Characterization of fungal pathogens (*Diaporthe angelicae* and *D. eres*) responsible for umbel browning and stem necrosis on carrot in France

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>Plant Pathology</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>PP-15-485.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Bastide, Franck; GEVES  
Sérandat, Isabelle; GEVES  
Gombert, Julie; FNAMS  
Laurent, Emmanuelle; FNAMS  
Morel, Elise; FNAMS  
Kolopp, Juliette; Vilmorin  
Guillermin, Pierre-Louis; HMClause  
Hamon, Bruno; Université d’Angers  
Simoneau, Philippe; Université ANGERS  
Berruyer, Romain; Université ANGERS  
Poupard, Pascal; Université d’Angers, UFR Sciences |
| Topics: | aetiology |
| Organisms: | fungi |
| Other Keywords: | necrotrophic fungi, seed-borne pathogen |
Characterization of fungal pathogens (*Diaporthe angelicae* and *D. eres*) responsible for umbel browning and stem necrosis on carrot in France

F. Bastide\(^a\), I. Sérandat\(^b\), J. Gombert\(^c\), E. Laurent\(^d\), E. Morel\(^d\), J. Kolopp\(^e\), P.L. Guillermin\(^f\), B. Hamon\(^a\), P. Simoneau\(^a\), R. Berruyer\(^a\) and P. Poupard\(^a\)*

\(^a\)IRHS, Agrocampus-Ouest, INRA, Université d’Angers, SFR 4207 QuaSaV, 49071, Beaucouzé, France; \(^b\)GEVES, 25 rue Georges Morel, 49071 Beaucouzé, France; \(^c\)FNAMS, Impasse du Verger, 49800 Brain-sur-l’Authion, France; \(^d\)FNAMS, 45 voie Romaine, BP 23, 41240 Ouzouer-le-Marché, France; \(^e\)VILMORIN, Route du Manoir, 49250 La Ménitré, France; \(^f\)HM CLAUSE, 1 chemin Moulin des Ronzières, 49800 La Bohalle, France.

*E-mail address: pascal.poupard@univ-angers.fr*

Running head: carrot umbel browning and stem necrosis

Keywords: Apiaceae, *Daucus carota* subsp. *sativus*, fungus detection on seed, necrotrophic fungi, *Phomopsis dauci*, seed production
Abstract

A collection of 102 Diaporthe isolates was compiled from lesions on carrot, parsley and wild Apiaceae species in France from 2010 to 2014. Molecular typing based on ITS rDNA sequences resulted in the identification of 85 D. angelicae and 17 D. eres isolates. Based on sequences of the 3’ part of the IGS rDNA, intraspecific variability was analyzed for 17 D. angelicae and 13 D. eres isolates representing diverse plant species, locations in France, and plant tissues. The genetic diversity was greater for the D. angelicae isolates than the D. eres isolates. In vitro sensitivity to each of nine fungicides for five D. angelicae and four D. eres isolates was similar for isolates of both species, with a marked variation in fungicide sensitivity depending on the active ingredient. To assess the pathogenicity of D. angeliace and D. eres isolates on carrot, one isolate of each species was inoculated onto umbels in a controlled environment. Typical lesions were observed for both isolates. Carrot crop debris collected from a seed production field in France and placed in controlled conditions produced perithecia and ascospores typical of Diaporthe, that were further characterized molecularly as belonging to D. angelicae. Detection of Diaporthe species on seed lots from three carrot production fields in France was investigated. Both species were detected on seeds by conventional PCR assay, with a greater frequency for D. angelicae (67 vs 33%, respectively). Overall, the results highlighted that umbel browning in carrot seed crops in France was mainly caused by D. angelicae.
Introduction

In 2014, carrot (*Daucus carota* L.) seed production was conducted on 2500 ha of land in France, which represents approximately 25% of the area devoted to vegetable small seed production (Daviot, 2014). Since 2007, umbel browning symptoms have been observed regularly in carrot seed production areas in the Centre-Val de Loire Region (Laurent & Blancard, 2009). Triangular, necrotic lesions initially emerge on carrot umbels and can spread to the entire umbel, and often progress onto the stem. Diseased umbels dry prematurely, jeopardizing seed development. Seed production loss was estimated at approximately 8% of harvested carrot umbels during the 2007 and 2008 cropping seasons in France (Laurent & Blancard, 2009). A fungus isolated from the diseased tissues was identified as *Diaporthe angelicae* (Berk.) Farr and Castlebury, comb. nov. based on morphological and molecular criteria (Ménard *et al.*, 2014). The disease resembles the lesions described in the Netherlands in 1951 on carrot inflorescences as being caused by *Phomopsis dauci* (Bakker, 1951; von Arx, 1951). The presence of umbel browning and stem necrosis lesions on carrot, parsley, dill and parsnip has been reported in seed production fields located in southwestern, western and southeastern France; and seeds produced on diseased umbels were less numerous and smaller than those that developed on asymptomatic umbels, at least partly explaining the seed production losses reported (J. Gombert, Fédération Nationale des Agriculteurs Multiplicateurs de Semences (FNAMS), Brain-sur-l’Authion, France, personal communication).

*Diaporthe* (*=Phomopsis, Ascomycota, Diaporthales*) corresponds to a very large fungal genus comprising at least 800 taxon names, while around 900 species have been described in the anamorphic *Phomopsis* genus (Rossman *et al.*, 2007; Uecker, 1988). *Diaporthe/Phomopsis* species are known to be endophytic fungi, especially on woody
plants (Rossman et al., 2007). The species *D. angelicae* (syn. *Mazzantia angelicae*) was described precisely in the taxonomic study of Castlebury et al. (2003). The fungus can develop typical pycnidia containing α- and β-conidia (asexual stage), and perithecia producing ascospores (sexual stage). The same study highlighted the presence of *D. angelicae* in Europe, Canada and USA on decaying stems belonging to various wild plants in the Apiaceae, such as *Angelica* spp., *Anthriscus* spp., *Daucus* spp. or *Eryngium* spp. The host range of this fungal species is not restricted to cultivated Apiaceae. Umbel lesions similar to those described on cultivated carrot and caused by *Phomopsis diachenii* were reported on caraway (*Carum carvi*) in Bulgaria (Rodeva & Gabler, 2004) and Hungary (Nagy, 2010). Umbel blight symptoms have also been described on wild and cultivated fennel and were assigned to *Diaporthe foeniculacea* in France (Du Manoir & Vegh, 1981), Portugal (Philips, 2003) and Bulgaria (Rodeva & Gabler, 2011). Santos & Philips (2009) isolated a complex of four *Diaporthe* species from fennel: *D. ambigua*, *D. angelicae*, *D. lusitanicae* and *D. neotheicola*. The situation could be the same on cultivated carrot, although only *D. angelicae* and *P. dauci* have been described to date on this plant species (Bakker, 1951; von Arx, 1951; Ménard et al., 2014).

Sequences of the large subunit nuclear ribosomal DNA (rDNA) and internal transcribed spacer (ITS) rDNA regions are available for *D. angelicae* characterization and phylogenetic studies (Castlebury et al., 2003). The four *Diaporthe* species present on wild fennel, including *D. angelicae*, were characterized by Santos & Philips (2009) using microsatellite-primed PCR profiles (MSP-PCR) and phylogenetic analysis based on ITS rDNA sequences. More recently, mating-type (MAT) genes in *Diaporthe* and *Phomopsis* were considered as phylogenetic markers for biological species definition by comparison with ITS rDNA and elongation factor 1α sequences (Santos et al., 2010). The results of
this latter study showed that *D. angelicae* and *P. dauci* isolates were in the same cluster, suggesting that these species may correspond to the same fungal pathogen that causes umbel browning and stem necrosis on carrot.

Different inoculum sources responsible for carrot umbel browning were suggested on the basis of the studies of Bakker (1951) and von Arx (1951) focused on *P. dauci* epidemiology: infested crop debris, infected seeds, infected wild or cultivated Apiaceae species near carrot seed crops and pollinator insects serving as vector of the fungus. However, to our knowledge, transmission of *P. dauci* to carrot seeds and from seeds to seedlings has never been demonstrated. More generally, a better understanding of the *D. angelicae* life cycle is required to develop adequate control methods and reduce the pathogen impact on carrot seed production. Prophylactic methods, including elimination or burying of infested crop debris, could be efficient (Bakker, 1951). Field experiments to select fungicides that limit *D. angelicae* development in carrot seed crops were performed recently in France by the FNAMS. Based on these experiments, fungicide control has been proposed to carrot seed growers (E. Morel, FNAMS Ouzouer-le-Marché, France, personal communication), while taking into account the importance of reducing fungicidal treatments for the environment and human health protection (Ecophyto plan proposed by the French Ministry of Agriculture, Agrifood and Forestry, http://agriculture.gouv.fr/ecophyto-kesako-0).

The general aim of this study was to characterize the fungal pathogen(s) causing umbel and stem lesions in carrot seed crops in France using a collection of isolates originating from different seed production fields in France that was larger than previously reported (Ménard *et al.*, 2014). The specific objectives were to describe (i) these isolates using phenotypic and molecular criteria for fungal diversity assessments, including
molecular analyses for species identification, (ii) pathogenicity on carrot umbels in a controlled environment, (iii) the production of perithecia and ascospores from carrot crop debris, (iv) sensitivity of the isolates to fungicides commonly used in carrot seed crops in France, and (v) the ability to detect the fungal pathogens on carrot seed lots based on seed plating on an agar medium or by PCR assay. A hypothetical disease cycle was proposed based on the results of this study.
Materials and Methods

Collection of field isolates and reference isolates

Between 2010 and 2014, 102 *Diaporthe* spp. isolates were collected: 99 isolates originated from carrot or parsley plants from seed production fields in different French locations, and three strains (DA080, DE014, and DE015) were isolated from wild Apiaceae species (Table 1). Fungal strains were isolated from lesions on umbel involucres or stems, and from black spots on carrot debris (Fig. 1). Plant tissues with lesions (umbel browning or stem necrosis) or sections of crop debris with black spots were surface-sterilized for 2 min in 1% sodium hypochlorite, and rinsed in two changes of sterilized distilled water for 5 min each. Approximately 5 mm$^2$ of tissue from the boundary of a lesion and healthy tissue on each section of plant tissue were excised in a laminar flow cabinet, plated in a Petri dish onto malt (1%) agar (1.7%) medium (MA) amended with streptomycin (0.005%), and incubated for 1 week at 20°C in the dark. For long-term storage, all isolates were kept in a 30% glycerol solution as cryoprotectant at -80°C. Three representative isolates of *D. angelicae* were obtained from the Centraalbureau voor Schimmelcultures (CBS), the Netherlands (DA001 to DA003, ref. CBS111592, CBS123214, and CBS123215, respectively) (Table 1). One isolate from this French *D. angelicae* collection (DA004) was deposited at the CBS in 2013 (CBS137262) (Table 1).

Species identification using ITS rDNA sequences

Fungal isolates were first identified at the species level in a part of the fungal collection (29 isolates). The isolates were selected on the basis of their origin (host plant, organ, location) and year of isolation. DNA was extracted from a fungal colony of each fungal
isolate according to Goodwin & Lee (1993). PCR amplification of the ITS1-5.8S-ITS2 region of rDNA was carried out with ITS1 and ITS4 universal primers (Table 2) (White et al., 1990). The reaction mixture (25 µL) consisted of 1 µL of 10-fold diluted fungal DNA, 0.5 U of GoTaq® polymerase (Promega), 1× reaction buffer, 1.4 mM MgCl₂, 10 pmol of each primer, and 0.2 mM dNTPs. PCR amplification was performed with the following parameters: initial denaturation at 94°C for 3 min; 40 denaturation cycles at 94°C for 30 s, annealing at 56°C for 50 s, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. Amplification products were resolved on 1.2% agarose gels in 0.5× TAE buffer (20 mM Tris-acetate, pH 8, 0.5 mM EDTA) followed by ethidium bromide staining. For species identification, PCR products were purified using a PCR purification kit (NucleoSpin Extract II, Macherey-Nagel) and sequenced using the commercial sequencing service of GATC Biotech AG (Konstanz, Germany). BLAST searches of repository information from GenBank were conducted using ITS sequences. ITS sequences were aligned using CLUSTAL W software to design specific primers at the genus level (DiapITSfw and DiapITSrv primers) or species level (AngelITSfw and AngelITSrv primers for D. angelicae; and EresITSfw and EresITSrv primers for D. eres) (Table 2). PCR amplification using these primers was performed with the same conditions described above for the universal primers. Phylogenetic analysis of the sequenced, amplified fragments was carried out using the Phylogeny.fr web service (Dereeper et al., 2008). Sequences were aligned with MUSCLE and a dendrogram was constructed using the neighbour joining algorithm (Saitou & Nei, 1987). Bootstrap confidence values were calculated from 1000 randomly re-sampled data sets. The sequences of the amplified products (GenBank acc. nos indicated in Table 1) were compared to the ITS sequences of three D. angelicae reference isolates (CBS111592, CBS123215 and CBS137262, referred to as DA001, DA003 and DA004 in Table 1) and one D. eres reference isolate (DLR12A,
Udayanga et al. 2014, see Table 1). Seven additional ITS sequences representing closely
related *Diaporthe* species (*D. foeniculacea*, *D. helianthi*, *D. lusitanicae*, *D. neotheicola*,
*D. phaseolorum* var. *sojae*, *D. rudis*, *D. subordinaria*) were also included in this analysis
(Table 1). The ITS sequence of *Diaporthe fibrosa* CBS113830 was used to root the tree.

**Morphological characterization and fungicide sensitivity**

Growth rates of five replicate cultures of each of 15 *D. angelicae* and 15 *D. eres* isolates
(see underlined accessions in Table 1) were measured after 7 days on MA medium at 20°C
in the dark, and the mean ± standard deviation (SD) was calculated. For each isolate,
colony colour and appearance were recorded. Pycnidia were produced on MA medium
after 1 to 3 weeks at 20°C under near-ultraviolet light (0.5 W m⁻²; Coaxel, L18/73) and a
12 h photoperiod/day. The size (length and width) of conidia produced in pycnidia was
measured for the 30 isolates, using three α-conidia and three β-conidia per isolate. These
data were analyzed using analysis of variance (ANOVA) to assess the isolate and species
effect. Residual normality was checked post hoc using Q-Q plots. Residuals were found to
be normally dispersed for all of the data. All morphological data are presented as the mean
± SD.

The *in vitro* mycelial sensitivity of five *D. angelicae* and four *D. eres* isolates
(accessions indicated in bold in Table 1) to each of nine fungicides (formulated and tested
as technical grade) was tested. Isolates of this sub-collection were selected according to
their geographical origin and the lesion organ on the host; all isolates were collected from
carrot lesions, except one isolate (DA035, from parsley). The active ingredients used were
picoxystrobin (Acanto, DuPont, Wilmington, USA), fludioxonil (Geoxe WG, Syngenta,
Basel, Switzerland), boscalid (Pictor Pro, BASF, Ludwigshafen, Germany), iprodione
(Rovral, BASF), pyrimethanil (Scala, BASF), difenoconazole (Score, Syngenta),
fenhexamid (Teldor, Bayer CropScience, Leverkusen, Germany), methyl thiophanate
(Topsin, Certis Europe, Utrecht, The Netherlands) and cyprodinil (Unix Max, Syngenta).
An *Alternaria dauci* isolate (FRA017, isolated in 2000 from naturally infected carrot
leaves in Gironde, France), was used as a reference pathogen for the experiments dealing
with picoxystrobin, fludioxonil, boscalid and difenoconazole. In France, *Alternaria* leaf
blight is controlled mainly by fungicide applications. Some of the fungicides used or tested
in France are composed of these four active ingredients (E. Morel, FNAMS Ouzouer Le
Marché, France, personal communication). Mycelial disks (8-mm dia.) removed from the
margins of a 7 day old culture of each isolate were transferred to MA medium for each
active ingredient except pyrimethanil and cyprodinil. For these latter two fungicides,
fungal cultures were prepared on minimal Vogel medium (Vogel, 1956) to avoid the
presence of amino acids, as pyrimethanil and cyprodinil act as inhibitors of amino acid
synthesis. MA or Vogel media were amended with the appropriate fungicide at final
concentrations of 0.1, 1, 10, 100 and 1000 mg L$^{-1}$. For the picoxystrobin sensitivity assay,
salicylhydroxamic acid (SHAM) was added at 28 mg L$^{-1}$ to the MA medium to prevent the
fungus from overcoming the fungicide toxicity through an alternative oxidative pathway
(Wise *et al.*, 2008). Three replicate plates were used per treatment combination (fungal
isolate - fungicide) and arranged in a completely randomized design. For each fungicide
concentration tested, inhibition of radial growth compared with the non-amended control
plate was calculated after 3 and 6 days of incubation at 20°C in the dark for each active
ingredient except pyrimethanil and cyprodinil, and after 6 and 13 days of incubation at
20°C in the dark for pyrimethanil and cyprodinil. The mycelial growth was slower on
Vogel medium than on MA. The results were expressed as half maximal effective
concentration (EC$_{50}$; the concentration which reduced mycelial growth by 50%), which determined by regressing the log$_{10}$ values of the fungicide concentrations against the inhibition of radial growth (% control). All statistical analyses were performed using R-2.6.1 software (R Development Core Team, 2010).

Intraspecific genetic variability based on IGS rDNA sequences

The complete IGS sequences of *D. angelicae* isolate DA057 (4485 bp) and of *D. eres* isolate DE009 (3673 bp) (GenBank acc. nos KP406523 and KT071711, respectively) were obtained from PCR fragments by using the 26S3111F and IGS27 primer set as described by Hong *et al.* (2005) and Subbarao *et al.* (1995). PCR fragments were cloned using the pGEM-T vector (Promega) according to the manufacturer’s protocol, and clones were sequenced at GATC Biotech AG. The IGS sequences were aligned using CLUSTAL W software in order to design internal primers for the intraspecific genetic variability study. A 403 bp portion in the 3’ variable domain from the IGS sequences of 17 *D. angelicae* and 13 *D. eres* isolates (accessions indicated by an asterisk in Table 1) was amplified using the primers VariabIGS3’fw and VariabIGS3’rv (Table 2) with the PCR conditions described above. In order to analyse the intraspecific genetic variability in the 5’ domain from the IGS sequences of the same 17 *D. angelicae* and 13 *D. eres*, a 810 bp or 461 bp portion was amplified, respectively, using the primers VariabIGS5’fw and VariabIGS5’rv1 or VariabIGS5’fw and VariabIGS5’rv2 (Table 2). Phylogenetic analysis of the sequenced amplified fragments (Table 1) was carried out as described above for species identification using ITS rDNA sequences.

Pathogenicity testing
In the summer of 2013, cuttings comprising umbels and attached 20 cm length of the stem of a carrot Nantes type open pollinated cultivar, were collected at developmental stage 65 (Feller et al., 1995) in an experimental seed production field located at FNAMS, Brain-sur-l’Authion, France (47.472132°N, 0.397789°W). Cuttings were planted in boxes (30 cm × 30 cm × 6 cm, with 3 cuttings per box) containing a 4 cm depth of sand moistened with 400 mL nutrient solution (Hakaphos-Vert, COMPO) at 2 g L⁻¹ (w/v) in distilled water. Boxes were placed in a growth cabinet at 20°C, with white light (OSRAM, L58W/830) and a 12 h photoperiod day⁻¹ and 80% relative humidity. Diaporthe angelicae isolate DA016 and D. eres isolate DE003 were grown as described above for pycnidia production in order to obtain conidia. The conidial suspensions were prepared to a final concentration of 1 × 10⁷ α-conidia per mL in sterilized distilled water containing 1% Tween 20 and 10% glycerol (WTG solution). Two inoculation methods were used: either the umbel was sprayed with the inoculum to runoff (around 3 mL of inoculum per umbel) or 10 µL of inoculum were deposited with a micropipette at the base of the umbel (involucre) onto a wound created on the involucre using a disinfected scalpel blade. Three replicate umbels were used for each inoculation method and isolate. Control umbels were sprayed with WTG solution to runoff (three umbels) or by depositing 10 µL WTG solution at the wounded involucre (three umbels). Inoculated umbels were placed in boxes in the same growth chamber, arranged in a randomized design. The time (number of days) for development of the first lesions on umbels was assessed after inoculation. The fungus was re-isolated from umbels using the conditions described above, and PCR assays of the isolates were performed using the species-specific ITS primers designed for D. angelicae and D. eres (Table 2). The whole experiment was carried out twice.

Perithecia production
In 2012, crop debris showing black spots were collected in a naturally infected carrot seed production field in Loir-et-Cher, France (47.654.766°N, 1.363743°E). The debris was deposited in a closed Plexiglas box containing moist Fontainebleau sand in a controlled environment [20°C, with white light (OSRAM, L58W/830) and a 12 h photoperiod day\(^{-1}\) and 80% relative humidity]. In these conditions, the crop debris was observed regularly for 35 days, and the durations until pycnidia, perithecia and cirrhi formation were recorded. A fragment of debris (10 cm length) was thoroughly observed to determine the number of pycnidia and perithecia. Cirrhi produced from the perithecial ostioles were collected using a micropipette in order to observe the morphology and size of ascospores microscopically (Nikon Eclipse 80i, G.E.O. Micro Service). Ascospore ejection from perithecia was supposed to occur before cirrhi development on perithecia. To study ascospore ejection from perithecia, 90-mm dia., opened Petri dishes containing MA medium, and cellotape pieces were placed in the Plexiglas box 40, 60 and 80 cm above the perithecia-producing debris at day 12 of the experiment. The presence of colonies in the Petri dishes and of ascospores on the cellotape pieces was assessed after 14 days. Fungal identification was performed using the morphological and molecular criteria described above (appearance and colour of colonies, morphology and size of ascospores, and PCR assay identification based on ITS species-specific primers). The whole experiment was carried out twice.

**Pathogen detection on carrot seeds**

In 2009, carrot seeds were collected at harvest from three carrot seed production fields (named I, II and III) in Loir-et-Cher, France (47.720341°N, 1.485171°E; 47.888470°N, 1.405769°E; and 47.655874°N, 1.359911°E, respectively). Typical umbel browning and
stem necrosis caused by *D. angelicae* were observed in these fields, as previously reported (Ménard *et al.*, 2014). For each field, three seed samples were harvested from symptomatic umbels (sample A), three from asymptomatic umbels collected from symptomatic plants (sample B), and three from asymptomatic umbels collected from asymptomatic plants (sample C). As a positive control, seeds were harvested from umbels that were artificially contaminated with strain DA016 (*D. angelicae* positive control) or DE003 (*D. eres* positive control). For pathogen detection on MA amended with streptomycin (0.005%), 200 seeds of each of the nine samples (three samples per field × three fields) were surface-sterilized for 2 min in 1% sodium hypochlorite and rinsed in two changes of sterilized distilled water for 5 min each. In a laminar flow cabinet, disinfected seeds were dried on sterilized blotting paper, placed on the surface of the MA Petri dishes, and incubated for 1 week at 20°C in the dark. The same experiment was performed using 200 non-disinfected seeds of each sample. Fungal colonies that developed from the seeds were inspected using morphological criteria for *Diaporthe* spp. isolates (morphology and colour of mycelia). For detection using molecular tools, 25 non-disinfected seeds of each of the nine samples were freeze-dried in a Christ Alpha 1–4 LSC apparatus (Fisher Bioblock Scientific), ground in a mortar in liquid nitrogen and stored at –20°C. DNA extractions from these seed samples were performed according to Briard *et al.* (2000), with an additional DNA precipitation step in isopropanol for 5 min on ice. Alternatively, DNA was extracted from macerates of the seed prepared using 1000 non-disinfected seeds (ca. 1.85 g) of each of the nine samples. Seed batches were each placed in a 50 mL tube (VWR North America) and incubated with 10 mL phosphate buffer saline (PBS, Sigma-Aldrich) containing 0.02% Tween 20 (v/v) for 12 h at 4°C with shaking (140 rpm). Seed macerates were centrifuged (6000 g for 15 min at 4°C), the supernatant was discarded and the pellet was dissolved in 1 mL supernatant. DNA was extracted from the concentrated macerate using the Powersoil
DNA isolation kit (MO-BIO, Novozymes) according to the manufacturer’s recommendations. A tissue crusher (MM30, Retsch France) was used at 300 Hz for 2 min. The last DNA elution step was achieved using 60 µL elution buffer (instead of 100 µL, as recommended in the manufacturer’s protocol). The DNA samples were stored at –20°C.

For DNA obtained from entire seeds or seed macerates, PCR amplifications using 1/10 diluted-DNA in water, were carried out as described above for Diaporthe species identification. Four primer sets were used (Table 2): ITS1 and ITS4 (as PCR control samples), DiapITSfw and DiapITSrv for identification to the Diaporthe genus level, AngelITSfw and AngelITSrv for D. angelicae identification, and EresITSfw and EresITSrv for D. eres identification. All PCR experiments were carried out twice.
Results

Isolation of *Diaporthe* spp. from field samples

The 102 fungal isolates from carrot and parsley infected plants sampled in French seed crop productions fields from 2010 to 2014 all developed white pigmentation and a woolly aerial mycelium on MA medium (Table 1, Fig. 2a). The mycelium was white on the top and on the reverse side of the agar colonies. Isolates were collected mainly from carrot lesions (87 isolates) vs. parsley (12 isolates). Three isolates originated from lesions observed on wild Apiaceae species. Pycnidia (Fig. 2b) appeared between 6 to 23 days after incubation in culture conditions described above for spore induction, with the exact duration depending on the isolate (n=78 isolates). Typical $\alpha$- and $\beta$-conidia were produced in these pycnidia (Fig. 2c), with a longer time for $\alpha$-conidia (between 12 and 35 days of incubation vs. 6 to 32 days for $\beta$-conidia production). These morphological criteria were in agreement with those previously reported in Ménard *et al.* (2014) for characterization of four *Diaporthe* isolates collected from carrot plants in France.

Identification and characterization of *D. angelicae* and *D. eres* isolates

Twenty nine isolates of *Diaporthe* from the French field collection were identified to the species level using ITS rDNA sequencing. They grouped into two clusters (Fig. 3): one group was composed of 21 isolates showing 100% identical sequences to the ITS rDNA sequences of *D. angelicae* reference isolates, DA001, DA003, and DA004, while the other group consisted of eight isolates exhibiting 100% sequence identity with the ITS sequence of the *D. eres* reference isolate (DLR12A). PCR assay using ITS primers designed for species detection of *D. angelicae* or *D. eres* (Table 2) identified 85 of the 120 isolates as *D.
angelicae and 17 as D. eres (Fig. 4). Interestingly, although most isolates belonged to D. angelicae, in three locations where one or two isolates were collected, all of the isolates were D. eres.

The ANOVA for mycelial growth rate on MA medium of 15 D. angelicae isolates (DA074 to DA088) and 15 D. eres isolates (DE001 to DE003; DE005; DE007 to DE017) revealed a significant isolate effect (F test p < 2.2.10^{-7}) but no statistical difference (p = 0.44) between the mean growth rate of the two species (D. angelicae: 7.65 ± 0.10 mm·day^{-1}, D. eres: 7.54 ± 0.10 mm·day^{-1}). Growth rates ranged from 6.22 ± 0.19 mm·day^{-1} for isolate DE002 to 8.91 ± 0.20 mm·day^{-1} for DA087. Pycnidia formation was induced on MA medium using the same D. angelicae and D. eres isolates. The width of α- and β-conidia were not significantly different based on the ANOVA (p > 0.05) among the isolates nor between the two species of Diaporthe. The mean α- and β-conidial lengths however, were significantly greater for D. eres (mean ± SE) than for D. angelicae (mean ± SE) (p < 1·10^{-4}, Table 3). Nevertheless, these criteria were not diagnostic as the range in α- and β-conidial lengths of the two species overlapped (Table 3).

Intraspecific variability within D. angelicae and D. eres

Analysis of the aligned nucleotide sequences in the 3’ part of the IGS rDNA sequences (403 bp) revealed that: i) all isolates of D. eres, except DE011, grouped within the same cluster (Fig. 5a); and ii) D. angelicae isolates grouped within two main clusters, with one comprising isolates DA016, DA046 and DA072 (from carrot and parsley), and the other including the remaining 14 isolates of this species that were sequenced (Fig. 5b). In this latter group, three sub-clusters were evident: one cluster included DA057 and DA080 from
carrot and wild *Daucus* species, respectively; one comprised the CBS reference isolates DA001 and DA003 from *Heracleum sphondylium* and *Foeniculum vulgare*, respectively; and one included 10 isolates, seven from carrot and three from parsley. There was no evidence of clustering according to host or geographic origin with the *D. angelicae* isolates. This also seemed to be the case concerning *D. eres*, as two isolates collected from wild Apiaceae species, DE014 and DE015, were grouped with isolates from carrot plants collected at different locations in France. Phylogenetic analysis of a portion of the 5’ part of the amplified IGS rDNA for the same sub-collection of 17 *D. angelicae* isolates (810 bp), using the sequence of *D. eres* isolate DE009 to root the tree, showed that all the *D. angelicae* isolates were in the same cluster, except the reference isolate DA001 (results not shown). A similar result was obtained when analysing a portion of the 5’ part of the amplified IGS rDNA of 13 *D. eres* isolates (461 bp) using the sequence of DA057 to root the phylogenetic tree. Twelve of the thirteen *D. eres* isolates clustered together, the exception being DE011 (results not shown).

**In vitro fungicide sensitivity of *D. angelicae* and *D. eres***

The EC$_{50}$ of the *D. angelicae* and *D. eres* isolates with the different active ingredients was highly variable, in a 0.01 to 1000 mg.L$^{-1}$ (1 to 100 000) range (Fig 6, Table S1). Isolates of both species were usually highly sensitive to difenoconazole (EC$_{50}$ < 0.15 mg.L$^{-1}$) and very weakly sensitive to boscalid and pyrimethanil (EC$_{50}$ > 188 mg.L$^{-1}$), while intermediate EC$_{50}$ values were obtained with other fungicides. Contrastingly, the EC$_{50}$ variations were in a somewhat narrower range when each active ingredient was considered separately. Narrow sensitivity ranges were observed for some of the tested fungicides, such as iprodione (1 to 2.3), boscalid (1 to 2.7), methyl thiophanate (1 to 2.8) and fenhexamid (1 to
4.66). By contrast, the EC$_{50}$ values obtained with difenoconazole (< 1 to 15) and fludioxonil (< 1 to 360) were more variable, depending on the isolates. The sensitivity of the reference isolate FRA017 of *A. dauci* to pycoxystrobin, difenoconazole and fludioxonil was within the range of the *Diaporthe* isolates. However FRA017 was very sensitive to boscalid (EC$_{50}$ < 0.01 mg.L$^{-1}$).

**Pathogenicity of *D. angelicae* and *D. eres***

Both *D. angelicae* isolate DA016 and *D. eres* isolate DE003 caused lesions on inoculated carrot umbels. Lesions appeared on average of 11.5 days after the conidial spray, while the first symptoms were observed at 21.5 days and 23.0 days after a spore suspension was deposited in a wound on the umbel involucre for DE003 and DA016, respectively. The results from the two repetitions of the experiment were significantly different between the two inoculation methods (p=0.000266 with 11.50 ± 0.711 days for spraying a spore suspension vs, 22.25 ± 0.711 days for the wound inoculation). However there was no statistical difference between isolates for the same inoculation method when comparing the duration for lesions to develop. The symptoms i.e., flower and involucre browning, were very similar after inoculation with DA016 or DE003. Both isolates were also able to cause stem necrosis under these conditions. The inoculated fungi were re-isolated from lesions and identified to species by molecular characterization (PCR assay) using the species-specific ITS primers for *D. angelicae*/*D. eres* (results not shown).

**Sexual stage of *D. angelicae***
The carrot residue fragments developed pycnidia from 5 days after incubation in the controlled environment chamber and a total of 41 pycnidia were observed on the 10 cm-long residue section during the 35-day observation period. On the fragments of debris, perithecia formed 12 days after incubation, and cirrhi were first observed at 25 days (Fig. 7a). A total of 82 perithecia developed on the 10 cm fragment during the experiment. The ascospores collected in cirrhi averaged $12.3 \pm 4.9 \mu m \times 2.3 \pm 0.4 \mu m$ (n=85) (Fig. 7b). Ascospores showing a similar aspect and size as those presented in Fig. 7b were observed microscopically at day 26 of the experiment on the cellotape pieces placed 40 and 60 cm above the symptomatic carrot residues incubated in the environmental chamber. No fungal spores were present on the cellotape pieces placed 80 cm above the perithecia. Typical *Diaporthe* colonies (white colour and wolly mycelium) developed in the Petri dishes placed 40 and 60 cm above the residues. No mycelial growth was observed in the dishes placed at 80 cm. Molecular characterization of fungal colonies that developed in Petri dishes was achieved by PCR amplification with the *D. angelicae*-specific ITS primers. The same results were obtained with the repeat of the whole experiment. Although some of the *D. eres* isolates in this collection originated from crop debris (DE003 to DE007 and DE010), perithecia production by this species was not observed from the residue samples collected and incubated in the conditions described above.

*Diaporthe angelicae and D. eres detection on carrot seeds*

For all tested carrot seed lots (disinfected or not), no fungal colonies exhibiting morphological criteria of *Diaporthe* spp. developed on agar medium (Table 4). Molecular detection of *Diaporthe* sp. by conventional PCR assays of subsamples of the same seed samples, with DNA extracted from entire seeds or from seed macerates, using ITS primers
specific for *D. angelicae* or *D. eres* resulted in detection of *D. angelicae* on all three seed lots corresponding to diseased umbels whether using whole seed or seed macerates (A samples), and only one seed lot from an asymptomatic umbels using the seed macerate protocol (II-B). *D. eres* was not detected in any of these seed lots when the whole seed protocol was used for DNA extraction but was detected in one seed lot from asymptomatic umbels with the seed macerate protocol for DNA extraction (II-B). *Diaporthe angelicae* was detected by PCR assay from I-C lot from asymptomatic plants using the whole seed protocol, and both I-C and II-C lots using the seed macerates protocol, while *D. eres* was detected only on II-C lot from asymptomatic plants using the seed macerates protocol (table 4).
Discussion

Using a collection of 87 isolates of *Diaporthe* established from carrot seed crop samples in France from 2010 to 2014, two *Diaporthe* species responsible for umbel browning and stem necrosis were identified: *D. angelicae*, which was previously reported by Ménard *et al.* (2014) and *D. eres*. In this study, Koch’s postulates were verified on carrot umbels for an isolate of both fungal species in a controlled environment. The pathogenicity of 3 other isolates of *D. angelicae* was previously tested in a greenhouse (Ménard *et al.*, 2014). Further work is needed to confirm the results by testing the pathogenicity of *D. eres* using several isolates. *Diaporthe angelicae* was previously described as the main pathogen responsible for umbel browning on wild fennel in Portugal (Santos & Phillips, 2009). In that study, based on a collection of 128 isolates from 10 locations in Portugal, 86 isolates were identified as *D. angelicae* from 9 of the 10 locations. A similar situation occurred in French carrot seed production areas surveyed in this study, as *D. angelicae* was identified from 83% of the isolates obtained (Fig. 4). To our knowledge, *D. eres* has never been isolated from Apiaceae species. This species was first described by Wehmeyer (1933) on different genera of ligneous plants. More recently, *D. eres* was identified as a pathogen causing shoot blight and canker of peach trees (Thomidis & Michailides, 2009) and was present in a complex of fungal species responsible for wood cankers of grape (Baumgartner *et al.*, 2013).

As colonies of both *Diaporthe* species exhibited a very similar growth on the agar media conditions tested in this study, other phenotypical criteria were investigated but also generally did not differentiate isolates of *D. angelicae* from those of *D. eres*. PCR-based identification using species-specific ITS primers developed in this study was a much more effective option. Another characteristic evaluated for these isolates was their sensitivity to
a panel of nine fungicides with different modes of action. The efficacy of the fungicides at
limiting mycelial growth was similar statistically when comparing several isolates of both
*Diaporthe* species. The high inhibition activity of difenoconazole (EC50 < 0.15) observed
was in accordance with results obtained in fungicide experiments performed in carrot seed
production fields (E. Morel, FNAMS Ouzouer-Le-Marché, France, personal
communication). In the present study, isolate DA035 of *D. angelicae* was much less
sensitive to difenoconazole or pyrimethanil than other *D. angelicae* isolates tested, as also
noted for isolate DE001 of *D. eres* in response to cyprodinil. In a collection of 24 *D. eres*
isolates from blighted shoots of peach, six isolates were either resistant or less sensitive to
methyl thiophanate than the other isolates (Thomidis & Michailides, 2009). These results
strongly suggest that the development of isolates of *Diaporthe* resistant to fungicides in the
field could be expected under conditions of intensive fungicide use. In this study, boscalid,
a fungicide commonly used in carrot seed production in France, was highly effective
against the *A. dauci* strain tested, while isolates of both *D. angelicae* and *D. eres* isolates
were quite resistant (EC > 270 mg.mL$^{-1}$). This latter result highlighted the importance of
selecting appropriate fungicides to control specific fungal pathogens on carrot and may, at
least partly, explain the emergence of carrot umbel browning as a cause of significant
losses in carrot seed production in France.

Another aim of this study was to explore the intraspecific diversity in *D. angelicae*
and *D. eres* isolates from carrot seed crops in France. Hence, the variability of IGS rDNA
sequences was studied through phylogenetic analysis using 30 isolates selected to represent
diversity in host plant and geographic origin. Sequence analysis of the variable domain in
the 3’ part of the IGS region demonstrated that the diversity in *D. angelicae* isolates was
significantly greater than that observed in *D. eres*. Perithecia of *D. angelicae*, previously
described by Castlebury et al. (2003), were produced in a controlled environment in this study on carrot crop debris collected in France (Fig. 7), and also in field experiments in France (unpublished data). The occurrence of the sexual stage in the life cycle of *D. angelicae* in carrot seed field may, at least partly, be responsible for the greater sequence polymorphism in this species than in the *D. eres* isolates. Although Wehmeyer (1933) published a thorough description of *D. eres* perithecia, their development in carrot seed production fields in France or elsewhere has, to our knowledge, never been described. Further investigations of this are needed. Phylogenetic analyses of the variable domain in the 5′ part of IGS sequences and the same set of isolates of *D. angeliace* and *D. eres* in this study did not show much variability within each fungal species, with only one sequence diverging from others in *D. angelicae* and in *D. eres*. In contrast, studies of intraspecific diversity based on portions of the 5′ IGS sequences allowed differentiation of several groups of isolates in a collection of 26 *Diaporthe helianthi* isolates (Pecchia et al., 2004), and a study of 27 *Alternaria dauci* isolates (Boedo et al., 2012). Those two fungal collections were, however, composed of isolates from different countries, whereas all the *Diaporthe* isolates in this carrot and parsley collection originated from French seed production fields.

As seed transmission was suspected and could constitute a major role in umbel browning epidemiology in carrot seed fields in France, the presence of *D. angelicae* and *D. eres* on carrot seeds also was investigated in this study. *Diaporthe* species were not detected using a traditional seed plating technique on agar medium, even for seed samples harvested from umbels with well-developed lesions. It seems that the competitiveness of *D. angelicae* or *D. eres* against other fungi for growth on the agar medium used was too low to detect these two species. Isolation of *D. angelicae* was, however, possible in one
seed sample collected from symptomatic umbels in the field (results not shown) when seed
disinfection was achieved before plating using a greater sodium hypochlorite concentration
(5%). In contrast to the seed plating results, D. angelicae and D. eres were respectively
detected on a total of six and three carrot seed lots using conventional PCR assays
performed on DNA extracted from whole seeds or seed macerates. The results showed that
the seed maceration protocol before DNA extraction improved the PCR protocol by
comparison to running the PCR assay on DNA extracted from whole seeds. Indeed, 1000
seeds were tested in the same subsample vs 25 seeds to compare both protocols. Another
advantage of using seed maceration for PCR diagnosis is the detection of living-fungal
tissue. The seed maceration protocol derived from the BIO-PCR technique, which was
firstly described to detect Pseudomonas syringae in bean seed extracts (Schaad et al.,
1995). BIO-PCR has then been developed for detection of different seed-borne pathogens
(for a review, see Walcott, 2003), including Alternaria radicina on carrot seeds (Pryor &
Gilbertson, 2001). After seed maceration, D. angelicae was detected in seed lots harvested
from symptomatic umbels and from asymptomatic umbels, the latter harvested from
diseased or healthy plants. The PCR results demonstrated the prevalence of D. angelicae
DNA on carrot seeds compared to D. eres, and were similar to previous analyses in this
study, based on the isolation of both fungal species from field samples. Further
investigations are needed to: i) localize the fungal pathogens in carrot seeds, and ii) study
the fungal transmission from the seed to the seedling.

This is the first report of D. angelicae and D. eres as seed-borne pathogens of carrot.
As a next step, molecular detection of Diaporthe species in seed macerates using real-time
PCR assays could be used to quantify the fungal biomass in seeds, and perhaps even to
lower the pathogen detection threshold by comparison with conventional PCR assays.
Detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* on soybean seeds has been performed using real-time PCR assays with Taqman chemistry by comparison with other methods, including seed plating (Zhang et al., 1999). In the latter study, results related to real-time PCR assays and seed plating were comparable with respect to total infection and individual species detected in each seed lot. The total *Diaporthe/Phomopsis* detected on soybean seed lots ranged from 0 to 32%, indicating a very high infection level for some lots. The percentage of seed infection was not calculated in this study as the carrot seed plating assay was inefficient for *D. angelicae* and *D. eres* detection.

Based on results of this study, a hypothetical disease cycle for *D. angelicae* is proposed (Fig. 8). Perithecia are produced on black spots lesions that develop on carrot crop debris or on non-carrot crop debris. Once mature, these perithecia are able to project ascospores as demonstrated in both controlled (this study) and field (unpublished data) conditions. This suggests that ascospores of *D. angelicae* are involved as primary inoculum in carrot seed production fields and may, at least partly, explain the presence of this fungal species in umbel browning epidemiological studies. The germ tubes formed by ascospores probably penetrate the plant through a flower and the fungus can then progress to the umbel involucre. *Diaporthe angelicae* may then infect proximal flowers from the involucre, progressively leading to typical triangular necroses and later to stem necrosis. Secondary contamination may be caused mainly by conidia that develop in pycnidia that have been observed on carrot stem lesions in the field. These lesions mature to the perithecia-producing black spots on crop residues, which initiate a new fungal life cycle during the next growing season. Infected carrot seeds may transmit the pathogen to carrot seedlings, but this step has not been demonstrated. The involvement of non-carrot Apiaceae as alternative hosts of *D. angelicae* is also a possibility. On non-carrot Apiaceae,
ascospores that develop in perithecia on debris (Castlebury et al., 2003) are potentially a
source of primary inoculum. The role of *D. eres* was not included in Fig. 8 as its
contribution to the epidemiology of umbel browning in carrot remains to be determined.
Interestingly, *D. eres* was isolated from two wild Apiaceae species in this study (DE014
and DE015), suggesting that this fungal species can develop and/or survive on volunteer
plants, similar to *D. angelicae* (Castlebury et al., 2003; this study: DA080). By contrast, *D.
eres* was never isolated from cultivated parsley in this study, but the fungi isolated from
that plant species were much more restricted in number than those obtained from carrot (12
vs 87 isolates, respectively).

In conclusion, the results of this study highlighted that umbel browning and stem
necrosis in carrot seed crops in France, which can lead to significant losses in seed
production, are caused mainly by *D. angelicae*, and to some degree by *D. eres*. To our
knowledge, the disease has so far only been reported in France and may be a re-emerging
disease, as similar attacks were described previously on carrot in 1951 in the Netherlands
(Bakker, 1951). The greater genetic variability shown for the most prevalent species, *D.
angelicae*, than *D. eres* should be taken into account in the development of suitable control
methods, especially when considering fungicide treatments. Some isolates in this study
were identified as less sensitive to specific fungicides than other active ingredients. Lastly,
the study contains preliminary results justifying a more comprehensive assessment of the
transmission of *D. angelicae* and *D. eres* from carrot seeds. Both species were detected on
seeds harvested from production fields, and a greater frequency of *D. angelicae* was
detected on the tested seed lots than *D. eres*. Further studies are needed to gain greater
insight into this potentially crucial stage in the development of both *Diaporthe* species in
carrot.
Acknowledgements

We would like to thank M. Barret, N. Bataillé-Simoneau, P.E. Brandéis, N. Cazanove, H. Dumas, S. Leclerc, L. Mauge, L. Ménard, V. Odeau, S. Rezki and L. Robbes for their help. D. Manley is also gratefully acknowledged for reviewing the English. This work was supported by the French Ministry of Agriculture, Agrifood and Forestry under the call for projects for the plant breeding program in 2012 (Appel à projet développement agricole et rural du programme semences et sélection végétale, DIAPOCAR 2012-2015).
References


Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S,

Du Manoir J, Vegh I, 1981. Phomopsis foeniculi spec. nov. on fennel (Foeniculum vulgare

193-206.

Goodwin DC, Lee SB, 1993. Microwave miniprep of total genomic DNA from fungi, plants,
protists and animals for PCR. BioTechniques 15, 438-44.

reveals variable and conserved domains. Mycological Research 109, 87-95.

Laurent E, Blancard D, 2009. Phomopsis dauci, un nouveau pathogène des multiplications de

Ménard L, Brandeis PE, Simoneau P, Poupard P, Sérandat I, Detoc J, Robbes L, Bastide F,
Laurent E, Gombert J, Morel E, 2014. First report of umbel browning and stem necrosis
caused by Diaporthe angelicae on carrot in France. Plant Disease 98, 421-2.


**Figure Legends**

**Figure 1** Typical symptoms on carrot seed crop plants in France infected with *Diaporthe angelicae* or *D. eres*. (a) Triangular necrotic lesion on an umbel. (b) Necrosis progressing down a stem from the umbel. (c) Black spots on stem debris collected in a carrot seed production field after harvest.

**Figure 2** Morphological criteria used for identification of *Diaporthe* isolates collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. (a) Adaxial side of a culture growing on malt-agar medium. (b) Production of pycnidia on malt-agar medium. (c) α- (black arrowheads) and β- (white arrowheads) conidia produced in pycnidia. Black scale bar = 20 µm.

**Figure 3** Molecular identification of *Diaporthe angelicae* and *D. eres* isolates collected from carrot and parsley seed crops in France from 2010 to 2014. A phylogenetic tree reconstructed by the neighbor-joining method was obtained via alignment of the ITS rDNA sequences of 24 *D. angelicae* and nine *D. eres* isolates. ITS sequences from a seven additional *Diaporthe* spp. isolates were included. Bootstrap confidence measures > 50% from 1000 bootstrap replicates are indicated above the horizontal lines of the tree. The ITS sequence of *Diaporthe fibrosa* was used to root the tree. The strain identity and GenBank account numbers for ITS sequences are given in Table 1. *Diaporthe angelicae* reference isolates are indicated by asterisks. The *Diaporthe eres* reference isolate corresponds to DLR12A isolate.

**Figure 4** Geographic origin of *Diaporthe* isolates collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. *Diaporthe angelicae* (white
circles) and *D. eres* (black circles) isolates originated from plant lesions or infested crop debris at locations corresponding to the main carrot seed production areas in France.

**Figure 5** Intraspecific variability within *Diaporthe angelicae* and *D. eres* collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. The dendrograms were reconstructed by the neighbor-joining method from the alignment of the 3’ variable domain from IGS rDNA regions of (a) 13 *D. eres* isolates and (b) 17 *D. angelicae* isolates. Bootstrap confidence measures > 50% from 1000 bootstrap replicates are indicated above the horizontal lines of the trees. *Diaporthe scabra* was used as an outgroup to root the trees. Strain identity and GenBank account numbers for IGS sequences are given in Table 1.

**Figure 6** Sensitivity of *Diaporthe angelicae* and *D. eres* to nine fungicides collected from carrot and parsley seed crops in France from 2010 to 2014. EC$_{50}$ values (mg L$^{-1}$) of the fungicides (indicated as active ingredients) were measured to assess the sensitivity of five *D. angelicae* isolates (white circles) and four *D. eres* isolates (dark circles) to each active ingredient. The isolate reference numbers are indicated in the graph (left column); An *Alternaria dauci* isolate (hatched square) was included as a reference carrot pathogen. Commercial fungicide products corresponding to the nine active ingredients are specified in the Materials and Methods section. Each data point represents the mean and standard error of three replicate plates.

**Figure 7** Sexual stage of *Diaporthe angelicae* that developed in a controlled environment. (a) Perithecia (white arrowheads), one of which is showing a cirrhus (black arrowhead), observed on carrot stem debris (white scale bar = 5 mm). (b) Ascospores produced in a perithecium (black scale bar = 10 µm). (c) Experimental chamber used to induce perithecia formation and to measure the heights of ascospore ejection. Stem debris (red arrowheads)
was placed at the bottom of a closed, transparent, plexiglas box. An upside down, Petri dish with malt-agar medium and without the lid is indicated between the black arrowheads, and a piece of cellotape is indicated between the white arrowheads. Petri dishes and cellotapes were placed at 40, 60, and 80 cm above the crop residues to assess the height of the ascospore dispersion.

**Figure 8** Diagram describing the disease cycle of *Diaporthe angelicae* in carrot seed crops in France. The different steps of fungal development on carrot are indicated on the left side of the figure, while the involvement of non-carrot plants in the life cycle is presented on the right side. Parts of the life cycle that were demonstrated in this study are indicated by solid arrows, while elements derived from the published literature are indicated by hatched arrows. The dotted arrows correspond to hypothetical steps in the fungal development. a = ascospores; c = conidia; m = mycelium.
Characterization of fungal pathogens (*Diaporthe angelicae* and *D. eres*) responsible for umbel browning and stem necrosis on carrot in France

F. Bastide\(^a\), I. Sérandat\(^b\), J. Gombert\(^c\), E. Laurent\(^c\), E. Morel\(^d\), J. Kolopp\(^e\), P.L. Guillermin\(^f\), B. Hamon\(^a\), P. Simoneau\(^a\), R. Berruyer\(^a\) and P. Poupard\(^a\)*

\(^a\)Université d’Angers, UMR 1345 Institut de Recherche en Horticulture et Semences, SFR 4207 QUASAV, 49045 Angers, France; INRA, UMR 1345 Institut de Recherche en Horticulture et Semences, 49071 Beaucouzé, France; Agrocampus Ouest, UMR 1345 Institut de Recherche en Horticulture et Semences, 49045 Angers, France; \(^b\)IRHS, Agrocampus-Ouest, INRA, Université d’Angers, SFR 4207 QuaSaV, 49071, Beaucouzé, France; \(^c\)GEVES, 25 rue Georges Morel, 49071 Beaucouzé, France; \(^d\)FNAMS, Impasse du Verger, 49800 Brain-sur-l’Authion, France; \(^e\)FNAMS, 45 voie Romaine, BP 23, 41240 Ouzouer-le-Marché, France; \(^f\)VILMORIN, Route du Manoir, 49250 La Ménitré, France; \(^1\)HM CLAUSE, 1 chemin Moulin des Ronzières, 49800 La Bohalle, France.

*E-mail address: pascal.poupard@univ-angers.fr*

Running head: carrot umbel browning and stem necrosis

Keywords: Apiaceae, *Daucus carota* subsp. *sativus*, fungus detection on seed, necrotrophic fungi, *Phomopsis dauci*, seed production
Abstract

A collection of 102 Diaporthe isolates was compiled from lesions (umbel browning, stem necrosis or black spots on crop debris) on carrot, parsley and wild Apiaceae species, in France from 2010 to 2014. Molecular typing based on ITS rDNA sequences allowed the identification of 85 D. angelicae and 17 D. eres isolates, whereas phenotypical criteria (mycelial growth, and length and width of conidia) were not useful for species discrimination. Based on sequences in the IGS rDNA, intraspecific variability was analyzed on a sub-collection of 17 D. angelicae and 13 D. eres isolates representing diverse plant species, locations in France, and plant tissues. The genetic diversity analyzed was clearly greater for the D. angelicae isolates than in the D. eres. Experiments of in vitro sensitivity to each of nine fungicides using five D. angelicae and four D. eres isolates showed a similar response trend for isolates of both species, with a marked variation in effectiveness depending on the active ingredient. To respectively confirm and assess the pathogenicity of D. angeliace and D. eres isolates on carrot, one isolate from each species was tested on inoculated carrot umbels in a controlled environment. Typical umbel browning lesions were observed for both isolates. Carrot crop debris collected from seed production fields in France and placed in controlled conditions was shown to produce typical perithecia and ascospores typical of Diaporthe, that were further characterized molecularly as belonging to D. angelicae. Detection of both Diaporthe species on seed lots from three carrot production fields in France was investigated. Both species were detected on seeds by conventional PCR assay, with a higher frequency for D. angelicae (67 vs 33%, respectively), but not by traditional plating of seeds on an agar medium. Overall, our results highlighted...
that the disease umbel browning in carrot seed crops in France was mainly caused by *D. angelicae* and secondarily, but also can be caused by *D. eres*. 
Introduction

In 2014, carrot (*Daucus carota* L.) seed production was conducted on 2500 ha of land in France, which represents approximately 25% of the area devoted to vegetable seed production ([Daviot, 2014](#)). Since 2007, umbel browning symptoms have been observed regularly in carrot seed production areas in the Centre-Val de Loire Region ([Laurent & Blancard, 2009](#)). Triangular, necrotic lesions initially emerge on carrot umbels and can later spread to the entire umbel and often progress onto the stem. Diseased umbels become dry prematurely, thus jeopardizing seed development. Seed production loss was estimated at approximately 8% of harvested carrot umbels during the 2007 and 2008 spring and summer cropping seasons in France ([Laurent & Blancard, 2009](#)). A fungus was isolated from the diseased tissues and was identified as *Diaporthe angelicae* (Berk.) Farr and Castlebury, comb. nov. based on morphological and molecular criteria ([Ménard *et al.*, 2014](#)). The current name of *D. angelicae* proposed in the Index Fungorum ([www.indexfungorum.org/names/names.asp](http://www.indexfungorum.org/names/names.asp)) is *Mazzantia angelicae* (Berk.) lar. N. Vassiljeva (1993), but this name has not been used in the recent literature. The disease resembles the lesions described in the Netherlands in 1951 on carrot inflorescences and as being caused by *Phomopsis dauci* ([Bakker, 1951](#); [von Arx, 1951](#)). The presence of typical umbel browning and stem necrosis lesions on carrot, parsley, dill and parsnip has been reported in seed production fields located in southwestern, western and southeastern France; and seeds produced on diseased umbels were less numerous and smaller than those that developed on asymptomatic umbels, therefore at least partly explaining the seed production losses reported above ([J. Gombert, Fédération Nationale des Agriculteurs Multiplicateurs de Semences (FNAMS), Brain-sur-l’Authion, France, personal communication](#)).
Diaporthe (=Phomopsis, Ascomycota, Diaporthales) corresponds to a very large fungal genus comprising at least 800 taxon names, while around 900 species have been described in the anamorphic Phomopsis genus. (Rossman et al., 2007; Uecker, 1988). Diaporthe/Phomopsis species are known to be endophytic fungi, especially on woody plants (Rossman et al., 2007). The species D. angelicae (syn. Mazzantia angelicae) was described precisely in the taxonomic study of Castlebury et al. (2003). It is a member of the Ascomycota group, which can develop typical fungal structures, with pycnidia containing α- and β-conidia (asexual stage) and perithecia producing ascospores (sexual stage). In the same study, the authors highlighted the presence of D. angelicae in Europe, Canada and USA on decaying stems belonging to various wild plants in the Apiaceae family, such as Angelica spp., Anthriscus spp., Daucus spp., or Eryngium spp. The host range of this fungal species is not restricted to cultivated Apiaceae species. Umbel lesions similar to those described on cultivated carrot and caused by Phomopsis diachenii were reported on caraway (Carum carvi) in Bulgaria (Rodeva & Gabler, 2004) and Hungary (Nagy, 2010). Umbel blight symptoms have also been described on wild and cultivated fennel and were assigned to Diaporthe foeniculacea/Phomopsis foeniculi in France (Du Manoir & Vegh, 1981), Portugal (Philips, 2003) and Bulgaria (Rodeva & Gabler, 2011). Santos & Philips (2009) isolated a complex of four Diaporthe species from fennel: D. ambigua, D. angelicae, D. lusitanicae and D. neotheicola. This suggests that the situation could be the same on cultivated carrot, although only D. angelicae and P. dauci have been described to date on this plant species (Bakker, 1951; von Arx, 1951; Ménard et al., 2014).

Some molecular data based on sequences of the large subunit nuclear ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions are available...
for *D. angelicae* characterization and phylogenetic studies (Castlebury et al., 2003). The four *Diaporthe* species present on wild fennel, including *D. angelicae*, were characterized by Santos & Philips (2009) using microsatellite-primed PCR profiles (MSP-PCR) and phylogenetic analysis based on ITS rDNA ITS sequences. More recently, mating-type (MAT) genes in *Diaporthe* and *Phomopsis* were considered as phylogenetic markers for biological species definition, by comparison with ITS rDNA and elongation factor 1α sequences (Santos et al., 2010). The results of this latter study showed that *D. angelicae* and *P. dauci* isolates were in the same cluster, highly suggesting that these species may correspond to the same fungal pathogen that causes umbel browning and stem necrosis on carrot.

Different *infection inoculum* sources responsible for carrot umbel browning were suggested on the basis of the studies of Bakker (1951) and von Arx (1951) focused on *P. dauci* epidemiology: *infested* crop debris, infected seeds, *infected* wild or cultivated Apiaceae species near carrot seed crops and pollinator insects, *serving as vector of the fungus*. However, to our knowledge, *fungal transmission* of *P. dauci* to carrot seeds and from seeds to seedlings has never been demonstrated and has yet to be studied. More generally, a better understanding of the *D. angelicae* life cycle is required to develop adequate control methods and reduce the pathogen impact *on carrot seed production*. Prophylactic methods, including elimination or burying of *infested* crop debris, could be efficient (Bakker, 1951). Field experiments to select fungicides that could limit *D. angelicae* development *in carrot seed crops* were recently performed in France by the Fédération Nationale des Agriculteurs Multiplicateurs des Semences (FNAMS). Based on these experiments, *chemical fungicide* control has already been proposed to carrot seed growers (E. Morel, FNAMS Ouzouer-le-Marché, France, personal communication), while
taking into account the importance of reducing fungicidal treatments required for the environment and human health protection (Ecophyto plan proposed by the French Ministry of Agriculture, Agrifood and Forestry, [http://agriculture.gouv.fr/ecophyto-kesako-0](http://agriculture.gouv.fr/ecophyto-kesako-0)).

The general aim of this study was to better characterize the fungal pathogen(s) causing umbel and stem lesions on carrot. A seed crop in France using a collection of isolates originating from different seed production fields in France was thus compiled, corresponding to a much larger plant sample than previously reported (Ménard et al., 2014). The collected isolates specific objectives were described according to different assessments, including molecular analyses for species, i.e. *D. angelicae* and *D. eres*. In order to gain further insight into the pathogenesis processes, we observed fungal development after two modes of artificial infection identification, (i) pathogenicity on carrot cuttings grown in a controlled environment. The production of perithecia and ascospores from carrot crop debris was also investigated in controlled conditions. Lastly, (iv) sensitivity of the isolates to fungicides commonly used in carrot seed crops in France, and (v) the detection of the fungal pathogens on carrot seed lots was tested using different assays based on seed plating on an agar medium or by PCR diagnosis assay. A hypothetical disease cycle was proposed based on the basis of these different results of this study.
Materials and Methods

Collection of field isolates and reference isolates

Between 2010 and 2014, a total of 102 Diaporthe spp. isolates were collected: 99 isolates originated from carrot or parsley plants from seed production fields in different French localities, and three strains (DA080, DE014, and DE015) were isolated from wild Apiaceae species (Table 1). Fungal strains were isolated from lesions developed on umbel involucres or stems, and from black spots on crop debris (Fig. 1). Lesions Plant tissues with lesions (umbel browning or stem necrosis) or sections of crop debris with black spots on debris were surface-sterilized for 2 min in 1% sodium hypochlorite, and rinsed in two changes of sterilized distilled water for 5 min each. Approximately 5 mm² of tissue from the boundary of a lesion and healthy tissue on each section of plant tissue were excised in a laminar flow cabinet, plated in a Petri dish onto malt (1%) agar (1.7%) medium (MA) amended with streptomycin (0.005%), and incubated for 1 week at 20°C in darkness. For long-term storage, all isolates were kept under cryogenic conditions (using a 30% glycerol solution as cryoprotectant) at -80°C. Three representative cultures of D. angelicae isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS), the Netherlands (DA001 to DA003, ref. CBS111592, CBS123214, and CBS123215, respectively) (Table 1). One isolate from this French D. angelicae collection (DA004) was deposited at the CBS in 2013 (ref. CBS137262) (Table 1).

Species identification using ITS rDNA sequences
Fungal isolates were first identified at the species level in a part of the fungal collection (29 isolates). The isolates were selected on the basis of their origin (host plant, organ, location) and year of isolation. DNA was extracted from a fungal colony of each fungal isolate according to Goodwin & Lee (1993). PCR amplification of the ITS1-5.8S-ITS2 region of rDNA was carried out with ITS1 and ITS4 universal primers (Table 2) (White et al., 1990). The reaction mixture (25 µL) consisted of 1 µL of 10-fold diluted fungal DNA, 0.5 U of GoTaq® polymerase (Promega), 1× reaction buffer, 1.4 mM MgCl₂, 10 pmol of each primer set, and 0.2 mM dNTPs. PCR amplification was performed with the following parameters: initial denaturation at 94°C for 3 min; 40 denaturation cycles at 94°C for 30 s, annealing at 56°C for 50 s, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. Amplification products were resolved on 1.2% agarose gels in 0.5× TAE buffer (20 mM Tris-acetate, pH 8, 0.5 mM EDTA) followed by ethidium bromide staining. For species identification, PCR products were purified using a PCR purification kit (NucleoSpin Extract II, Macherey-Nagel) and sequenced using the commercial sequencing service of GATC Biotech AG. (Konstanz, Germany). BLAST searches of repository information from GenBank were conducted using the ITS sequences (see GenBank acc. nos in Table 1). ITS sequences were aligned using CLUSTAL W software to design specific primers at the genus level (DiapITSfw and DiapITSrv primers) or species level (AngelITSfw and AngelITSrv primers for D. angelicae; and EresITSfw and EresITSrv primers for D. eres) (Table 2). PCR amplification using these primers was performed with the same conditions as described above for the universal primers. Phylogenetic analysis from the sequenced, amplified fragments was carried out using the Phylogeny.fr web service (Dereeper et al., 2008). Sequences were aligned with MUSCLE and a dendrogram was constructed using the neighbour joining algorithm (Saitou & Nei, 1987). Bootstrap confidence values were
calculated from 1000 randomly re-sampled data sets. The sequences of the amplified products (GenBank acc. nos indicated in Table 1) were compared to the ITS sequences of three *D. angelicae* reference isolates (CBS111592, CBS123215 and CBS137262, referred to as DA001, DA003 and DA004 in Table 1) and one *D. eres* reference isolate (DLR12A, Udayanga *et al.* 2014, see Table 1). Seven additional ITS sequences representing closely related *Diaporthe* species (*D. foeniculacea*, *D. helianthi*, *D. juxtaniciae*, *D. neotheicola*, *D. phaseolorum* var. *sojae*, *D. rudis*, *D. subordinaria*) were also included in this analysis (Table 1). The ITS sequence of *Diaporthe fibrosa* CBS113830 was used to root the tree.

**Morphological characterization and fungicide sensitivity**

Growth rates of five replicate cultures of each of 15 *D. angelicae* and 15 *D. eres* isolates (see underlined accessions in Table 1) were measured after 7 days on MA medium at 20°C in darkness, and the mean (± standard deviation (SD) was calculated. For each isolate, colony colour and aspect were recorded. Pycnidia were produced on MA medium after 1 to 3 weeks at 20°C under near-ultraviolet light (0.5 W m⁻²; Coaxel, L18/73) under a 12 h photoperiod/day. The size (length and width) of conidia produced in pycnidia was measured for the 30 isolates, using a total of 45 α-conidia and 45 β-conidia per species. All of these data were analyzed using analysis of variance (ANOVA) to assess the isolate and species effect. Residual normality was checked *post hoc* using Q-Q plots. Residuals were found to be normally dispersed for all of the data presented here. All morphological data are given as the mean ± SD.
The *in vitro* mycelial sensitivity of five *D. angelicae* and four *D. eres* isolates (accessions indicated in bold in Table 1) to each of nine fungicides (formulated and tested as technical grade) was tested. An *Alternaria dauci* isolate (FRA017, isolated in 2000) was used as reference pathogen for some experiments. *Alternaria* leaf blight is indeed mainly controlled by fungicide applications except one isolate (DA035, from parsley). The active ingredients used were picoxystrobin (Acanto, DuPont, Wilmington, USA), fludioxonil (Geoxe WG, Syngenta, Basel, Switzerland), boscalid (Pictor Pro, BASF, Ludwigshafen, Germany), iprodione (Rovral, BASF), pyrimethanil (Scala, BASF), difenoconazole (Score, Syngenta), fenhexamid (Teldor, Bayer CropScience, Leverkusen, Germany), methyl thiophanate (Topsin, Certis Europe, Utrecht, The Netherlands) and cyprodinil (Unix Max, Syngenta). An *Alternaria dauci* isolate (FRA017, isolated in 2000 from naturally infected carrot leaves in Gironde, France), was used as a reference pathogen for the experiments dealing with picoxystrobin, fludioxonil, boscalid and difenoconazole. In France, *Alternaria* leaf blight is controlled mainly by fungicide applications. Some of the fungicides used or tested in France are composed of these four active ingredients (E. Morel, FNAMS Ouzouer Le Marché, France, personal communication). Mycelial disks (8-mm dia.) removed from the margins of a 7 day old culture of each isolate were transferred to MA medium for each active ingredient except pyrimethanil and cyprodinil. For these last two fungicides, fungal cultures were prepared on minimal Vogel medium (Vogel, 1956) to avoid the presence of amino acid sources, as pyrimethanil and cyprodinil act as inhibitors of amino acid synthesis. MA or Vogel media were amended with the appropriate fungicide at final concentrations of 0.1, 1, 10, 100 and 1000 mg L\(^{-1}\). For the
picoxystrobin sensitivity assay, salicylhydroxamic acid (SHAM) was added at 28 mg L\(^{-1}\) was added into the MA medium to prevent the fungus from overcoming the fungicide toxicity through an alternative oxidative pathway (Wise et al., 2008). Three replicate plates were used per treatment combination (fungal isolate - fungicide) and arranged in a completely randomized design. For each fungicide concentration tested, inhibition of radial growth compared with the untreated control plate was calculated after 3 and 6 days of incubation at 20°C in darkness for each active ingredient except pyrimethanil and cyprodinil, and after 6 and 13 days of incubation at 20°C in darkness for pyrimethanil and cyprodinil. The mycelial growth was slower on Vogel medium than on MA. The results were expressed as half maximal effective concentration (EC\(_{50}\); the concentration which reduced mycelial growth by 50\%\,), which determined by regressing the log10 values of the fungicide concentrations against the inhibition of radial growth values (% control). All statistical analyses were performed using R-2.6.1 software (R Development Core Team, 2010).

**Intraspecific genetic variability based on IGS rDNA sequences**

The complete IGS sequences of *D. angelicae* isolate DA057 (4485 bp) and of *D. eres* isolate DE009 (3673 bp) (GenBank acc. nos KP406523 and KT071711, respectively) were obtained from PCR fragments by using the 26S3111F and IGS27 primer set as previously described in Hong et al. (2005) and Subbarao et al. (1995). PCR fragments were cloned using the pGEM-T vector (Promega) according to the manufacturer’s protocol, and clones were sequenced at GATC Biotech AG. The IGS sequences were aligned using CLUSTAL W software in order to design internal primers for the intraspecific genetic variability study. A 403 bp portion in the 3’ variable domain from the IGS sequences of 17 *D.*
angelicae and 13 D. eres isolates (accessions indicated by an asterisk in Table 1) was amplified using the primers VariabIGS3’fw and VariabIGS3’rv (Table 2) in the PCR conditions described above. In order to analyse the intraspecific genetic variability in the 5’ domain from the IGS of sequences of the same 17 D. angelicae and 13 D. eres, a 810 bp or 461 bp portion was amplified, respectively, using the primers VariabIGS5’fw and VariabIGS5’rv1 or the primers VariabIGS5’Tfw and VariabIGS5’rv2, respectively (Table 2). The same set of isolates as described above for the 3’ IGS domain analysis was used for these amplifications. Phylogenetic analysis from the sequenced amplified fragments (see GenBank acc. nos in Table 1) was carried out as described above for species identification using ITS rDNA sequences.

Pathogenicity testing

In the summer of 2013, cuttings comprising umbels and attached 20 cm stemlength of the stem of a carrot Nantes type open pollinated cultivar, were collected at developmental stage 65 (Feller et al., 1995) in an experimental seed production field located at FNAMS, Brain-sur-l’Authion, France (GPS coordinates: 47.472132°N, 0.397789°W). Cuttings were planted in boxes (30 cm × 30 cm × 6 cm, with 3 cuttings per box) containing a 4 cm depth of sand moistened with 400 mL nutrient solution (Hakaphos-Vert, COMPO) used at 2 g L⁻¹ (w/v) in distilled water. Boxes were placed in a growth cabinet at 20°C, white light (OSRAM, L58W/830) under a 12 h photoperiod day⁻¹ and 80% relative hygrometry-humidity. Diaporthe angelicae isolate DA016 and D. eres isolate DE003 were grown in the conditions as described above for pycnidia production in order to obtain conidia. The conidial suspensions were prepared at a final
concentration of $1 \times 10^7 \alpha$-conidia per mL in distilled water containing 1% of Tween 20 and 10% of glycerol (WTG solution). Two inoculation methods were used: either the umbel was sprayed with the inoculum to runoff (around 3 mL of inoculum per umbel) or 10 µL of inoculum were deposited with a micropipette at the base of the umbel (involucre, which was wounded before inoculation) onto a wound created on the involucre using a disinfected scalpel blade. Three replicate umbels were used for each inoculation method and isolate. Control umbels were sprayed with WTG solution to runoff (three umbels) or by depositing 10 µL WTG solution at the wounded involucre (three umbels). Inoculated umbels were placed in boxes in the same growth chamber, arranged in a randomized design. The time (in day numbers) for development of the first lesions on umbels was assessed after plant inoculation. The fungus was re-isolated from umbels using the conditions described above, and PCR identification assays of the isolates were performed using the species-specific ITS primers designed for *D. angelicae* and *D. eres* (Table 2). The whole experiment was repeated twice.

### Perithecia production

In 2012, crop debris showing characteristic black spots were collected in a naturally infected carrot seed production field in Loir-et-Cher, France, GPS coordinates: (47.654.766°N, 1.363743°E). The debris was deposited in a closed Plexiglas box containing moist Fontainebleau sand in a controlled environment [growth cabinet at 20°C, with white light (OSRAM, L58W/830) under a 12 h photoperiod day$^{-1}$ and 80% relative humidity]. In these conditions, the crop debris was observed regularly.
observed for 35 days, and the time required for durations until pycnidia, perithecia and cirri formation were recorded. A fragment of debris (10 cm length) was thoroughly observed to determine the number of pycnidia and perithecia. Cirri produced from the perithecia to top perithecial ostioles were collected using a micropipette in order to observe the morphology and size of ascospores and measure their size under a microscopically (Nikon Eclipse 80i, G.E.O. Micro Service). In order to study ascospore ejection from perithecia, 90-mm diameter opened Petri dishes (containing MA medium) and cellulose pieces were placed in the Plexiglas box at different heights (40, 60 and 80 cm) above the perithecia-producing debris (40, 60 and 80 cm) at day 12 of the experiment. The presence of colonies in the Petri dishes and of ascospores on the cellulose pieces was assessed after 14 days. Fungal identification was performed using the morphological and molecular criteria as described above (aspect, appearance and colour of colonies, morphology and size of ascospores, and PCR assay identification using based on ITS sequences species-specific primers). The whole experiment was carried out twice.

Pathogen detection on carrot seeds

In 2009, carrot seeds were collected at harvest stage from three carrot seed production fields (named I, II and III, and located) in Loir-et-Cher, France (GPS coordinates: 47.720341° N, 1.485171° E; 47.888470° N, 1.405769° E; and 47.655874° N, 1.359911° E, respectively). Typical umbel browning and stem necrosis damage caused by D. angelicae were observed in these carrot fields, as previously reported (Ménard et al., 2014). For each
field, three seed samples were harvested from diseased symptomatic umbels (sample A), three from asymptomatic umbels collected from diseased symptomatic plants (sample B), or on and three from asymptomatic umbels collected from asymptomatic plants (sample C).

As a positive control, seeds were harvested from umbels that were artificially contaminated with strain DA016 (D. angelicae positive control) or DE003 (D. eves positive control). For pathogen detection on MA amended with streptomycin (0.005%) amended plates, 200 seeds of each of the nine samples (three samples per field × three fields) were surface-sterilized for 2 min in 1% sodium hypochlorite and rinsed in two changes of sterilized distilled water for 5 min each. In a laminar flow cabinet, disinfected seeds were dried on a sterilized blotting paper, placed on the surface of the MA Petri dishes and incubated for 1 week at 20°C in darkness. The same experiment was performed using 200 non-disinfected seeds of each sample. Fungal colonies that developed from the seeds were inspected using basic characteristic morphological criteria for Diaporthe spp. isolates (aspect morphology and colour of mycelia). For pathogen detection using molecular tools, 25 non-disinfected seeds of each of the nine samples were freeze-dried in a Christ Alpha 1–4 LSC apparatus (Fisher Bioblock Scientific), ground in a mortar in the presence of liquid nitrogen and stored at –20°C. DNA extractions from these seed samples were performed according to Briard et al. (2000), with an additional DNA precipitation step in isopropanol for 5 min on ice. Alternatively, DNA was extracted from macerates of the seed prepared using 1000 non-disinfected seeds (ca. 1.85 g) of each of the nine samples. Seed batches were each placed in a 50 mL tube (VWR North America) and incubated with 10 mL phosphate buffer saline (PBS, Sigma-Aldrich) containing 0.02% Tween 20 (v/v) for 12 h at 4°C under shaking (140 rpm). Seed macerates were centrifuged (6000 g, for 15 min, at 4°C), the supernatant was discarded and the pellet was dissolved in 1 mL supernatant. DNA was extracted from the concentrated macerate using
the Powersoil DNA isolation kit (MO-BIO, Novozymes) according to the manufacturer’s recommendations. A tissue crusher (MM30, Retsch France) was used at 300 Hz for 2 min. The last DNA elution step was achieved using 60 µL elution buffer (instead of 100 µL, as recommended in the manufacturer’s protocol). The DNA samples were stored at 0–20°C until use. For both DNA sources (obtained from entire seeds or seed macerates), PCR amplifications using 1/10 diluted-DNA in water, were carried out as described above for Diaporthe species identification. Four primer sets were used (Table 2): ITS1 and ITS4 (for PCR control samples), DiapITSfw and DiapITSrv (for identification at the genus level), AngelITSfw and AngelITSrv (for D. angelicae identification), and EresITSfw and EresITSrv (for D. eres identification). All PCR experiments were carried out twice.
Results

Isolation of *Diaporthe* spp. from field samples

A collection of 102 fungal isolates was compiled from carrot and parsley infected plants sampled in different French seed crop productions fields between 2010 and 2014 (Table 1). A total of 102 fungal colonies showing all developed white pigmentation and a woolly aspect were isolated aerial mycelium on MA medium (Table 1, Fig. 2a). The same mycelium was white color was observed on the top and on the reverse side of the agar colonies. Isolates were mainly collected mainly from carrot lesions (87 isolates) vs. parsley (12 isolates on parsley). Three isolates originated from lesions observed on wild Apiaceae species. Pycnidia (Fig. 2b) appeared between 6 to 23 days after incubation in culture conditions described above for sporulation, with the exact duration depending on the isolate (n=78 isolates (n=78)). Typical \( \alpha \) and \( \beta \) conidia were produced in these pycnidia (Fig. 2c), with a longer time for \( \alpha \)-conidia (between 12 and 35 days after incubation vs. 6- to 32 days for \( \beta \)-conidia production). These morphological criteria were in agreement with those previously reported in Ménard et al. (2014) for characterization of a few Diaporthe isolates collected on carrot plants in France.

Identification and characterization of *D. angelicae* and *D. eres* isolates

Fungal isolates were first identified at the species level in a part of the fungal collection (29 isolates). The isolates were selected on the basis of their origin (host plant, organ, location) and year of isolation. DNA was extracted from mycelium of the 29 isolates and the ITS region of the ribosomal DNA was amplified using universal primers (Table 2). The sequences of the amplified products (GenBank acc. nos indicated in Table 1) were aligned
and compared to the ITS sequences of three *D.* _angelicae_ reference isolates (CBS111592, CBS123215, and CBS137262, referred to as DA001, DA003, and DA004 in Table 1) and one *D.* _eres_ reference isolate (DLR12A, Udayanga et al. 2014, see Table 1) through phylogenetic analysis (Fig. 3). Eight additional ITS sequences representing closely related *Diaporthe* species (*D.* _fibrosa_ from the French *foeniculacea*; *D.* _helianthi; D.* _luteojae; D.* _neotheicola; D.* _phaseolorum var. sojae; D._ _rudis; D._ _subordinaria_) were also included in this analysis (see Table 1 for references and GenBank acc. nos of the relevant isolates). The ITS sequence of *Diaporthe paraeparisi* CBS133184 was used to root the tree. The 29 isolates of our field collection were identified to the species level using ITS rDNA sequencing. They grouped into two clusters (Fig. 3): one group was composed of 21 isolates, showing 100% identical sequences with the ITS rDNA sequences of *D.* _angelicae_ reference isolates, DA001, DA003, and DA004, while the other group consisted of eight isolates exhibiting 100% sequence identity with the ITS sequence of the *D.* _eres_ reference isolate. As a second step, in order to identify all isolates of the collection at the species level, specific (DLR12A) PCR assay using ITS primers were designed for species detection of *D.* _angelicae_ or *D.* _eres_ (Table 2). DNA was extracted from the mycelium and typing of fungal isolates was performed by PCR amplification, which allowed us to identify 85 of the 120 isolates as *D.* _angelicae_ isolates and 17 as *D.* _eres_ isolates in the collection (Fig. 4). Interestingly, although most isolates belonged to the *D.* _angelicae_ species, in some of the localities three locations where a few one or two isolates were collected, all of them belonged to the isolates were *D.* _eres_.

The ANOVA for mycelial growth rate on MA medium was measured for of 15 *D.* _angelicae_ isolates (DA074 to DA088) and 15 *D.* _eres_ isolates (DE001 to DE003;
DE005; DE007 to DE017). Observed growth rates were analyzed using ANOVA with either species or isolate used as a factor. Although a clear effect was observed (F test p < 2.2 \times 10^{-7}) but no statistical difference (p = 0.44) was noted between the mean growth rate of both the two species (D. angelicae: 7.65 ± 0.10 mm·day\(^{-1}\), D. eres: 7.54 ± 0.10 mm·day\(^{-1}\)), with growth rates ranging from 6.22 ± 0.19 mm·day\(^{-1}\) (for isolate DE002) to 8.91 ± 0.20 mm·day\(^{-1}\) (for DA087) on an isolate basis. Pycnidia formation was induced on MA medium using the same D. angelicae and D. eres isolates. The length and width of α- and β-conidia were measured and the results were also analyzed using not significantly different based on the ANOVA with either species or isolate used as a factor. No significant difference (p > 0.05) in width was observed among the isolate isolates nor between the two species levels of Diaporthe. The mean of mean α- and β-conidial lengths however, were significantly greater for D. eres (mean ± SE) than for D. angelicae (mean ± SE) (p < 1 \times 10^{-4}, Table 3). Nevertheless, these criteria were not diagnostic, as the range in α- and β-conidial lengths of the two species overlapped; some D. angelicae isolates sported longer α- and β-conidia than those from some D. eres isolates (Table 3).

Intraspecific variability within D. angelicae and D. eres

In order to assess the isolate diversity within D. angelicae and D. eres, the complete IGS sequence of one isolate for each species was determined. Sequences of DA057 and DE009 isolates (4485 bp and 3673 bp respectively) were aligned. Primers were designed in the variable 3' part and 5' part of the IGS region (Table 2). The corresponding amplified PCR products were sequenced for 17 D. angelicae isolates and 13 D. eres isolates selected in
the collection (see Table 1). Analysis of the aligned nucleotide sequences in the 3’ part of the IGS rDNA sequences (403 bp) revealed that: i) all isolates of D. eres, except DE011, were grouped within the same cluster (Fig. 5a); and, ii) D. angelicae isolates were grouped within two main clusters, with one comprising isolates DA016, DA046 and DA072 (isolated from carrot and parsley), and the other including the remaining 14 other isolates of this species that were sequenced (Fig. 5b). In this latter group, three sub-clusters were evident: one including DA057 and DA080 (isolated from carrot and wild Daucus species, respectively); one comprising the CBS reference isolates DA001 and DA003 (isolated from Heracleum sphondylium and Foeniculum vulgare, respectively); and one including 10 isolates (seven isolated from carrot and three from parsley). There was no evidence of clustering according to host or geographic origin in the D. angelicae isolates. This also seemed to be the case concerning D. eres, as two isolates collected from wild Apiaceae species (DE014 and DE015) were grouped with isolates from carrot plants collected at different locations in France.

A Phylogenetic analysis of a portion of the 5’ part of the IGS was amplified using IGS rDNA for the same sub-collection of 17 D. angelicae isolates (810 bp) and 13 D. eres isolates (461 bp). Phylogenetic analysis was carried out with aligned D. angelicae sequences, using the sequence of D. eres isolate DE009 to root the tree. All showed that all the D. angelicae isolates were in the same cluster, except the reference isolate DA001 (results not shown). A similar result was obtained when comparing a portion of the sequences of 5’ part of the amplified IGS rDNA of 13 D. eres isolates (461 bp) using the sequence of DA057 to root the phylogenetic tree. As already observed above in Fig. 7,
all Twelve of the thirteen D. eres isolates clustered together, with the exception of DE011 (results not shown).

In vitro fungicide sensitivity of D. angelicae and D. eres

The in vitro sensitivity to nine fungicides was determined for five EC$_{50}$ of the D. angelicae isolates and four D. eres isolates. Isolates of this sub-collection were selected according to their geographical origin and the lesion organ on the host (Table 1); all isolates were collected from carrot lesions, except one isolate (DA035, from parsley). The IC$_{50}$ results (Fig. 6) showed that isolates of both Diaporthe species generally exhibited a similar response trend to the nine fungicides. As expected, the sensitivity of D. angelicae and D. eres isolates to the different active ingredients was highly variable, in a 0.01 to 1000 mg L$^{-1}$ (1 to 100 000) range (Fig 6). Isolates of both species were usually highly sensitive to difenoconazole (EC$_{50}$ < 0.15 mg L$^{-1}$) and very weakly sensitive to boscalid and pyrimethanil (EC$_{50}$ > 188 mg L$^{-1}$), while intermediate IC$_{50}$ values were obtained with other fungicides. At the intraspecific level, the different isolates showed quite similar sensitivity. Contrastingly, the EC$_{50}$ variations were in a somewhat narrower range when each active ingredient was considered separately. Narrow sensitivity ranges were observed for some of the tested fungicides, such as iprodione (1 to 2.3), boscalid (1 to 2.7), methyl thiophanate, iprodione or (1 to 2.8) and fenhexamid, (1 to 4.66). By contrast, the IC$_{50}$ values obtained with difenoconazole or (< 1 to 15) and fludioxonil (< 1 to 360) were more variable, depending on the isolates. As some active ingredients were present in fungicides currently used or tested against A. dauci, the sensitivity of a reference isolate of this pathogen against pycoxystrobin, difenoconazole, and fludioxonil and boscalid was also tested. The IC$_{50}$ values of the A. dauci reference isolate FRA017 of A. dauci.
*dauci* reference isolate FRA017 relative to pycoxystrobin, fludioxonil and difenoconazole were included within those obtained with *Diaporthe* isolates, but this was not the case for boscalid: was within the range of the *Diaporthe* isolates. However FRA017 was indeed very sensitive to this fungicide boscalid (EC$_{50}$ < 0.01 mg L$^{-1}$).

Pathogenicity of *D. angelicae* and *D. eres*

The pathogenicity of one isolate for each species (Both *D. angelicae* isolate DA016 and *D. eres* isolate DE003) was tested on carrot cuttings in a controlled environment. Two inoculation methods were used, i.e. either by spraying a conidial suspension on the umbel or by depositing the conidial suspension on the umbel involucre. Lesion development was checked regularly after plant inoculation and the time, in number of days, to obtain the first flower and involucre necrosis was assessed in the different experimental conditions. Statistical analysis was carried out on values obtained from two repetitions of the whole experiment. For both isolates, the first caused lesions on inoculated carrot umbels. Lesions appeared on average of 11.5 days after the conidial spray, while the first symptoms were observed at 21.5 days and 23.0 days after inoculum deposit using a spore suspension was deposited in a wound on the umbel involucre for DE003 and DA016 isolates, respectively. The results related to each inoculation mode from the two repetitions of the experiment were significantly different, while between the two inoculation methods (p=0.000266 with 11.50 ± 0.711 days for spraying a spore suspension vs. 22.25 ± 0.711 days for the wound inoculation). However there was no statistical difference was obtained between isolates for the same inoculation method when comparing the time duration for lesion development between both isolates; lesions to develop. The lesion aspect symptoms i.e. typical flower and involucre browning, was were
very similar after *plant*-inoculation with *isolate* DA016 or *isolate*-DE003. Both isolates were also able to *develop* cause stem necrosis under these conditions. The inoculated *isolates* fungi were re-isolated from lesions and their *identified* to *species* by molecular characterization was performed by (PCR *assay*) using the *species*-specific ITS primers for *D. angelicae*/*D. eres* (results not shown).

**Sexual stage of *D. angelicae***

Carrot crop debris collected in a naturally infected field was placed in controlled conditions in a Plexiglas box (Fig. 7c). The debris was regularly inspected for 35 days for pycnidia and perithecia formation. A fragment of debris (10 cm length) was thoroughly observed to measure the time for the development of these fungal structures and their number. On this fragment, pycnidia (carrot residue fragments) developed pycnidia from 5 days after incubation in the box-controlled environment chamber and a total of 41 pycnidia were assessed observed on the 10 cm-long residue section during the 35-day observation period.

On the same fragment of debris, perithecia were formed from 12 days after incubation, and cirri were first observed at the top of these structures at day 25 (Fig. 7a). A total of 82 perithecia developed on the 10 cm fragment during the experiment. A cirrus was collected to observe ascospore morphology (Fig. 7b). The size of the ascospores was measured under a microscope (collected in cirri averaged 12.3 ± 4.9 µm × 2.3 ± 0.4 µm, *n=85*).

Ascospore ejection from perithecia was supposed to occur before cirri development on perithecia. In order to verify this assumption, opened Petri dishes containing MA medium and cellotape pieces were placed in the Plexiglas box 40, 60 and 80 cm above perithecia.
producing debris at day 12 of the experiment. Two weeks later, ascospores) (Fig. 7b).

Ascospores showing a similar aspect and size as those presented in Fig. 7b were observed microscopically observed at day 26 of the experiment on the cellotape pieces placed at 40 and 60 cm and 60 cm above the symptomatic carrot residues incubated in the environmental chamber. No fungal spores were present on the cellotape pieces at 80 cm above the perithecia. The presence of typical Typical Diaporthe colonies (white colour and wolly aspect) was evident in mycelium) developed in the Petri dishes placed 40 and 60 cm above the Petri dishes placed at 40 and 60 cm-residues. No mycelial growth was observed in the dishes placed at 80 cm. Molecular characterization of fungal colonies that developed in Petri dishes was achieved by PCR amplification with the D. angelicae-specific ITS primers allowing the identification of D. angelicae isolates (results not shown). The same results were obtained after repetition with the repeat of the whole experiment. Although some of the D. eres isolates of our in this collection originated from crop debris (DE003 to DE007 and DE010), perithecia production by this species was not observed in the experimental from the residue samples collected and incubated in the conditions described above.

Diaporthe angelicae and D. eres detection on carrot seeds

Seed samples were collected in three carrot seed production fields (fields referred to I, II and III). For each field, three types of seed lots were tested for D. angelicae and D. eres detection: seeds of sample A were harvested on diseased umbels, while samples B and C were composed of seeds harvested on asymptomatic umbels, from diseased plants and asymptomatic plants, respectively. Using a conventional mycelial growth test, 200 seeds from each of the nine samples (three samples per field × three fields) were disinfected or
not disinfected and plated on MA medium. Developing fungal colonies were inspected for *Diaporthe* sp. identification on the basis of their aspect and colour. For all tested carrot seed lots (disinfected or not), no fungal colonies exhibiting morphological criteria of *Diaporthe* spp. developed on agar medium (Table 4). Molecular detection of *Diaporthe* sp. was investigated by conventional PCR on assays of subsamples of the same seed samples, with DNA was extracted from entire seeds or from seed macerates and PCR amplifications were achieved, using specific ITS primers for specific for *D. angelicae* or *D. eres* resulted in detection of *D. angelicae* or *D. eres* identification (Table 4). Using the first protocol based on entire seeds, *D. angelicae* was detected in all three seed lots corresponding to diseased umbels (whether using whole seed or seed macerates (A samples A)), and in only one seed lot from an asymptomatic umbels (field I), while *D. eres* was not detected in any of these seed lots tested in the same conditions. *Diaporthe angelicae* detection in A samples was also evident using the second protocol based on seed macerates. Seeds harvested on asymptomatic umbels were sometimes also infected by *D. angelicae* (see results from fields I and II). This also held true for *D. eres* macerate protocol (II-B). *D. eres* was not detected in any of these seed lots when considering the whole seed samples from fields I protocol was used for DNA extraction but was detected in one seed lot from asymptomatic umbels with the seed macerate protocol for DNA extraction (II-B). *Diaporthe angelicae* was detected by PCR assay from I-C lot from asymptomatic plants using the whole seed protocol, and both I-C and II-C lots using the seed macerates protocol, while this fungal species *D. eres* was surprisingly not-detected only on seeds collected on diseased umbels in the same fields—II-C lot from asymptomatic plants using the seed macerates protocol (table 4).
Using a fungal collection of 87 isolates of *Diaporthe* established from field carrot crop samples in France from 2010 to 2014, two *Diaporthe* species responsible for umbel browning and stem necrosis were identified: *D. angelicae*, which was previously reported by Ménard et al. (2014) and *D. eres*. In the present study, Koch’s postulates were verified on carrot umbels for an isolate of both fungal pathogens species in a controlled environment. The pathogenicity of 3 other isolates of *D. angelicae* was previously tested in a greenhouse (Ménard et al. 2014). Further work is needed to confirm the results by testing the pathogenicity of *D. eres* using several isolates. *Diaporthe angelicae* was previously described as the main pathogen responsible for umbel browning on wild fennel in Portugal (Santos & Phillips, 2009). In this latter study, based on a collection of 128 isolates from 10 localities in Portugal, 86 were *D. angelicae* isolates were identified as *D. angelicae* from 9/10 of the localities. A similar situation seemed to occur on carrot in French carrot seed production areas surveyed in this study, as *D. angelicae* was identified in around 83% of isolates obtained (Fig. 4). To our knowledge, *D. eres* has never been isolated from Apiaceae species in previous studies. This species was first described by Wehmeyer (1933) on different genera of ligneous plants. More recently, *D. eres* was identified as a pathogen causing shoot blight and canker disease in peach trees (Thomidis & Michailides, 2009) and was present in a complex of fungal species responsible for wood cankers on grape (Baumgartner et al., 2013).

As colonies of both *Diaporthe* species exhibited a very similar aspect in growth on the agar media conditions tested in this study, other phenotypical criteria were investigated here but also generally unable to differentiate isolates of *D. angelicae* from...
those of *D. eres*. PCR-based *species*-identification using *species*-specific ITS primers developed in this study was a much better more effective option. Another criterion used here-characteristic evaluated for these isolates was the their sensitivity of fungal isolates to a panel of nine fungicides with different modes of action. The results showed that efficacy of the effectiveness of fungicides on at limiting mycelial growth inhibition was in the same range similar statistically when comparing several isolates of both *Diaporthe* species. The relatively high inhibition activity of difenoconazole (EC50 < 0.15) observed here was in accordance with results obtained in fungicide experiments performed in carrot seed production fields (E. Morel, FNAMS Ouzouer-Le-Marché, France, personal communication), while methylthiophanate or pycoxystrobin, which were classified as intermediate in terms of effectiveness in). In the present study, were respectively inactive or active against umbel browning in the same field experiments. Thomidis & Michailides (2009) showed that methylthiophanate significantly inhibited both the *in vitro* development of *D. eres* isolates and disease symptoms on peach shoots or fruits, whereas iprodione was not or much less effective in the same conditions. In our study, methylthiophanate- and iprodione-induced *in vitro* growth inhibition of *D. eres* isolates was roughly comparable, relative to some other tested fungicides. All of the above reported studies highlighted some discrepancies between the *in vitro* and field experiment results.

Here isolate DA035 of *D. angelicae* was especially much less sensitive to difenoconazole or pyrimethanil than other *D. angelicae* isolates tested. As also noted for isolate DE001 of *D. eres* in response to cyprodinil. In a collection of 24 *D. eres* isolates from blighted shoots of peach, six isolates were shown to be either resistant or less sensitive to methylthiophanate compared to than the other isolates (Thomidis & Michailides, 2009). These overall results strongly suggest that the development of isolates...
of *Diaporthe* resistant *Diaporthe* isolates to fungicides in the field could be expected in cases under conditions of their-intensive fungicide use. In the present study, boscalid, a fungicide commonly used in carrot seed production in France, was interestingly highly effective against the tested *A. dauci* strain tested, while isolates of both *D. angelicae* and *D. eres* isolates were quite resistant. (EC > 270 mg mL⁻¹). This latter result highlighted the importance of selecting well-adapted active ingredients appropriate fungicides to control specific fungal pathogens on carrot and may, at least partly, explain the re-emergence of carrot umbel browning as a cause of significant losses in carrot seed production in France.

Another aim of our study was to explore the intraspecific diversity in *D. angelicae* and *D. eres* isolates from carrot seed crops in France. Hence, the variability of IGS rDNA sequences was studied through phylogenetic analysis using 30 isolates selected from our collection to represent diversity based on host and geographic origin. Sequence analysis of the variable domain in the 3’ part of the IGS region clearly indicated that the diversity in *D. angelicae* isolates was significantly higher than that observed in *D. eres*. Perithecia of *D. angelicae*, previously described by Castlebury *et al.* (2003), were produced in a controlled environment in the present study on carrot crop debris collected in France (Fig. 7), and also in field experiments in France (unpublished data). The occurrence of the sexual stage in the life cycle of *D. angelicae* in the carrot seed field may, at least partly, be responsible for the higher sequence polymorphism in this fungal species. Contrastingly, although than in the *D. eres* isolates. Although Wehmeyer (1933) published a thorough description of *D. eres* perithecia, their development in carrot seed production fields in France or elsewhere has, to our knowledge, never been described. Further investigations are needed to clarify this point. In a subsequent step, phylogenetic...
analyses achieved using of the variable domain in the 5’ part of IGS sequences and the same sub-collection of isolates of D. angelicae and D. eres in this study did not show much variability within each fungal species, with only one sequence diverging from others in D. angelicae and in D. eres. In contrast with these results, studies of intraspecific diversity based on portions of the 5’ part of IGS sequences allowed us to differentiate several groups of isolates in a collection of 26 Diaporthe helianthi isolates (Pecchia et al., 2004) or, and a study of 27 Alternaria dauci isolates (Boedo et al., 2012). Those two fungal collections were, however, composed of isolates from different countries, whereas all the Diaporthe isolates of our in this carrot and parsley collection originated from French seed production fields.

As seed transmission was suspected and could constitute a major role in umbel browning epidemiology we investigated in carrot seed fields in France, the presence of D. angelicae and D. eres on carrot seeds. Here no detection also was investigated in this study. Diaporthe species were not detected using a traditional seed plating technique on agar medium, even in the case of seed samples harvested from umbels with well-developed lesions. It seems that the competitiveness of D. angelicae or D. eres against other fungi for growth on the agar medium was too low to detect them. Isolation of D. angelicae was, however, possible in some cases one seed sample collected from symptomatic umbels in the field (results not shown) when seed disinfection was achieved before plating was achieved using a higher percentage of sodium hypochlorite concentration (5%). In contrast to the seed plating results, D. angelicae and D. eres were both respectively detected on several total of six and three carrot seed lots using conventional PCR assays performed on DNA extracted from whole seeds or seed macerates. Our results clearly showed that the seed maceration
stage protocol before DNA extraction greatly improved the PCR protocol by comparison to running the PCR assay on DNA extracted from entire seeds. Indeed, 1000 seeds were tested in the same batch vs 25 seeds when comparing both protocols. Moreover, another advantage of using seed maceration for PCR diagnosis is the detection of living fungal tissue. The seed maceration protocol derived from the BIO-PCR technique, which was firstly described to detect Pseudomonas syringae in bean seed extracts (Schaad et al., 1995). BIO-PCR has then been developed for detection of different seed-borne pathogens (For a review, see Walcott, 2003), including Alternaria radicina on carrot seeds (Pryor & Gilbertson, 2001). After seed maceration, D. angelicae was detected in all types of seed lots, i.e. seeds harvested from symptomatic umbels or from asymptomatic umbels, the latter harvested from diseased or healthy plants. The PCR results clearly demonstrated the prevalence of D. angelicae DNA on carrot seeds compared to D. eres, and were in line with previous analyses in this study, based on the isolation of both fungal species from field samples. Further investigations are needed to: i) thoroughly localize the fungal pathogens in carrot seeds, and ii) study the fungal transmission from the seed to the plantlet.

This is the first report of D. angelicae and D. eres as seed-borne pathogens in carrot. In a next step, molecular detection of Diaporthe species in seed macerates using qPCR assays could be useful to quantify the fungal biomass in seeds, and perhaps even to lower the pathogen detection threshold by comparison with conventional PCR assays. Detection of Diaporthe phaseolorum and Phomopsis longicolla on soybean seeds has been performed using qPCR assays with Taqman chemistry by comparison with other methods, including seed plating (Zhang et al., 1999). In this latter study, results related to qPCR assays and seed plating were
comparable with respect to total infection and individual species detected in each seed lot. The total Diaporthe/Phomopsis detected on soybean seed lots ranged from 0 to 32%, indicating a very high infection level for some lots. We did not calculate the percentage of seed infection was not calculated in this study as the carrot seed plating assay was inefficient for D. angelicae and D. eures detection.

Based on the different results mentioned above of this study, a hypothetical disease cycle for D. angelicae is proposed in (Fig. 8). Perithecia are produced on black spots developed lesions that develop on carrot crop debris or on non-carrot crop debris. At a mature stage, we showed that these perithecia were able to project ascospores as demonstrated in both controlled (this study) and field (unpublished data) conditions. This highly suggests that ascospores of D. angelicae were involved in primary contamination inoculum in carrot seed production fields and may, at least partly, explain the main presence of this fungal species in umbel browning epidemiological studies. The germ tubes formed by ascospores could probably penetrate the plant through a flower or a flower group and the fungus can then progress to the umbel involucre. Diaporthe angelicae may then infect proximal flowers from the involucre, progressively leading to typical triangular necroses and later to stem necrosis. Secondary contamination may mainly be caused by conidia develop in pycnidia that have been observed on carrot stem lesions in the field. These lesions evolve to the perithecia-producing black spots on crop residues, which are needed to initiate a new fungal life cycle during the next growing season. Infected carrot seeds are supposed to transmit the pathogen to carrot seedlings, but this step has not yet been demonstrated. The involvement of non-carrot plants Apiaceae as alternative hosts of D. angelicae is also indicated. On non-carrot Apiaceae, ascospores
developed in perithecia on debris (Castlebury et al., 2003) are potentially a source of primary inoculum. To simplify, the role of D. eres was not included in Fig. 8 as its contribution to the epidemiology of umbel browning in carrot remains to be specified. Interestingly, here D. eres was isolated from two wild Apiaceae species in this study (DE014 and DE015 isolates), suggesting that this fungal species could develop and/or survive on volunteer plants, similar to D. angelicae (Castlebury et al., 2003; this study: DA080 isolate). By contrast, D. eres was never isolated from cultivated parsley in this study, but the fungal collection obtained from this plant species was much more restricted in number than that obtained from carrot (only 12 isolates vs 87 isolates). These different points should be specified using a larger fungal collection on parsley and wild Apiaceae species, respectively).

In conclusion, our results highlighted that umbel browning and stem necrosis in carrot, leading seed crops in France, which can lead to significant losses in seed production, were caused mainly by D. angelicae, and secondarily to some degree by D. eres. To our knowledge, the disease has so far only been reported in France and may be considered as a re-emerging pathology, as similar attacks were described previously on carrot in 1951 in the Netherlands (Bakker, 1951). The higher genetic variability shown here in the most prevalent species responsible for the disease, D. angelicae, than D. eres should be taken into account in the development of adapted control methods, especially when considering fungicide treatments. Some isolates studied in this study were identified as being less sensitive to specific fungicides than other active ingredients. Lastly, the present paper contains preliminary results for justifying a more comprehensive survey of the pathogenicity of the fungus on a larger number of Apiaceae species.
transmission of *D. angelicae* and *D. eres* to/from carrot seeds. Both fungal species were detected on seeds harvested in/from production fields, and a higher frequency of *D. angelicae* was clearly shown detected on the tested seed lots, than *D. eres*. Further studies are needed to gain greater insight into this potentially crucial stage in the development of both *Diaporthe* species in carrot.
Acknowledgements

We would like to thank M. Barret, N. Bataillé-Simoneau, P.E. Brandéis, N. Cazanove, H. Dumas, S. Leclerc, L. Mauge, L. Ménard, V. Odeau, S. Rezki and L. Robbes for their help. D. Manley is also gratefully acknowledged for reviewing the English. This work was supported by the French Ministry of Agriculture, Agrifood and Forestry under the call for projects for the plant breeding program in 2012 (Appel à projet développement agricole et rural du programme semences et sélection végétale, DIAPOCAR 2012-2015).
References


Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S,

Du Manoir J, Vegh I, 1981. *Phomopsis foeniculi* spec. nov. on fennel (*Foeniculum vulgare*

193-206.

Goodwin DC, Lee SB, 1993. Microwave miniprep of total genomic DNA from fungi, plants,
protists and animals for PCR. *BioTechniques* **15**, 438-44.


Laurent E, Blancard D, 2009. *Phomopsis dauci*, un nouveau pathogène des multiplications de

Ménard L, Brandeis PE, Simoneau P, Poupard P, Sérandat I, Detoc J, Robbes L, Bastide F,


**Figure Legends**

**Figure 1** Typical disease symptoms on carrot seed crop plants in France infected with *Diaporthe angelicae* or *D. eres*. (a) triangular necrotic lesions on an umbel.; (b) necrosis progression on a stem from the umbel.; (c) black spots developed on stem debris collected in a carrot seed production field after harvest.

**Figure 2** Morphological criteria used for characterization of *Diaporthe* isolates collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. (a) upper adaxial side of a culture growing on malt agar medium.; (b) production of pycnidia on malt agar medium.; (c) α- (black arrowheads) and β- (white arrowheads) conidia produced in pycnidia. Black scale bar = 20 µm.

**Figure 3** Molecular identification of *Diaporthe angelicae* and *D. eres* isolates collected from carrot and parsley seed crops in France from 2010 to 2014. A phylogenetic tree reconstructed by the neighbor-joining method was obtained via the alignment of the ITS rDNA sequences of 24 *D. angelicae* and nine *D. eres* isolates. ITS sequences from eight seven additional *Diaporthe* spp. isolates were added. Bootstrap confidence measures greater than 50% from 1000 bootstrap replicates are indicated above the horizontal lines of the tree. The ITS sequence of *Diaporthe paranensis-fibrosa* was used to root the tree. The strain identity and GenBank account numbers for ITS sequences are given in Table 1. *Diaporthe angelicae* reference isolates are indicated by an asterisk. The *Diaporthe eres* reference isolate corresponds to DLR12A isolate (SMML culture collection, USDA-ARS, Beltsville, MD, USA).
Figure 4 Geographic origin of *Diaporthe* isolates collected from carrot and parsley seed crops or from wild Apiaceae species in the present study France from 2010 to 2014. *Diaporthe angelicae* (white circles) and *D. eres* (black circles) isolates originated from plant lesions or infested crop debris collected at localities corresponding mostly to the main carrot seed production areas in France.

Figure 5 Intraspecific variability within *Diaporthe angelicae* and *D. eres*. Dendrograms were reconstructed by the neighbor-joining method from the alignment of the 3’ variable domain from rDNA regions of (A) 13 *D. eres* isolates and (B) 17 *D. angelicae* isolates and 13 *D. eres* isolates. Bootstrap confidence measures greater than 50% from 1000 bootstrap replicates are indicated above the horizontal lines of the trees. *Diaporthe scabra* was used as an outgroup to root the trees. Strain identity and GenBank account numbers for IGS sequences are given in Table 1.

Figure 6 Sensitivity of *Diaporthe angelicae* and *D. eres* to nine fungicides. IC_{50} collected from carrot and parsley seed crops in France from 2010 to 2014. EC_{50} values (mg L^{-1}) of the fungicides (indicated as active ingredients) were measured to assess the sensitivity of five *D. angelicae* isolates (white circles) and four *D. eres* isolates (dark circles) to each active ingredient. The isolate reference numbers are indicated in the graph (left column); An *Alternaria dauci* isolate (hatched square) was included in some experiments as a reference carrot pathogen. Commercial fungicide products corresponding to the nine fungicides-active ingredients are specified in the Materials and Methods section. Each data point represents the mean and standard error of three replicate plates.

Figure 7 Sexual stage of *Diaporthe angelicae* obtained in a controlled environment. (a) perithecium (white arrowheads), one of which is showing a
cirrhous (black arrowhead), observed on carrot stem debris (white scale bar = 5 mm); (b) ascospores produced in perithecia (black scale bar = 10 µm); (c) experimental system used to induce perithecia formation and to measure the heights of ascospore ejection. Stem debris (red arrowheads) was placed at the bottom of a closed, transparent, plexiglas box. An upside-down Petri dish with malt agar medium and without the lid is indicated between the black arrowheads, and a piece of cellophane is indicated between the white arrowheads. Petri dishes and cellophane could be placed at variable height in order 40, 60, and 80 cm above the crop residues to assess the height of the ascospore dispersion ability.

Figure 8 Diagram describing the disease cycle of Diaporthe angelicae in carrot seed crops in France. The different steps of fungal development on carrot are indicated on the left-hand side of the figure, while the involvement of non-carrot plants in the life cycle is presented on the right-hand side. Parts of the life cycle that were demonstrated in this study are indicated by solid arrows, while elements originating from the published literature are indicated by hatched arrows. The dotted arrows correspond to hypothetical steps in the fungal development: ascospores; conidia; mycelium; perithecia; pycnidia.
Figure 1 Typical symptoms on carrot seed crop plants in France infected with *Diaporthe angelicae* or *D. eres*. (a) Triangular necrotic lesion on an umbel. (b) Necrosis progressing down a stem from the umbel. (c) Black spots on stem debris collected in a carrot seed production field after harvest.

66x49mm (300 x 300 DPI)
Figure 2 Morphological criteria used for identification of *Diaporthe* isolates collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. (a) Adaxial side of a culture growing on malt-agar medium. (b) Production of pycnidia on malt-agar medium. (c) α- (black arrowheads) and β- (white arrowheads) conidia produced in pycnidia. Black scale bar = 20 µm. 88x88mm (300 x 300 DPI)
Figure 3 Molecular identification of *Diaporthe angelicae* and *D. eres* isolates collected from carrot and parsley seed crops in France from 2010 to 2014. A phylogenetic tree reconstructed by the neighbor-joining method was obtained via alignment of the ITS rDNA sequences of 24 *D. angelicae* and nine *D. eres* isolates. ITS sequences from seven additional *Diaporthe* spp. isolates were included. Bootstrap confidence measures > 50% from 1000 bootstrap replicates are indicated above the horizontal lines of the tree. The ITS sequence of *Diaporthe fibrosa* was used to root the tree. The strain identity and GenBank account numbers for ITS sequences are given in Table 1. *Diaporthe angelicae* reference isolates are indicated by asterisks. The *Diaporthe eres* reference isolate corresponds to DLR12A isolate.
Figure 4 Geographic origin of *Diaporthe* isolates collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. *Diaporthe angelicae* (white circles) and *D. eres* (black circles) isolates originated from plant lesions or infested crop debris at locations corresponding to the main carrot seed production areas in France.

90x93mm (300 x 300 DPI)
Figure 5 Intraspecific variability within *Diaporthe angelicae* and *D. eres* collected from carrot and parsley seed crops or from wild Apiaceae species in France from 2010 to 2014. The dendrograms were reconstructed by the neighbor-joining method from the alignment of the 3' variable domain from IGS rDNA regions of (a) 13 *D. eres* isolates and (b) 17 *D. angelicae* isolates. Bootstrap confidence measures > 50% from 1000 bootstrap replicates are indicated above the horizontal lines of the trees. *Diaporthe scabra* was used as an outgroup to root the trees. Strain identity and GenBank account numbers for IGS sequences are given in Table 1.

96x105mm (300 x 300 DPI)
Figure 6 Sensitivity of Diaporthe angelicae and D. eres to nine fungicides collected from carrot and parsley seed crops in France from 2010 to 2014. EC50 values (mg L⁻¹) of the fungicides (indicated as active ingredients) were measured to assess the sensitivity of five D. angelicae isolates (white circles) and four D. eres isolates (dark circles) to each active ingredient. The isolate reference numbers are indicated in the graph (left column); An Alternaria dauci isolate (hatched square) was included as a reference carrot pathogen. Commercial fungicide products corresponding to the nine active ingredients are specified in the Materials and Methods section. Each data point represents the mean and standard error of three replicate plates.

255x741mm (300 x 300 DPI)
Figure 7 Sexual stage of *Diaporthe angelicae* that developed in a controlled environment. (a) Perithecia (white arrowheads), one of which is showing a cirrhus (black arrowhead), observed on carrot stem debris (white scale bar = 5 mm). (b) Ascospores produced in a perithecium (black scale bar = 10 µm). (c) Experimental chamber used to induce perithecia formation and to measure the heights of ascospore ejection. Stem debris (red arrowheads) was placed at the bottom of a closed, transparent, plexiglas box. An upside down, Petri dish with malt-agar medium and without the lid is indicated between the black arrowheads, and a piece of cellotape is indicated between the white arrowheads. Petri dishes and cellotapes were placed at 40, 60, and 80 cm above the crop residues to assess the height of the ascospore dispersion. 186x390mm (300 x 300 DPI)
Figure 8 Diagram describing the disease cycle of *Diaporthe angelicae* in carrot seed crops in France. The different steps of fungal development on carrot are indicated on the left side of the figure, while the involvement of non-carrot plants in the life cycle is presented on the right side. Parts of the life cycle that were demonstrated in this study are indicated by solid arrows, while elements derived from the published literature are indicated by hatched arrows. The dotted arrows correspond to hypothetical steps in the fungal development. a = ascospores; c = conidia; m = mycelium.

47x26mm (300 x 300 DPI)
Table 1 Origin of *Diaporthe* spp. isolates and DNA sequences used for species identification and genetic variability in this study on the causal agents of umbel browning in carrot seed crops in France

<table>
<thead>
<tr>
<th>Isolate accession</th>
<th>Host plant</th>
<th>Plant</th>
<th>Country (province or department)</th>
<th>Year of isolation</th>
<th>GenBank accession ITS rDNA, 5’ rDNA IGS, 3’ rDNA IGS sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA001*</td>
<td><em>Heracleum sphondylium</em></td>
<td>Decaying stems</td>
<td>At (Kaernten)</td>
<td>2001</td>
<td>KC343027, KR060025, KR060055</td>
</tr>
<tr>
<td>DA002*</td>
<td><em>Foeniculum vulgare</em></td>
<td>NA</td>
<td>Pt (Alenquer)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>DA003*</td>
<td><em>F. vulgare</em></td>
<td>NA</td>
<td>Pt (Sintra)</td>
<td>NA</td>
<td>KC343028, KR060026, KR060056</td>
</tr>
<tr>
<td>DA004</td>
<td><em>Daucus carota</em></td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2010</td>
<td>KF240772</td>
</tr>
<tr>
<td>DA005</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2010</td>
<td>KF240773</td>
</tr>
<tr>
<td>DA006</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2010</td>
<td>KF240774</td>
</tr>
<tr>
<td>DA007</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA008</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loiret)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA009</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loiret)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA010</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA011</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loiret)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA012</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Loiret)</td>
<td>2010</td>
<td></td>
</tr>
</tbody>
</table>

* D. angelicae
<table>
<thead>
<tr>
<th>Code</th>
<th>Species</th>
<th>Type</th>
<th>Location</th>
<th>Year</th>
<th>Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA013</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA014</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA015</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>DA016*</td>
<td><em>D. carota</em></td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2011</td>
<td>KJ934653, KR060027, KR060057</td>
</tr>
<tr>
<td>DA017</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA018</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td>KJ934654</td>
</tr>
<tr>
<td>DA019</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Eure-et-Loire)</td>
<td>2012</td>
<td>KJ934655</td>
</tr>
<tr>
<td>DA020</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Eure-et-Loire)</td>
<td>2012</td>
<td>KJ934656</td>
</tr>
<tr>
<td>DA021*</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loiret)</td>
<td>2012</td>
<td>KJ934657, KR060028, KR060058</td>
</tr>
<tr>
<td>DA022</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA023*</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Indre-et-Loire)</td>
<td>2012</td>
<td>KJ934658, KR060029, KR060059</td>
</tr>
<tr>
<td>DA024</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA025*</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Eure-et-Loire)</td>
<td>2012</td>
<td>KJ934659, KR060030, KR060060</td>
</tr>
<tr>
<td>DA026</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loiret)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA027</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>Code</td>
<td>Species</td>
<td>Part</td>
<td>Location</td>
<td>Year</td>
<td>Accession</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td>DA028</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td>KJ934660</td>
</tr>
<tr>
<td>DA029</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA030</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA031</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Maine-et-Loire)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA032</td>
<td><em>D. carota</em></td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>DA033</td>
<td><em>D. carota</em></td>
<td>Involucre</td>
<td>Fr (Maine-et-Loire)</td>
<td>2012</td>
<td>KJ934661</td>
</tr>
<tr>
<td>DA034</td>
<td><em>P. crispum</em></td>
<td>Involucre</td>
<td>Fr (Eure-et-Loire)</td>
<td>2012</td>
<td>KJ934662</td>
</tr>
<tr>
<td>DA035*</td>
<td><em>P. crispum</em></td>
<td>Stem</td>
<td>Fr (Loiret)</td>
<td>2012</td>
<td>KR060031, KR060061</td>
</tr>
<tr>
<td>DA036</td>
<td><em>P. crispum</em></td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td>KJ934663</td>
</tr>
<tr>
<td>DA037</td>
<td><em>P. crispum</em></td>
<td>Involucre</td>
<td>Fr (Eure-et-Loire)</td>
<td>2012</td>
<td>KJ934664</td>
</tr>
<tr>
<td>DA038</td>
<td><em>D. carota</em></td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934665</td>
</tr>
<tr>
<td>DA039*</td>
<td><em>D. carota</em></td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KR060032, KR060062</td>
</tr>
<tr>
<td>DA040</td>
<td><em>D. carota</em></td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934666</td>
</tr>
<tr>
<td>DA041*</td>
<td><em>D. carota</em></td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KR060033, KR060063</td>
</tr>
<tr>
<td>DA042</td>
<td><em>D. carota</em></td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934667</td>
</tr>
<tr>
<td>DA043</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>-------------</td>
<td>------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>DA044</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934668</td>
</tr>
<tr>
<td>DA045</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA046*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Gers)</td>
<td>2013</td>
<td>KR060034, KR060064</td>
</tr>
<tr>
<td>DA047*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2013</td>
<td>KR060035, KR060065</td>
</tr>
<tr>
<td>DA048</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA049</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934669</td>
</tr>
<tr>
<td>DA050</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA051</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA052*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Lot-et-Garonne)</td>
<td>2013</td>
<td>KR060036, KR060066</td>
</tr>
<tr>
<td>DA053</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934670</td>
</tr>
<tr>
<td>DA054</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA055</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA056</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
</tr>
<tr>
<td>DA057*</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KR060037, KR060067</td>
</tr>
<tr>
<td>Code</td>
<td>Species</td>
<td>Location</td>
<td>Year</td>
<td>Additional Info</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>--------------------</td>
<td>------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>DA058</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA059</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA060</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA061</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA062</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA063</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA064</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Eure-et-Loire)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA065</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA066</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA067</td>
<td><em>D. carota</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA068*</td>
<td><em>P. crispum</em></td>
<td>Involucre Fr (Loir-et-Cher)</td>
<td>2013</td>
<td>KJ934671, KR060038, KR060068</td>
<td></td>
</tr>
<tr>
<td>DA069</td>
<td><em>P. crispum</em></td>
<td>Stem Fr (Loir-et-Cher)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA070</td>
<td><em>P. crispum</em></td>
<td>Stem Fr (Vienne)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA071</td>
<td><em>P. crispum</em></td>
<td>Involucre Fr (Vienne)</td>
<td>2013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA072*</td>
<td><em>P. crispum</em></td>
<td>Stem Fr (Vienne)</td>
<td>2013</td>
<td>KR060039, KR060069</td>
<td></td>
</tr>
<tr>
<td>Code</td>
<td>Taxon</td>
<td>Part</td>
<td>Location</td>
<td>Year</td>
<td>Accession Numbers</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
<td>-------</td>
<td>-------------------</td>
<td>------</td>
<td>-------------------</td>
</tr>
<tr>
<td>DA073*</td>
<td><em>P. crispum</em></td>
<td>Stem</td>
<td>Fr (Eure-et-Loire)</td>
<td>2013</td>
<td>KR060040, KR060070</td>
</tr>
<tr>
<td>DA074*</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA075</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA076</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA077</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA078</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Loir-et-Cher)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA079</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Gers)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA080*</td>
<td>Wild <em>Daucus</em> sp.</td>
<td>Involucre</td>
<td>Fr (Gard)</td>
<td>2014</td>
<td>KR060041, KR060071</td>
</tr>
<tr>
<td>DA081</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA082</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA083</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA084</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA085</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA086</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DA087</td>
<td><em>P. crispum</em></td>
<td>Stem</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>Sample Code</td>
<td>Species</td>
<td>Part</td>
<td>Location</td>
<td>Year</td>
<td>Accession Numbers</td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td>------</td>
<td>----------</td>
<td>------</td>
<td>-------------------</td>
</tr>
<tr>
<td>DA088</td>
<td>P. crispum</td>
<td>Stem</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>D. eres</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE001*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Loir-et-Cher)</td>
<td>2010</td>
<td>KJ934672, KR060042, KP895818</td>
</tr>
<tr>
<td>DE002*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2010</td>
<td>KJ934673, KR060043, KP895819</td>
</tr>
<tr>
<td>DE003*</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2011</td>
<td>KJ934674, KR060044, KP895820</td>
</tr>
<tr>
<td>DE004*</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2011</td>
<td>KJ934675, KR060045, KP895821</td>
</tr>
<tr>
<td>DE005*</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2012</td>
<td>KJ934676, KR060046, KP895822</td>
</tr>
<tr>
<td>DE006*</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Côtes-d'Armor)</td>
<td>2012</td>
<td>KJ934677, KR060047, KP895823</td>
</tr>
<tr>
<td>DE007*</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Côtes-d' Armor)</td>
<td>2012</td>
<td>KJ934678, KR060048, KP895824</td>
</tr>
<tr>
<td>DE008*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Ardèche)</td>
<td>2012</td>
<td>KJ934679, KR060049, KP895825</td>
</tr>
<tr>
<td>DE009*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2013</td>
<td>KR060050, KP895826</td>
</tr>
<tr>
<td>DE010</td>
<td>D. carota</td>
<td>Crop debris</td>
<td>Fr (Loir-et-Cher)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DE011*</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Gers)</td>
<td>2014</td>
<td>KR060051, KP895827</td>
</tr>
<tr>
<td>DE012</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Gers)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DE013</td>
<td>D. carota</td>
<td>Involucre</td>
<td>Fr (Aude)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>Code</td>
<td>Species</td>
<td>Type</td>
<td>Location</td>
<td>Year</td>
<td>GenBank Accession Numbers</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------</td>
<td>----------</td>
<td>---------------</td>
<td>-------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>DE014*</td>
<td>Wild Daucus sp.</td>
<td>Stem</td>
<td>Fr (Isère)</td>
<td>2014</td>
<td>KR060052, KP895828</td>
</tr>
<tr>
<td>DE015*</td>
<td>Silaum silaus</td>
<td>Stem</td>
<td>Fr (Isère)</td>
<td>2014</td>
<td>KR060053, KP895829</td>
</tr>
<tr>
<td>DE016*</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td>KR060054, KP895830</td>
</tr>
<tr>
<td>DE017</td>
<td>D. carota</td>
<td>Stem</td>
<td>Fr (Maine-et-Loire)</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>DLR12A</td>
<td>Vitis vinifera</td>
<td>NA</td>
<td>Fr</td>
<td>NA</td>
<td>KJ210518</td>
</tr>
<tr>
<td></td>
<td>D. fibroSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS113830</td>
<td>Rhamnus cathartica</td>
<td>NA</td>
<td>Se (Uppland)</td>
<td>1987</td>
<td>KC343100</td>
</tr>
<tr>
<td></td>
<td>D. foeniculacea</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS603.88</td>
<td>Bougainvillea spectabilis</td>
<td>Flower peduncles</td>
<td>Pt</td>
<td>NA</td>
<td>KC343111</td>
</tr>
<tr>
<td></td>
<td>D. helianthi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS592.81</td>
<td>Helinathus annuus</td>
<td>Overwintering stem</td>
<td>RS (Vojvodina)</td>
<td>1980</td>
<td>KC343115</td>
</tr>
<tr>
<td></td>
<td>D. lusitanicae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS123212</td>
<td>F. vulgare</td>
<td>NA</td>
<td>Pt (Oeiras)</td>
<td>NA</td>
<td>KC343136</td>
</tr>
<tr>
<td></td>
<td>D. neotheicola</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBS123209</td>
<td>F. vulgare</td>
<td>NA</td>
<td>Pt (Evora)</td>
<td>2007</td>
<td>GQ250192</td>
</tr>
</tbody>
</table>
### D. phaseolorum var. sojae

| CBS25780 | NA | NA | NA | NA | KC343178 |

### D. rudis

| CBS794.96 | *Aucuba japonica* | NA | GB (Sheffield) | NA | KC343243 |

### D. scabra

| CBS127746 | *Platanus × acerifolia* | NA | NA | NA | HQ450374 |

### D. subordinaria

| CBS104.84 | *Plantago lanceolata* | NA | NA | NA | GQ922519 |

---

*a* Asterisks correspond to isolates used for the intraspecific genetic variability study.

*b* Isolates indicated in bold font correspond to isolates tested for fungicide sensitivity.

*c* Isolates underlined correspond to isolates used for morphological characterization.

*d* Isolates DA001, DA002 and DA003 were obtained from the Centraalbureau voor Schimmelcultures (CBS) and correspond to isolates CBS111592, CBS123214 and CBS123215, respectively. DA004 was deposited at CBS under the reference CBS137262. Isolate DLR12A was obtained from the SMML culture collection, USDA-ARS, Beltsville, MD, USA. NA: not available.
<table>
<thead>
<tr>
<th>rDNA region</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Direction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>ITS1</td>
<td>TCCGTAGGGTGAACCTGCGG</td>
<td>Forward</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>DiapITSfw</td>
<td>TACTGTTGCCTCGGC</td>
<td>Forward</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>AngelITSfw</td>
<td>TTTCTCGGTAAGGCCCCTT</td>
<td>Forward</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>EresITSfw</td>
<td>GGCCAACCCAACCTTCTT</td>
<td>Forward</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>ITS4</td>
<td>TCCTCCGCTATTGATATGC</td>
<td>Reverse</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>DiapITSrv</td>
<td>TAATCTACTCGCTCGG</td>
<td>Reverse</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>AngelITSrv</td>
<td>AGATTTCAAGGCGCTGCCC</td>
<td>Reverse</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>EresITSrv</td>
<td>GCCTGCTCCTTGGAAGA</td>
<td>Reverse</td>
<td>This study</td>
</tr>
<tr>
<td>IGS</td>
<td>26S3111F</td>
<td>AGGGAACGTGAGCTGGGTTAG</td>
<td>Forward</td>
<td>Hong et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>VariablIGS3’fw</td>
<td>GCCGCTGGCAACGGCTGAAA</td>
<td>Forward</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>VariablIGS5’fw</td>
<td>CCTCGCCTAGATTTCAC</td>
<td>Forward</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>IGS27</td>
<td>AATGAGCGATTCCATGT</td>
<td>Reverse</td>
<td>Subbarao et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>VariablIGS3’rv</td>
<td>GAGACAAGCATATGACTACTG</td>
<td>Reverse</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>VariablIGS5’rv1</td>
<td>CTGTCCTAAACACGAAGG</td>
<td>Reverse</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>VariablIGS5’rv2</td>
<td>CCTGCAAATCACGAGGG</td>
<td>Reverse</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 3 Mean ± standard deviation of α and β conidia lengths in *Diaporthe angelicae* and *D. eres* isolates collected from carrot or parsley seed crops or from wild Apiaceae species in France from 2010 to 2014

<table>
<thead>
<tr>
<th>Species</th>
<th><em>Diaporthe angelicae</em></th>
<th><em>Diaporthe eres</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean α conidia length</td>
<td>6.289 ± 0.13</td>
<td>7.111 ± 0.13</td>
</tr>
<tr>
<td>Longest α conidia (isolate name)</td>
<td>7.00 ± 0.32</td>
<td>8.66 ± 0.32</td>
</tr>
<tr>
<td>Shortest α conidia (isolate name)</td>
<td>5.67 ± 0.32</td>
<td>5.67 ± 0.32</td>
</tr>
<tr>
<td>Mean β conidia length</td>
<td>22.89 ± 0.22</td>
<td>24.18 ± 0.22</td>
</tr>
<tr>
<td>Longest β conidia (isolate name)</td>
<td>25.00 ± 0.71</td>
<td>26.00 ± 0.71</td>
</tr>
<tr>
<td>Shortest β conidia (isolate name)</td>
<td>20.67 ± 0.71</td>
<td>22.67 ± 0.71</td>
</tr>
</tbody>
</table>

*Refer to Table 1 for details of the isolates. Each value is noted as the mean ± standard deviation of 15 isolates of *D. angelicae* and 15 isolates of *D. eres*. }
**Table 4** Detection of *Diaporthe* spp. isolates from carrot seed field samples in France using a classical fungal growth test on agar medium in Petri dishes or a PCR assay with DNA extracted from whole seeds or after seed maceration.

<table>
<thead>
<tr>
<th>Seed sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Classical growth test: presence of <em>Diaporthe</em> spp.</th>
<th>Whole seeds</th>
<th>Seed macerates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR detection using <em>D. angelicae</em> specific primers</td>
<td>PCR detection using <em>D. eres</em> specific primers</td>
<td>PCR detection using <em>D. angelicae</em> specific primers</td>
</tr>
<tr>
<td>I-A</td>
<td>-&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>I-B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I-C</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>II-A</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>II-B</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>II-C</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>III-A</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>III-B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>I, II and III correspond to three seed production fields in France; A, B and C are different seed samples collected in fields I, II and III: seeds were harvested from symptomatic umbels (A), from asymptomatic umbels collected from diseased plants (B), or from asymptomatic umbels collected from asymptomatic plants (C). See Materials and Methods for details.

<sup>b</sup>- or + corresponds to a negative or positive result, respectively, using the relevant test. PCR assays were carried out twice.