Pathogenic *Alternaria* spp. associated with seed of *Daucus carota* Plant Introduction lines of the United States Department of Agriculture National Plant Germplasm System

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Introduction

Alternaria leaf blight and black rot of carrot (*Daucus carota*) are caused by *Alternaria dauci* and *A. radicina*, respectively. These economically important diseases have been found in most areas where carrots are produced (11,13). *A. dauci* and *A. radicina*, as well as the closely related species *A. carotiincultae* that is also pathogenic on carrot, can be seedborne and seed transmitted (4,11,13). Alternaria leaf blight is characterized by necrotic foliar lesions, each of which usually is surrounded by a chlorotic halo (13). Alternaria leaf blight can reduce photosynthetic activity and hinder harvest of fresh market carrots if the roots are pulled from the soil by weakened foliage. Symptoms of black rot typically begin as a black decay at the base of the petioles, resulting in rot at the crown (11). *A. radicina* may also cause foliar lesions similar to Alternaria leaf blight, and can impede harvest of fresh market carrots if decayed petioles break when roots are pulled from the soil by the foliage. These fungi also can cause damping-off of carrot seedlings. An important step in controlling *A. dauci* and *A. radicina* in carrot crops is planting pathogen-free seed or seed treated with fungicides effective against these fungi (4).

Damping-off was observed on seedlings grown from seed of carrot Plant Introduction (PI) lines from the United States Department of Agriculture (USDA) National Plant Germplasm System (NPGS) during a greenhouse trial in 2012 at Washington State University (3). Isolations from symptomatic seedlings produced conidia resembling those of *A. dauci* and *A. radicina*. Given the seedborne nature and potential economic importance of these pathogens, as well as the distribution of seed of *Daucus* spp. from the USDA NPGS collection, the objective of this study was to assess the incidence of *Alternaria* spp. pathogenic on carrot associated with seed of a subset of *Daucus* germplasm from the USDA NPGS.

Materials and Methods

Twenty-five seeds of each of 66 carrot PI lines being used in a bacterial blight resistance screening project (3), were assayed using the malt agar protocol of the International Seed Testing Association for detection of *A. dauci* and *A. radicina* (6,7). The seeds were incubated at 20 ± 2°C with a 12 h/12 h night/day cycle, and both near-UV and cool-white fluorescent light by day. After five days, each seed was examined microscopically (x40 to x100) for conidia resembling those of *A. dauci* and *A. radicina*. The number of seeds infested with *A. dauci* and/or *A. radicina* was recorded for each PI line (Table 1). Individual spores of putative *A. dauci* and *A. radicina* isolates were removed from seed of 23 PI lines with a sterilized needle, placed on a plate of potato dextrose agar amended with chloramphenicol at 100 ppm (cPDA), incubated at room temperature (~22°C), and used to generate single-spore isolates tentatively identified as *A. dauci* (n = 17) and *A. radicina* (n = 27).

DNA of each isolate was extracted using the DNeasy Plant Minikit (Qiagen Inc., Valencia, CA), the FastDNA kit (MP Biomedicals, LLC, Santa Ana, CA), or protocols based on that of Dobinson (2) and Peever et al. (10). DNA extracts were then used in PCR assays with the Alt a1 forward primer (5'-ATGCAGTTCACCACCATCGC-3') and reverse primer (5'-ACGAGGGTGAYGTAGGCGTC-3') designed by Hong et al. (5), and PCR reaction mixture and conditions used by Park et al. (9). The forward and reverse sequences of each PCR product were determined by ELIM Biopharmaceuticals, Inc. (Hayward, CA), proofread, aligned manually, and edited (BioEdit 7.2.5, Ibis Biosciences, Carlsbad, CA). The consensus sequence for each isolate was submitted to GenBank (accession numbers KJ732975-KJ733010).

Based on the species identification determined by sequencing and morphology, 27 isolates of *A. radicina*, 2 isolates of *A. carotiincultae*, and 1 isolate of *A. petroselini* obtained from seed of the PI lines were tested for pathogenicity on carrot root discs to assess whether the isolates could cause black rot of

root discs based on the method described by Pryor et al. (12). One isolate of *A. dauci* and non-colonized agar discs were included as negative control treatments. A completely randomized design was used with three replicate root discs/isolate. Pathogenicity was rated using a 0-to-9 scale, where: 0 = no disc discoloration; 1 = slight discoloration; 2 = softening and necrosis of the disc; 3 = disc rotted slightly on the surface and edges, with sporulation typical of *A. radicina* observed microscopically; 6 = disc rotted moderately with part of the disc succumbing to soft rot, and conidia typical of *A. radicina* observed on the disc; and 9 = disc rotted entirely with conidia typical of *A. radicina* observed microscopically.

In spring 2014, seeds of the carrot cultivar Big Sur (Nunhems USA, Inc.; Parma, ID) were sown in Sunshine Mix #1 in 12.7 cm-diameter pots (STD, Anderson Die and Manufacturing Inc., Portland, OR) in a greenhouse (7 seeds/pot). After emergence, plants were thinned to five/pot. After 35 days, the foliage was inoculated with a conidial suspension (2.0×10^3 conidia/ml) of each of three isolates of *A. dauci,* five isolates of *A. radicina,* three isolates of *A. carotiincultae,* and one isolate of *A. petroselini*. The species were determined based on sequencing of the Alt a1 gene or in a previous study (9). A randomized complete block design (RCBD) was used with four replicate plants/isolate, including a non-inoculated control treatment. The foliage of each plant was rated for severity of symptoms weekly for 4 weeks post-inoculation (wpi) based on the percentage foliage with lesions. Similarly, a parsley foliar pathogenicity test was completed with a RCBD of two replicates/isolate, using an isolate of each of *A. dauci*, *A. radicina*, and *A. carotiincultae*, and three isolates of *A. petroselini*, a pathogen of parsley but not carrot. One *A. petroselini* isolate was identified from a seed of the PI 280706 based on comparison of the Alt a1 sequence with sequences in the NCBI database (see Results below). The pathogenicity trial data were analyzed using PROC MIXED in SAS Version 9.3 (SAS Institute, Cary, NC).

Results

Of 25 seeds tested/PI, the average number of seeds infested with putative *A. radicina* and *A. dauci* was 1.9 and 3.1, respectively, among the 66 PI lines assayed. Putative *A. radicina* conidia were detected on seed of 37 of the 66 PIs, at a range of 4 to 80% infested seed/PI (mean of 14%). Putative *A. dauci* conidia were detected on seed of 34 PIs at a range of 4 to 100% infested seed/PI (mean of 24%). Both *A. radicina* and *A. dauci* conidia were detected on seed of 21 PIs. Seed of only 16 PI lines had no *A. radicina* or *A. dauci* conidia (Table 1). A total 27 putative *A. radicina* isolates were obtained from seed of 17 PIs, and 17 putative *A. dauci* isolates were obtained from seed of 12 PIs. The species identities of these 44 isolates were determined with molecular and pathogenicity studies.

The Alt a1 PCR assay produced an amplicon of ~510 bp for each of 36 of the 44 *Alternaria* isolates suspected of being pathogenic on carrot. A BLAST search of GenBank with the consensus sequence of each isolate revealed 1 isolate was *A. petroselini*, 1 isolate was *A. carotiincultae,* 18 isolates were *A. radicina,* and 16 isolates were *A. dauci*. The species of the remaining 8 isolates could not be verified by sequencing.

All 27 *A. radicina* isolates (18 confirmed by sequencing and 9 based on morphology) collected from carrot seed caused rotting of carrot root discs (*data not shown*). The *A. dauci* isolate did not rot carrot discs. Disc rot ratings ranged from 1 to 9 and averaged 5.4 ± 0.2 among the 27 *A. radicina* isolates and 1 *A. dauci* isolate. However, the Kruskal-Wallis test of ranked carrot disc rot ratings showed no significant effect of isolate on rot severity ($P = 0.0846$). All five *A. radicina* isolates, three *A. carotiincultae* isolates, and three *A. dauci* isolates caused blight symptoms on carrot foliage, but the *A. petroselini* isolate was not pathogenic (*data not shown)*. Repeated measures analysis using the compound symmetry covariance structure on ranked percentage carrot foliage blighted showed a significant effect ($P < 0.0001$) of isolates of *Alternaria* spp. on severity of symptoms over 4 weeks following inoculation. All three *A. petroselini* isolates, the *A. radicina* isolate, and the *A. dauci* isolate caused symptoms on parsley foliage (*data not shown*). Repeated measures analysis on ranked percentage parsley foliage blighted showed that isolates of *Alternaria* spp. had a significant effect ($P < 0.0001$) on severity of blight over 4 weeks following inoculation. The *A. petroselini* isolate caused the most severe disease on parsley foliage 4 wpi (average 5.0% blighting vs. 0.9% for the other isolates).

Discussion

To our knowledge, this is the first report of *A. carotiincultae*, *A. dauci*, and *A. radicina* detected on seed of PI lines from the USDA NPGS, and of *A. petroselini* isolated from carrot seed. All but one of 36 isolates, the *A. petroselini* isolate, were confirmed as species pathogenic on carrot by sequencing the Alt a1 gene. However, no attempt was made to obtain isolates from the PI seed of *Alternaria* spp. that are not pathogenic on carrot, e.g., *A. alternata*, a common saprophyte on carrot seed (6,7). A majority of the 36 isolates were *A. dauci* (16 isolates) or *A. radicina* (18 isolates), two common seedborne pathogens of carrot (4). One isolate of *A. carotiincultae*, which causes similar symptoms on carrot as those caused by *A. radicina*, was isolated from a seed of PI 418967. However, only 25 seeds/PI were assayed in this study because of the limited number of seeds distributed by the USDA NPGS, most of which were used in greenhouse trials to screen the PIs for resistance to the bacterial blight pathogen, *Xanthomonas hortorum* pv. *carotae* (3).

A subset of isolates of *A. carotiincultae*, *A. petroselini*, and *A. radicina* evaluated in this study were able to colonize and cause mild to severe rot of carrot root discs. Despite the relatively limited number of isolates of *A. carotiincultae* (n = 3), *A. dauci* (n = 3), and *A. radicina* (n = 5) evaluated in the carrot foliar pathogenicity test, all of the isolates were pathogenic on carrot. The *A. petroselini* isolate was not pathogenic on carrot but was pathogenic on parsley. Isolates of *A. dauci* and *A. radicina* also caused foliar blight symptoms on parsley foliage. However, symptoms caused by *A. dauci* and *A. radicina* generally were less severe, with two of the three *A. petroselini* isolates causing significantly more severe blighting on parsley foliage than isolates of the former two species. The *A. petroselini* isolate from a PI seed was significantly more virulent on parsley foliage than the *A. carotiincultae* and *A. radicina* isolates, with no significant difference between isolates of the latter two species; and the *A. carotiincultae* and *A. radicina* isolates were significantly more virulent on carrot foliage than the *A. petroselini* isolate.

The foliar pathogenicity tests revealed some variation in virulence among isolates on both carrot and parsley foliage. The carrot PI lines evaluated were collected from all over the world, so the *Alternaria* isolates may have originated from sites where the plants or seed were collected, or may have infested the PI seed during seed increases by the NCRPIS in Ames, IA from local sources of inoculum or inoculum from other areas where carrot PI seed is increased for the USDA NPGS (e.g., commercial seed companies sometimes increase seed of PI lines).

For curators of the USDA NPGS, management of fungal pathogens such as *Alternaria* spp. on plants of carrot PIs grown for seed (e.g., with fungicide applications, planting pathogen-free seed, removal or incorporation of crop debris into soil, reducing leaf wetness, and rotating to non-umbelliferous crops), sterilizing harvest and seed cleaning equipment between seed lots, and monitoring seed and plants of the PIs for pathogenic *Alternaria* spp. when new accessions are acquired from areas of the world, is important to minimize the risk of distributing pathogen-infested carrot seed (4,8,11,12,13). Recipients of carrot PI seed could treat the seed with fungicides (e.g., azoxystrobin, fludioxonil, or iprodione) or with hot-water or disinfectants to reduce the risk of spread and transmission of pathogenic *Alternaria* spp. on PI seed (1,4). This should facilitate effective utilization of the carrot PI collection while reducing risks associated with distributing seedborne *Alternaria* spp. pathogenic on carrot.

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Table 1. Detection of *Alternaria dauci* and *A. radicina* on carrot seed of each of 66 Plant Introduction (PI) lines[a]

		% Seed infested[d]	
Line[b]	**Origin[c]**	***A. dauci***	***A. radicina***
PI 163238	India	0	4
PI 164136	India	0	36
PI 169482	Turkey	0	0
PI 169488	Turkey	100	28
PI 174206	Turkey	24	4
PI 174828	India	0	4
PI 175132	India	4	0
PI 175715	Turkey	0	16
PI 175716	Turkey	0	0
PI 175718	Turkey	0	8
PI 175719	Turkey	0	0
PI 176557	Turkey	0	0
PI 176558	Turkey	52	4
PI 176559	Turkey	0	0
PI 176969	Turkey	0	0
PI 177381	Turkey	32	4
PI 180834	Turkey	20	40
PI 181052	Pakistan	4	0
PI 181766	Lebanon	0	0
PI 181767	Lebanon	8	0

Table 1. *continued…*

Line[b]	Origin[c]	% Seed infested[d]	
		A. dauci	***A. radicina***
PI 182204	Turkey	0	12
PI 182207	Turkey	48	4
PI 183401	India	0	4
PI 187237	Belgium	20	4
PI 207480	Afghanistan	0	0
PI 220014	Afghanistan	12	0
PI 222723	Iran	48	4
PI 223360	Iran	12	4
PI 223777	Afghanistan	16	8
PI 226636	Iran	8	0
PI 234621	South Africa	0	4
PI 249535	Spain	0	12
PI 251228	Afghanistan	16	80
PI 261614	Spain	8	8
PI 263601	France	32	0
PI 264236	France	8	0
PI 264237	France	0	0
PI 264543	Japan	0	0
PI 271471	India	0	20
PI 274909	Turkey	8	4
PI 277710	Netherlands	8	28
PI 280706	Chile	0	4
PI 284773	Sweden	4	0
PI 288458	India	0	0
PI 288461	India	4	28
PI 294084	Japan	0	0
PI 326010	Tajikstan	4	0
PI 326011	Lithuania	0	4
PI 344072	Turkey	0	0
PI 344447	Iran	60	0
PI 357984	Former Serbia and Montenegro	96	0
PI 390885	Israel	0	8
PI 390889	Israel	4	8
PI 390893	Israel	68	4
PI 390900	Israel	24	4
PI 390901	Israel	12	0
PI 418967	China	12	48
PI 430524	Azerbaijan	8	12
PI 390887	Israel	36	8
PI 478863	Germany	0	4
PI 478873	Italy	0	0
PI 478883	France	4	0
PI 432900	China	0	28
PI 432905	China	0	0
PI 432906	China	0	0
PI 436674	China	0	4

[a] Seed assays were carried out using the malt agar protocols for detection of *Alternaria dauci* and *A. radicina* on *Daucus carota* seed as published by the International Seed Testing Association (6,7).

[b] Seed of carrot PI lines were obtained from the North Central Regional Plant Introduction Station (NCPIS) of the United States Department of Agriculture (USDA) National Plant Germplasm System in Ames, IA.

[c] PI lines originally were collected from various countries, and are maintained at the USDA NCRPIS in Ames, IA.

[d] Percentage of seeds infested with *A. dauci* or *A. radicina*, out of 25 seeds assayed/PI line.