Establishment of Alternaria Leaf Blight on Carrots in Controlled Environments

J. O. STRANDBERG, Professor and Plant Pathologist, University of Florida, Institute of Food and Agricultural Sciences, Central Florida Research and Education Center, Sanford 32771

ABSTRACT
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Carrot plants in the 4- to 6-leaf stage were sprayed with conidia of *Alternaria dauci*. Incubation at 16–28 C in 100% relative humidity with wetted leaves for 24 hr was required for infection. Temperatures above 12 C during night hours along with wetted leaves were necessary for disease development. Responses of plants to disease development were evaluated with a damage index key that estimated the percent of leaf area damaged. Intermittent misting of plants during night hours made disease development in a greenhouse possible. Surfactants improved the efficiency of collection of conidia produced in culture and their application to plants.

Additional keywords: *Daucus carota*, inoculation, leaf wetness

Alternaria leaf blight of carrot, caused by *Alternaria dauci* (Kühn) Groves & Skolko, is an economically important

disease of carrot (*Daucus carota* L.). It is controlled by frequent applications of protectant foliar fungicides. Recently, sources of horizontal resistance to *A. dauci* have been identified (12). Incorporation of disease resistance into carrot could provide additional opportunities to manage Alternaria leaf blight, but reliable and efficient assessment of test plants for response to the pathogen will be required. Field evaluation in a region where Alternaria leaf blight commonly occurs was used to identify sources of resistance (12), but a controlled environment test offers increased reliability and efficiency and could be used where weather is unfavorable for disease development (3,4,6,10, 11,13).

Inoculation of carrot plants with *A. dauci* has been reported (2,3,5,10,11), but only one investigator inoculated seedlings in a controlled environment (2). Disease-resistant plants were not available to earlier workers and, with one exception (2), efficient establishment of disease and assessment of disease resistance were not primary goals.

This study identified factors important in establishing Alternaria leaf blight in controlled environments or greenhouses. Methods for isolation and storage of the

pathogen and production of inoculum have been reported previously (11).

MATERIALS AND METHODS

Inoculum preparation and inoculation. Conidia of *A. dauci* were spread on carrot leaf agar (CLA) plates. Plates were kept for 5–7 days at 22 C with an 18-hr daylength (150 mol sec^{-1} m^{-2}) provided by eight 40W incandescent bulbs and four FC-40 cool white fluorescent tubes (General Electric Co.) 0.3 m from the plates. Production of inoculum and preparation of CLA has been previously described (11). Conidia were recovered by washing them from the agar with 50 ml of water or water containing 50–150 ppm of Triton CS-7 or Triton AG-98 wetting agent (Rohm & Haas Co., Philadelphia, PA). Throughout this study, plants were inoculated by spraying 5 ml of a suspension of conidia with an atomizer onto five carrot plants growing in a 15-cm pot. Numbers of conidia in suspensions were estimated by counting at least four 20-μl samples at 60×. Numbers applied to plants were estimated by placing 25-mm-diameter filter disks (Type GA-1, Gelman Instrument Co., Ann Arbor, MI) on the soil surface or by supporting them on paper disks suspended within the carrot foliage. Following inoculation, conidia deposited on filter disks were counted at 60×.

The cultivar Chantenay was used in all experiments. Seeds were planted in a peat moss-vermiculite medium (Terra-Lite Vegetable Tray Mix, W. R. Grace & Co., Cambridge, MA) in 15-cm-plastic pots and seedlings were thinned to five per pot. Three times weekly, plants were watered with half-strength Hoagland's solution (14) and, on alternate days, with tap water. Plants were grown in a greenhouse (20–30 C) and were inoculated in the 4- to 6-leaf stage unless otherwise specified.

Conditions during infection. Immediately after inoculation with a suspension of 5 × 10^4 conidia/ml, plants were placed in humidity chambers constructed of black or transparent plastic film (0.33 m^3 chamber volume). A metal pan covered the bottom of each chamber. It was filled with 3–4 cm of water 5–10 C warmer than the chamber temperature just before inserting the plants. Chambers were kept in controlled environment rooms. When light was used, measured intensity in the plastic chambers was approximately 45 mol sec^{-1} m^{-2} from fluorescent lights. Infection periods ranged from 12 to 72 hr.

Effect of postinoculation environment. Following a 24-hr infection period in humidity chambers, pots holding plants inoculated with 5 × 10^4 conidia/ml were divided at random into groups of 10. Each group was placed in one of the following postinoculation environments 1) controlled environment room (24 C) in a 14-hr light period (150 mol sec^{-1} m^{-2},

eight 40W incandescent bulbs and four FC-40 cool white fluorescent tubes), where minimum relative humidity (RH) varied from 60 to 72% during light periods, maximum measured RH was 85% during dark periods; 2) greenhouse (20–31 C), with minimum RH of 72% daytime, maximum RH of 100% for 3–5 hr at night; 3) humid greenhouse (22–34 C), with minimum RH of 70%, maximum RH of 100% for 6–12 hr at night; 4) outdoors (22–30 C), with minimum RH of 50–60% during daylight hours with periods of 100% RH at night ranging from 4 to 14 hr; and 5) humid greenhouse, as in number 3 above, with intermittent mist provided for 30 sec at 1-hr intervals from 1800 to 0600 hr. Relative humidities were measured with a dew point hygrometer (Model 91, Yellow Springs Instrument Co., Inc., Yellow Springs, OH). In greenhouses and outdoors, hygrothermographs were used.

To examine effects of postinoculation temperature, plants inoculated with 5 × 10^4 conidia/ml then incubated 24 hr in 100% RH at 22 C were kept in controlled environment rooms during daylight hours at 20 C (13-hr daylength, 40–85% RH). During each night period, plants were transferred to dew chambers (100% RH) for 11-hr wet periods in the dark at 11, 16, 21, and 26 C. The temperature-controlled dew chambers were similar to those described by Tuite (14). The experiment was repeated twice. Plants were evaluated for disease damage at 10 or 12 days after inoculation. Leaves were numbered serially from the first to emerge (the oldest) to the youngest leaf just emerging and expanding. Leaf four was removed from each plant and evaluated for Alternaria leaf blight damage with a damage index key (Fig. 1).

Disease damage index key. The development of the key follows. Images of selected leaves damaged in the field by *A. dauci* were reproduced on a xerographic copier. Healthy and damaged areas of selected leaves were estimated by cutting out the corresponding areas on the paper images. The ratio of diseased and healthy areas were estimated by weighing the two classes of removed paper. Leaf images with 0, 5, 10, 15, 25, and 40% of leaf area damaged were selected for the key. On copies of an image of a representative healthy leaf, areas were darkened to simulate damage levels corresponding to these densities on images in the desired damage classes. Areas of simulated damage on prepared images were verified to 5% accuracy by cutting and weighing as before. Selected images were photographed to produce the damage index key. Sampled leaves were compared to the key, assigned to damage classes, and an average damage index was calculated.

Age and number of conidia used for inoculum. The age of conidia (4–14 days)

that could be used to infect plants was examined, with conidia produced on CLA plates at 22 C in a 16-hr daylength (incandescent and fluorescent light 150 mol sec^{-1} m^{-2}). At 4, 6, 8, 10, 12, and 14 days after inoculation of CLA plates, conidia were collected from each of three plates selected at random, suspended in water, and adjusted to 10^4 conidia/ml, then sprayed on plants. Inoculated plants were kept 24 hr in 100% RH at 22 C then placed in a warm greenhouse with intermittent misting during night hours. Ten days later, the fourth leaf was collected and the leaf area damaged was estimated with the damage index key.

Numbers of conidia needed to establish disease were also examined. Conidia from 5-day-old CLA plates were collected in water containing 120 ppm AG-98. Conidia were counted in four 20-μl samples and the suspension was diluted by 1:1, 1:10, 1:100, 1:1,000, and 1:10,000 and sprayed on plants. Filter disks were supported within the leaf canopy of half the pots to estimate the number of conidia applied. After 24 hr at 100% RH and 24 C, inoculated plants were placed in a greenhouse with misting during night hours. Numbers of infection sites on leaf four or leaf five were counted 6 days after inoculation. Leaf area was measured with a leaf area meter (Model LF-3000, LiCor Instruments, Lincoln, NE). Damage index ratings were made 10 days after inoculation.

Use of wetting agents. Conidia were washed from 6-day-old CLA plates with 50 ml of water, or water with 50 ppm CS-7 or 120 ppm AG-98. Suspensions were diluted to 1:1, 1:9, and 1:99 with the washing solution. Conidia were counted before and after dilution, and errors between expected and observed numbers were calculated. In a similar experiment,
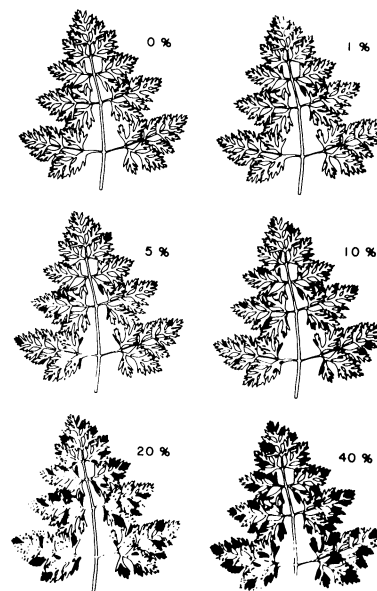

Fig. 1. Disease damage index key for damage to carrot leaves by *Alternaria dauci*.

suspensions were sprayed on 25-mm-filter disks placed horizontally at a distance (50 cm) and angle (45°) to simulate application to plants, and conidia deposited on the filter disks were counted at 60×.

AG-98 seemed to wet and disperse conidia most effectively, so conidia were washed from CLA plates with 50, 100, 200, 400, and 800 ppm of AG-98. Suspensions were diluted with the washing solution to 5×10^4 conidia/ml and sprayed on three pots of plants. Plants were incubated at 100% RH at 24 C for 24 hr then placed in a warm
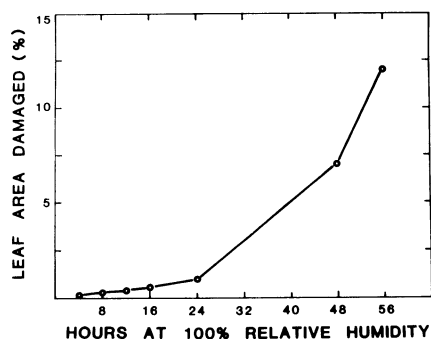

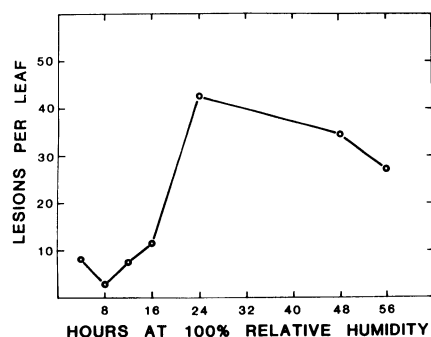**Fig. 2.** Disease damage on leaves of carrot plants inoculated with conidia of *Alternaria dauci*.

Fig. 3. Effect of postinoculation wet period (24 C in the dark) on number of lesions produced by *Alternaria dauci*.

greenhouse with intermittent night misting. After 9 or 10 days, damage index ratings were made on leaf five or leaf six. Conidia in six 20-μl droplets of the suspensions were incubated in plastic petri dishes in the laboratory and examined after 1, 2, 4, and 8 hr for germination.

Effect of plant age on disease establishment. Plants grown for 4, 5, 6, 7, and 8 wk after seeding were inoculated (3.5×10^4 conidia/ml water with 50 ppm AG-98), incubated 24 hr in 100% RH at 24 C, then moved to a warm greenhouse with intermittent night misting. Two weeks after inoculation, disease damage on leaves one through six was estimated with the damage index key.

RESULTS AND DISCUSSION

Effect of environment during infection and postinfection periods. Over the range of 4–56 hr, disease damage increased as plants were held for longer periods at 100% RH and leaves remained wet following inoculation (Fig. 2). Apparently, the pathogen was able to more extensively colonize leaf tissue during the longer, uninterrupted wet periods following infection. Wet periods longer than 56 hr (up to 72 hr were used in one experiment) resulted in the death and collapse of entire leaves within 1–3 days after removal from moisture chambers. Lesions per cm^2 of leaf increased as the time plants were held at 100% RH was increased from 8 to 24 hr (Fig. 3); maximum numbers were observed after 24 hr. Numbers of lesions declined on plants held longer than 24 hr, probably because some multiple lesions coalesced to form single infection sites (Fig. 3).

In the field, infection by *A. dauci* occurs overnight (usually within 12–16 hr) when leaves are wet (3,4,10). In this study, postinoculation moist periods longer than 24 hr were unnecessary to establish disease levels that approximated field conditions.

Following inoculation, plants held in 100% RH for 24 hr at 24 C with constant light developed significantly less disease damage, 29 vs. 43% of leaf area damaged,

respectively ($P = 0.05$, Student's t test), than plants held in darkness. Since rigorous control of RH was not possible in this experiment, the difference may have been due to failure to maintain constant levels of free moisture on leaves in the lighted treatment and not to effects of light on spore germination or infection.

Numbers and age of conidia used for disease establishment. Conidia collected from CLA plates at 4, 6, 8, 10, 12, or 14 days after the plates were inoculated all produced abundant lesions (Table 1). However, there were significant differences in both number of conidia deposited per cm^2 of leaf and percent of leaf area damaged. Regressions of these variables against age of conidia were not significant, and no close relationship between these factors was apparent. Regression of age vs. leaf area damaged was significant, indicating that the infectivity of conidia increased with age (Table 1); this trend was observed in two experiments. For the experimental data reported here, inoculations were made on different dates to plants of similar age from inoculum sources all initiated on the same day. In a second experiment (data not reported), all plants were inoculated on the same day with conidia of appropriate ages. Results were similar, but there were still significant differences in disease damage and estimated numbers of conidia per cm^2 of leaf. These experiments were done before wetting agents were tested, and apparently large (and unexplained) variations can be expected with these methods. Age of conidia probably contributed to this variation, but the effect did not seem to be a large one. Age of conidia (up to 14 days) would probably not greatly affect disease establishment if this factor were held constant within a study.

Numbers of lesions and percent of leaf area damaged were closely related to number of conidia deposited per cm^2 of filter disks, and numbers deposited on disks were closely related to inoculum levels (Table 2). Reduced variation in these results were, in part, attributed to

Table 1. Disease damage to carrot plants inoculated with *Alternaria dauci* conidia of different ages

Age of conidia (days)	Conidia applied[a,b] per cm^2	Leaf area damaged (%)[a,c]
4	7.6	13.1
6	14.4	6.9
8	21.2	42.5
10	11.9	23.3
12	40.8	17.0
14	5.6	48.5
	F value = 11.98^d	

[a] Average for fourth leaf from each of 10 plants.
[b] Regressions for number of conidia vs. leaf age or area damaged were not significant.
[c] X = Percent leaf area damaged, Y = age of conidia ($Y = 1.63X + 2.65$, $r^2 = 0.20$).
[d] Significant at $P = 0.05$ level.

Table 2. Disease damage and numbers of lesions on carrot leaves inoculated with different densities of *Alternaria dauci* conidia

Inoculum dilution	Conidia per ml		Conidia deposited[a] per cm^2 of leaf	Lesions produced		Leaf area damaged (%)
	Expected	Observed		per cm^2	per leaf	
...	47,105	147,105	314.7	20.9	112.6	22.2
1:1	75,553	42,750	138.6	9.3	65.1	9.6
1:10	14,711	5,210	21.1	1.4	5.3	0.9
1:100	1,471	870	2.7	0.2	0.3	0.4
1:1,000	147	155	2.6	0.2	0.5	0.2
1:10,000	15	0	1.6	0.1	0.5	0.0

[a] Estimated by conidia deposited on 25-mm filter disks. All values are averages for five leaves in each of six replicates. X = conidia/ml, Y = conidia deposited/cm^2 of leaf, $Y = 0.00214X + 9.98$, $r^2 = 0.96$. X = conidia/cm^2 of leaf, Y = lesions/cm^2 of leaf, $Y = 0.38X + 0.60$, $r^2 = 0.7$. X = conidia/cm^2 of leaf, Y = percent of disease damage, $Y = 0.07X - 0.2$, $r^2 = 0.64$. X = lesions/leaf, Y = percent of disease damage, correlation coefficient = 0.94.

the use of AG-98. The average leaf area in this experiment was 15.4 cm^2. A calculated value of only 8.79% (SD 4.0) of conidia in inoculum suspensions were deposited on leaves (estimated from deposition on filter disks). The average infection efficiency of conidia (ability to produce a lesion) for all inoculum levels was 6.84% (SD 0.43). Densities of 138 and 314 conidia deposited per cm^2 of leaf obtained from spraying a 1:1 dilution and an undiluted suspension of conidia (1 plate/50 ml) produced nine and 21 lesions per cm^2 and 10 and 22% of leaf area damaged, respectively (Table 2). Thus, undiluted suspensions recovered from CLA plates, or low dilutions of them contain adequate numbers of conidia to establish moderate levels of disease. Results from an identical experiment that used 11% lower original spore concentration were similar but more variable at lower conidial densities than those presented in Table 2.

Use of wetting agents. Wetting agents CS-7 and AG-98 increased the number of spores recovered from CLA plates by 68 and 114%, respectively, compared with water alone. AG-98 increased by tenfold the number of conidia deposited by spray inoculation on filter disks placed in carrot foliage or sprayed directly to simulate inoculation. When water alone was used, a large proportion of conidia were not effectively wetted and many remained on the surfaces of equipment used to contain them.

Average inoculum dilution and spore counting errors for expected and observed numbers were −56.5, 20.13, and 61.8% for water alone, AG-98, and CS-7, respectively. Largest errors were at the greatest dilutions. Clearly, some of the difficulties in relating numbers of conidia in inoculum suspensions to number of lesions produced, to disease damage, or to numbers of spores applied to plants could be due to dilution and counting errors. Wetting agents, especially AG-98, reduced these errors.

Germination of conidia was not significantly affected by suspending conidia in water containing 0, 50, 100, 200, 400, and 800 ppm AG-98. Identical suspensions sprayed on plants resulted in 13.0, 19.4, 15.8, 12.7, 29.2, and 24.3% of leaf area damaged, respectively. The regression equation was significant ($Y = 0.015X + 15.6$, $r^2 = 0.45$) and indicated some increase in disease damage with increasing levels of AG-98. Although an optimum use level of AG-98 was not demonstrated, A. dauci was not affected by levels of AG-98 well above those (100–150 ppm) that improved the efficiency of disease establishment.

Effect of postinoculation environment. Following inoculation and infection, plants kept in a 24 C environment room, a dry greenhouse, and a humid greenhouse developed 1.5, 2.3, and 1.5% of leaf area damaged on sampled leaves, respectively

(not significantly different according to Duncan's multiple range test at $P = 0.05$). Plants kept outdoors or in a greenhouse with intermittent misting at night developed 11.4 and 13.5% of leaf area damaged, respectively. These values did not differ significantly from each other, but were significantly different from the environment room and greenhouse treatments ($P = 0.05$). Experiments in the greenhouse with night hour misting were performed several times with similar results. However, disease development was noticeably reduced when night hour greenhouse temperatures fell below 12 C (author, *unpublished*). Controlled environment tests confirmed this observation. When 11-hr dew periods were provided each night at different temperatures, disease development was normal (night temperatures of 16, 21, and 26 C); average disease damage ratings were 14.2, 12.6, and 16.7%, respectively (not significantly different, $P = 0.05$ level). However, at 11 C the average disease damage rating was 3.1%. This was significantly different from the other three temperatures.

Effect of plant age on disease establishment. Four- to 8-wk-old plants all developed typical disease symptoms and damage. Lesions were produced on all leaves except the youngest, which emerged and expanded after the plants were inoculated; these leaves were not included in damage assessments. There appeared to be more disease damage on all leaves from plants 5–8 wk old than from plants 4 wk old (Fig. 4). On plants 4 wk old, leaves five and six were just emerging when plants were inoculated and were not heavily infected (Fig. 4). The age of plants did not seem to be an important factor, but 5- to 6-wk-old plants appeared to be more suitable for evaluating response to disease.

Previous work on the biology of *A. dauci* has shown that growth and sporulation occur over a wide temperature range (2,4,10,12). This study demonstrated that a temperature between 20 and 24 C is adequate to establish disease and to allow sufficient disease development to assess the response of carrot cultivars and breeding lines.

Moisture requirements were more critical. Leaf wetness, dew, and water vapor are all important for sporulation and for the progress of epidemics involving *A. dauci* (3–6,10,13). In this study, a moist environment that produced wet leaves for a part of each day was essential for both infection and disease development. Infected plants growing in the greenhouse developed symptoms and disease damage levels similar to those on plants grown outdoors, when supplied with intermittent misting at night. Barksdale (1) and Rowell (9) reported a similar relationship between moisture and disease development for *A. solani* Sorauer on tomato plants. They found
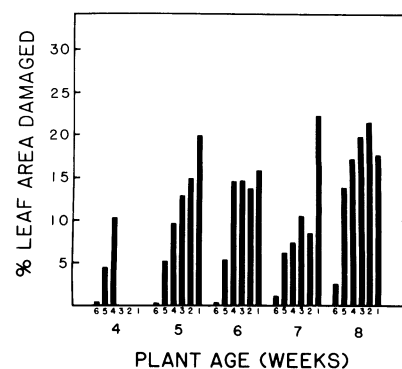

Fig. 4. Effect of plant age on percent of leaf area damaged by *Alternaria dauci*. Numbers refer to leaf number: 1 = oldest, 6 = youngest leaf.

that misting tomato plants enabled the establishment of leaf blight caused by *A. solani* in the greenhouse. The present study demonstrated the importance of moisture to disease development for the closely related pathogen *A. dauci*. The disease damage key was useful to assess host response and to estimate levels of disease damage. It helped to provide more uniform assessment between different experiments.

Alternaria dauci is highly variable in culture and has shown a tendency to change growth characteristics, sporulation ability, and virulence in culture (2,5,7,8, 11). It is likely that pathogen variation contributed to some of the unexplained variation in results reported here. However, problems in preparing and applying known numbers of spores to plants and inadequate control of RH and temperature were probably more important factors that contributed to unexpected differences in infection efficiency and disease damage obtained from apparently similar inoculations.

Although goals to establish more quantitative relationships between inoculum levels, environmental factors, and disease establishment were not fully achieved, this study identified some important factors in disease establishment. It also provided useful techniques and better insight into improving methods to establish Alternaria leaf blight in controlled environments.

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