Eur J Plant Pathol (2008) 121:55–66
DOI 10.1007/s10658-007-9241-6

Impact of carrot resistance on development of the *Alternaria* leaf blight pathogen (*Alternaria dauci*)

Cora Boedo · Valérie Le Clerc · Mathilde Briard · Philippe Simoneau · Michel Chevalier · Sonia Georgeault · Pascal Poupard

Received: 10 May 2007 / Accepted: 25 October 2007 / Published online: 15 January 2008

Abstract The interaction between *Alternaria dauci* and two carrot cultivars differing in their resistance to leaf blight was investigated by microscopy. The fungal development between 1 and 15 days post-inoculation was quite similar in the susceptible cv. Presto and the partially resistant cv. Texto: After conidial germination, leaf adhesion of the pathogen was achieved with mucilaginous filaments; hyphae penetrated the leaves directly with/without the formation of *appressoria*-like structures or *via* stomata; the fungus spread by epiphytic hyphae with hyphopodia and subcuticular mycelia. Intense necrotic plant cell reactions occurred under the fungal structures. At 21 days post-inoculation, typical features of fungal development were noted for each cultivar: growing hyphae emerged from stomata in cv. Presto, whereas conidiophores without conidia were observed in cv. Texto. Leaf tissues of both cultivars were strongly damaged and vesicle-like structures (assumed to be plant phenolics) were abundantly present between mesophyll cells. A real-time PCR method was developed for *in planta* quantification of *A. dauci*. Between 1 and 15 days post-inoculation, the fungal biomass was equivalent in the two cultivars and was about fourfold higher in cv. Presto than cv. Texto at 21 and 25 days post-inoculation. Taken together, our results indicated that *A. dauci* was able to colonize both cultivars in a similar manner during the first steps of the interaction, then fungal development in the partially resistant cultivar was restricted due to putative plant defence reactions. The results of this study enhance the overall understanding of infection processes in the *A. dauci*-carrot pathosystem.

Keywords *Daucus carota* · Histology · Scanning electron microscopy · Real-time quantitative PCR

Abbreviations

ALB	*Alternaria* leaf blight
Ct	cycle threshold
Dpi	days post-inoculation

C. Boedo · P. Simoneau · P. Poupard (✉)
UMR A77 PaVé (INRA-INH-UA),
UFR Sciences, Université d'Angers,
2 boulevard Lavoisier,
49045 Angers Cedex 01, France
e-mail: pascal.poupard@univ-angers.fr

C. Boedo · V. Le Clerc · M. Briard · M. Chevalier
UMR 1259 GenHort (INRA-INH-UA),
Institut National d'Horticulture,
2 rue Le Nôtre,
49045 Angers Cedex 01, France

S. Georgeault
Service Commun d'Imagerie et d'Analyses Microscopiques, UFR Sciences Médicales,
Université d'Angers,
Rue Haute de Reculée,
49045 Angers Cedex 01, France

ITS	internal transcribed spacers
6-MM	6-methoxymellein
gpd	glyceraldehyde-3-phosphate dehydrogenase
qPCR	quantitative PCR
SEM	scanning electron microscopy
TEM	transmission electron microscopy

Introduction

Alternaria dauci is a necrotrophic fungus responsible for *Alternaria* leaf blight (ALB) of carrot (*Daucus carota*). The disease is present worldwide in all carrot growing areas and is the most common destructive foliar disease on this crop (Souza et al. 2001). Inoculum sources include mycelium in over-wintering carrot debris, diseased volunteer or wild carrots and infected seeds. ALB symptoms in petioles and limbs involve greenish-brown lesions, irregular in shape and size, which usually form along the leaflet margins. Older lesions become dark brown and may be surrounded by a chlorotic halo. Under favourable conditions, the lesions ultimately coalesce, giving the leaf tissue a blighted appearance, thus considerably reducing the photosynthetic activity (Farrar et al. 2004). In field experiments, the disease severity (proportion of foliage area showing ALB symptoms), after infection by natural inoculum, may range from around 5 to 99%, depending on the fertilization rate and fungicide application conditions (Vintal et al. 1999; Ben-Noon et al. 2001). Severe epidemics of ALB jeopardize efficient mechanical harvesting by fan gripping, and carrot yields may be significantly reduced, e.g. from 28 to 74% in commercial fields in Israel (Ben-Noon et al. 2001).

Many control measures, including prophylactic methods, fungicide applications and use of partially resistant carrot cultivars are currently used to control ALB. Under high disease pressure, none of these measures is completely satisfactory for managing ALB (Farrar et al. 2004). However, Ben-Noon et al. (2003) demonstrated experimentally that the joint action of two control methods (e.g. chemical control and host resistance) reduced ALB *via* additive or synergistic effects. Among the control methods, selection of cultivars with higher levels of resistance to ALB and with durable resistance to *A. dauci* is of major interest for carrot breeders. In order to improve methods for selecting such carrot cultivars, accurate knowledge of plant-pathogen interaction mechanisms is crucial. *A. dauci* infection processes were previously investigated at the cellular level by transmission electron microscopy (TEM) using leaves of cv. Fancy and wild carrots, which are respectively susceptible and partially resistant to *A. dauci* (Dugdale et al. 2000). The TEM observations at 5 days post-inoculation (dpi) showed plant cell wall disintegration, breakdown of the cell membrane structure, loss of cytoplasmic organization and of chloroplast structure in both cultivated and wild carrots. To our knowledge, except for this study of Dugdale et al. (2000), very little literature is currently available describing interaction processes between *A. dauci* and its host. Further work is thus needed to characterize these processes using microscopic approaches.

In order to evaluate carrot resistance to leaf blight, previous studies in controlled or natural environments involved estimation of *A. dauci* development on leaves according to visual assessment of symptoms on a disease rating scale (Simon and Strandberg 1998; Pawelec et al. 2006; Gugino et al. 2007). In these studies, susceptible and less susceptible carrot cultivars were differentiated in resistance screening tests. However, visual scoring is still a subjective and time-consuming way to estimate fungal development *in planta*. The real-time quantitative PCR (qPCR) method has already proved to be a valuable tool for accurate quantification of fungal pathogen biomass in plant extracts, e.g. quantification of *Alternaria brassicicola* in *Arabidopsis thaliana* (Brouwer et al. 2003). Quantitative qPCR measurement of *A. dauci* in carrot leaves should be a reliable way to compare fungal development in different cultivars.

The present study was carried out to characterize the different steps of fungus infection and development in carrot leaves in order to set the stage for a future comprehensive survey of key ALB-resistance features. Two cultivars showing different levels of resistance to ALB were studied, i.e. cv. Presto, which is highly susceptible to *A. dauci* (Ben-Noon et al. 2001; Pawelec et al. 2006) and partially resistant cv. Texto. First, plant-fungus interaction mechanisms were explored using microscopic methods. Then the *in planta* fungal biomass was quantified by qPCR over an infection time course with *A. dauci*. Microscopic characterization of the infection processes and quantification of the fungus in carrot leaves enabled us to gain greater

insight into the interaction between the plant and the pathogen.

Materials and methods

Plant and fungal material

The carrot cvs Presto (Vilmorin, France) and Texto (Vilmorin, France) were grown in boxes (60×40×23 cm) with 50 plants per box containing peat moss (Traysubstrat Klasmann)/sand (67:33, *v*/*v*) mixture in a greenhouse with day/night temperatures of 20°C±2°C, 16 h photoperiod and 80–85% RH. Different fungal strains were used in this study: the *A. dauci* P2 strain, reportedly aggressive (Pawelec et al. 2006), was isolated in 2000 from naturally infected carrots (Gironde, France). The R65–52–1 strain of *Alternaria radicina* and the 004926F strain of *Alternaria alternata*, both isolated from carrot, were obtained from the Station Nationale d'Essais de Semences (SNES), Beaucouzé, France. Fungal strains were grown in Petri dishes on malt (2%) agar (2%) medium in the dark at 20°C±2°C for 10 days and then incubated in the conditions indicated by Pawelec et al. (2006) for conidial production.

Inoculation of plants

Young plants were inoculated at the three to eight true leaf stage with a conidial suspension adjusted to 100,000 conidia ml^{-1}. Conidial suspensions were prepared according to Pawelec et al. (2006) by using sterile water amended with 0.05% Tween 20 (Sigma, USA). Control plants were sprayed with 0.05% Tween 20 in distilled water. Inoculated and control plants were incubated under plastic covers for 2 days with day/night temperatures of 24°C±2°C. Boxes and plastic covers were sprayed twice a day for 2 days with sterile water in order to maintain high RH. After removal of plastic covers, the day/night temperatures were maintained at 24±2°C.

Histological and cytological analyses

Five leaf samples were randomly collected from the second true leaf of two plants (i.e. 10 samples of 5 mm^2 each) at 0, 1, 2, 4, 10, 15 and 21 dpi or from the second true leaf of two control plants at 0 and 21 days after water spraying. Specimens were fixed under vacuum in 4% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 [80% buffer 1 (2.4% Na_2HPO_4, 12 H_2O *w*/*v*), 20% buffer 2 (0.9% Na_2HPO_4, H_2O *w*/*v*)] for 3 h at 4°C and stored overnight at 4°C in the same fixative buffer. Specimens were rinsed three times in 0.2 M phosphate buffer (pH 7.2). For histological observations, leaf samples were dehydrated in a graded ethanol series (50, 70, 80, 95% for 10 min each). The dehydration process was completed by treating the leaf samples three times in 100% ethanol for 15 min. Specimens were then embedded in Technovit 7100 resin (Kulzer Histo-Technique kit, Labonord, Templemars, France) according to the manufacturer's instructions. Sections of 4 μm made using a microtome (RM 2165, Leica, France) were treated with 10% sodium hypochlorite for 1 min and then with 5% acetic acid for 1 min. Finally, the sections were stained overnight using a 'Fasga' solution diluted in distilled water (1:8, *v*/*v*). The 'Fasga' solution was composed of 0.05% Safranin O, 0.2% Alcian Blue, 1.5% acetic acid and 46% glycerine in distilled water. Alternatively sections were stained for 5 min using a toluidine blue solution (0.25% in distilled water *w*/*v*, pH 2.6). After staining, sections were rinsed in distilled water for 1 min, dried for 5 min at 40°C and mounted in Entellan (Merck, Darmstadt, Germany). Sections were examined under a microscope (Axioskop 2 plus, Zeiss, Germany) coupled with an Axiocam Hrc camera (Zeiss, Germany). Fifteen to twenty leaf sections of cv. Presto or cv. Texto were observed for each point of the infection time course between 0 and 21 dpi. For fluorescence microscopy, unstained sections were examined using a FITC filter (absorption, 490 nm; emission, 520 nm).

For scanning electron microscopy (SEM) observations, specimens were rinsed twice in distilled water for 1 min after glutaraldehyde fixation and post-fixed in 2% osmium tetroxide in 0.2 M phosphate buffer (pH 7.2) in the dark for 2 h. Samples were rinsed three times with distilled water, dehydrated in a graded ethanol series (50, 70, 80, 90, 95 and 100% for 10 min each). The dehydration process was completed by treating the leaf samples in ethanol/acetone (1:1, *v*/*v*) for 15 min and then twice in acetone for 15 min. Specimens were dried by the critical-point method. Leaf samples were then sputter-coated with a thin carbon layer and examined using a JEOL JSM 6301-F scanning electron microscope.

Material sampling and DNA extractions for real-time PCR assays

Samples were composed of the second and third true leaves from five inoculated plants collected at 0, 4, 10, 15, 21 and 25 dpi or from five control plants collected at 0 and 25 days after water spraying. Plant samples (pools of 10 leaves from five plants) were freeze-dried in a Christ Alpha 1–4 LSC apparatus (Fisher Bioblock Scientific), ground for 30 s with a TissueLyser crusher (Mixeur-Mill, Qiagen) and stored at −80°C. DNA extractions from inoculated or control plant samples were performed according to Briard et al. (2000). For the qPCR assays, DNA concentrations were measured by fluorometric assay (Turner Design Inst. 700, Sunnyvale, CA) and all DNAs were diluted to a final concentration of 6.5 ng μl^{-1} in order to minimize the effect of PCR inhibitors. This concentration was determined according to the results of preliminary experiments. Fungal DNA from pure cultures on malt agar medium was extracted according to Möller et al. (1992). In order to trace calibration curves and to evaluate the specificity of our qPCR assay, the DNA concentrations of mycelium or of non-inoculated carrot leaves were determined by fluorometry.

Real-time PCR for *in planta* fungal quantification

DNA levels of the *A. dauci* gene glyceraldehyde-3-phosphate dehydrogenase (*gpd*) relative to the DNA levels of the carrot-*ITS1* sequence were determined by qPCR using *SYBR*Green® chemistry on an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Specific primer sequences were designed: (1) after alignment of *gpd* or *ITS1* sequences of different *Alternaria* species and of *D. carota* with Clustal W software (Thompson et al. 1994), (2) according to the requirements for qPCR using ABI Prism Primer Express software (Applied Biosystems, Foster City, CA, USA). The GenBank accession numbers used for alignment of *gpd* sequences were AY278803 for *A. dauci*, AY278798 for *A. radicina*, AY278808 for *A. alternata*, AY278809 for *A. tenuissima* and AY491512 for *D. carota*. The GenBank accession numbers used for alignment of *ITS1* sequences were AY536369 for *A. dauci*, AY781081 for *A. radicina*, AJ853759 for *A. alternata*, AJ301709 for *A. tenuissima* and AY552527 for *D. carota*. The following primers were used: *gpd*-sense (5′AAGCTT CCCCAAGCACTCACAA3′) and *gpd*-antisense (5′CTGCGTTCTGCAGCTGTA GAGA3′), *ITS1*-sense (5′TTGGTCCCCTGTCTGTG AAC3′) and *ITS1*-antisense (5′TCTAGCGCC CAGTTGATTTTATT3′). The qPCR reactions were carried out in a 25 µl final volume containing 2.5 µl of diluted DNA, 12.5 µl of *SYBR*Green® PCR Master Mix (Applied Biosystems, Courtaboeuf, France), 900 nM *gpd* primers or 600 nM *ITS1* primers and water up to 25 µl. PCR reactions were performed according to the manufacturer's instructions. Each run included a no-template control in order to rule out test reagent contamination. After each run, a dissociation curve was plotted to check the amplification specificity (absence of primer dimers and of non-specific amplification products) by cooling samples to 55°C after the last cycle and then increasing the temperature to 95°C at 0.2°C/s. Serial dilutions of pure genomic DNA from *A. dauci* and *D. carota* were used to plot a calibration curve, which was necessary to quantify fungal and plant DNA in each sample (assay). Three replicates of the qPCR assay were used for each sample and the results were expressed as *gpd*/*ITS* ratios ± SE. Each qPCR experiment was repeated twice. Statistical analyses (Mann-Whitney test) were performed using Graphpad Instat 3 software.

To ensure that qPCR-inhibitors potentially present in plant DNA extracts would not induce significant variations in the qPCR results, the *Ypr10*Md.b* gene [Genbank accession number AY026908 (Ziadi et al. 2001)] encoding a pathogenesis-related PR-10 protein from *Malus* x *domestica* Borkh was cloned in the DH5α F'IQ *Escherichia coli* using the pGEM-T vector (Promega, Madison, WI, USA) and was used as external control in the qPCR assay. A standard plasmid quantity (0.1 ng) containing the *PR-10* sequence was added to each DNA sample from inoculated or control plant leaves and qPCR was performed in the conditions described above using the following primer pair: *PR-10* sense (5′CATGGGTG TTTTCACATACGAATC3′) and *PR-10* antisense (5′ TTCACTGCTTGTGGTGCAATC3′). A ΔCt corresponding to the Ct of the external control in the plant sample tested − Ct of the external control in distilled water was determined for each sample. Plant samples exhibiting a ΔCt value of <1 were considered as PCR-inhibitor free, as previously reported by Gao et al. (2004).

Results and discussion

The different steps of fungal infection of carrot leaves were studied using SEM and histological analysis after inoculation of susceptible cv. Presto and partially resistant cv. Texto with the *A. dauci* P2 strain. The SEM observations concerning cv. Presto are shown in Fig. 1. After conidial germination, hyphae exhibited mucilaginous filaments that allowed fungal adhesion to the leaf surface (Fig. 1a). Fungal adhesion to the host with mucilaginous secretions was also described for the entomophagic *Alternaria alternata* (Hatzipapas et al. 2002) and for several fungal plant pathogens, including *Alternaria eichhorniae* (Shabana et al. 1997) and *Phoma macdonaldii* (Roustaee et al. 2000). Besides its role in fungal adhesion, mucilaginous secretions could protect the pathogen against abiotic stress (Gold and Mendgen 1984). In contrast to the findings of Dugdale et al. (2000), our observations indicated that hyphae could enter leaves *via* stomata, particularly in the lower limb region (Fig. 1b). Direct penetration of *A. dauci* hyphae generally occurred at the cellular junctions of host epidermal cells with or without the formation of *appressoria*-like structures (Fig. 1c). Our observations indicated that direct penetration by *A. dauci* hyphae was much more frequent than pathogen entry through stomata. This is the first report of *appressoria*-like structures developed by *A. dauci* on carrot leaves. Further work is needed to better characterize these structures using TEM. The presence of non-melanised *appressoria*-like structures was previously described for other *Alternaria* species, including Brassicaceae pathogens (McRoberts and Lennard 1996) and the *A.*

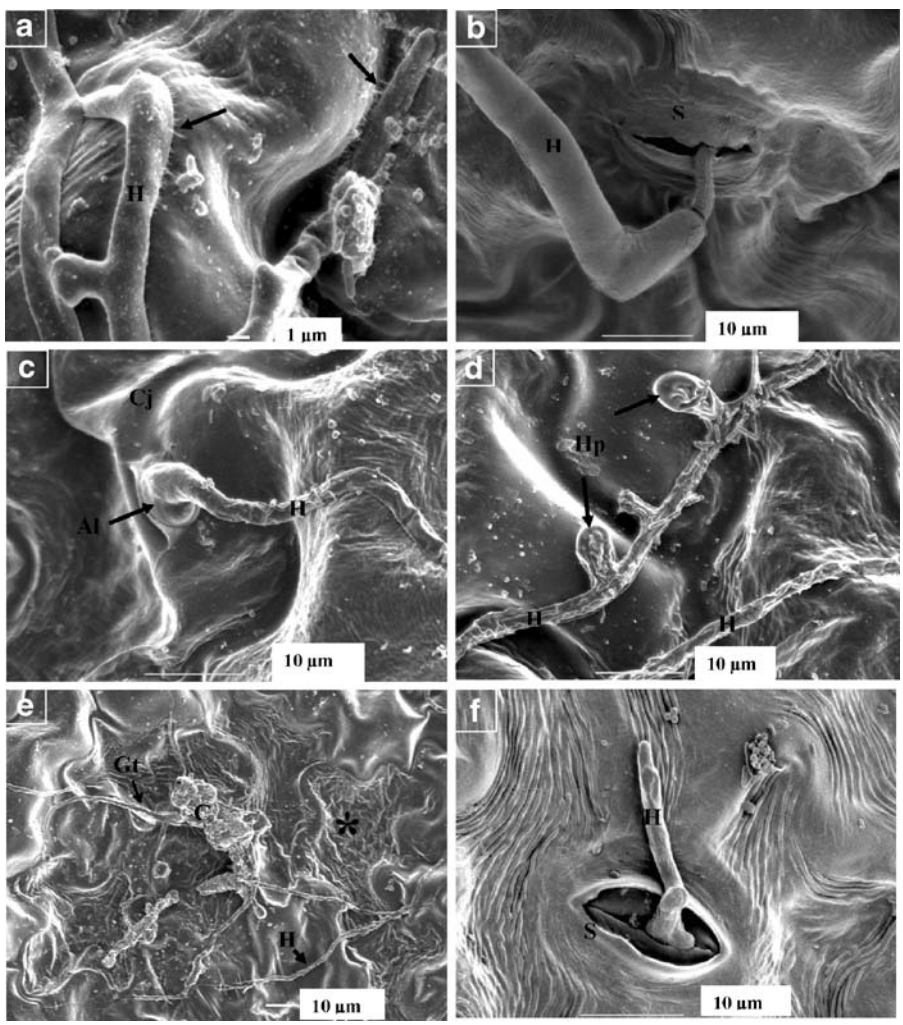

Fig. 1 Scanning electron microscopy images of *A. dauci* on the leaf surface of the susceptible carrot cv. Presto. **a** Adhesion of hyphae to the leaf cuticle with mucilaginous filaments (*arrows*) at 1 dpi; **b** mycelium entering the leaf tissue through a stoma at 1 dpi; **c** *pseudo-appressorium* formed at the cellular junction between two epidermal leaf cells at 9 dpi; **d** typical examples of hyphopodia formed at 9 dpi; **e** necrotic reactions of plant cells (*asterisk*) under multiple germ tubes developing from a conidium at 9 dpi; **f** growing mycelium emerging from a stoma at 21 dpi; *Al appresorium*-like, *C* conidium, *Cj* cellular junction, *Gt* germ tube, *H* hypha, *Hp* hyphopodia, *S* stoma

alternata groundnut pathogen (Prasad et al. 1990). The formation of non-melanised *appressoria*-like structures by fungi suggests an enzymatic rather than mechanical mode of plant penetration (Tanabe et al. 1995). Different enzymes were described for plant infection by *Alternaria* pathogen species, such as lipases and cutinases synthesized by *A. brassicicola* (Berto et al. 1999; Yao and Köller 1995) and endopolygalacturonases produced by *A. citri* (Isshiki et al. 2001). To our knowledge, such enzyme activities have not yet been reported for *A. dauci* and should be investigated to gain insight into the interaction processes between the *Alternaria* leaf blight pathogen and carrot. Structures on epiphytic hyphae previously described as hyphopodia by Howard (1997) were observed on *A. dauci* (Fig. 1d). Hyphopodia have been defined as structures that allow epiphytic spreading of the fungus after infection of the plant, or they might enhance penetration or survival (Howard 1997; Solomon et al. 2006). Intense necrotic reactions of plant cells were observed under germinating conidia and hyphae of *A. dauci* (Fig. 1e). These necrotic reactions are likely to be the consequence of fungal enzyme and/or toxin activities.

Observations similar to those presented in Fig. 1a to e for cv. Presto were obtained with partially resistant cv. Texto. Some SEM observations relevant to this latter cultivar are shown in Fig. 2. At 1 dpi, multiple germ tubes were produced from each conidium (Fig. 2a) and this holds also true for cv. Presto. The necrosis of plant cells under the fungal structures was also evident in the partially resistant cultivar (Fig. 2b). Both epiphytic hyphae and subcuticular mycelial growth were observed during *A. dauci* infection processes in cv. Texto (Fig. 2c) and cv. Presto. The presence of subcuticular hyphae, which was also previously described in *Alternaria brassicae* on rapeseed (Tewari 1986), highly suggests the synthesis of cutinolytic enzymes by *A. dauci* and could correspond to a fungal development latency phase before deeper colonization of plant tissues, as suggested by Roustaee et al. (2000) for sunflower infection by *P. macdonaldii.* As illustrated above, although there were apparently no obvious differences in *A. dauci* development between the two carrot cultivars, growing hyphae emerging from stomata were sometimes observed at 21 dpi in cv. Presto leaf samples (Fig. 1f) but not in cv. Texto. At 21 dpi, conidiophores emerging from subcuticular hyphae were observed on leaves from cv. Texto but not from cv. Presto (Fig. 2d). No conidium was observed on these emerging conidiophores. Either conidiogenesis could not (or only weakly) occur on the partially resistant cultivar or conidiogenesis had not yet been

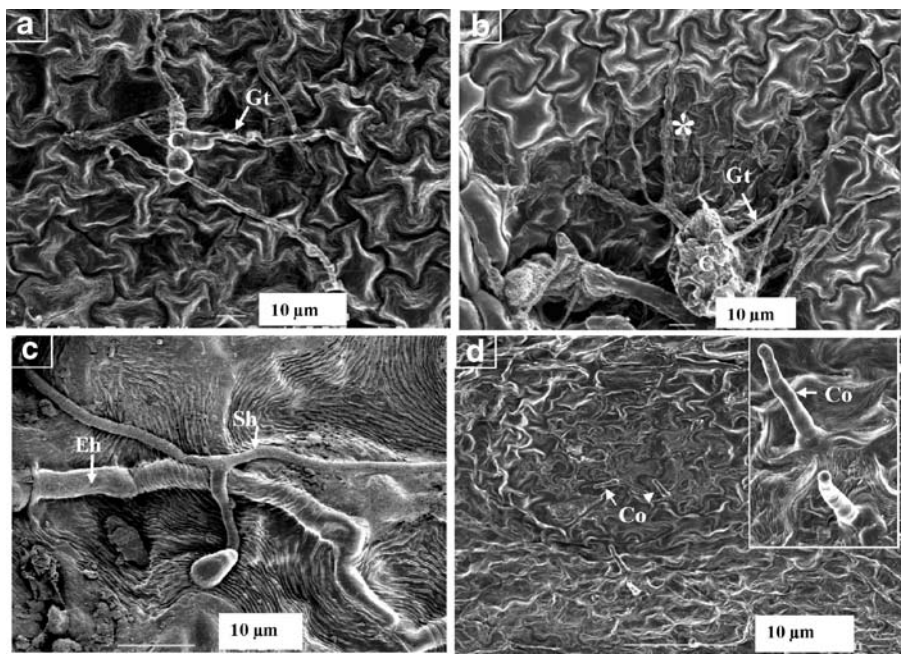

Fig. 2 Scanning electron microscopy images of *A. dauci* on the leaf surface of the partially resistant carrot cv. Texto. **a** Germinating conidium at 1 dpi; **b** necrotic reactions of plant cells (*asterisk*) at 1 dpi; **c** co-presence of subcuticular hyphae and epiphytic hyphae spreading on the leaf surface at 15 dpi; **d** conidiophores emerging from subcuticular hyphae at 21 dpi; *C* conidium, *Co* conidiophore, *Eh* epiphytic hypha, *Gt* germ tube, *Sh* subcuticular hypha

induced at the observation time. In future experiments, it would be worthwhile studying conidial production in both carrot cultivars in terms of conidiogenesis timing after plant inoculation and according to conidial abundance on each leaf surface. The SEM observations of fungal development in cv. Presto and cv. Texto are summarized in Table 1.

By comparison to the control plants (Fig. 3a), our histological investigations showed that the different carrot leaf tissues (i.e. upper epidermis, palisade and spongy parenchyma, lower epidermis) of cv. Presto (Fig. 3b) or cv. Texto (results not shown) were substantially damaged by *A. dauci* at 21 dpi. These observations indicate that cellular disintegration events similar to those described by Dugdale et al. (2000) could also occur in cv. Presto and cv. Texto. In the current study, vesicle-like structures of unknown nature were present between necrotic mesophyll host cells in the two cultivars at 21 dpi (Fig. 3b). Fluorescence microscopy revealed significant autofluorescence of these structures (Fig. 3c), thus suggesting the presence of plant phenolic compounds (Nicholson and Hammerschmidt 1992). As phenolic compounds are known to have potential antimicrobial activities (Bennett and Wallsgrove 1994), the vesicle-like structures observed here are assumed to be defence compounds synthesized by carrot leaves in response to *A. dauci*. In the present study, there were no clear-cut differences between the two cultivars in terms of size and abundance of the vesicle-like structures or time of appearance of these structures after plant inoculation with the fungus. Using Fasga staining, we never observed the fungus in the leaf sections of cv. Presto or cv. Texto (although, as shown by preliminary experiments, *A. dauci* mycelia collected from a pure culture of the fungus stained intensely with Fasga). Using toluidine blue staining, we very rarely observed *A. dauci* hyphae (at 15 dpi) growing in the palisade parenchyma in leaf sections of susceptible cv. Presto (results not shown). The fungus was never clearly observed in sections of partially resistant cv. Texto. Our histological observations were thus poorly informative in terms of fungal development *in planta*. We concluded that the histological methods used here were inadequate to investigate *A. dauci* infection processes.

In a subsequent step, we developed a real-time qPCR protocol based on the *SYBR*Green® methodology to measure *A. dauci* development in carrot leaf samples. This qPCR tool was used to further characterize the plant pathogen interaction by comparing fungal development in susceptible cv. Presto and in partially resistant cv. Texto. The specificity of *gpd* and *ITS1* primers was evaluated by comparing the amplification of DNA extracted from *A. dauci*, *D. carota, A. radicina* and *A. alternata*. *A. radicina* has been mainly reported as a pathogen on carrot root and crown, while causing foliar blight only under certain specific conditions (Farrar et al. 2004). *A. alternata* is a plant saprophyte that can be present on seeds and aerial parts of carrot (Joly 1964). The results in Table 2 indicate that no amplification was observed using *gpd* primers with *D. carota* and *A. alternata* DNAs. By contrast, the *gpd* primers were able to amplify *A. radicina* DNA at high concentrations. The dissociation curves were similar when comparing the *gpd* amplification products of *A. dauci* and *A. radicina*: melting temperatures of PCR products ranged from around 0.8°C to 1°C in the two *Alternaria* species, but significant ΔCt values (>15) were calculated. Using *ITS1* primers, no amplification products were obtained with *A. dauci* or *A. radicina*

Table 1 Comparison of *A. dauci* development (P2 strain) between 1 to 21 dpi in carrot leaves of susceptible cv. Presto and partially resistant cv. Texto on the basis of SEM observations

Steps of *A. dauci* infection of carrot leaves	cv. Presto	cv. Texto
Fungal adhesion to host with mucilaginous filaments (1 dpi[a])	Yes	Yes
Fungal penetration through stomata (1 dpi)	Yes	Yes
Direct penetration of the fungus at cellular junctions with/without *appressoria*-like structures (9 dpi)	Yes	Yes
Presence of hyphopodia: epiphytic spreading, penetration and/or survival (9 dpi)	Yes	Yes
Subcuticular mycelial growth (15 dpi)	Yes	Yes
Emergence of hyphae through stomata (21 dpi)	**Yes**	**No**
Presence of conidiophores (21 dpi)	**No**	**Yes**

[a] Day(s) post-inoculation

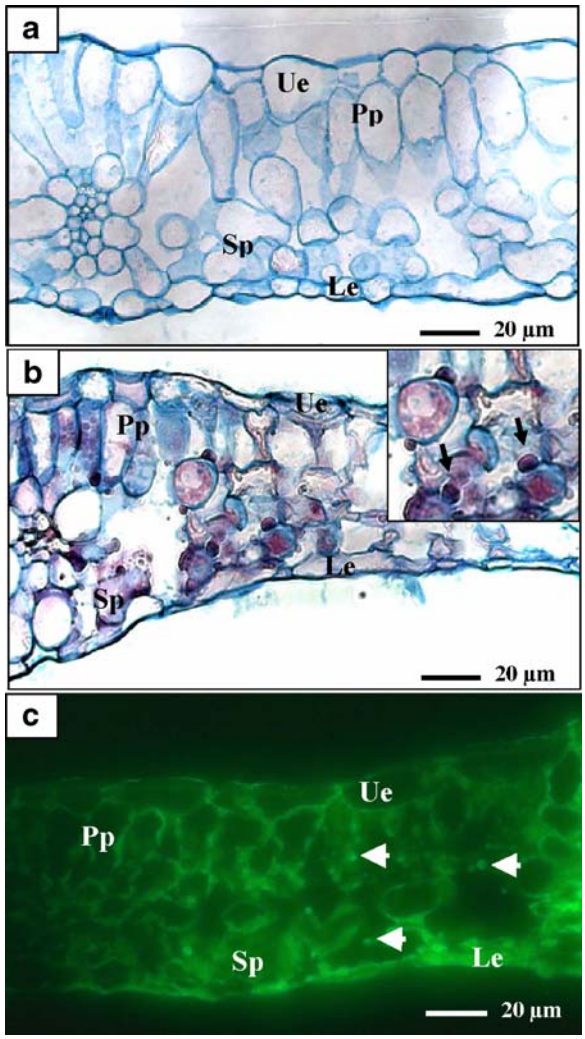

Fig. 3 Histological observations of plant leaf tissues of the susceptible carrot cv. Presto after *A. dauci* inoculation. **a** Aspect of leaf tissues in non-inoculated control plants (Fasga-stained section); **b** damage to different leaf tissues at 21 dpi (Fasga-stained section). Vesicle-like structures (*arrows*) were observed in the spongy and palisade parenchyma; **c** unstained leaf section at 21 dpi observed under UV light revealing autofluorescence of vesicle-like structures (*arrows*); *Le* lower epidermis, *Pp* palisade parenchyma, *Sp* spongy parenchyma, *Ue* upper epidermis

DNAs, whereas the different *A. alternata* DNA concentrations tested led to *ITS1* amplification. The relevant dissociation curves were very close when comparing *ITS1* amplification products of carrot and *A. alternata*, with melting temperatures ranging from 0.1 to 0.3°C in the two items, but the ΔCt were high (>12). The cross reactions shown in Table 2 after *gpd* or *ITS1* amplification therefore did not upset the reliability of our molecular tool because the primers used in this study were developed for PCR analysis of plant samples artificially infected with *A. dauci* under controlled conditions. Figure 4a and b show the regression curves obtained after amplification of serial dilutions of *A. dauci* and *D. carota* pure genomic DNAs with *gpd* and *ITS1* primers, respectively. The correlation coefficients (R^2) of the calibration curves were close to 1 in both cases, indicating that the amplification linearity allows reliable DNA quantification over a wide dynamic range.

Quantification of the fungal biomass by qPCR using *gpd* and *ITS1* primers was achieved in cv. Presto and cv. Texto between 0 and 25 dpi with the *A. dauci* P2 strain. No aspecific amplification products were obtained in all qPCR experiments performed with the relevant plant samples. The use of *gpd* and *ITS1* primers for DNA amplification of *A. dauci* and *D. carota*, respectively, can thus be considered as reliable. In order to experimentally check for the absence of an inhibitor effect in our qPCR results, a standard quantity of a plasmid containing an apple *PR-10* sequence was added to each plant DNA sample, and this external control was amplified using specific *PR-10* primers in the same qPCR conditions as those used for *gpd* and *ITS1* amplification. For all plant samples analysed in the present study, the ΔCt values (i.e. Ct of external control in a plant sample – Ct of external control in distilled water) were < 0.6, indicating that PCR-inhibitors did not affect qPCR reactions (Gao et al. 2004). Results of fungal growth quantification in cv. Presto and cv. Texto are shown in Fig. 4c.

Between 0 and 15 dpi, similar fungal development was observed in the two cultivars, although the *gpd*/*ITS1* ratios were significantly different between cv. Presto and cv. Texto at 0 and 10 dpi. The most striking differences in fungal biomass between the two cultivars were obtained at 21 and 25 dpi: the development of *A. dauci* was significantly higher in cv. Presto, i.e. about fourfold that of cv. Texto. The *gpd/ITS1* ratio remained quite stable over the time-course of infection in cv. Texto. This suggests that the pathogen colonization in this partially resistant cultivar was limited by plant defence reactions. These reactions may differ from the vesicle-like structures described above in both cultivars, which were assumed to be phenolics (Fig. 3b–c). Further investigations are needed to determine the kind of compounds that may be synthesized by cv. Texto

Table 2 Evaluation of the specificity of primers used in the qPCR assays

Source and concentration of DNA (pg μl^{-1})	Primers *gpd* sense/antisense[a]		Primers *ITS1* sense/antisense[b]	
	Ct[c]	ΔCt[d]	Ct[c]	ΔCt[e]
D. carota 2500	Und[f]	–	16.28	–
D. carota 1000	Und	–	17.06	–
D. carota 200	Und	–	18.92	–
A. dauci 2500	19.79	–	Und	–
A. dauci 1000	20.72	–	Und	–
A. dauci 200	22.94	–	Und	–
A. radicina 2500	35.82	16.03	Und	–
A. radicina 1000	36.37	15.65	Und	–
A. radicina 200	Und	–	Und	–
A. alternata 2500	Und	–	29.2	12.92
A. alternata 1000	Und	–	29.48	12.42
A. alternata 200	Und	–	31.67	12.75

Cycle threshold values obtained after pure genomic DNA amplification from mycelium of different *Alternaria* species and from healthy carrot leaves (cv. Presto).

[a] The primer pair *gpd* sense/antisense was designed for specific amplification of *A. dauci* DNA.

[b] The primer pair *ITS1* sense/antisense was designed for specific amplification of carrot DNA.

[c] Mean of Ct values obtained from three replicates. Ct: PCR threshold cycle number at which the fluorescent signal is greater than the minimal detection level

[d] Variation between the mean of three Ct values obtained after amplification of *A. dauci* DNA and the mean of Ct values obtained after amplification of *Alternaria* sp. or carrot DNA

[e] Variation between the mean of three Ct values obtained after amplification of carrot DNA and the mean of Ct values obtained after amplification of *Alternaria* sp. DNA

[f] Undetermined Ct (Ct>40)

against *A. dauci*. Little is known about the activity of defence metabolites against fungal pathogens attacking carrot aerial parts. The phytoalexin 6-methoxymellein (6-MM) accumulates in carrot leaves infected by *Cercospora carotae* (Mercier and Kuc 1997). According to Dugdale et al. (2000), high constitutive levels of polyphenols could explain the partial resistance of wild carrot to *A. dauci*. The potential role of 6-MM and polyphenols against *A. dauci* attack should be investigated in cv. Presto and cv. Texto. Although the qPCR analyses were only performed on two different cultivars, our *A. dauci* quantification results clearly differentiated the susceptible cultivar from the partially resistant cultivar. The qPCR method described here is a promising tool for early selection of ALB resistance in carrot cultivars. Further experiments are necessary to determine if qPCR could be used as an alternative to visual scoring for resistance evaluation in controlled environments. In the *A. brassicicola–Arabidopsis thaliana* pathosystem, symptom development is not always correlated with *in planta* pathogen colonization (Thomma et al. 1999). This suggests that, at least in some cases, estimation of plant resistance against a fungal pathogen should be assessed by measuring pathogen growth rather than disease symptoms. Interestingly, Mercado-Blanco et al. (2003) noted a correlation between the development of *Verticillium dalhiae* in olive genotypes measured by qPCR and the degree of host susceptibility to this pathogen.

In conclusion, one main purpose of the present work was to better understand the interaction processes between *A. dauci* and *D. carota* at different stages of ALB development using two carrot cultivars with different levels of disease resistance. The light microscopic analysis clearly revealed the presence of putative defence reactions (vesicle-like structures) in the two cultivars infected by the fungus. Using histology, poor information was finally obtained in terms of characterization of the infection processes. On the basis of SEM observations, differences in *A. dauci* development between the cultivars were only obvious at 21 dpi and not at the very first steps of fungal infection (Table 1). The specific presence of

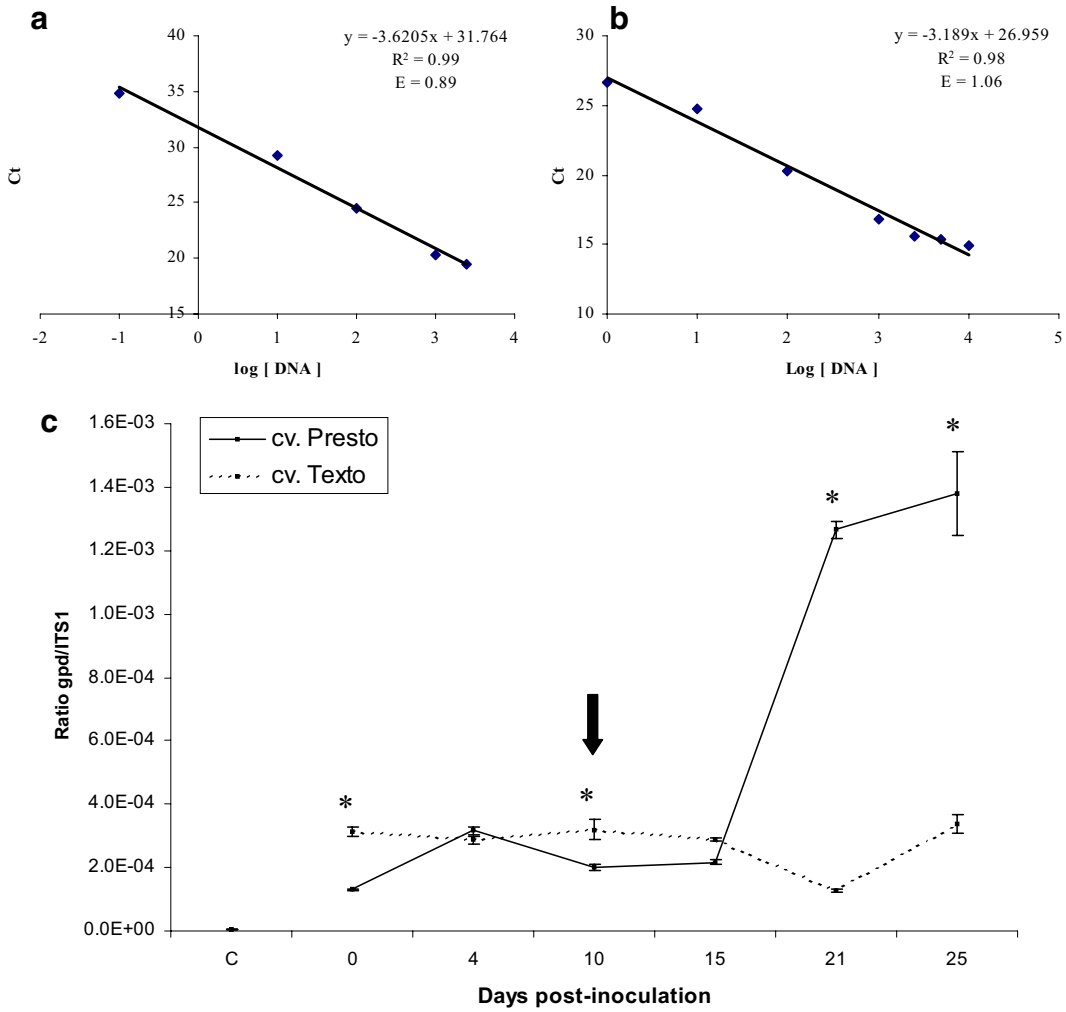

Fig. 4 Quantification of *A. dauci* **a** and *D. carota* **b** genomic DNA. Serial dilutions of *A. dauci* and *D. carota* genomic DNA were quantified using qPCR with *gpd* sense/antisense and *ITS1* sense/antisense primer pairs for amplification of the glyceraldehyde-3-phosphate dehydrogenase gene and the *ITS1* sequence, respectively. The regression curves were plotted using the logarithm of known DNA concentrations against the Ct values. Ct is the cycle number at which fluorescence emission of the PCR product significantly differed from the background. The linear regression analysis showed an almost perfect correlation between the recorded fluorescence signal and the initial DNA amount in both cases. The PCR efficiency (E) was deduced from the slope of regression line using the formula $E = (10^{(-1/\text{slope})} - 1)$; **c** fungal growth quantification over an infection time-course with *A. dauci* in the susceptible carrot cv. Presto or the partially resistant cv. Texto. The abundance of *A. dauci* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) was quantified in infected samples and standardized with *D. carota ITS1* using real-time qPCR. The fungal biomass was expressed as the *gpd/ITS1* ratio between 0 and 25 dpi. *C* Non-infected control plants. The first symptoms were macroscopically present at 10 dpi on both cultivars (*arrow*). The data are the mean and standard error of *gpd/ITS1* ratios calculated from triplicates. Values that differed significantly between the two cultivars are indicated by an *asterisk* above the graph

conidiophores on the partially resistant cultivar (Fig. 2d) but not on the susceptible cultivar was surprising at first, but could be considered as a stress response of *A. dauci* (sporulation attempt) against the putative host defence reactions developed by cv. Texto. These defence reactions were able to efficiently limit pathogen growth, as the fungal biomass measured by qPCR in the partially resistant cultivar was about fourfold lower than in cv. Presto (Fig. 4c). By contrast, *A. dauci* was able to rapidly invade and spread through leaf tissues of the susceptible cultivar, as illustrated by the growing hyphae observed to be emerging from stomata at 21 dpi (Fig. 1f), and by the significantly higher fungal biomass than in cv. Texto

at and after 15 dpi (Fig. 4c). We suggest that in cv. Presto, too weak and/or too late host defence reactions allowed extensive colonization of leaf tissues by the fungus. In future investigations, *A. dauci* strains with different levels of aggressiveness should be compared in susceptible and partially resistant carrot cultivars.

Acknowledgements We are grateful to Bruno Hamon (UMR PaVé), Marie Tellier and Anita Suel (UMR GenHort) for their excellent technical assistance and to David Manley for revising the English in the manuscript. We are also grateful to Nathalie Leduc (UMR Sagah) for advice in histology and Robert Filmon from the Service Commun d'Imagerie et d'Analyses Microscopiques (SCIAM, Université Angers).

References

Bennett, R. N., & Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New Phytologist*, *127*, 617–633.

Ben-Noon, E., Shtienberg, D., Shelvin, E., & Dinoor, A. (2003). Joint action of disease control measures: a case study of *Alternaria* leaf blight of carrot. *Phytopathology*, *93*, 1320–1328.

Ben-Noon, E., Shtienberg, D., Shelvin, E., Vintal, H., & Dinoor, A. (2001). Optimization of chemical suppression of *Alternaria dauci*, the causal agent of *Alternaria* leaf blight in carrots. *Plant Disease*, *85*, 1149–1156.

Berto, P., Commenil, P., Belingheri, L., & Dehorter, B. (1999). Occurrence of a lipase in spores of *Alternaria brassicicola* with a crucial role in the infection of cauliflower leaves. *FEMS Microbiology Letters*, *180*, 183–189.

Briard, M., Le Clerc, V., Grzebelus, D., Senalik, D., & Simon, P. W. (2000). Modified protocols for rapid genomic DNA extraction and AFLP analysis using silver stain or radioisotopes. *Plant Molecular Biology Reporter*, *18*, 235–241.

Brouwer, M., Lievens, B., Van Hemelrijck, W., Van den Ackerveken, G., Cammue, B. P. A., & Thomma, B. P. H. J. (2003). Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiology Letters*, *228*, 241–248.

Dugdale, L. J., Mortimer, A. M., Isaac, S., & Collin, H. A. (2000). Disease response of carrot and carrot somaclones to *Alternaria dauci*. *Plant Pathology*, *49*, 57–67.

Farrar, J. J., Pryor, B. M., & Davis, R. M. (2004). *Alternaria* diseases of carrot. *Plant Disease*, *88*, 776–784.

Gao, X., Jackson, T. A., Lambert, K. N., Li, S., Hartman, G. L., & Niblack, T. L. (2004). Detection and quantification of *Fusarium solani* f. sp. *glycines* in soybean roots with real-time quantitative polymerase chain reaction. *Plant Disease*, *88*, 1372–1380.

Gold, R. N., & Mendgen, K. (1984). Cytology of basidiospore germination, penetration, and early colonization of *Phaseolus vulgaris* by *Uromyces appendiculatus* var. *appendiculatus*. *Canadian Journal of Botany*, *62*, 1989–2002.

Gugino, B. K., Carroll, J. E., Widmer, T. L., Chen, P., & Abawi, G. S. (2007). Field evaluation of carrot cultivars for susceptibility to fungal leaf blight diseases in New York. *Crop Protection*, *26*, 709–714.

Hatzipapas, P., Kalosaka, K., Dara, A., & Christias, C. (2002). Spore germination and appressorium formation in the entomophagic *Alternaria alternata*. *Mycological Research*, *106*, 1349–1359.

Howard, R. J. (1997). Breaching the outer barriers: Cuticle and cell wall penetration. In Carroll, & Tudzynski (Eds.) *The Mycota: Plant relationships* (pp. 43–60). Berlin: Springer.

Isshiki, A., Akimitsu, K., Yamamoto, M., & Yamamoto, H. (2001). Endopolygalacturonase is essential for Citrus Black Rot caused by *Alternaria citri* but not Brown Spot caused by *Alternaria alternata*. *Molecular Plant–Microbe Interaction*, *14*, 749–757.

Joly, P. (1964). *le genre Alternaria: recherches physiologiques, biologiques et systématiques*. Paris: Paul Lechevalier.

McRoberts, N., & Lennard, J. H. K. (1996). Pathogen behaviour and plant cell reactions in interactions between *Alternaria* species and leaves of host and nonhost plants. *Plant Pathology*, *45*, 742–752.

Mercado-Blanco, J., Collado-Romero, M., Parilla-Araujo, S., Rodriguez-Jurado, D., & Jomenez-Diaz, R. M. (2003). Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiological and Molecular Plant Pathology*, *63*, 91–105.

Mercier, J., & Kuc, J. (1997). Elicitation of 6-methoxymellein in carrot leaves by *Cercospora carotae*. *Journal of the Science of Food and Agriculture*, *73*, 60–62.

Möller, E. M., Bahnweg, G., Sandermann, H., & Geiger, H. H. (1992). A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research*, *22*, 6115–6116.

Nicholson, R. L., & Hammerschmidt, R. (1992). Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology*, *30*, 369–389.

Pawelec, A., Dubourg, C., & Briard, M. (2006). Evaluation of carrot resistance to *Alternaria* leaf blight in controlled environments. *Plant Pathology*, *55*, 68–72.

Prasad, P., Devi, S., & Reddy, M. N. (1990). Studies of early stages of infection of *Alternaria alternata* on groundnut (*Arachis hypogaea* L.). *Journal of Basic Microbiology*, *30*, 349–352.

Roustaee, A., Dechamp-Guillaume, G., Gelie, B., Savy, C., Dargent, R., & Barrault, G. (2000). Ultrastructural studies of the mode of penetration by *Phoma macdonaldii* in sunflower seedlings. *Phytopathology*, *90*, 915–920.

Shabana, Y. M., Baka, Z. A. M., & Abdel-Fattah, G. M. (1997). *Alternaria eichhorniae*, a biological control agent for water-hyacinth: Mycoherbicidal formulation and physiological and ultrastructural host responses. *European Journal of Plant Pathology*, *203*, 99–111.

Simon, P. W., & Strandberg, J. O. (1998). Diallel analysis of resistance in carrot to *Alternaria* leaf blight. *Journal of the American Society of Horticultural Science*, *123*, 412–415.

Solomon, P. S., Wilson, T. J. G., Rybak, K., Parker, K., Lowe, R. G. T., & Oliver, R. P. (2006). Structural characterisation of the interaction between *Triticum aestivum* and the

dothideomycete pathogen *Stagonospora nodorum*. *European Journal of Plant Pathology*, *114*, 275–282.

Souza, R. T., Forcelini, C. A., Reis, M. E., & Calvete, E. O. (2001). Frequency of *Alternaria dauci* and *Cercospora carotae* as causal agents of carrot leaf blights in Passo Fundo, RS. *Fitopatologia Brasileira*, *26*, 614–618.

Tanabe, K., Park, P., Tsuge, T., Kohmoto, K., & Nishimura, S. (1995). Characterization of the mutants of *Alternaria alternata* Japanese pear pathotype deficient in melanin production and their pathogenicity. *Annals of the Phytopathological Society of Japan*, *61*, 27–33.

Tewari, J. P. (1986). Subcuticular growth of *Alternaria brassicae* in rapeseed. *Canadian Journal of Botany*, *64*, 1277–1281.

Thomma, B. P. H. J., Eggermont, K., Tierens, K. F. M. J., & Broekaert, W. F. (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiology*, *121*, 1093–1102.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680.

Vintal, H., Ben-Noon, E., Shelvin, E., Yermiyahu, U., Shtienberg, D., & Dinoor, A. (1999). Influence of rate of soil fertilization on *Alternaria* leaf blight (*Alternaria dauci*) in carrots. *Phytoparasitica*, *27*, 1–8.

Yao, C., & Köller, W. (1995). Diversity of cutinases from plant pathogenic fungi: different cutinases are expressed during saprophytic and pathogenic stages of *Alternaria brassicicola*. *Physiological and Molecular Plant Pathology*, *8*, 122–130.

Ziadi, S., Poupard, P., Brisset, M. N., Paulin, J. P., & Simoneau, P. (2001). Characterization in apple leaves of two subclasses of PR-10 transcripts inducible by acibenzolar-*S*-methyl, a functional analogue of salicylic acid. *Physiological and Molecular Plant Pathology*, *59*, 33–43.