Detection of *Alternaria dauci* on Carrot Seed

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ABSTRACT

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Alternaria dauci was detected within 7–10 days at 20 C when infested seeds were incubated on filter paper moistened with water or carrot leaf extract. The pathogen was identified by its characteristic conidia. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used to inhibit seed germination. Carrot leaf extract did not improve the efficiency of detection. Concentrations of 2,4-D amine greater than 0.1% inhibited growth and sporulation of *A. dauci*, but concentrations of 2,4-D amine or reagent-grade 2,4-D between 0.0001 and 0.01% had no significant effects on the pathogen or efficiency of detection. Surface disinfestation of seeds with 0.1% $Ca(OCl)_2$ before testing eliminated many saprophytic fungi and improved the sensitivity of the test by twofold. Hot water treatment with or without 1% $Ca(OCl)_2$ reduced the proportion of infested seeds detected but greatly enhanced growth and sporulation of the pathogen on seeds where *A. dauci* survived this treatment. The test was demonstrated to be useful over the range of 0.001–14% infested seeds.

Additional keywords: *Daucus carota* L., seed testing, seedborne disease

Alternaria dauci (Kühn) Groves & Skolko is an important pathogen of carrot (*Daucus carota* L.) that can be seedborne (8–12,14,17). The etiology of seed infection and colonization has been studied (9,13,14), and methods to eradicate the pathogen from seeds have been reported (6,7,15). Detection of *A. dauci* on seeds is essential to establish seed health and to monitor and evaluate disease management and pathogen eradication activities.

Several methods of testing carrot seed for *A. dauci* have been reported (1–4,10,11,15,17,18). All employ modifications of seed germination blotter methods where seeds are incubated under conditions similar to those established for testing seed germination. Seeds and seedlings (commonly 100–400) are then examined for the pathogen. A major disadvantage of the seed germination procedure is that each seed and seedling must be examined under magnification. Seed germination and seedling development make inspection difficult and time-consuming. Moreover, light is an important factor in promoting sporulation by *A. dauci* (16,18,19). Light duration and quality used for seed germination tests are not always conducive to sporulation by the pathogen (1–4,18).

Seed germination blotter methods are adequate to test seed lots containing large proportions of infested seeds, and levels of 2–25% or more have been reported (4,8–11,17). However, contemporary carrot seed production in regions unfavorable for Alternaria leaf blight coupled with other effective disease management activities have greatly reduced the incidence of seedborne *A. dauci*, and more sensitive testing methods are needed.

Other approaches to detect seedborne pathogens on live, but ungerminated, seed have been reported. Maguire et al (5) resolved a problem similar to testing for *A. dauci* on carrot seed. They used 2,4-dichlorophenoxyacetic acid (2,4-D) to inhibit germination of cabbage seeds so reproductive structures of *Phoma lingam* (Tode ex Fr.) Desm. produced on the

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seed surface could be identified without interference from seed germination and seedling development. Pregermination followed by freezing has also been proposed or utilized to prevent germination during tests for other pathogens (1,2,5).

This study reports a method to detect *A. dauci* on carrot seeds in which 2,4-D was used to inhibit seed germination. Carrot leaf extract (CLE), which has promoted sporulation of the pathogen on agar media (16), and mild surface disinfestation to reduce saprophytic fungi were also evaluated as possible enhancements to the assay.

MATERIALS AND METHODS

Seeds of the carrot cultivar Hicolor 9 were planted in December 1985 at Zellwood, FL, and grown with methods commonly used to produce market carrots. Winter temperatures were cold enough to induce flowering by early May. Alternaria leaf blight, present in nearby crops, quickly colonized seed stalks and flower parts of seed plants. Umbels were harvested in June and dried for 3 wk in an air-conditioned laboratory. Seeds were separated and cleaned by hand. About 500 g of seed (hereafter referred to as the infested seed lot) was stored at ambient laboratory conditions until tested. A 1.0% aqueous stock solution of 2,4-D was prepared from a 2,4-D dimethylamine herbicide formulation (DMA 4, 41.5% 2,4-D acid equivalent), hereafter referred to as 2,4-D amine, or from reagent-grade 2,4-D (0.3 mg/ml) prepared by dissolving 300 mg of 2,4-D (No. 5532, Eastman Kodak Co.) in 50 ml of ethyl alcohol, then slowly adding this solution to 950 ml of water with constant stirring.

Effects of 2,4-D on seed germination were examined with aqueous solutions of 0.0001, 0.0005, 0.001, 0.005, and 0.01% 2,4-D and 2,4-D amine. The solutions were used to moisten filter papers lining the bottom of plastic petri dishes. Seeds from a commercial lot of Hicolor 9 were uniformly distributed on the moist filter papers (100 seeds per plate, four plates per treatment), and plates were placed at 20 C in a 16-hr daylength (cool-white fluorescent light, 50 $mol \cdot m^{-1} \cdot s^{-2}$). Numbers of seeds germinated after 7 and 14 days were recorded.

To test for seedborne *A. dauci*, seeds were distributed on a double thickness of filter paper (two disks) lining the bottom of plastic petri dishes. Initially, 11-cm-diameter dishes were used to test seeds for the pathogen and for effects of 2,4-D on germination. Later, dishes 15 cm in diameter and 25 mm deep were used; the large dishes permitted greater seed spacing and reduced chances of secondary colonization of seeds close to infested ones. Just before seed placement, sterile water or sterile CLE with or without 2,4-D was applied to the filter paper. Properties of CLE (an extract made from 20 g/L of dried carrot leaves in water) and the preparation of carrot leaf agar (CLA) have been described in a previous paper (16). Three milliliters of liquid were added to 11-cm dishes and 8 ml to 15-cm dishes. Dishes were sealed in large, airtight plastic boxes (32× 24× 12 cm) or 2-mil polyethylene bags and placed at 20 C in a 16-hr daylength (30–50 $mol \cdot s^{-1} \cdot m^{-2}$). No water or CLE was added after initial preparation; plates kept in plastic bags or boxes maintained sufficient moisture to allow sporulation by *A. dauci*. At 7, 10, and, in some experiments, 14 days after plating, seeds were examined for conidia of *A. dauci*.

The sensitivity of the test was determined with samples prepared by mixing seeds from the infested lot with a commercial lot of Hicolor 9. The commercial lot was tested twice for *A. dauci* (10,000 seeds each test) and the pathogen was not detected. The infested lot was assumed to contain an average value of 14.2% *A. dauci*-infested seed on the basis of four tests (500 seeds per test). The basic assay method with CLE and 0.01% 2,4-D amine was used for these tests. Seeds were counted and mixed in proportions of 50:50, 40:60, 30:70, 20:80, 10:90, 5:95, 2:98, and 1:99 to provide an expected range of 14–0.0014% infested seed in the prepared samples. Ten replicates of 100 seeds from each mixture were tested in 15-cm dishes on filter paper moistened with CLE containing 0.01% 2,4-D.

To test for effects of 2,4-D on growth and sporulation of *A. dauci*, 2,4-D amine or 2,4-D was added to CLA before sterilization to provide concentrations of 0.005, 0.01, 0.05, 0.1, and 1.0% 2,4-D amine and 0.001, 0.05, and 0.01% 2,4-D. Five small disks (8 mm diameter) were punched from 47-mm cellulose filter pads, sterilized, and placed on CLA plates. Disks were inoculated with 10 μl of water that contained approximately 50 conidia. Plates were incubated at 24 C in a 16-hr daylength (fluorescent and incandescent lights, 150 $mol \cdot s^{-1} \cdot m^{-2}$); these conditions were previously shown to be optimum for growth and sporulation (16). After 5 days, disks were transferred to vials containing 5 ml of a detergent solution (50 ppm AG-98) and five glass beads and shaken vigorously for 30 sec to dislodge and disperse conidia. Conidia in each of five 50-μl subsamples were counted at 100×.

Effects of 2,4-D on efficiency of detection were also examined. CLE with 0, 0.01, 0.05, 0.1, 0.2, and 0.5% 2,4-D amine and 0.01, 0.05, and 0.1% 2,4-D was used to moisten filter papers in 15-cm dishes as already described. Small, randomly selected increments of the infested seed lot were combined until a 150-mg sample (approximately 150 seeds) was obtained. Samples were weighed rather than counted to eliminate bias toward larger and heavier seeds. Samples were distributed uniformly in each of four plates for each 2,4-D concentration and placed at 20 C in a 16-hr daylength (50 $mol \cdot s^{-1} \cdot m^{-2}$). After 7 and 10 days, seeds were examined for the pathogen. Detection efficiency with CLE was evaluated with identical methods; 0.1, 0.5, 1, and 2× CLE (with 0.01% 2,4-D amine) were compared with 0.01% 2,4-D amine alone.

Mild surface disinfestation at 20 C was performed on 150-mg seed samples held in small, loose-fitting cheesecloth bags. Seeds were treated for 10 min in 500 ml of 0.05, 0.1, or 0.2% $Ca(OCl)_2$ or water alone, with constant agitation. Seeds were washed four times in tap water, dried for 1 hr on paper towels in an air-conditioned room, then tested for *A. dauci*; 0.01% 2,4-D was used without CLE. Heat was also used to surface-disinfest seeds. Seeds were soaked, while constantly agitated, for 12 min in water or 1.0% $Ca(OCl)_2$ maintained at 50 C in a temperature-controlled water bath. Treated seeds were bagged, dried, and tested with methods identical to those described for mild surface disinfestation.

RESULTS AND DISCUSSION

Seed germination occurred at low concentrations of 2,4-D but was completely inhibited at the 0.01% level. Some seeds germinated at 0.01% 2,4-D, but emerged radicles turned brown and did not elongate. The regression equation for 2,4-D (0.0001–0.01%) and seed germination at 20 C was $Y = -0.1135X + 0.728$, $r^2 = 0.47$. The lowest effective concentration (0.01%) was used for all subsequent tests. Experiments with 2,4-D amine produced similar results.

After 7 days on filter paper moistened with CLE and 0.01% 2,4-D amine, conidia of *A. dauci* were visible on the surfaces of infested seeds. However, saprophytic fungi also colonized seed surfaces, often obscuring the conidia of *A. dauci* and making identification difficult. After 10 days, additional *A. dauci*-infested seeds were observed but

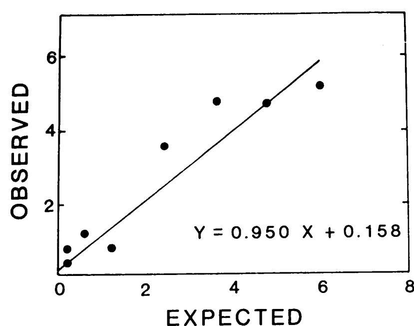

Fig. 1. Observed and expected values of *Alternaria dauci*-infested seeds in prepared seed mixtures containing calculated proportions of 0.001–14% infested seeds.

growth of saprophytic fungi had also increased. By 14 days, profuse growth of saprophytes made identification of the pathogen very difficult. Thus, for all subsequent tests in this study, seeds were examined only at 7 and 10 days after plating. Later work (*unpublished*) demonstrated that about 50% of commercial seed lots tested with this method were not heavily colonized by saprophytes and could be evaluated after 14 days. From four experiments (with CLE and 0.01% 2,4-D amine), it was concluded that the infested seed lot contained 14.2% infested seeds.

Prepared samples with smaller ratios of infested to noninfested seeds were tested with CLE and 0.01% 2,4-D. Numbers of infested seeds observed at 10 days agreed closely with expected values over the calculated range of 0.0014–14% (Fig. 1). The regression equation for expected vs. observed values over this range was $Y = 0.950X + 0.158$, $r^2 = 0.90$.

Maguire et al (5) reported that different formulations and sources of 2,4-D affected the results of their assay for *P. lingam* on crucifer seeds. Because 2,4-D amine was soluble in water and readily available, it was used during early development of the seed assay. However, reagent-grade 2,4-D is soluble in water at levels needed to inhibit seed germination and could provide more uniform test results. Thus, it was important to demonstrate that for 2,4-D concentrations used in the carrot seed assay, results obtained with 2,4-D amine and reagent-grade 2,4-D were not significantly different.

Added to CLA, 2,4-D amine (0.01–1.0%) reduced growth and sporulation by *A. dauci*. Mycelial growth observed on agar and cellulose disks was greatly reduced as 2,4-D amine was increased above 0.05%. Spore production on cellulose disks decreased accordingly. The regression equation over the range of 0.01–1.0% 2,4-D amine and conidia produced per square millimeter of disk was $Y = -270.3X + 238.5$, $r^2 = 0.68$. Concentrations of 2,4-D amine from 0.0001 to 0.01% did not significantly affect growth and sporulation; the regression equation for 2,4-D amine level and conidia per square millimeter was $Y = -31.23X + 21.92$, $r^2 = 0.0002$ ($P > F = 0.99$). It was not possible to examine effects of 2,4-D levels >0.01% because of its low solubility, but 2,4-D levels from 0.0001 to 0.01% did not significantly affect sporulation. It was concluded that the level of 2,4-D needed to inhibit germination (0.01%) did not affect growth and sporulation of *A. dauci*.

Results of early experiments with 2,4-D amine were similar to those obtained later with 2,4-D. Numbers of infested seeds detected when 2,4-D was used at 0.01% or less were not significantly different ($P = 0.05$) from numbers found with 2,4-D amine. For example, at the 0.01% level, there were 16.2% (SD = 4.16) infested seeds detected with 2,4-D amine and 14.0% (SD = 3.14) with 2,4-D; values were not significantly different ($P = 0.05$). Although 2,4-D amine could be used in this assay, reagent-grade 2,4-D should provide more uniform results.

Although CLE incorporated into an agar medium (CLA) effectively promoted sporulation by *A. dauci* (14) and enhanced sporulation on infected plant materials placed on filter paper moistened with CLE (*unpublished*), its use in the seed assay was not beneficial. There were no significant differences between numbers of infested seeds detected when CLE with 0.01% 2,4-D or 2,4-D alone was used. For 10 paired replicates of 100 seeds, 10.20% (SD = 2.39) and 12.20% (SD = 2.94) infested seeds were found with CLE plus 2,4-D and 2,4-D alone, respectively. The values were not significantly different (*t* test or ANOVA, $P = 0.05$). When CLE was used, saprophytic fungi were observed to produce more mycelia and sporulation by *A. dauci* was slightly reduced.

Unexpected variation in numbers of infested seeds detected in treatment replications prompted an examination of environmental factors affecting the assay. When dishes were stacked five high in clear plastic boxes, more infested seeds were detected in the first and second dish from the top of the stack than in dishes in lower positions. This was apparently due to differences in light intensity caused by stacking the dishes. When dishes were placed unstacked in plastic bags or boxes, the effect was eliminated. With this modification, overall detection efficiency was improved and the apparent level of infestation in the infested seed lot was found to be 22% instead of 14.2%.

Saprophytic fungi also colonized seed surfaces when CLE was not used. Mild surface disinfestation of seeds greatly reduced colonization by saprophytes. Sporulation by *A. dauci* was more profuse and the conidia were more easily seen on disinfested seeds (Table 1). When seeds were partially disinfested, infested seeds bearing only a few (one to five) conidia of *A. dauci* could be identified at 7 days after plating. Seeds that received mild disinfestation treatments were mostly free from saprophytic fungi at 7 and 10, but not 14, days after plating. After treatment with 0.05, 0.1, and 0.2% $Ca(OCl)_2$, 42.2, 34.4, and 40.7% infested seeds were detected, respectively. These values were significantly greater than those for untreated seeds ($P = 0.05$) but did not differ significantly from each other. The regression equation for $Ca(OCl)_2$ between 0.05 and 0.5% and percent infested seed detected was $Y = 0.196X + 30.2$, $r^2 = 0.11$; the slope of the regression equation was not significant ($P > F = 0.34$). Treatment with 0.05, 0.1, or 0.2% $Ca(OCl)_2$ for 10 min at 20 C improved the efficiency of detection by about twofold (Table 1) and demonstrated that the infested seed lot contained between 35 and 42% infested seeds.

Hewett (4) reported that surface disinfestation of carrot seeds with 1% available chlorine for 10 min reduced numbers of bacteria and saprophytes but also reduced numbers of *A. dauci*-infested seeds detected in his assay. Maguire et al (5) found no significant effects from disinfestation of cabbage seeds with 0.1% $Ca(OCl)_2$ in their assay for *P. lingam*. In this study, numbers of *A. dauci*-infested seeds increased after disinfestation with 0.1% $Ca(OCl)_2$, but the large improvement in detection efficiency seemed to be due to suppression of saprophytes. This apparent increase in efficiency could have obscured small deleterious effects of $Ca(OCl)_2$ on the pathogen.

Hot water treatment reduced populations of saprophytic fungi but suppressed *A. dauci* as well. Numbers of infested seeds were reduced to 49.3% of those detected on untreated seeds and to 33% of the number detected on seeds treated with 0.1% $Ca(OCl)_2$ at 20 C (Table 1). Treatment with 1.0% $Ca(OCl)_2$ at 50 C almost eliminated saprophytic fungi but did not eradicate *A. dauci*. Numbers of infested seeds detected were about half the number found after mild disinfestation but were not significantly different from numbers detected after hot water treatment alone (Table 1). Within 5 days after plating seeds treated with hot water

Table 1. Effects of some surface-disinfestation treatments on detection of *Alternaria dauci*-infested carrot seed in the 2,4-D test

Treatment	Percent infested[a]	Comments
None	22.3[b]	Many saprophytes, sparse sporulation
0.05% $Ca(OCl)_2$	42.2[c]	Reduced saprophytes, enhanced sporulation
0.1% $Ca(OCl)_2$	34.4[c]	Reduced saprophytes, enhanced sporulation
0.2% $Ca(OCl)_2$	40.7[c]	Reduced saprophytes, enhanced sporulation
Hot water, 50 C, 12 min	11.0[d]	Few saprophytes, enhanced sporulation
Hot water with 1.0% $Ca(OCl)_2$	14.4[d]	Very few saprophytes, enhanced sporulation

[a] Average for four replicates, approximately 165 seeds per replicate.
[b] Significantly different from others (*t* test, $P = 0.05$).
[c] Not significantly different from each other (slope of regression line not significant, $P = 0.05$).
[d] Not significantly different from each other (*t* test, $P = 0.05$).

or hot water + $Ca(OCl)_2$, propagules of *A. dauci* that survived these treatments grew profusely on infested seeds and colonized the filter paper. Some colonies reached 1 cm in diameter and were visible without magnification. This effect was more striking with seeds treated with hot water + $Ca(OCl)_2$. Failure of hot water or hot water + $Ca(OCl)_2$ to eradicate *A. dauci* from seeds demonstrated that the pathogen could survive hot water treatments (10–12 min at 50 C) currently used to eradicate *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson from carrot seed. Moreover, these results reenforce the conclusion that mild disinfestation with $Ca(OCl)_2$ does not reduce populations of the pathogen to levels unacceptable for seed testing.

Responses of the pathogen to disinfestation by heat and $Ca(OCl)_2$ cannot be entirely explained by the suppression of saprophytic fungi that obscured identification of *A. dauci* conidia. It is likely that populations of fungi, and possibly bacteria, antagonistic to *A. dauci* were reduced. The possible occurrence of biotic factors antagonistic to *A. dauci* indicates mild surface disinfestation should be routinely applied to seeds tested for this pathogen and may suggest opportunities for biological control as well.

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