv. Luesia probably impedes the safe dissipation of light energy in this cultivar, and therefore does not avoid the production of harmful species, such as singlet oxygen, which are produced in the photosynthetic apparatus of plant cells in response to abiotic stresses and fungal diseases (Fryer *et al.*, 2002).

These results underline the convenience of monitoring physiological parameters, such as the photochemical process of photosynthesis (Fig. 2) since morphological parameters, such as f. wt (Fig. 1a) and total leaf surface (Fig. 1b–d), do not accurately reflect all the disturbances which occur in peppers during *Verticillum* wilts. These results also suggest that, especially in the case of cv. Padrón, the early response characterized by stunted growth and responsible for the 'dwarf-like' phenotype, may be due to the creation of a novel sink (the fungal hyphae) for carbon partitioning, as is suggested by the increase in both Φ_{PSII} and ETR values (Fig. 2a,d). This is not surprising since plants, including pepper (Goicoechea *et al.*, 2000), infected by endophytic microbes show a much increased carbohydrate metabolism and a host solute efflux at the interface between the host plant and the microorganism, the exported energy to the apoplast being ready for use by the endophytic microbe (Bishop *et al.*, 2002).

Strategies to avoid/reduce damage caused by fungal colonization in cv. Padrón could be achieved in different ways. One of them involves phenylpropanoid compounds, since they may constitute preformed or inducible physical and chemical barriers against infection (Calderón *et al.*, 1993). Phenylpropanoids may also act as signal molecules involved in local and systemic signaling for defense gene induction (Dixon *et al.*, 2002). Although the phenolic metabolism of pepper is altered during the *Verticillium* wilt (Fig. 3), the levels of chlorogenic acid, the main free phenolic in peppers (Díaz *et al.*, 1997), showed no significant changes during the disease (Fig. 3b). In the case of the bound phenolic fraction, levels of ferulic acid (a C_6C_3 compound) and vanillin (a C_6C_1 compound) increased significantly (Fig. 3c) in diseased plants.

Verticillium dahliae inoculation of the three pepper lines produced also an increase in total stem lignin content, which became significant after 14 dpi. At 28 dpi, the degree of lignification reached in diseased plants was greater in cv. Padrón than in cvs Yolo Wonder and Luesia (Table 1). This suggests that lignification may be a mechanism through which Padrón peppers could restrict the growth of *V. dahliae* hyphae in the xylem, and thus retard the rapid damage of leaf photosynthetic tissues. The lignification response of cv. Padrón was studied by the analysis of nitrobenzene oxidation product and thioacidolysis. Nitrobenzene oxidation solubilizes both the condensed and noncondensed lignin fractions present in primary and secondary cell walls (Anterola & Lewis, 2002), while thioacidolysis solubilizes only the noncondensed lignin fraction (i.e. the lineal β-*O*-4 chains) (Anterola & Lewis,

2002) located mainly in secondary cell walls (Ruel *et al.*, 2002). The results obtained from nitrobenzene oxidation product analyses (Table 2), supported the above claim, and revealed that the (H + G + S) content of diseased cv. Padrón stems was 1.15-fold greater than that of healthy plants. Furthermore, stem lignins from healthy plants were mainly composed of guaiacyl/syringyl units (the H : G : S ratio was 6 : 60 : 34, Table 2), while stem lignins from diseased plants contain greater amounts of *p*-hydroxyphenyl units (the H : G : S ratio was 55 : 34 : 11). That is, stem lignins from diseased plants were putatively more crosslinked since they exhibited higher contents of H units derived from *p*-coumaryl alcohol. This observation is in accordance with the view that total lignin levels and putative crosslinking degree is increased in pepper in order to restrict *V. dahliae* hyphal growth to the xylem apoplastic cavity.

The results obtained by thioacidolysis (Table 3) also revealed two additional striking features. First, levels of β-*O*-4-linked thioethylated monomers were significantly reduced in diseased plants with respect to the controls, despite the fact that total lignin levels were increased (Tables 1 and 2). Furthermore, *O*-4-linked terminal units were reduced by 40%, which indicates that the polymerization degree was increased 1.65-fold. All these results suggest that the lineal β-*O*-4 lignin fraction was further crosslinked and insolubilized in response to infection during this *Verticillium* disease. Second, the results obtained by thioacidolysis (Table 3) also suggest that levels of the *O*-4-linked *p*-hydroxybenzaldehydes, vanillin and syringaldehyde, increased in response to inoculation. This result is in accordance with those obtained by alkaline hydrolysis of the bound phenolic fraction (Fig. 3c), and it is not surprising since the conjugated carbonyl group (CO) of benzaldehydes may form Schiff bases with free NH_2 groups of proteins, thus inactivating enzymes (Grabber *et al.*, 1998). Through this mechanism, pepper plants could retard the *Verticillium* hyphal growth beyond the apoplastic xylem cavity by inactivating hydrolase-type enzymes.

That the levels of total conjugated carbonyl groups were increased in cv. Padrón cell walls during disease was confirmed by FT-IR spectroscopy analyses. Such techniques are suitable for this purpose since *p*-hydroxycinnamyl aldehydes (Table 3, peaks 9 and 11), *p*-hydroxybenzaldehydes (Table 3, peaks 3 and 5), and compounds with aryl-α-CO-containing moieties, which are found in keto-enolic equilibrium with C_6C_2-enol ether structures (Table 3, peaks 4 and 7, Ar–CH=CHO–Ar), represent all the possible carbonyl units present in lignifying cell walls (Tsai *et al.*, 1998), and therefore the conjugated carbonyl signal of cell walls during FT-IR spectroscopy is unequivocal and clear (Stewart *et al.*, 1997; Pomar *et al.*, 2002). In this context, inoculation of cv. Padrón with *V. dahliae* provoked an increase in the level of conjugated/aromatic aldehydes in cell walls since the area of the corresponding borohydride-reducible IR peak increased 6.6-fold (Table 4). Increases in cell wall-bound aromatic aldehydes have also been described during the interaction of several others plant species with, especially, fungal pathogens (Kauss *et al.*, 1993). The involvement of pepper peroxidase in the crosslinking reaction of *p*-hydroxycinnamylaldehydes appears to be plausible since, in both healthy and diseased plants (Fig. 7), peroxidase not only showed a capacity to oxidize coniferyl aldehyde (the putative precursor of vanillin), but also its level increased during disease.

That peroxidase was located in the same tissues as *p*-hydroxy cinnamylaldehydes, and therefore is the perfect candidate for driving the cross-linking of these aromatic aldehydes in the xylem cell walls was deduced from histochemical probes. In cv. Padrón stems, *p*-hydroxycinnamylaldehyde-containing lignins are exclusively located in xylem vessels, as revealed by the Wiesner test (Fig. 5a), which is specific for cinnamylaldehydes (Pomar *et al.*, 2002). Also, in cv. Padrón stems, peroxidase is exclusively located in pro-lignifying (outermost) xylem cell layers, as revealed by both TMB or syringaldazine (Fig. 5b,c). Additional proof for this fine localization of peroxidase was obtained from competitive inhibitor-dissected histochemistry (Fig. 6). Since the TMB staining of the lignifying xylem was sensitive to both ferulic acid and ferrocyanide (Fig. 6), the inhibition observed in these histochemical probes can only be caused by a target enzyme of an oxidoreductase nature, such as peroxidase, which is capable of accepting either hydrogen atoms (in the case of ferulic acid) or only electrons (in the case of ferrocyanide) during its catalytic cycle.

As would be expected from its putative effect on *p*-hydroxycinnamylaldehyde crosslinking reactions, *V. dahliae* infection produced a similar increase in stem peroxidase activity in the three pepper lines used, but the joint change in both peroxidase (data not shown) and stem lignins (Table 1) was greater in cv. Padrón. However, it should be remembered that it is uncertain to what extent these changes in peroxidase may condition the defense response of pepper to *V. dahliae*, and whether the levels of cell wall peroxidases really are limiting factors for the rate of cell wall crosslinking of *p*-hydroxycinnamylaldehydes. To cast light on the latter point, overexpression of a lignin-related tobacco peroxidase in transgenic tomato has been described as conferring resistance to *V. dahliae* (Lagrimini *et al.*, 1993).

The strong increases in peroxidase activity in cv. Padrón as a consequence of *V. dahliae* inoculation (Fig. 7) have also been observed for other *Verticillium* wilts (Reuveni & Ferreira, 1985), and in peppers in response to other fungi (Alcázar *et al.*, 1995). However, the increases in peroxidase activity were not accompanied by drastic changes in the peroxidase isoenzyme pattern. Only minor, although significant, changes for a strong basic peroxidase (Fig. 8) were observed. This basic peroxidase isoenzyme is located in cell walls (Bernal *et al.*, 1993) and is also responsive to copper stress, which induces a lignification response in pepper seedlings (Díaz *et al.*, 2001).

In the case of acidic peroxidases, no changes were observed in either stems or roots in response to infection. Only in leaves was a decrease in the activity of a weakly acid peroxidase observed (Fig. 8). This observation is striking since an increase in acidic peroxidases has frequently been observed during suberization induced by wounding in potato (Bernards, 2002) and by *V. albo-atrum* infection in tomato (Mohan & Kolattukudy, 1990), and reinforces the idea that suberin layers play a minor role in restricting the growth of *V. dahliae* in its interaction with *C. annuum*. This view is further supported by the undetectable levels of tyramine in the bound-phenolic fraction.

Taken together, these results suggest that through changes in stem lignins (monomer composition and crosslinking) and peroxidase (one of the enzymes responsible for their crosslinking), compatible peppers retard the penetration by *V. dahliae* hyphae, thus maintaining leaf photosynthetic integrity as shown by the sustained Φ_{PSII} and NPQ values. These results also suggest that, especially in the cv. Padrón, the early response characterized by stunted growth, and responsible for the 'dwarf-like' phenotype, may be due to the creation of a novel sink (the fungal hyphae) for carbon partitioning, as suggested by the increase in both Φ_{PSII} and ETR values.

Acknowledgements

This work was supported by grants from MCYT (BOS2002-03550), CICYT (AGF99-031) and Xunta de Galicia (project # XUGA 10303A97).

References

Alcázar MD, Egea C, Espín A, Candela MA. 1995. Peroxidase isoenzymes in the defense response of *Capsicum annuum* to *Phytophthora capsici*. *Physiologia Plantarum* **94**: 736–742.

Anterola A, Lewis NG. 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. *Phytochemistry* **61**: 221–294.

Bernal MA, Pedreño MA, Calderón AA, Muñoz R, Ros Barceló A, Merino F. 1993. The subcellular localization of isoperoxidases in *Capsicum annuum* leaves and their different expression in vegetative and flowered plants. *Annals of Botany* **72**: 415–421.

Bernards MA. 2002. Demystifying suberin. *Canadian Journal of Botany* **80**: 227–240.

Bilger W, Björkman O. 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. *Photosynthesis Research* **25**: 173–185.

Bishop DL, Chatterton NJ, Harrison PA, Hatfield RD. 2002. Changes in carbohydrate coordinated partitioning and cell wall remodeling with stress-induced pathogenesis in wheat sheaths. *Physiological and Molecular Plant Pathology* **61**: 53–63.

Boudet AM. 1998. A new view of lignification. *Trends in Plant Science* **3**: 67–71.

Calderón AA, Zapata JM, Muñoz R, Pedreño MA, Ros Barceló A. 1993. Resveratrol production as a part of the hypersensitive-like response of grapevine cells to an elicitor from *Trichoderma viride*. *New Phytologist* **124**: 455–463.

Dang JL, Jones JDG. 2001. Plant pathogens and integrated defence responses to infection. *Nature* **411**: 826–833.

Díaz J, Bernal A, Pomar F, Merino F. 2001. Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annuum* L.) seedlings in response to copper stress and its relation to lignification. *Plant Science* **161**: 179–188.

Díaz J, Ros Barceló A, Merino F. 1997. Changes in shikimate dehydrogenase and the end products of the shikimate pathway, chlorogenic acid and lignins, during the early development of seedlings of *Capsicum annuum*. *New Phytologist* **136**: 183–188.

Dixon RA, Achnine L, Kota P, Liu CJ, Srinivasa Reddy MS, Wang L. 2002. The phenylpropanoid pathway and plant defence – a genomics perspective. *Molecular Plant Pathology* **3**: 371–390.

Fryer MJ, Oxborough K, Mullineaux PM, Baker NR. 2002. Imaging of photo-oxidative stress responses in leaves. *Journal of Experimental Botany* **53**: 1249–1254.

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CMT. 2000. Fungal pathogen protection in potato by expression of a plant defensin peptide. *Nature Biotechnology* **18**: 1307–1310.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and the quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**: 87–92.

Goicoechea N, Aguirreolea J, Cenoz S, García-Mina JM. 2000. *Verticillium dahliae* modifies the concentrations of proline, soluble sugars, starch, soluble protein and abscisic acid in pepper plants. *European Journal of Plant Pathology* **106**: 19–25.

Grabber JH, Ralph J, Hatfield RD. 1998. Severe inhibition of maize wall degradation by synthetic lignins formed with coniferylaldehyde. *Journal of the Science of Food and Agriculture* **78**: 81–87.

Kauss H, Franke R, Krause K, Conrath U, Jeblick W, Grimmig B, Matern U. 1993. Conditioning of parsley (*Petroselinum crispum* L.) suspension cells increases elicitor-induced incorporation of cell wall phenolics. *Plant Physiology* **102**: 459–466.

Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ, Fischer R, Prüfer D. 2001. Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Sciences, USA* **98**: 6511–6515.

Kim H, Ralph J, Yahiaoui N, Pean M, Boudet AM. 2000. Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Organic Letters* **2**: 2197–2200.

Lagrimini LM, Vaughn J, Erb WA, Miller SA. 1993. Peroxidase overproduction in tomato: wound-induced polyphenol deposition and disease resistance. *Hort Science* **28**: 218–221.

Lapierre C, Pollet B, Rolando C. 1995. New insights into the molecular architecture of hardwood lignins by chemical degradative methods. *Research Chemistry Intermediaries* **21**: 397–412.

Lewis NG, Davin LB, Sarkanen S. 1999. The nature and function of lignins. In: Barton DHR, Nakanishi K, eds. *Carbohydrates and their derivatives including tannins, cellulose and related lignins. Comprehensive natural products chemistry, Vol. 3*. New York, NY, USA: Elsevier Science, 617–745.

López-Serrano M, Ros Barceló A. 2000. Histochemical localization and developmental expression of peroxidase and polyphenoloxidase in strawberry fruit. *Journal of the American Society for Horticultural Science* **126**: 27–32.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence – a practical guide. *Journal of Experimental Botany* **51**: 659–668.

Mohan R, Kolattukudy PE. 1990. Differential activation of expression of a suberization-associated anionic peroxidase gene in near-isogenic resistant and susceptible tomato lines by elicitors of *Verticillium albo-atrum*. *Plant Physiology* **92**: 276–280.

Müller P, Li XP, Niyogi KK. 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiology* **125**: 1558–1566.

Pomar F, Merino F, Ros Barceló A. 2002. *O*-4-linked coniferyl and sinapyl aldehyde in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction. *Protoplasma* **220**: 17–28.

Ralph J, MacKay JJ, Hatfield RD, O'Malley DM, Whetten RW, Sederoff RR. 1997. Abnormal lignin in loblolly pine mutant. *Science* **277**: 235–239.

Reuveni R, Ferreira JF. 1985. The relationship between peroxidase activity and the resistance of tomatoes (*Lycopersicum esculentum*) to *Verticillium dahliae*. *Phytopathologische Zeitschrift* **112**: 193–197.

Robb J, Lee S, Mohan R, Kolattukudy PE. 1991. Chemical characterization of stress-induced vascular coating in tomato. *Plant Physiology* **97**: 528–836.

Ros Barceló A. 1997. Lignification in plant cell walls. *International Review of Cytology* **176**: 87–132.

Ros Barceló A. 1998. Use and misuse of peroxidase inhibitors. *Trends in Plant Science* **3**: 418.

Ros Barceló A, Pomar F. 2001. Oxidation of cinnamyl alcohol and aldehydes by a basic peroxidase from lignifying *Z. elegans* hypocotyls. *Phytochemistry* **57**: 1105–1113.

Ros Barceló A, Pomar F, Pedreño MA. 2000. Competitive inhibitor-dissected histochemistry of the peroxidase responsible for syringyl lignin biosynthesis in the *Z. elegans* xylem. *Australian Journal of Plant Physiology* **27**: 1101–1107.

Ruel K, Montiel MD, Goujon T, Jouanin L, Burlat V, Joseleau JP. 2002. Interrelation between lignin deposition and polysaccharide matrices during the assembly of plant cell walls. *Plant Biology* **4**: 2–8.

Sadras VO, Quiroz F, Echarte L, Escande A, Pereyra VR. 2000. Effect of *Verticillium dahliae* on photosynthesis, leaf expansion and senescence of field-grown sunflower. *Annals of Botany* **86**: 1007–1015.

Smit F, Dubery IA. 1997. Cell wall reinforcement in cotton hypocotyls in response to a *Verticillium dahliae* elicitor. *Phytochemistry* **44**: 811–815.

Stewart D, Yahiaoui N, McDougall GJ, Myton K, Marque Ch, Boudet AM, Haigh J. 1997. Fourier-transform infrared and Raman spectroscopic evidence for the incorporation of cinnamylaldehydes into the lignin of transgenic tobacco (*Nicotiana tabacum* L.) plants with reduced expression of cinnamyl alcohol dehydrogenase. *Planta* **201**: 311–318.

Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, Zhu T, Zou G, Katagiri F. 2003. Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* **15**: 317–330.

Tsai CHJ, Popko JL, Mielke MR, Hu WJ, Podila GK, Chiang VL. 1998. Suppression of *O*-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes. *Plant Physiology* **117**: 101–112.

Veljovic-Jovanovic SD, Pignocchi C, Noctor G, Foyer CH. 2001. Low ascorbic acid in the vtc-1 mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system. *Plant Physiology* **127**: 426–435.

Williams JS, Hall SA, Hawkesford MJ, Beale MH, Cooper RM. 2002. Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. *Plant Physiology* **128**: 150–159.