Pathogenicity of *Alternaria angustiovoidea* on Leafy Spurge

SHAW-MING YANG, Research Plant Pathologist, D. R. JOHNSON, Research Biologist, and W. M. DOWLER, Supervisory Plant Pathologist, Foreign Disease-Weed Science Research Unit, USDA-ARS, Ft. Detrick, Bldg. 1301, Frederick, MD 21701

ABSTRACT

Yang, S.-M., Johnson, D. R., and Dowler, W. M. 1990. Pathogenicity of *Alternaria angustiovoidea* on leafy spurge. Plant Dis. 74: 601-604.

Experiments were conducted as a preclude to the use of *Alternaria angustiovoidea* as a biological control agent for leafy spurge (*Euphorbia esula*). A single dew period of at least 12 hr was needed for spores to cause slight infection (a few lesions) on inoculated leaves. To obtain severe infection (dead plants), a single dew period of at least 48 hr was needed. Two successive dew periods of up to 12 hr each did not increase infection over that of a single, 24-hr dew period. Twenty-two out of 25 collections of leafy spurge were susceptible to a single culture of *A. angustiovoidea* when they were incubated in dew chambers at 20–25 C for 48 hr after inoculation. Minor infection also occurred on globe artichoke, corn, cowpea, okra, safflower, and zinnia under the same conditions. *A. angustiovoidea* produced phytotoxins that caused chlorosis and wilting of leaves on cuttings of leafy spurge placed in the culture filtrate.

Leafy spurge (*Euphorbia esula* L.), a polymorphic complex of many biotypes or species (4), is a perennial weed of the grasslands of North America. The economic losses due to the weed are estimated to be more than 50 million dollars annually in the United States (C. Messersmith, *personal communication*). Chemical control of the weed is both difficult and costly. The most effective herbicides often cannot be used near other desirable broadleaf species. Therefore, biological control using plant pathogens is being considered as a potential alternative control method.

Alternaria angustiovoidea Simmons (5) has been considered for biological control of leafy spurge (2). We reported (8) preliminary studies on infection of several collections of leafy spurge by this fungus. Ten out of 14 collections of leafy spurge showed resistance to *A. angustiovoidea* when the inoculated plants were incubated in the dew chamber at 20 C for 12 hr (8). Whether or not these resistant collections would become susceptible to *A. angustiovoidea* when a longer dew period is given to the inoculated plants is unknown. *A. alternata* (Fries) Keissler, a causal agent of black leaf blight of spotted knapweed (*Centaurea maculosa* Lam.), produced a secondary metabolite, maculosin, that is phytotoxic only to spotted knapweed (6). However, it is also unknown whether or not secondary metabolites produced by *A. angustiovoidea* are phytotoxic to leafy spurge.

The objectives of this study were to: 1) determine the effect of different lengths of dew periods on infection of leafy spurge by *A. angustiovoidea*, 2) study the reaction of different collections of leafy spurge and other plant species to this fungus, and 3) obtain preliminary evidence as to whether this pathogen produces phytotoxins in culture.

Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Accepted for publication 3 January 1990 (submitted for electronic processing).

MATERIALS AND METHODS

Preparation of inoculum. A culture of *A. angustiovoidea*, originally isolated by Krupinsky and Lorenz (2), was grown on potato-dextrose agar (PDA) or on autoclaved (210 C for 30 min) leaves of leafy spurge on moistened filter paper in petri dishes and incubated in an incubator at 20 C (12 hr light, 90 $\mu E \cdot sec^{-1} \cdot m^{-2}$). Conidia of *A. angustiovoidea* were collected from 4- to 5-wk-old cultures on PDA or from 2-wk-old cultures on autoclaved leaves of leafy spurge by flooding cultures with a sterile, aqueous solution (distilled water + 0.1% polyoxyethylene sorbitan monolaurate [Tween 20] + 2% gelatin + 2% sucrose) and rubbing the surface with a spatula. The combined conidial suspension was filtered through two layers of cheesecloth and adjusted to contain 1×10^4 to 1×10^5 spores per milliliter. Inoculum was atomized onto the plants (3 ml for 10 shoots), and the inoculated plants, unless otherwise stated, were immediately incubated in a dew chamber at 20–25 C for 48 hr before being moved to greenhouse benches. Control plants were atomized with the sterile aqueous solution.

Growth of leafy spurges. All leafy spurges used in this study were propagated from two to three root buds in greenhouse mixed soil in 10-cm-diameter clay pots in the containment greenhouse at the Foreign Disease-Weed Science Research Unit at Frederick, MD. The greenhouse soil was prepared by mixing 40 shovels of field soil, 40 shovels of peat moss, 40 shovels of vermiculite, 30 shovels of perlite, 20 shovels of sand, 2 L of lime stone, 1 L of fertilizer (10-10-10), and 1 L of Aqua Grow "G" granular (blended nonionic soil wetting agent, polyethylene ester of cyclic acid [18.8%], polyethylene ether of alkylated phenols [18.8%], silicone antiform emulsion [2.4%], and vermiculite [60%]; Aquatrols Corp. of America, 1423 Union Ave., Pennsauken, NJ). Water was given every other day. Leafy spurge, at different growth stages before flowering, was used for the inoculation studies. Preliminary studies indicated that the older plants were as susceptible as young plants to *A. angustiovoidea*.

Effect of dew periods on infection of leafy spurge. After inoculation with a conidia suspension of *A. angustiovoidea*, the leafy spurge plants (1982MT000) were placed on greenhouse benches (0 hr dew) or in dew chambers (25 C) in darkness for 2, 4, 6, 12, or 24 hr before moving to the greenhouse. Each initial dew period was followed by a second dew period of either 0, 2, 4, 6, or 12 hr, administered 1, 2, 3, or 4 days after the initial dew period. Each treatment had six plants (total of 126 treatments and 756 plants in one test), and the experiment was repeated once.

Disease severity was rated 2 wk after inoculation. A numerical system, 0–4, was used to rate the severity where: 0 = no infection; 1 = chlorosis or one to two small brown spots on the leaves; 2 = three to 15 spots on the leaves; 3 = leaves wilted and easily detached; and 4 = leaves brown and the plants dying or dead. A disease index score was then calculated by [summation of (severity rating × number of plants in that rating)]/total number of plants. A disease index at or below 2.9 was considered resistant and that above 2.9 was considered susceptible. After readings were taken, attempts were made to reisolate the pathogen on PDA from tissues cut from selected slightly and severely infected leaves. The tissue pieces (0.3 × 0.8 cm) were surface sterilized in 3% NaOCl solution for 5–10 min, washed in sterile distilled water for 5–10 min, placed on PDA plates (four

in each plate), and incubated at 20 C for 3–4 wk. Presence of *A. angustiovoidea* was determined by examining conidia under the microscope.

In another experiment, inoculated plants of collection 1982MT000 were either given a dew period of 0, 6, 12, 24, or 48 hr, or they were placed in the greenhouse for 1, 2, 3, or 4 days before they were given a dew period of 0, 6, 12, 24, or 48 hr. Each treatment had five plants (total of 125 plants per test), and the test was repeated once. Disease severity was determined 2 wk after inoculation.

Inoculation of collections of leafy spurge and other plant species. Leafy spurge was collected from Europe and the United States by W. L. Bruckart, D. G. Davis, R. M. Hosford, S. K. Turner, and R. Vonmoos. The collections were numbered following the system proposed by Davis (1): the year of collection followed by a one- or two-letter designation for country or state of origin and a 3-digit number showing the sequence of collections. Sixteen to 24 plants of each collection in two tests were inoculated with *A. angustiovoidea* and incubated in dew chambers at 25 C for 48 hr.

Seedlings of other plant species, alfalfa (*Medicago sativa* L.) cv. Williamburg, artichoke (*Cynara scolymus* L.) cv. Green Globe, corn (*Zea mays* L.) cv. 3369A, cowpea (Vigna sinensis (L.) Engl.) cv. California blackeye, jute (*Corchorus capsularis* L.), mung bean (*Phaseolus aureus* Roxb.), oats (*Avena sativa* L.) cv. Clinton, okra (*Hibiscus esculentus* L.) cv. Cleanson spineless, peanut (*Arachis hypogaea* L.) cv. Wilco 1, rice (*Oryza sativa* L.) cv. M201, safflower (*Carthamus tinctorius* L.) cv. Pacific 1, sorghum (*Sorghum bicolor* L.) cv. Top Hand, sunflower (*Helianthus annuus*) cv. 894, wheat (*Triticum aestivum* L.) cv. Max, and zinnia (*Zinnia grandiflora* Nutt.) cv. Cupid, were also inoculated with *A. angustiovoidea* and incubated in dew chambers at 25 C for 12, 24, or 48 hr. Five plants of each species were inoculated each time, and the test was repeated once. Readings were taken 2 wk after inoculation.

Bioassay for phytotoxin production. A small piece of agar with mycelium taken from the edge of 3- to 4-day-old cultures on PDA was placed in each 250-ml flask (total of 10 flasks in each test) containing 50 ml of Fries liquid medium (7) minus lithium chloride. Flasks containing only Fries medium served as

Table 1. Effect of length of initial dew period and length and time of second dew periods on severity of disease[x] on leafy spurge inoculated with *Alternaria angustiovoidea* at 20–25 C

Second dew period		Initial dew length (hr)						LSD[y]
Days after initial dew	Length (hr)	0	2	4	6	12	24	($P = 0.05$)
0		0	0	0	0	1.5[z] cd	2.5 c	0.6
1	0	0	0	0	0	1.4 cd	2.5 c	0.6
	2	0	0	0	0	1.8 bc	2.5 c	0.6
	4	0	0	0	0	1.9 bc	2.9 b	0.8
	6	0	0	0	0	2.8 a	2.8 bc	0.6
	12	0	0	0	0	2.3 ab	3.3 a	0.9
2	0	0	0	0	0	1.3 cd	2.5 c	0.9
	2	0	0	0	0	1.5 cd	2.7 bc	0.9
	4	0	0	0	0	1.3 cd	2.8 b	0.9
	6	0	0	0	0	1.3 cd	2.9 b	0.9
	12	0	0	0	0	1.2 cd	2.9 b	0.6
3	0	0	0	0	0	1.5 cd	2.7 bc	0.6
	2	0	0	0	0	1.3 cd	2.7 bc	0.6
	4	0	0	0	0	1.4 cd	2.5 c	0.6
	6	0	0	0	0	1.5 cd	2.8 bc	0.6
	12	0	0	0	0	1.4 cd	2.9 b	1.4
4	0	0	0	0	0	1.6 c	2.5 c	0.9
	2	0	0	0	0	1.2 d	2.5 c	0.6
	4	0	0	0	0	1.6 c	2.9 b	0.6
	6	0	0	0	0	1.5 cd	2.7 bc	0.9
	12	0	0	0	0	1.5 cd	2.9 b	0.9

[x] Disease index was calculated from [summation of (severity rating × number of plants in the rating)]/total number of plants. A numerical system, 0–4, was used to rate the severity of disease. Average of 12 plants in each treatment in two tests.
[y] LSD ($P = 0.05$) used to compare the paired means of dew at 12 and 24 hr in the same row.
[z] Numbers followed by the same letter in a column are not significantly different according to Duncan's new multiple range test ($P = 0.05$).

Table 2. Effect of delaying initial dew period on severity of disease on leafy spurge inoculated with *Alternaria angustiovoidea*

Initial dew given after inoculation (days)	Hours in dew chamber at 25 C				
	0	6	12	24	48
0	0 z[a]	0 z[b]	1.5 ay	2.4 ax	4.0 aw
1	0 z	0 z	0.6 by	1.5 ax	4.0 aw
2	0 y	0 y	0.0 cy	2.1 ax	3.9 aw
3	0 y	0 y	0.0 cy	1.9 ax	3.8 aw
4	0 y	0 y	0.0 cy	1.9 ax	4.0 aw

[a] Disease index was calculated from [summation of (severity rating × number of plants in the rating)]/total number of plants. A numerical system, 0–4, was used to rate the severity of disease. Average of 10 plants in two tests.
[b] Numbers followed by the same letter (abc for same column, and wxyz for same row) are not significantly different according to Duncan's new multiple range test ($P = 0.05$).

Table 3. Reaction of leafy spurge (*Euphorbia esula*) collections to inoculation with *Alternaria angustiovoidea*[a]

Collection[b]	Origin (country or state)	Disease index[c]
1977A001	Austria	3.6
1978A001	Austria	2.3
1981I002	Italy	3.8
1982TU001	Turkey	4.0
1982TU002	Turkey	2.3
1982YU001	Yugoslavia	4.0
1978ID001	Idaho	4.0
1978ID002	Idaho	3.8
1982MA001	Massachusetts	4.0
1982MD001	Maryland	4.0
1978MI001	Michigan	3.9
1979MN001	Minnesota	3.7
1979MN007	Minnesota	4.0
1979MN008	Minnesota	4.0
1982MT000	Montana	3.5
1979NE001	Nebraska	4.0
1979NE002	Nebraska	4.0
1988NE001	Nebraska	4.0
1978NV001	Nevada	3.7
1982NJ001	New Jersey	3.5
1982NJ002	New Jersey	3.9
1979ND006	North Dakota	3.5
1986ND001	North Dakota	3.5
1978OR001	Oregon	3.8
1982BC001	British Columbia, Canada	2.8

[a] Inoculated plants were incubated in dew chambers at 25 C for 48 hr before being placed on greenhouse benches for an additional 12 days.
[b] Collection numbers consist of the year of collection, followed by a one- or two-letter designation for country or state of origin, and by a 3-digit number showing the sequence of the collection.
[c] Disease index was calculated from [summation of (severity rating × number of plants in the rating)]/total number of plants. A numerical system, 0–4, was used to rate the severity of disease. (Ratings ≤ 2.9 = resistant reaction). Average of 16–24 plants of each collection in two tests.

controls. The flasks were placed in an incubator (12 hr light, 20 $\mu E \cdot sec^{-1} \cdot m^{-2}$/day) at 25 C. After 1–2 wk of incubation, the cultures in each flask were separately centrifuged at 6,600 rpm for 10 min to pellet the mycelium. The supernatant in each centrifuge tube was separately passed through a membrane filter (pore size 0.45 μm) and collected in 15-ml sterile polypropylene centrifuge tubes. The stem end of a healthy shoot of leafy spurge freshly cut from greenhouse-grown plants was immediately immersed in the solution and held at 20±2 C with 10 hr light, 9.5 $\mu E \cdot sec^{-1} \cdot m^{-2}$/day. Cuttings placed in the Fries medium from the control flasks or distilled water served as controls. Cuttings were examined 24–48 hr later to observe reactions. Chlorosis and wilting of leaves was assumed to indicate the presence of phytotoxins in the culture filtrate. The objective of this study was to obtain preliminary evidence of phytotoxins produced by this pathogen; therefore, only presence or absence of symptoms was scored. This test was repeated twice.

RESULTS AND DISCUSSION

Effect of dew periods on infection of leafy spurge. Both runs of the experiment produced similar results and data were pooled (Table 1). An initial dew period of 6 hr or less and a second dew period of 12 hr or less did not allow disease to develop (Table 1). A single dew period of at least 12 hr was needed to cause slight infection (a few spots on inoculated leaves) on leafy spurge (Tables 1 and 2). Leafy spurge given a 24-hr dew period after inoculation had significantly greater disease severity than that given a 12-hr dew period (Tables 1 and 2). Similarly, leafy spurge that received a second dew period of 24 hr (1–4 days after an initial 24-hr dew period given soon after inoculation) also had significantly greater disease severity than leafy spurge that received a second dew period of 12 hr. Severity of disease on leafy spurge receiving a second 12-hr dew period 1 day after an initial 12-hr dew period was similar to that of plants receiving a single 24-hr dew period. When the second 12-hr or 24-hr dew period was given 2, 3, or 4 days after inoculation, the severity of disease was significantly reduced.

To cause severe infection (dying or dead plants), a single dew period of at least 48 hr was needed (Table 2). When an initial 12-hr dew period was delayed 2 days or more after inoculation, there was no infection. When an initial 24-hr or 48-hr dew period was delayed up to 4 days after inoculation, the disease severity was similar to that of plants that received dew periods immediately after inoculation. Both runs of the experiment gave similar results, and data were pooled. *A. angustiovoidea* required dew periods of at least 48 hr at 20–25 C for severe disease development; this may be one of the reasons why the pathogen does not become epidemic on leafy spurge in the Great Plains. The pathogen was reisolated from all of the selected severely infected plants but from only 40% of slightly infected plants.

Inoculation of collections of leafy spurge and other plant species. Twenty-two out of 25 leafy spurge collections were rated susceptible to *A. angustiovoidea* when inoculated plants were incubated for 48 hr in the dew chamber (Table 3). Susceptible leafy spurge included those collections from Austria, Italy, Turkey, Yugoslavia, Idaho, Maryland, Massachusetts, Michigan, Minnesota, Montana, Nevada, New Jersey, North Dakota, and Oregon. Both resistant and susceptible individual plants were found in populations of one collection from Austria (1978A001), one from Canada (1982BC001), and one from Turkey (1982TU002). It is not known whether resistant individuals would show a susceptible reaction to a different culture of the fungus. The 10 collections of leafy spurge that showed resistance to *A. angustiovoidea* at 20 C in a dew chamber for 12 hr (8) also became susceptible to this pathogen when the plants were incubated in dew chambers at 25 C for 48 hr (*unpublished*). This result confirms the previous experiments that this pathogen requires an extended dew period for inducing severe disease on leafy spurge. Control plants showed no symptoms of the disease. The pathogen was reisolated from all of the selected severely infected plants. However, the pathogen was reisolated from only 30–50% of the selected leafy spurge plants that gave a resistant reaction. The pathogen was not isolated from the control plants. The repeat experiment produced similar results, and data were pooled (Table 3).

A. angustiovoidea did not infect alfalfa, jute, peanut, rice, sorghum, sunflower, or wheat, but it induced a few spots on globe artichoke, corn, cowpea, mung bean, oats, okra, safflower, and zinnia when the inoculated plants were incubated in dew chambers at 25 C for 48 hr. The pathogen also was reisolated from infected plants of these species. However, infection was not found on these species when the inoculated plants were given 12-hr and 24-hr dew periods.

The fungus used in this study infected plants other than leafy spurge only after an extended dew period. It will be important to compare disease severity on the various hosts under field conditions to determine their relative susceptibility if this fungus is to be used as a candidate for a mycoherbicide.

Bioassay for phytotoxin production. *A. angustiovoidea* appeared to produce phytotoxins in the Fries liquid medium (Fig. 1). The pathogen produced the toxin(s) in vitro within 1 wk at 25 C, but 2-wk-old cultures also had phytotoxic activity. Phytotoxic activity was seen in 27 of the 30 flask-cultures in three tests. The three flask-cultures that showed no activity were contaminated either with bacteria or other fungal species. Chlorosis of leaves and wilting were observed 24 hr after exposing the leafy spurge cutting to the culture filtrate. The phytotoxins are not apparently host specific because partially purified toxin also caused chlorosis on duckweed (*Lemna obscura* L.) (3). The characteristics, structure, and efficacy of the toxins for control of leafy spurge in the greenhouse are under investigation.

Fig. 1. Wilting of leaves on cuttings of leafy spurge when immersed in the culture filtrate of *Alternaria angustiovoidea*. Left, cutting in sterile Fries culture medium; center, two cuttings in culture filtrates; and right, cutting in sterile distilled water. Photo was taken 48 hr after exposure.

Our results with *A. angustiovoidea*, and similar results obtained by others (2), suggest that this fungus might be useful in biological control of leafy spurge. However, before considering large-scale field trials, knowledge should be gathered about 1) how to increase the effectiveness of *A. angustiovoidea* on leafy spurge, 2) potential secondary spread of the fungus, 3) overwintering ability, and 4) the potential for damage to nontarget crops.

LITERATURE CITED

1. Davis, D. G. 1985. The status of the leafy spurge numbering system. Pages 1-2 in: Proceedings of the Leafy Spurge Symposium, 1985. Montana State Univ., Bozeman. July 17 and 18.
2. Krupinsky, J. M., and Lorenz, R. J. 1983. An *Alternaria* sp. on leafy spurge (*Euphorbia esula*). Weed Sci. 31:86-88.
3. Leather, G. R., Yang, S. M., and Dowler, W. M. 1989. Control of leafy spurge with natural chemical products. Pages 46-50 in: Proceedings of the Leafy Spurge Symposium, 1989. Montana State Univ., Bozeman. July 12 and 13.
4. Radcliffe-Smith, A. 1985. Taxonomy of North American leafy spurge. Pages 14-25 in: Leafy Spurge. A. K. Watson, ed. Weed Science Society of America, Champaign, IL.
5. Simmons, E. G. 1986. *Alternaria* themes and variations (14-16). Mycotaxon 25:195-202.
6. Stierle, A., Cardellina, J., II, Park, S. H., and Strobel, G. 1989. Maculosin, a host specific toxin from *Alternaria alternata* on spotted knapweed. Agrochemicals Division 105. Book of Abstracts, 197th ACS National Meeting, Dallas, TX. April 9-14, 1989, ACS, Washington, D.C.
7. Tuite, J. 1969. Plant Pathological Methods. Fungi and Bacteria. Burgess Publishing Co., Minneapolis, MN. 239 pp.
8. Yang, S. M., Johnson, D. R., Dowler, W. M., and Krupinsky, J. M. 1988. Reaction of different biotypes of leafy spurge and other plant species to *Alternaria tenuissima* f. sp. *euphorbiae*. Pages 56-58 in: Proceedings of the Leafy Spurge Symposium, 1988. South Dakota State Univ., Brookings. July 12 and 13.