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A role for oxidative stress in the *Citrus limon*/*Phoma tracheiphila* interaction

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Phoma tracheiphila, the agent of citrus 'mal secco', releases a toxic complex called malseccin into the plant. Oxidative stress was studied, both regarding the reaction of lemon (*Citrus limon*) leaves to the infiltration of fungal extracellular proteins, and the analysis of the pathogen's ability to cause symptoms in leaves of lemon cultivars with different degrees of susceptibility. Studies were perfomed *in planta* on three lemon cultivars: cv. Interdonato, partially tolerant to 'mal secco'; cv. Monachello, tolerant; and cv. Femminello, susceptible. When inoculated with *P. tracheiphila* extracellular proteins, cv. Interdonato leaves showed chlorosis and necrosis symptoms and an increase in lipoxygenase and glutathione peroxidase of up to 100 and 76·5 fold, respectively. Furthermore, extracellular proteins of *P. tracheiphila* infiltrated into leaves induced lipoperoxide formation 10 fold in cvs Interdonato and Femminello and 3 fold in cv. Monachello compared to the control, with Monachello reacting earlier. *In vitro* analyses indicate that the presence of lyophilized twigs and leaves (LTV) of cv. Monachello in the fungal growth medium, stimulated concentrations of superoxide dismutases (~20–60 fold), glutathione peroxidase (~115 fold) and catalase (~15 fold) in fungal mycelia. When LTV of cv. Femminello was added, the pathogen produced a higher quantity of hydrolytic enzymes *in vitro*, polygalacturonase (~40 fold) and laccase (~110 fold). The MALDI-TOF and TOF/TOF MS analyses performed on 60–70 KDa extracellular proteins of *P. tracheiphila* after inoculation in malseccin conducive media showed the presence of a monoamine oxidase enzyme able to release NH_4^+ and H_2O_2 in the cell. Overall the results suggest oxidative stress occurs in this interaction.

Keywords: hydrolytic enzymes, lipoperoxidation, malseccin, 'mal secco' of citrus, monoamine oxidase, phytotoxin

Introduction

The 'mal secco' disease of citrus is caused by the mitosporic ascomycete *Phoma tracheiphila*. This disease has led to serious losses of crops of *Citrus* spp. in the Mediterranean areas, mainly in southern Italy where citrus crop production has decreased by 90% since 1900 (Perrotta & Graniti, 1988). The diseased plants show the classic symptoms of tracheomycosis such as chlorosis, phylloptosis, wilt and die-back of twigs and branches induced by malseccin, a phytotoxic extracellular complex produced in the xylem after exposure to *P. tracheiphila*. The pathogen penetrates plants through wounds or stomata by conidia produced from pycnidia present on twigs or from phialides. The optimum temperature for symptom expression and pathogen growth is 20–25°C with infection occurring between 14 and 28°C. The length of the infection period depends on the climatic and seasonal conditions (Solel, 1976; Somma & Scarito, 1986; Perrotta & Graniti, 1988). Recently, analysis of ITS sequences of *P. tracheiphila* has revealed a close relationship with the teleomorphic taxon *Leptosphaeria congesta* (Balmas, 2005). Three cultivars of *C. limon* described by Solel & Salerno (1988) are known, with different phenotypic reactions to the pathogen: cv. Monachello, is tolerant (i.e. not killed by the pathogen but suffers physiological and growth alterations); cv. Interdonato partially tolerant; and cv. Femminello, one of the most valuable crops, is susceptible.

Phoma tracheiphila is considered a necrotrophic pathogen, producing hydrolytic enzymes and toxins during the infection phase, and no peculiar structures of a biotrophic phase have been reported (Professor S.O. Cacciola, personal communication, cacciola@unipa.it). Glycoproteins of 93 KDa and 60 KDa (called Pt60) belonging to the malseccin complex have been isolated from culture filtrates and host plants infected by *P. tracheiphila* (Nachmias *et al*., 1977; Fogliano *et al*., 1994; Fogliano *et al*., 1998). Both were able to reproduce all the symptoms of the disease when injected into different plants (Fogliano *et al*., 1998). The toxic effects of the

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malseccin complex on citrus leaves are apparent only under illuminated conditions, suggesting that light plays a role in the toxin activity. Nachmias *et al*. (1980) reported that injection of the 93 KDa protein into the leaves induced chloroplast degeneration, and all the toxic effects were avoided when the leaves were shaded. Similar results were also obtained by Fogliano *et al*. (1998) using Pt60. There have been at least two reports on the role of light in plant-pathogen interactions. Tab-toxin, a well-known non host-selective toxin produced by *Pseudomonas syringae* pv. *tabaci* causes symptoms such as chlorosis and necrosis in tobacco leaves, similar to those induced by malseccin on leaves of *Citrus* spp. The toxin, tabtoxinin-β-lactam (TβL), released after hydrolysis from threonine by the plant amminopeptidases, inhibited glutamine synthetases by blocking both the synthesis of glutamine and cell detoxification of ammonia. The accumulation of ammonia results in the malfunction of chloroplasts and the uncoupling of photophosphorylation (Bender *et al*., 1999). Nachmias *et al*. (1980) demonstrated that chloroplasts and cellular membranes are damaged both in affected trees and also in leaves of *C. limon* plants affected by malseccin. Light also seems to drive the activity of cercosporin, a phytotoxic polyketide produced by many species of *Cercospora* (Daub & Ehrenshaft, 2000; Choquer *et al*., 2005). In light conditions, cercosporin was able to damage cellular structures through the induction of reactive oxygen species (ROS) formation. ROS can induce lipid membrane peroxidation leading to the loss of membrane integrity, electrolyte leakage and cell death.

Oxidative stress in plant pathology has been a general subject of investigation and its ability to drive the metabolism of both host and pathogen during their interaction has been demonstrated (Fedoroff, 2006). These considerations and the common features between these toxins and malseccin has led to question if oxidative stress may also play a role in the interaction between *P. tracheiphila* and *C. limon*. In *in planta* tests, the three above mentioned lemon cultivars with different resistance to mal secco disease were used to study the correlation between the necrosis event and the modulation of lipoperoxidation. In *in vitro* experiments, lyophilized twigs and leaves of the same lemon cultivars were assayed to verify the involvement of parameters related to the pathogen's attack strategy (e.g. the activation of hydrolytic and antioxidant enzymes, the formation of peroxides and toxic compounds in culture media). The results obtained also indicate a role for oxidative stress in this interaction, and it is proposed that a fungal monoamine oxidase has a direct role in the symptomatology of citrus 'mal secco'.

Materials and methods

Plant materials

Twigs and leaves from plants of the same age of the lemon cvs Monachello L26, Femminello L25 and Interdonato were used in this study (kindly supplied by Professor M.A. Germanà, University of Palermo, Italy). To study their effects on the pathogen, lyophilized lemon twigs and leaves (LTL) of cvs Monachello and Femminello (marked respectively as MON and FEM) were added (0·2% w/v) to the fungal growth medium before sterilization. Leaves of cv. Interdonato, which shows a partial tolerance to 'mal secco' (Solel & Salerno, 1988), were used for *in planta* toxicity testing and for enzymatic activity lipoxygenase (LOX) and glutathione peroxidase (GSH POX) analysis.

Fungal strains and culture conditions

Phoma tracheiphila wild type strain 2338 (highly pathogenic and a toxin producer), kindly supplied from the collection of Professor G.M. di San Lio (Institute of Plant Pathology, Università degli Studi di Reggio Calabria, Italy), was grown on potato dextrose agar (PDA, Difco) at the optimum growth temperature of 23°C. The fungus was pre-cultivated for 7 days in carrot agar (Fogliano *et al*., 1998), harvested with a cork borer (Ø 5 mm) and transferred to fresh, sterile, synthetic malseccin conducive medium (Nachmias *et al*., 1977), amended either with 20 g L^{-1} glucose (control, CON) or 2 g L^{-1} of sterilized LTL of *Citrus limon* (MON or FEM) as sole carbon source. This strategy reproduces conditions similar to the first step of the interaction between the pathogen and *C. limon*, as also reported in other experimental systems (e.g. *Aspergillus flavus – Zea maydis*, Maggio-Hall *et al*., 2005).

In planta toxicity test

Injections of *Citrus limon* (cv. Interdonato) leaves was carried out to test the presence of some phytotoxic proteinaceous components produced by the fungus in the culture filtrates after 21 and 30 days of growth in control medium, or in medium supplemented with LTL of *C. limon* (MON or FEM). The leaves were first surface-sterilized in 0·5 g L^{-1} sodium hypochlorite and then washed three times with sterile distilled water. Ten microlitres (containing 10 μg of proteins) of the different extracellular proteins of the fungal culture filtrates, dialyzed and lyophilized, or of culture medium (as control), were injected into the central vein of mature *C. limon* leaves using a micro syringe. Ten microliters of a similarly treated control medium filtrate, with the addition of 1 mM salicylhydroxamic acid (SHAM), prepared as described in Reverberi *et al*. (2005b), were also injected. The leaves were layered on paper filters in sterilized Petri dishes, humidified with 5 mL of bi-distilled sterile water and incubated for 4 and 6 days at room temperature under continuous illumination (20 μE m^{-2} s^{-1}), as reported by Fogliano *et al*. (1998).

Lipoxygenase activity

The activity of lipoxygenase (LOX, EC 1·13·11·12) was tested in cv. Interdonato leaves, at 0, 6, 12, 24 and 48 h after fungal extracellular protein (EP) injection, following the formation of the diene conjugates by the increase in absorbance at 234 nm, as reported by Reverberi *et al*. (2005b).

Analysis of 9 and 13-hydroxyoctadecadienoic acid (HODE) *in C. limon* leaves of different cultivars

Citrus limon leaves infiltrated with *P. tracheiphila* extracellular proteins (EP, 10 μg) incubated for 1, 24 and 48 h were lyophilised and homogenized in liquid nitrogen. Hydroperoxyoctadecadienoic acid (HPODE) present in the mycelia was analysed after extraction of the samples three times with chloroform: methanol (2:1 v/v) in the presence of 100 μg of butylated hydroxytoluene (BHT) as antioxidant, and reduced with $NaBH_4$. The hydroxides of linoleic acid (hydroxyoctadecadienoic acid, HODE) and the regioisomers (9-HODE and 13-HODE) were analysed by HPLC-APCI-MS as reported by Reverberi *et al.* (2006). Data were collected and analyzed using the Chemstation LC/MSD revision A·09·01 (Agilent Technologies). 13-HODE and 9-HODE were purchased from Cayman.

FOX 1 assay for testing peroxide formation by *P. tracheiphila*

The hydroperoxide produced by *P. tracheiphila* grown in different media (CON, FEM, MON) was analyzed in the filtrate by spectrophotometric assay by monitoring the oxidation of xylenol orange (FOX-1) at 560 nm. The sensitivity of the method was increased by the use of triphenylphosphine and stabilizing the reagent at pH 1·7–1·8, as reported by Banerjee *et al.* (2003).

Enzymatic activities of laccase, Mn peroxidase and polygalacturonase in *P. tracheiphila* culture filtrates

The filtrates were sampled after 15, 21 and 30 days incubation, from *P. tracheiphila* cultures grown as reported above. The laccase (LAC, EC1·10·3·2) and Mn peroxidase (Mn POX, EC 1·11·1·13) activities were performed as reported by Zjalic *et al.* (2002). To test the polygalacturonase activity (PG, EC 3·2·1·15), 1 mL of liquid culture medium of *P. tracheiphila* was sampled at 15, 21 and 30 days and precipitated with ammonium sulphate (final saturation 80%). The enzyme activity was analysed as reported by Cook *et al.* (1999).

Antioxidant enzymatic activities of SOD, CAT and GSH POX in *P. tracheiphila* mycelia

The activities of superoxide dismutase (SOD, EC 1·15·1·1), catalase (CAT, EC 1·11·1·6) and glutathione peroxidase (GSH POX, EC 1·11·1·9) were analyzed by spectrophotometric assay (Beckman DU530 spectrophotometer), as previously described by Reverberi *et al.* (2005a), in the homogenized mycelia of *P. tracheiphila* and *C. limon* (cv. Interdonato) leaves, after fungal filtrate injection (10 μL; 1 mg mL^{-1}).

Isolation of extracellular proteins

Culture filtrates were filtered through a 0·45 μm filter, lyophilized and resuspended in 5 mL of sterile Milli-Q water containing 5 mM PMSF and then dialyzed for 48 h at 4°C against distilled water using a Spectrachom membrane (cut-off > 3500 Da). Extracellular protein precipitation was carried out after resuspension of lyophilized filtrates in 20% TCA (trichloroacetic acid) in acetone containing 2 g L^{-1} DTT, and kept overnight at –20°C. The samples were then centrifuged at 20 000 ***g*** for 20 min, at 4°C. The resulting pellet was collected and washed three times with ice-cold acetone containing 2 g L^{-1} DTT, re-suspended in RB buffer (tris-HCl 0·5 M pH 6·8, glycerol 80 g L^{-1}, SDS 80 g L^{-1}, 2-β-mercaptoethanol 40 g L^{-1}, bromophenol blue 2·5 g L^{-1}), and subjected to SDS-PAGE directly. In other experiments extracellular proteins (1/5 of those used in previous experiments) were de-glycosylated by using an endo-N-glucanase H (Roche) in denaturing conditions: the samples were heated at 100°C for 5 min in the presence of 0·5% SDS and 1% β-mercaptoethanol. After cooling, appropriate buffer (10X) was added and the glycoprotein was incubated for 1 h at 37°C with 1 mU of enzyme. SDS-PAGE was performed running a 100 g L^{-1} polyacrylamide gel (11 cm) at 150 V for 1 h, and stained following the colloidal Comassie blue G-250 staining procedure (Invitrogen).

MALDI-TOF and MALDI-TOF/TOF MS analyses

Protein spots were excised from 1D gels and digested with trypsin using a ZipPlate with a vacuum manifold apparatus (Millipore). Briefly, the gel pieces were further diced into smaller pieces of about 1 mm^{-3} in size and placed into ZipPlate wells, then washed sequentially with 100 μL each of 25 mM NH_4HCO_3/5% acetonitrile (ACN) and 25 mM NH_4HCO_3/50% ACN for 30 min. The gel pieces were then dehydrated by 200 μL of ACN and 15 μL of sequencing grade porcine trypsin (Promega) (12·5 ng μL^{-1}) was added to each well. The ZipPlate was incubated at 37°C overnight. The reverse-phase resins at the bottom of ZipPlate wells were wetted by adding 8 μL of ACN directly onto them and further incubated at 37°C for 15 min. The tryptic peptides were extracted by adding 130 μL of 0·2% trifluoroacetic acid (TFA) and incubated for 30 min at room temperature. Vacuum was applied to allow for the extract to pass through and the tryptic peptides to bind to the resins. The resins were washed twice with 100 μL each of 0·2% TFA and the peptides were eluted with 20 μL 0·1% TFA/50% ACN and collected into a 96-well plate. After vacuum drying, the peptides were re-dissolved in 1 μL matrix solution containing 3 mg mL^{-1} of α-cyano-4-hydroxycinnamic acid (CHCA) in 0·1%TFA/50% ACN, and spotted onto a stainless-steel MALDI target plate (Applied Biosystems).

The mass spectra of these fragments were processed with MASCOT software (Matrix Science), which allows determination of the peptides sequence related to the inserted fragments. MS/MS analysis was carried out using a MALDI-TOF/TOF ABI 4700 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems). Peptide mixtures (0·5 μL) were spotted on a 192-well target

plate and crystallized with an equal volume of CHCA matrix solution (5 g L^{-1}). MS data were automatically acquired. Mass calibration was performed using trypsin autolysis fragments for internal calibration. The collision gas was atmospheric air and the energy was 1 kV. Interpretation was carried out using the GPS Explorer software (Applied Biosystems) and database searching was done using the in house MASCOT program (Matrix Science). Cysteine carbamidomethylation was selected as variable modifications. One missing cleavage was allowed. Precursor error tolerance was set to < 150 μg mL^{-1} and MS/MS fragment error tolerance < 0·4 Da. NSite and cellular localization predictions were performed by the software PSITE (© Softberry, Inc. 2000–2005).

Statistics

All the data presented are the mean value (± S.E.M.) of three determinations of three different experiments. In all the experiments, mean values were compared using Student's *t*-test.

Results

In planta experiments

Effect of the injection of extracellular proteins of P. tracheiphila *in cv. Interdonato leaves*

The toxicity tests indicated that leaves inoculated with the extracellular proteins (EP) from the fungus grown on FEM LTL, showed the first necrosis symptoms after 4 days of incubation (Fig. 1). The leaves inoculated with control EP showed necrosis symptoms after 6 days, whereas no symptoms were evident in leaves treated with MON LTL. According to studies on the role played by oxidative stress in plant-pathogen interactions (Feussner & Wasternack, 2002) and to the first results obtained, enzymatic activities were analyzed in cv. Interdonato. To evaluate the role of LOX in the necrosis, leaves were infiltrated with SHAM, an inhibitor of LOX (Fig. 1). The results showed that the addition of SHAM to *P. tracheiphila* EP advances and augments the formation of necrosis on the treated leaves (Fig. 1).

LOX and GSH POX activities in lemon leaves

Lemon leaves of cv. Interdonato inoculated with *P. tracheiphila* EP, obtained after 30 days of growth in the presence of MON LTL, showed increases in LOX and GSH POX activities of 100·6 (± 10·4) fold and 76·2 (± 8·5) fold, respectively, at 48 h, compared to the corresponding values at time 0. In contrast, these enzymatic activities were inhibited compared to the control when the leaves were inoculated with filtrates derived from fungal cultures on FEM LTL (Fig. 2a,b).

9 and 13-hydroxyactadecadienoic (HODE) formation in lemon leaves

The inoculation of EP of *P. tracheiphila* in the lemon leaves of all three cultivars stimulated a different

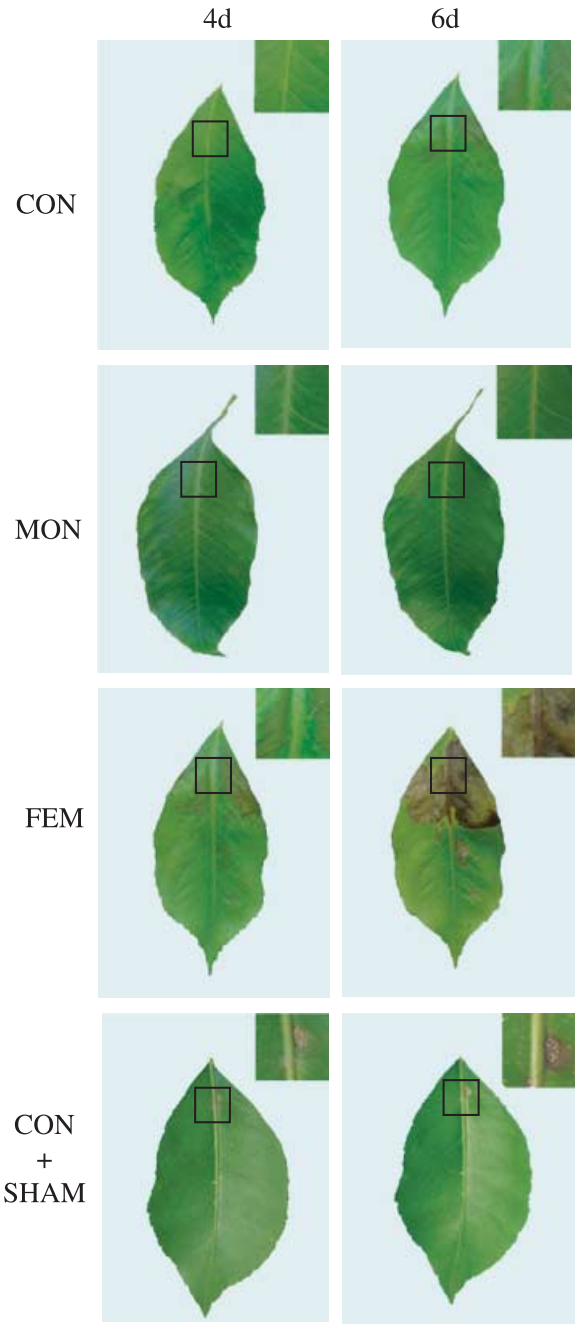

Figure 1 *In planta* toxicity test of the extracellular proteins secreted by *Phoma tracheiphila*. Effects of 10 μg of extracellular protein inoculated into the central vein of young, partially tolerant cv. Interdonato lemon leaves. The inoculum was obtained from the pathogen grown for 30 days in 20 g L^{-1} glucose (control medium, CON), or in the presence of 2 g L^{-1} tolerant (MON) or susceptible (FEM) lyophilized twigs and leaves, or control medium culture filtrate with added 1 mM salicylhydroxamic acid (CON+SHAM). Visual symptoms were evaluated 4 and 6 days after inoculation and incubation of leaves under continuous illumination (20 μE m^{-2} s^{-1}) to better analyze the light-activated toxin performance.

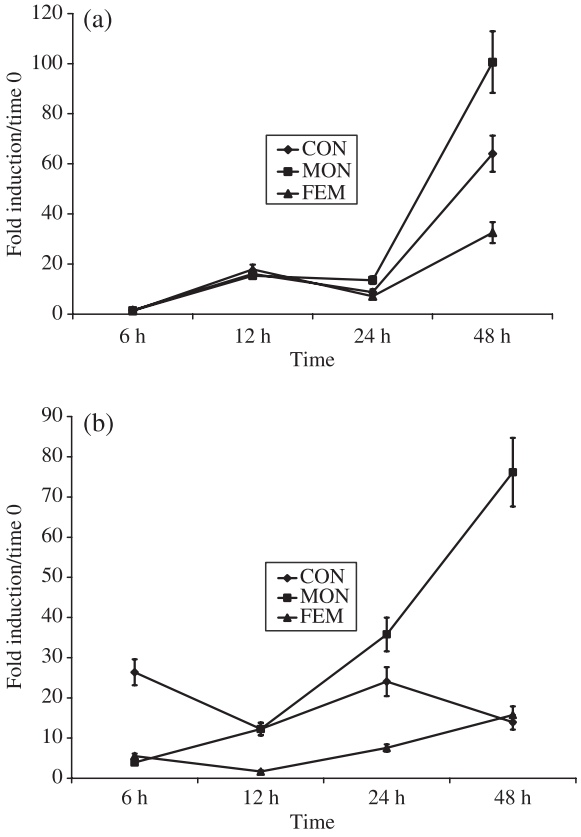

Figure 2 (a) Lipoxygenase (LOX) and (b) glutathione peroxidase (GSH POX) activities in partially tolerant lemon leaves cv. Interdonato, inoculated with 10 μg of the extracellular proteins from different fungal culture filtrates (CON, MON, FEM) at 6, 12, 24 and 48 h after injection and incubation. The results, reported as fold induction in respect to time 0, are the mean ± S.E.M. of three determinations from three separate experiments. Initial values were 10·7 U mg^{-1} protein for LOX and 1·5 U mg^{-1} protein for GSH POX.

pattern of lipoperoxide formation. 9 and 13-HODE are present at low levels in the three cultivars, in particular in FEM inoculated with sterile Milli-Q water (control, CON) (Table 1) with cv. Monachello attaining the highest level of 9 + 13 HODE after just 1 h of incubation whereas cvs Femminello and Interdonato took 48 h. The qualitative differences in HODE formation in the different samples and at different time intervals are shown in Table 1. After 1 h of treatment with EP, 13-HODE was prevalent (70%) in the bulk of lipoperoxides formed in cv. Monachello whereas only traces of 13 and 9-HODE were formed in the other two cultivars at this time. At 48 h after inoculation a prevalence of 9-HODE formation was evident in all the lemon cultivars, although levels of 9 and 13 HODE (65 and 35% for cv. Interdonato; 90 and 10% for cv. Femminello; 99 and 1% for Monachello, respectively) and their total quantities (21·8 ng mg^{-1} d.w.; 8·5 ng mg^{-1} d.w.; 2·4 ng mg^{-1} d.w. for these cultivars respectively) were different.

Table 1 9 and 13 hydroxyoctadecadienoic acid (9-HODE, 13-HODE) detected in *Citrus limon* cvs Interdonato, Femminello and Monachello after infiltration with extracellular proteins (EP) of *Phoma tracheiphila*, grown for 30 days in a malseccin conducive medium after 1, 24 and 48 h. The results are the mean ± S.E.M. of three determinations from three separate experiments

	CON[a]		EP	
Time	13-HODE	9-HODE	13-HODE	9-HODE
Interdonato				
1 h	1·73 ± 0·22[b]	1·02 ± 0·11	0·01 ± 0·005	0·01 ± 0·005
24 h	0·12 ± 0·02	0·01 ± 0·05	0·01 ± 0·006	0·01 ± 0·005
48 h	0·90 ± 0·08	0·10 ± 0·02	7·62 ± 0·85	14·16 ± 1·23
Femminello				
1 h	0·01 ± 0·005	0·01 ± 0·005	0·01 ± 0·005	0·01 ± 0·005
24 h	0·37 ± 0·04	0·01 ± 0·005	0·23 ± 0·03	0·01 ± 0·005
48 h	0·01 ± 0·005	0·01 ± 0·005	0·86 ± 0·09	7·65 ± 0·85
Monachello				
1 h	0·28 ± 0·03	0·21 ± 0·03	3·41 ± 0·32	1·41 ± 0·15
24 h	0·66 ± 0·07	0·62 ± 0·07	0·01 ± 0·005	0·01 ± 0·005
48 h	0·36 ± 0·04	0·35 ± 0·04	0·01 ± 0·005	2·40 ± 0·32

[a]CON = control experiment.
[b]Measured as ng mg^{-1} dry weight.

In vitro assays

Peroxide formation in cultures inoculated with the pathogen

A FOX 1-assay was performed for testing the formation of hydroperoxides during the growth of *P. tracheiphila* in different conditions. The presence of FEM LTL promoted output of peroxides in comparison with the control or MON after 7 days of incubation. The presence of MON LTL resulted in higher levels of hydroperoxides than FEM after 14 days, which was maintained for up to 28 days. The lowest quantity of hydroperoxides (7 and 14 days) was detected when *P. tracheiphila* was grown in the control medium. LTL treatments stimulated peroxide production by the pathogen for up to 28 days in comparison with the control (Table 2).

Antioxidant and hydrolytic enzymes in mycelia and filtrates of P. tracheiphila

To further investigate a possible involvement of oxidative stress in the *P. tracheiphila-C. limon* interaction, analyses of extracellular antioxidant (superoxide dismutase, SOD and catalase, CAT) and hydrolytic enzyme activities were performed in the filtrate of *P. tracheiphila*. Analyses of enzymatic activities showed that FEM LTL highly stimulated increases of fungal hydrolytic enzymes compared to control (PG 41·98 (± 3·92) fold after 21 days; laccase 112·02 (± 12·41) fold at 15 and 21 days; Mn POX 10·10 (± 1·20) fold at 21 days) in comparison with MON LTL (PG 0·25 ± 0·05; Lac 58·25 ± 6·23; MnPOX 2·30 ± 0·21 fold, respectively) (Fig. 3a). In contrast, the pathogen

Table 2 Hydroperoxide (ROOH) formation in *Phoma Tracheiphila*-culture medium analysed by spectrophotometric FOX 1-assay. The pathogen was grown in the presence of susceptible (FEM) and tolerant (MON) lemon twigs and leaves. The control (CON) was represented by a malseccin conducive medium. The results are the mean ± S.E.M. of three determinations from three separate experiments

	ROOH (μM)		
Time (days)	CON	FEM	MON
7	0·05 ± 0·01	0·11 ± 0·01	0·08 ± 0·01
14	0·09 ± 0·01	0·13 ± 0·02	0·15 ± 0·02
21	0·16 ± 0·03	0·07 ± 0·01	0·14 ± 0·02
28	0·14 ± 0·02	0·32 ± 0·04	0·45 ± 0·05

grown in the presence of MON LTL mainly produced increases, compared to control, in extracellular antioxidant activities in the filtrate (SOD 45·18 (± 4·13) fold and CAT 12·12 (± 1·53) fold) (Fig. 3a). SOD activity was lower in FEM samples than in MON (33·89 (± 8·52) fold increase over control).

Stimulation of enzymatic antioxidant activities was also detected inside the mycelia of *P. tracheiphila*, grown in the same conditions as previously shown (Fig. 3a). In particular, the mycelia developed in the presence of MON LTL showed a high stimulation of SOD at 15 days (63·71 (± 5·93) fold increase, pH 10·0), GSH POX (115·16 (± 10·35) fold increase) and a slight stimulation of CAT activity (15·2 (± 3·2) fold increase) in comparison with the control (Fig. 3b). The differential induction of antioxidant defenses of *P. tracheiphila* by MON LTL compared to susceptible FEM LTL might also indicate that oxidant (and/or antimicrobial) compounds were present in MON LTL medium, as described in Table 3. Intriguingly, this type of stress did not alter the dry weight of fungal growth which, after 30 days of incubation, was 86·5 ± 2·3 and 85·2 ± 3·2 mg d.w. 50 mL^{-1} in tolerant and susceptible LTL respectively, and 92·0 ± 7·4 mg d.w. in the control.

SDS-PAGE analysis of P. tracheiphila *extracellular proteins*

Fogliano *et al*. (1998) showed that when grown in a suitable conducive medium *P. tracheiphila* accumulated a 60 KDa protein complex in the culture filtrates from which malseccin was purified. Following the same

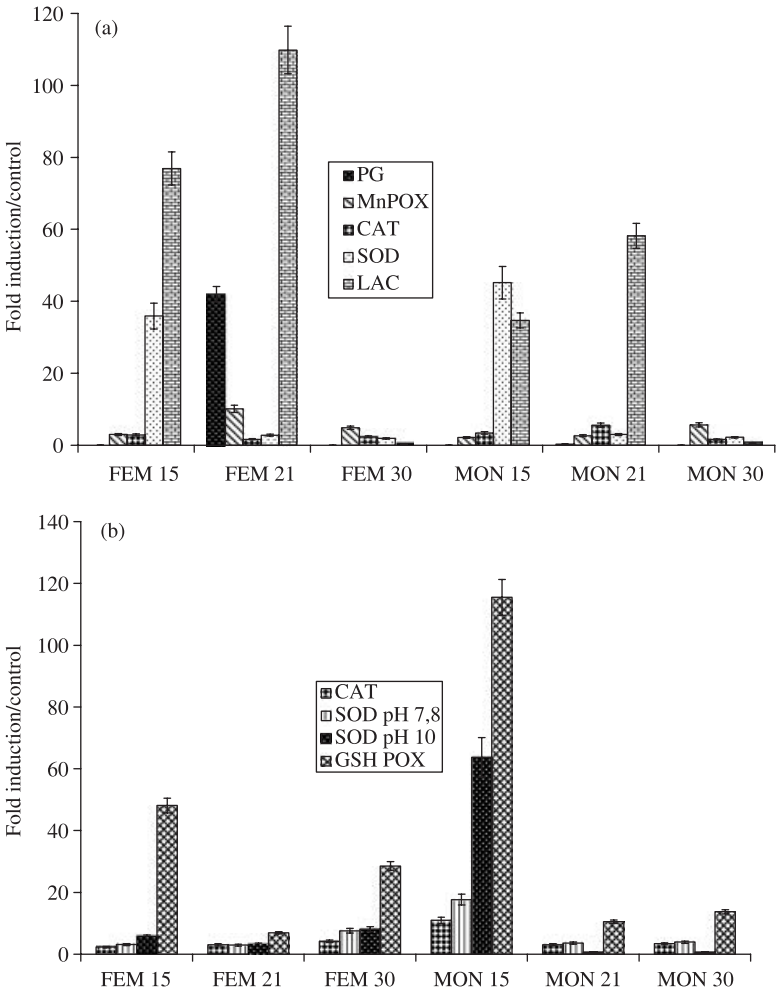

Figure 3 (a) Assays of extracellular enzyme activities in FEM & MON culture filtrates of *Phoma tracheiphila* grown in the presence of lyopholized twigs and leaves (LTL) of susceptible or tolerant lemon cultivars respectively, after 15, 21 and 30 days of incubation. Fungal hydrolytic enzymes laccase (LAC), polygalacturonase (PG) and Manganese dependent peroxidase (Mn POX) and antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD). (b) Analysis of antioxidant enzyme activities in the pathogen mycelia: glutathione peroxidase (GSH POX), CAT and SOD present in mycelia of *P. tracheiphila* after 15, 21, and 30 days of incubation in the presence of FEM or MON LTL. SOD activity was tested using two different buffer solutions (pH 7·8 and 10·0), to assess activity in different cell locations (putatively cytosolic and peroxisomal, respectively). The results are the mean ± S.E.M. of three determinations from three separate experiments.

Table 3 MALDI TOF MS and MALDI TOF/TOF MS analysis of the 55–60 KDa spots derived from the SDS-PAGE of different *Phoma tracheiphila*-secreted proteins. Each spot is numbered in Fig. 4a,b

Spot-sample	Mw[a]	pI[b]	Accession N°-Organism[c]	Protein Id[d]	P SITE[e]	Loc.[f]	Hom-Score (a)[g]	TOF/TOF Peptides[h]	Hom-Score (b)[i]
1 CON 30d	30·6	6·79	C95388 *Sinorhizobium meliloti*	Acetyltransferase	NGlyc-Phospho-Myrist	M	69–58		
	33·9	6·21	COG1234 *Methanosarcina barkeri*	metal dependent hydrolase	NGlyc-Phospho-Myrist- Amid.	E	57–49		
2 CON 30d EndoH	29·7	8·8	RS02485 *Ralstonia solanacearum*	Cytochrome C	Phospho-Myrist-Pren-Amid	E	69–64		
	23·8	9·0	COG3881 *Moorella thermoacetica*	β-glucosidase	Phospho-Myrist	E	66–40		
3 CON 30d EndoH	32·0	8·94	ST1194 *Escherichia coli*	Radical SAM enzyme	Phospho-Myrist	M	63–45		
4 CON 30d EndoH	29·8	8·94	MAP1574c *Mycobacterium avium*	Dehydrogenases	Phospho-Myrist	M	57–49	KCANLFTRADNLVCVHHAGR	98–34
								CKALNFTRADNLVCVHHAGR	94–35
5 CON 30d EndoH	30·8	7·71	COG1231 *Chloroflexus auriantiacus*	Monoamine oxidase	Phospho-Amid-NGlyc-cAMP PKC- CK2-NMyr-Prenyl- Microb tgt	E	94–67	VLMALRPR	75–60
								VNKSGIPPLPERRASESRKDVEAR	83–75
								NSPRFRFWGTTPFLWHR	94–78
								DCGEWSSAAELTR	90–81
								DIAPFIDIRNPEEEITK	97–89
6 FEM 30d	34·2	6·06	AAO44274·1 *Tropheryma whipplei*	ABC transporter	NGlyc-cAMP PKC-CK2-TK-Nmyr-Microb tgt-ATP/GTP BS-PK ATP BS	P	75–68	RLSGKAGSNTNFD	52–67
								YYKKPGNATVEPA	70–75
								SSHGGSAAKGAATTKFRTIHGVPGA	64–56
								KGNHVKDVINDAEKFNIAGR	75–78

[a]Predicted molecular weight.
[b]Isoelectric point.
[c]Accession number on NCBI dbase for the protein with the highest similarity to the spot sample.
[d]Identification name of the protein with maximum identity to the spot analysed.
[e]Identification of proteins functional motifs: Phospho: phosphorylation, Amid: amidation, NGlyc: N Glycosylation, BS: binding sequence, PK: phosphokinases, CK, TK: cysteine and tyrosine kinases, Microb tgt: microbodies target, Myr: mirystoilation, Pren: prenilation.
[f]Predicted localization for the identified proteins: M: mythocondrial, E: extracellular, P: plasma membrane (plant).
[g]Values of homology and score of the comparison between the predicted protein sequence and the spot analysed.
[h]Amino acid sequence of the peptides identified with the TOF/TOF analysis for each spot.
[i]Values referred to the TOF/TOF sequenced peptides homologies with the proteins indicated.

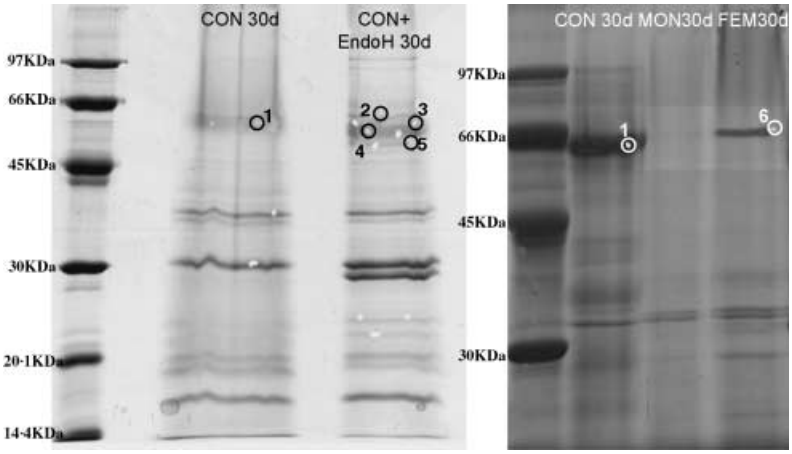

Figure 4 Characterization of *Phoma tracheiphila*-extracellular proteins. (a) Identification by SDS-PAGE of *P. tracheiphila*-extracellular proteins (EP) secreted in the culture medium after 30 days of growth (control, CON 30d), and of the same proteins treated with an endo-N-glucanase H (endoH 30d) to remove the N-glycosylated residues. (b) SDS-PAGE of extracellular proteins secreted by *P. tracheiphila* after 30 days of growth in medium with added tolerant (MON30d) or susceptible (FEM30d) lyophilized twigs and leaves. The 60 KDa protein is not present in MON, and shows an alteration in FEM compared to the control (CON 30d). Spots 1–6 were further analysed by MALDI-TOF/TOF as shown in Table 3.

experimental conditions described by Fogliano *et al.* (1998), an SDS-PAGE analysis was performed. The results showed a smeared band at 60 KDa (spot 1, Fig. 4a) which was subsequently digested with an endo-N-glucanase H in order to eliminate the glycosylic fraction of the toxin. The enzymatic digestion led to the release of different bands of slower electrophoretic mobility (Fig. 4a, Table 3) indicating that some selective deglycosylation had occurred. The multifaceted 60 KDa protein pattern suggested a composite nature of the toxin glycosylation (both N- and O- linked carbohydrates) which could have caused a partial enzymatic digestion. The nature of these and other spots were further investigated (2–5, Fig. 4a) using MALDI-TOF and TOF/TOF MS analyses (Table 3). The SDS-PAGE analysis of the extracellular proteins secreted by *P. tracheiphila* showed that the 60 KDa band was not apparent in the culture filtrates of the fungus grown in the presence of the tolerant cultivar after 30 days of incubation, but was present in the control (Fig. 4b). The same results were obtained after 21 days (data not shown).

MALDI-TOF and TOF/TOF MS analysis of 60 KDa extracellular proteins

MALDI-TOF MS analysis of the culture filtrates of *P. tracheiphila* showing the 60 KDa bands in the SDS-PAGE (Fig. 4a,b) indicated that the spot samples shared high homology with proteins involved in the oxidative burst cascade, such as radical SAM enzyme or proteins related to cell redox balance regulation (e.g. monoamine oxidases (MOX)). The amino acid sequences obtained with the TOF/TOF analysis had a high degree of homology with the conserved domains of an extracellular MOX (94%), an ABC transporter related to pathogenicity (Fetherson *et al.*, 1999) (75%) and a dehydrogenase (57%) (Table 3). All the ionised peaks of the spots at 60 KDa, presented in the different samples, were inserted together in the MASCOT database (Matrix Science) and showed a very high homology with a FAD binding oxidase domain, a common feature to MOX and other related enzymes (Jalkanen & Salmi, 2001).

Discussion

This study has furthered the understanding of the pathology of citrus 'mal secco'. Oxidative stress generally plays a crucial role in plant-pathogen interactions (Lamb & Dixon, 1997; Mithöfer *et al.*, 1997; Bézier *et al.*, 2002; Lev *et al.*, 2005; Fedoroff, 2006) and a role for lipoxygenase and the subsequent formation of lipoperoxides (LOOH) in creating an oxidative environment has been ascertained (Feussner & Wasternack, 2002). Lipoperoxides formed by the action of LOX on polyunsaturated fatty acids, such as linoleic and linolenic acid, might have different physiological targets in the cell (Feussner & Wasternack, 2002). At high concentrations they can display toxic effects and usually their excess is scavenged by the action of glutathione peroxidases (Palomero *et al.*, 2001). Nevertheless, a controlled formation of LOOH can serve as a source of secondary messengers (Vijayan *et al.*, 1998) and defensive compounds in the plant cell (Hamberg, 2000).

Necrosis obtained by inoculating *P. tracheiphila* extracellular proteins inside the central vein of lemon leaves of the cv. Interdonato suggests peroxidative events are involved. MON promoted an evident activation of LOX and GSH POX in leaves but no necrosis, whereas FEM inhibited these activities and necrosis advanced compared to the control (EP-treated leaves). It is believed that phytopathogenic bacteria can synthesize and release effector proteins in an attempt to suppress host defenses and/or cell death (Alfano & Collmer, 2004; Chang *et al.*, 2004). These may be enzyme inhibitors or proteases which can function in microbial counterdefence toward secreted plant-pathogenesis-related proteins (Dean *et al.*, 2005; Kamoun, 2006). Results suggest that *P. tracheiphila* grown in the presence of FEM secretes proteins that can contribute to bypassing host defenses such as LOX and GSH POX. This also suggests that lipoperoxidation and its modulation can have a role in establishing the appearance of symptoms in infected lemon leaves. The experiments performed with SHAM, although not conclusive, suggests that the inhibition of LOX causes the plant to react to infection, as occurs in FEM-treated leaves

where necrosis symptoms appear and the lowest LOX activity is shown. If the plant actively modulated the formation of LOOH (also by GSH POX activity), this can contribute to the avoidance of necrosis during fungal infection (as shown in MON treated leaves). This observation could indicate that lipoperoxide production favours a plant defence impairing the pathogen which in this situation can not produce MOX. Alternatively, non modulated oxidative stress conditions, probably induced by the ROS formed in light conditions through fungal MOX or radical SAM enzyme and low plant LOX activity, can favour pathogen infection as suggested by the reaction (necrosis) of the leaves following injection with CON or FEM.

The lipoperoxide formation in leaves of the three *C. limon* cultivars treated with proteins secreted by *P. tracheiphila* in the culture media, was quantified to correlate lipoperoxidative events with different plant responses. It has been shown that a different timing in 13 and 9-HODE formation occurred in the assayed cultivars, proposing a different role of the two regioisomers in the plant's reaction to pathogen protein. 13-HODE was mainly formed in cv. Monachello 1 h after EP injection, whereas the other two cultivars showed a later stimulation of 9-LOX metabolism. The high level of 13-HODE detected in cv. Monachello could indicate that the plant reacts rapidly to induce the formation of defensive compounds. In fact it has been reported that the 13 LOX pathway is activated early whatever the nature of the stress, leading to the peroxidation of chloroplast lipids that induces plant defences (Montillet *et al*., 2004). Thus cv. Monachello, early after injury, is able to activate the 13 oxylipins pathway, probably counteracting directly (antimicrobial effect of cyclopentenones, Hamberg, 2000) and indirectly (by producing signal molecules) the effect of pathogen infection. It is known that induced resistance can also be triggered by the pathogen after its contact with the plant, and a wide array of chemical signals (elicitors) such as fatty acid-amino acid conjugates are among those responsible for this response (Paré *et al*., 2005). Furthermore, it could be hypothesized that EP stimulates in cv. Monachello the release of some endogenous elicitor able to trigger a prompter defensive reaction compared to other cultivars.

The partially tolerant cv. Interdonato showed the highest level of LOOH after EP treatment, with a ratio between 9 and 13-HODE of 65:35 compared to 90:10 in the susceptible cv. Femminello. This suggests that cv. Interdonato activates a later response (also based on 13-LOX pathway) that does not allow a defensive reaction to occur as in cv. Monachello, but allows a higher tolerance to 'mal secco' than cv. Femminello. In tobacco, HR is characterized by the induction of 9-oxylipin metabolism (Rusterucci *et al*., 1999), with earlier and higher accumulation of divinyl ethers, and the late or rapid and early accumulation of oxylipins in the *P. infestans*-potato interaction has been correlated with compatible and incompatible reactions, respectively. The involvement of 9-LOX and their products in the plant-defence response can be due to a direct antimicrobial activity against the pathogen (Gobel *et al*., 2001).

How does the pathogen react to the presence of LTL of the different lemon cultivars? The presence of FEM in the culture media induces the pathogen to release peroxides and to activate hydrolytic enzymes and, at a low level, intra- and extracellular antioxidant enzymes whilst with *P. tracheiphila* grown in the presence of MON, antioxidant activities are highly augmented, and a higher level of peroxides is formed in the media from the 14th day of incubation. Considering that *P. tracheiphila* is stimulated to release peroxides in the media by the presence of LTL (Table 1), the secretion of SOD and CAT could also represent a way to modulate ROS generation and action *in planta*, where the pathogen is probably also engaged in counteracting plant defence with antioxidant enzymes. The production and release in the media of antioxidant enzymes such as SOD and CAT allows some necrotrophic pathogens, such as *Botrytis cinerea*, to resist ROS produced during the HR by the host (Gil-ad *et al*., 2000; Govrin & Levine, 2000; Rolke *et al*., 2004).

Previous studies concerning 'mal secco' disease indicate that the glycoprotein malseccin is the main pathogenic factor released by *P. tracheiphila* in the lemon vessels (Nachmias *et al*., 1977; Fogliano *et al*., 1998). From the BLASTP 2·2·13 analysis, the six peptides sequences identified by Fogliano *et al*. (1998) belonged mainly to the Fe-S oxidoreductase and the nitrite reductase families.

In the culture filtrate of *P. tracheiphila* there is a group of proteins with a MW ranging between 60 and 66 KDa (SDS-PAGE analysis) and more than one protein is present in this MW interval (MALDI-TOF/TOF analysis). Recently Raudino *et al*. (2001) have proposed that under light conditions (the same as in this study), malseccin can interfere with photo-phosphorylative processes by inducing an accumulation of nitrogen (NH_4^+) that blocks the glycine decarboxylase cycle. The authors hypothesize that malseccin utilizes NADH or FADH present in plant cells and displays its toxic effect by accumulating NH_4^+. By considering the results obtained in MALDI-TOF/TOF analysis of the secreted proteins, the interference with these processes can be due to monoamine oxidase (copper binding or FAD binding) that catalyzes oxidative deamination of primary amines forming aldehydes, H_2O_2 and NH_4^+, leading to a toxic accumulation of these reactive species inside the host cell (Jalkanen & Salmi, 2001).

The lack of the 60 KDa proteins in the culture media of *P. tracheiphila* grown in the presence of MON suggests that an alteration of the pathogen metabolism takes place interfering with the production of the biochemical weapons necessary for the development of the disease. In stress conditions, i.e. presence of tolerant LTL, *P. tracheiphila* could divert part of the resources necessary to invade the host, such as production of hydrolytic enzymes (Fig. 3) or toxic compounds (Fig. 4b), to inducing antioxidant activities that preserve fungal survival. In prokaryotic organisms such as *Clostridium perfringens*, stress conditions such as heat shock lead to the induction of a defense machinery (e.g. heat shock proteins) that alter the accumulation of enterotoxin (Heredia *et al*., 1998).

In conclusion, this study shows that the synchronous presence of hydrolytic enzymes, toxic compounds, oxidative stress inducers and membrane transporters in the fungus, and the differential ability to modulate the lipoperoxidative pathway in the host, can play a central function in *P. tracheiphila* infection of *C. limon*. The extracellular enzyme monoamine oxidase could be among the different compounds putatively involved in the 'mal secco' pathology. This research adds novel information on a severe but poorly studied disease, offering ways to elaborate control strategies to counteract this pathogen based on the use of antioxidants.

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References

Alfano JR, Collmer A, 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annual Review of Phytopathology* **42**, 385–414.

Balmas V, 2005. Characterisation of *Phoma tracheiphila* by RAPD-PCR, microsatellite-primed PCR and ITS rDNA sequencing and development of specific primers for *in planta* PCR detection. *European Journal of Plant Pathology* **111**, 235–47.

Banerjee D, Madhusoodanan UK, Sharanabasappa M, Ghosh S, Jacob J, 2003. Measurement of plasma hydroperoxide concentration by FOX-1 assay in conjunction with triphenylphosphine. *Clinica Chimica Acta* **337**, 147–52.

Bender CL, Alarcón-Chaidez F, Gross DC, 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetates. *Microbiology and Molecular Biology Review* **63**, 266–92.

Bézier A, Lambert B, Baillieul F, 2002. Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. *European Journal of Plant Pathology* **108**, 111–20.

Chang JH, Goel AK, Grant SR, Dangl JL, 2004. Wake of the flood: ascribing functions to the wave of type III effector proteins of phytopathogenic bacteria. *Current Opinion in Microbiology* **7**, 11–8.

Choquer M, Dekkers KL, Chen HQ *et al*., 2005. The CTB1 gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of *Cercospora nicotianae*. *Molecular Plant-Microbe Interactions* **18**, 468–76.

Cook BJ, Clay RP, Bergmann CW, Albersheim P, Darwill AG, 1999. Fungal polygalacturonases exhibit different substrate degradation patterns and differ in their susceptibilities to polygalacturonase-inhibiting proteins. *Molecular Plant-Microbe Interactions* **8**, 703–11.

Daub ME, Ehrenshaft M, 2000. The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. *Annual Review of Phytopathology* **38**, 461–90.

Dean RA, Talbot NJ, Ebbole DJ *et al*., 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **434**, 980–6.

Fedoroff N, 2006. Redox regulatory mechanisms in cellular stress responses. *Annals of Botany* **98**, 289–300.

Fetherson JD, Bertolino VJ, Perry RD, 1999. YbtP and YbtQ: two ABC transporters required for iron uptake in *Yersinia pestis*. *Molecular Microbiology* **32**, 289–99.

Feussner I, Wasternack C, 2002. The lipoxygenase pathway. *Annual Review of Plant Biology* **53**, 275–97.

Fogliano V, Graniti A, Marchese A, Ritieni A, Randazzo G, Visconti A, 1994. Purification of a phytotoxic glycoprotein from the 'malseccin' complex produced in culture by *Phoma tracheiphila*. In: Kumari S, ed. *Proceedings of the 9th Congress of the Mediterranean Phytopathological Union, 1994*. Kusadasi-Aydin, Turkey: WJAS/AEJAES, 143–5.

Fogliano V, Marchese A, Scaloni A *et al*., 1998. Characterization of a 60 KDa phytotoxic glycoprotein produced by *Phoma tracheiphila* and its relation to malseccin. *Physiological and Molecular Plant Pathology* **53**, 149–61.

Gil-ad NL, Bar-Nun N, Noy T, Mayer AM, 2000. Enzymes of *Botrytis cinerea* capable of breaking down hydrogen peroxide. *FEMS Microbiology Letters* **190**, 121–6.

Gobel C, Feussner I, Schmidt A *et al*., 2001. Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. *Journal of Biological Chemistry* **276**, 6267–73.

Govrin EM, Levine A, 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology* **10**, 751–7.

Hamberg M, 2000. New cyclopentenone fatty acids formed from linoleic and linolenic acids in potato. *Lipids* **35**, 353–63.

Heredia NL, Labbe RG, Garcia-Alavarado JS, 1998. Alteration in sporulation, enterotoxin production, and protein synthesis by *Clostridium prefringens* type A following heat shock. *Journal of Food Protection* **61**, 1143–7.

Jalkanen S, Salmi M, 2001. Cell surface monoamine oxidases: enzymes in search of a function. *EMBO Journal* **20**, 3893–901.

Kamoun S, 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology* **44**, 337–66.

Lamb C, Dixon RA, 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 251–75.

Lev S, Hadar R, Amedeo P, Baker SE, Yoder OC, Horwitz BA, 2005. Activation of an AP1-like transcription factor of the maize pathogen *Cochliobolus heterostrophus* in response to oxidative stress and plant signals. *Eukaryotic Cell* **4**, 443–54.

Maggio-Hall LA, Wilson RA, Keller NP, 2005. Fundamental contribution to β-oxidation to polyketide mycotoxin production *in planta*. *Molecular Plant-Microbe Interaction* **18**, 783–93.

Mithöfer A, Daxberger A, Fromhold-Treu D, Ebel J, 1997. Involvement of an NAD(P)H oxidase in the elicitor-inducible oxidative burst of soybean. *Phytochemistry* **45**, 1101–7.

Montillet JL, Cacas JL, Garnier L *et al*., 2004. The upstream oxylipin profile of *Arabidopsis thaliana*: a tool to scan for oxidative stresses. *The Plant Journal* **40**, 439–51.

Nachmias A, Barash I, Solel Z, Strobel GA, 1977. Translocation of mal secco toxin in lemons and its effect on electrolyte leakage, transpiration, and citrus callus growth. *Phytoparasitica* **5**, 94–103.

Nachmias A, Barash I, Solel Z, Strobel GA, 1980. Effect of mal secco toxin on lemon leaf cells. *Phytoparasitica* **8**, 51–60.

Palomero J, Galan AI, Munoz ME, Tunon MJ, Gonzalez-Gallego J, Jimenez R, 2001. Effects of aging on the susceptibility to the toxic effects of cyclosporin A in rats. Changes in liver glutathione and antioxidant enzymes. *Free Radicals in Biology and Medicine* **30**, 836–45.

Paré PW, Farag MA, Krishnamachari V, Zhang H, Ryu C, Kloepper JW, 2005. Elicitors and priming agents initiate plant defense responses. *Photosynthesis Research* **85**, 149–59.

Perrotta G, Graniti A, 1988. *Phoma tracheiphila* (Petri) Kanchaveli & Gikashvili. In: Smith IM, Dunez J, Lelliott RA, Phillips DH, Archer SA, eds. *European Handbook of Plant Diseases*. Oxford, UK: Blackwell Scientific Publications, 396–8.

Raudino F, Cacciola SO, Magnano di San Lio G, Fogliano V, Graniti A, 2001. Effetto della luce sull'espressione dei sintomi del mal secco degli agrumi. *Petria* **11**, 59–126.

Reverberi M, Fabbri AA, Zjalic S, Ricelli A, Punelli F, Fanelli C, 2005a. Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibit aflatoxin production. *Applied Microbiology and Biotechnology* **69**, 207–15.

Reverberi M, Fanelli C, Zjalic S *et al*., 2005b. Relationship among lipoperoxides, jasmonic acid and indole-3-acetic acid formation in potato tuber after wounding. *Free Radical Research* **39**, 637–47.

Reverberi M, Zjalic S, Ricelli A, Fabbri AA, Fanelli C, 2006. Oxidant/antioxidant balance in *Aspergillus parasiticus* affects aflatoxin biosynthesis. *Mycotoxin Research* **22**, 39–47.

Rolke Y, Liu S, Quidde T *et al*., 2004. Functional analysis of H_2O_2-generating systems in *Botrytis cinerea*: the major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable. *Molecular Plant Pathology* **5**, 17–27.

Rusterucci C, Montillet JL, Agnel JP *et al*., 1999. Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. *Journal of Biological Chemistry* **274**, 36446–55.

Solel Z, 1976. Epidemiology of mal secco disease of lemons. *Phytopathologische Zeitschrift* **85**, 90–2.

Solel Z, Salerno M, 1988. Mal secco. In: Whiteside JO, Garnsey SM, Timmer LW, eds. *Compendium of Citrus Diseases*. St Paul, MN, USA: APS Press, 18–19.

Somma V, Scarito G, 1986. Thirty years of observations on the periodicity of infection by *Phoma tracheiphila* in Sicily. *Phytopathologia Mediterranea* **25**, 103–6.

Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J, 1998. A role for jasmonate in pathogen defense of *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **95**, 7209–14.

Zjalic S, Fabbri AA, Ricelli A, Reverberi M, Galli E, Fanelli C, 2002. Effect of olive oil mill waste waters on edible and medicinal mushroom *Lentinus edodes* (Berk.) Sing. growth and lignin degrading enzymes. *International Journal of Medicinal Mushrooms* **4**, 85–93.