Cultural Variance of *Alternaria carthami* Isolates and Their Virulence on Safflower

K. MORTENSEN, Former Postdoctoral Research Associate, Montana State University, Bozeman 59717, and J. W. BERGMAN, Associate Professor of Agronomy, Montana Agricultural Experiment Station, Eastern Agricultural Research Center, Sidney 59270

ABSTRACT

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Thirty-five isolates of *Alternaria carthami* from leaves of safflower (*Carthamus tinctorius*) in field plots and from naturally infected safflower seeds were variable in culture on potato-carrot agar but all were virulent on safflower cultivars and breeding lines tested. Symptoms appeared as irregular necrotic lesions on leaves and stems, similar to stem and leaf spots occurring under natural conditions. Lesions often coalesced, leaving large areas of the plants necrotic. Higher levels of resistance to *A. carthami* were detected in some breeding lines compared with safflower cultivars US-10, Gila, and S208. *A. carthami, A. solani, A. alternata,* and *Stemphylium vesicarium* penetrated healthy leaves of safflower. Infections by *A. solani, A. alternata,* and *S. vesicarium* did not cause leaf spots on healthy safflower leaves but the fungi remained dormant until leaf senescence.

Leaf spot disease on safflower (*Carthamus tinctorius* L.) is caused mainly by *Alternaria carthami* Chowdhury (1,3,6,9), although in the latter part of the growing season, *A. alternata* (Fr.) Keissler can frequently be isolated from leaf spots as well as from mature safflower seeds (9). *A. alternata* is reported to be a saprophyte on dead and dying plant material of a wide host range (5) but has also, along with several other fungi, been shown capable of penetrating healthy tobacco leaves. Endophytic hyphae were observed in epidermal, mesophyll, and vascular tissues without visible symptoms (10,12,13).

The objectives of this work were 1) to study cultural variances of *A. carthami* isolates and their virulence on different safflower cultivars and breeding lines and 2) to compare infections of *A. carthami* with infections of *A. solani* Sorauer and the saprophytes *A. alternata* and *Stemphylium vesicarium* (Wallr.) Simmons on safflower in a histological study. A preliminary abstract on these studies has been published (8).

MATERIALS AND METHODS

Thirty-five isolates of *A. carthami* were collected from safflower (*C. tinctorius*) at the Eastern Agricultural Research Center, Sidney, MT. Eighteen isolates were collected in 1976 from irrigated safflower field plots in the lower Yellowstone River valley, including a plot continuously cropped since 1961 to a composite of 555 introductions from the 1960 USDA World Safflower Collection. Four isolates originated from safflower in dryland field plots in 1976. Three isolates came from dry safflower leaves collected from field plots in 1975. One isolate, ME-74, was collected from field plots in 1974 and kept in culture on potato-carrot agar (PCA) for 2 yr. The remaining nine isolates originated from naturally infected seedlings grown in the greenhouse or on PCA from safflower seeds produced in Montana. *A. alternata* isolates 2-b and 42-4, collected from safflower in the field at Sidney, MT, and 133-2, collected from naturally infected safflower seedlings in the greenhouse; *S. vesicarium* isolates 136, collected from naturally infected safflower seedlings in the greenhouse, and 319, collected from a tomato plant in the greenhouse; and one *A. solani* isolate supplied by G. A. Strobel, Montana State University, Bozeman, were included in the studies.

Single-conidial cultures from these isolates were cultured on PCA at 22–24 C with a 12-hr period of cool fluorescent light (3,200 lux). PCA was prepared by macerating 20 g each of peeled white potatoes and carrots in a blender. The macerated tissue was then mixed with 30 g of commercial potato-dextrose agar in 1,000 ml of distilled water and autoclaved.

Mycelial growth (mm diameter) of each *A. carthami* isolate was measured 8 days after transfer of a 5-mm circular agar block of 8- to 10-day-old mycelium to a PCA plate (four replicates per isolate). Conidia produced in 18-day-old cultures on the PCA plates were counted by washing the conidia from each plate in 20 ml of water and counting two samples per plate with a hemacytometer.

The safflower breeding lines used were selected in a safflower breeding program at the Eastern Agricultural Research Center, Sidney, MT. The nine breeding lines selected (Table 1) all showed less severe leaf spot symptoms under field conditions than the cultivars US-10, S208, and Gila.

Plants for inoculation with *A. carthami* were grown in autoclaved topsoil (sandy loam) mixed with peat moss (3:1) in metal trays (35 × 25 × 10 cm) on greenhouse benches under 1,000 W metal halide lamps (7,120 microwatts per square centimeter) with a 16-hr light period at temperatures of 18–24 C for about 30 days before inoculation. Each tray contained 6 cultivars or breeding lines for testing virulence of the 35 isolates and 12 cultivars or breeding lines for testing virulence of two selected isolates (5 plants per entry). Inoculum of *A. carthami* isolates was prepared by spreading 1 ml of a sterile water suspension of conidia and mycelial fragments of the fungus over the entire surface of a PCA plate and culturing it for 12–15 days as described. Conidia were harvested by pouring 20 ml of distilled water on the plate, scraping the surface gently with a glass rod, and straining the

Table 1. Reaction of safflower cultivars and breeding lines to two *Alternaria carthami* isolates under greenhouse conditions

Cultivar or line	Leaf spot rating[a]	
	8-a[b]	5-a[b]
US-10	6.4	3.4
Gila	6.0	3.5
S208	5.8	2.4
74B141	5.6	3.2
74B169	5.2	2.5
74B312	4.8	2.6
87-42-3	4.6	2.6
74B164	4.4	1.8
74B236	4.3	1.6
88-74-2	4.3	2.0
74B233 (Sidwill)	4.2	1.8
75B202	4.2	2.0

[a] Rating scale based on percentage of leaf area covered with symptoms: 0 = no symptoms, 1 = 1–5, 2 = 6–15, 3 = 16–30, 4 = 31–45, 5 = 46–59, 6 = 60–69, 7 = 70–79, 8 = 80–89, 9 = 90–99, and 10 = plants totally wilted.

[b] Inoculum concentrations: 8-a = 3.6×10^5 and 5-a = 4.5×10^5 conidia and/or mycelial fragments per inoculation.

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Present address of first author: Agriculture Canada, Research Station, Regina, Sask. S4P 3A2, Canada.

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suspension through a single layer of cheesecloth.

Inoculum concentration was determined by counting conidia and mycelial fragments with a hemacytometer. The inoculum suspension (15 or 20 ml) was atomized onto each tray until runoff with an airbrush (Paasche Airbrush, type HS No. 5, Paasche Airbrush Co., Chicago, IL 60614) operated by a constant air pressure (207 kPa). Inoculated plants were placed in a mist chamber for 40 hr after inoculation, where they were kept wet without runoff with a cold-mist humidifier. One uninoculated tray was included with each inoculation. The plants were placed on greenhouse benches in the daytime (8 hr) and returned to the mist chamber at night until disease rating was done about 12 days after inoculation. Infected plants were rated on a scale of 0–10, modified after James (7), based on the percentage of leaf area covered with lesions where 0 = no symptoms, 1 = 1–5, 2 = 6–15, 3 = 16–30, 4 = 31–45, 5 = 46–59, 6 = 60–69, 7 = 70–79, 8 = 80–89, 9 = 90–99, and 10 = plants totally wilted.

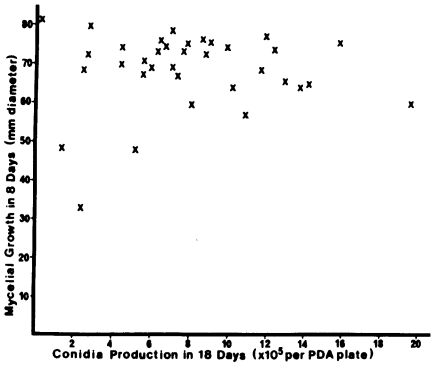

Fig. 1. Mycelial growth of 35 *Alternaria carthami* isolates on potato-dextrose agar in 8 days plotted against their conidia production after 18 days (mean of four replicates per isolate). No correlation was detected (r^2 = 0.08).

To study the infection process of *Alternaria* spp. and *S. vesicarium* on safflower leaves, infected leaves were cleared in one part glacial acetic acid to one part 95% ethanol for 2 days, then transferred to lactophenol for 2–4 days (4). Cleared leaf pieces were placed in lacto-fuchsin (2) on microscope slides and examined with a light microscope. Plants used to study the infection process were incubated 42 hr after inoculation in a temperature-controlled dew-simulation chamber to ensure uniform conditions for germination and infection. Temperature in this dew chamber was 16 C ± 1 with no lights. Enough dew was formed on the plants to keep them wet without runoff.

RESULTS

Considerable variation in mycelial growth on PCA was observed for 35 isolates of *A. carthami*, ranging from 81 to as low as 32-mm-diameter radial growth in 8 days. Conidia production after 18 days ranged from 0.1×10^5 to 19.3×10^5 per PCA plate (Fig. 1). Isolates 4 and 43-1 had rapid mycelial growth (81 and 79 mm) and low conidia production (0.1×10^5 and 2.7×10^5 per plate), and isolates 5-a, 22, and 49-1 showed relatively slow mycelial growth (65, 64, and 60 mm, respectively) but high conidia production (14.0×10^5, 13.6×10^5, and 19.3×10^5 per plate, respectively). Isolates 3 and 9 had rapid growth (76 and 77 mm) and high conidia production (15.6×10^5 and 11.8×10^5 per plate), and isolates 11 and 37-2 had slow growth (32 and 48 mm) and low conidia production (2.3×10^5 and 1.3×10^5 per plate). No correlation ($r^2 = 0.08$) was detected between rapid mycelial growth and high conidia production (Fig. 1).

Slight differences in morphology of conidia among isolates were observed. Isolates 4, 8-a, and 52-1 produced more uniform and slightly longer conidia than isolates 3, 5-a, and 9. Conidia of the latter isolates were more variable in size and had beaks that were more branched.

All 35 isolates of *A. carthami* were virulent on the safflower cultivars and breedling lines tested (Table 2). Leaf spots obtained after inoculations were similar to leaf spots occurring under natural conditions, appearing as irregular necrotic lesions. Lesions would often coalesce, leaving large areas of leaves necrotic. Stem infections also occurred, appearing as sunken brown necrotic lesions. Under heavy inoculation and long incubation periods with wet conditions, entire plants became necrotic.

Inoculation of three safflower cultivars and nine breeding lines with *A. carthami* isolates 8-a and 5-a (Table 1) showed that 8-a caused more severe leaf spot symptoms than 5-a. Breeding lines 74B233 and 74B236 had higher resistance to the two isolates than cultivars US-10, Gila, and S208. No significant interactions between isolates and cultivars or breeding lines were detected.

Table 2. Virulence of isolates of *Alternaria carthami* on safflower cultivars and breeding lines[a]

Isolate	Inoculum concentration[b]	Leaf spot rating[c]					
		US-10	74B141	S208	87-42-3	74B236	Gila
Field 76							
1	0.4	6	6	6	5	4	6
3	2.0	8	...	8	4	6	8
4	0.5	7	6	5	4	4	8
5-a	1.2	4	4	4	4	4	6
6	1.9	8	7	8	8	8	7
7	1.8	8	9	...	4	4	8
8-a	1.9	5	...	5	6	7	7
9	2.2	6	7	7	6	5	8
10	0.6	4	3	4	5	3	6
11	1.9	8	7	7	7	7	8
21	4.6	8	8	7	8	8	8
22	5.4	7	7	7	7	7	7
23	3.4	8	7	7	8	8	8
26-1-a	1.1	5	7	6	3	4	6
26-3-a	4.7	6	6	6	7	7	8
30-a-4	1.9	6	6	5	5	3	5
30-a-5	2.1	8	8	8	7	7	7
42-3	1.1	5	5	5	4	5	5
32-1	1.3	7	7	6	8	6	6
34-1	3.1	8	8	8	9	8	9
35-1	3.4	8	8	7	9	7	7
36-2	4.0	7	7	7	7	5	7
Field 75							
37-1	1.2	8	8	8	8	8	8
37-2	0.3	4	3	3	4	2	2
49-1	2.2	8	8	8	7	8	9
Field 74							
ME-74	2.0	3	2	2	2	1	...
Seedlings							
40-1	2.3	3	3	4	3	2	7
40-2	1.5	3	4	4	3	4	5
41-1	1.3	3	4	5	3	3	5
43-1	1.3	3	3	3	3	2	...
44-2	1.4	4	4	4	4	3	5
44-3	1.9	4	4	5	3	4	...
52-1	2.6	6	5	6	5	5	6
53-2	0.3	5	4	5	3	3	5
133	3.0	8	8	7	8	7	8
Mean		6.0	5.8	5.8	5.5	5.1	6.7

[a] Five plants per cultivar or breeding line per inoculation.
[b] Conidia and/or mycelial fragments per inoculation ($\times 10^6$).
[c] Rating scale based on percentage of leaf area covered with symptoms: 0 = no symptoms, 1 = 1–5, 2 = 6–15, 3 = 16–30, 4 = 31–45, 5 = 46–59, 6 = 60–69, 7 = 70–79, 8 = 80–89, 9 = 90–99, and 10 = plants totally wilted.

Figure 2A,B shows infection of a safflower leaf by *A. carthami* 42 hr after inoculation. Seventy to 80% of the germinated conidia and mycelial fragments of three isolates of *A. carthami* (Table 3) penetrated either directly through the epidermis or through stomata of safflower leaves. *A. solani* penetrated safflower leaves through the epidermis as well as through stomata (Table 3). Infections by *A. solani* (Fig. 2C,D) and *A. carthami* (Fig. 2A,B) on safflower were similar when observed 42 hr after inoculation, but *A. solani* did not cause larger lesions on safflower leaves until leaf senescence began. *A. alternata* (Fig. 2E) and *S. vesicarium* (Fig. 2F) infected safflower leaves within 42 hr of inoculation but at a low frequency (Table 3). The two fungi remained dormant in safflower leaves until senescence began. *A. alternata* penetrated directly through the epidermis as well as through stomata, whereas *S. vesicarium* only penetrated through stomata (Table 3).

Isolates of *A. alternata* in this study varied in penetration frequency (Table 3). *A. alternata* isolates from field-grown safflower as well as from safflower seeds also varied in culture appearance (color and growth of mycelium) and size of conidia.

DISCUSSION

All isolates of *A. carthami*, although quite variable in culture, were virulent on the safflower cultivars and breeding lines tested. Our results support field observations at Sidney, MT, where Alternaria leaf spots have been observed on the same cultivars and breeding lines grown in field trials, but in this study, higher resistance was detected in some of the breeding lines compared with cultivars US-10, Gila, and S208. Breeding line 74B233, now registered as cultivar Sidwill, showed resistance to two isolates of *A. carthami* (Table 1), which concurs with findings in the field where the line was selected for resistance to leaf spot disease.

Inoculations in tests shown in Table 2 were done with very high concentrations of conidia and/or mycelial fragments. Use of equal concentrations of inoculum from each isolate was difficult because of differences in conidia production and rate of mycelial development between the *A. carthami* isolates. Because inoculations and incubations in the mist chamber were made with only four or five isolates at one time, a time span of about 6 mo resulted between the first and the last inoculations. Slight differences in incubation conditions between inoculation trials might have occurred during that time. Thus, differences in inoculum densities and incubation conditions might account for some of the differences in leaf spot ratings among isolates of *A. carthami*. From data on leaf spot ratings in Table 2, it is obvious that when high inoculum concentrations ($>3\times10^6$ conidia and/or mycelial fragments per inoculation) were used, it became difficult to detect differences in leaf spot severity between safflower cultivars or lines, whereas lower inoculum concentrations ($<1\times10^6$) showed better differentiation. The purpose of the studies in Table 2 was to test virulence versus nonvirulence of collected isolates of *A. carthami* rather than to test pathogenicity levels. For the latter purpose and in screening tests for resistance to *A. carthami*, it is important that equal inoculum concentrations and incubation conditions are used to obtain

Table 3. Germination and infection of *Alternaria carthami*, *A. solani*, *A. alternata*, and *Stemphylium vesicarium* on inoculated safflower leaves[a]

Fungi Isolate no.	Total germinated spores or mycelial fragments observed (no.)	Percent penetration		
		Through epidermis	Through stomata	Total
A. carthami				
5-a	427	69.5[b]	4.5[b]	75.4
8-a	474	59.4[b]	7.8[b]	69.6
49-1	210	55.7	25.2	81.0
A. solani	227	28.2	22.9	51.1
A. alternata				
2-b	310	1.3	5.8	7.1
42-4	253	9.5	5.1	14.6
133-2	186	1.1	0.5	1.6
S. vesicarium				
136	352	0	9.9	9.9
319	241	0	7.1	7.1

[a] 42 Hr after inoculation.
[b] Not distinguished between penetration directly through epidermis and through stomata on total counts, percentage given is only based on 200 germinated conidia and/or mycelial fragments.

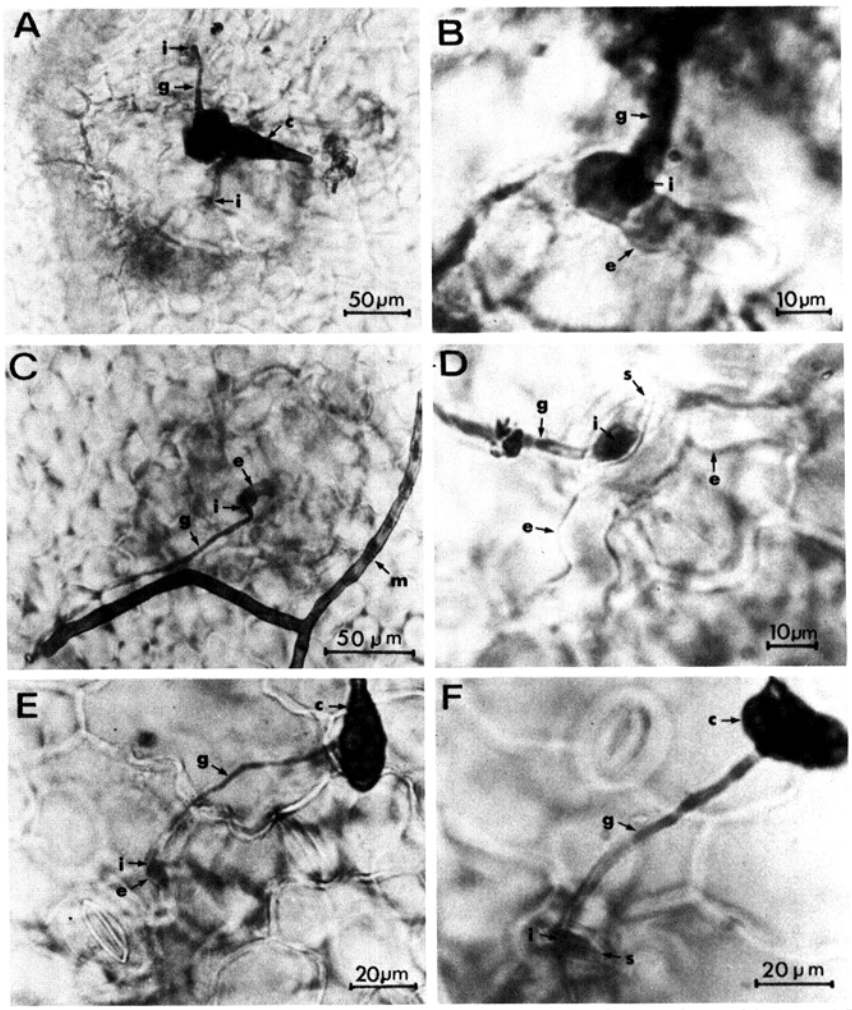

Fig. 2. Infection in leaves of safflower cultivar US-10 48 hr after inoculations with **(A and B)** *Alternaria carthami*, **(C and D)** *A. solani*, **(E)** *A. alternata*, and **(F)** *Stemphylium vesicarium* cleared and stained with lacto-fuchsin. c = Conidia, m = mycelial fragment, g = germ tubes, i = infection site, and e = endophytic hyphae. Note dark area around infection sites.

reliable results.

Isolates 5-a and 8-a were similar in penetration frequency (Table 3) but showed obvious differences in leaf spot development (Table 1). Thus, the lower level of pathogenicity for isolate 5-a appears to result from poor development in the leaf tissue rather than lack of penetration ability.

Even though *A. alternata* and *S. vesicarium* are reported as saprophytes (5), our study showed that both fungi are capable of penetrating healthy leaf tissue of safflower (Fig. 1E,F). Thus, they could be termed weakly pathogenic facultative saprophytes or endophytic fungi, as similar fungi isolated from tobacco leaves have been termed (13). The variability among isolates of *A. alternata* in penetration frequencies (Table 3) and observed morphological differences are comparable to the findings of Spurr (11) regarding this species. We made no effort to distinguish further among isolates of *A. alternata*.

Penetration of *A. carthami* directly through the epidermis was more frequent than through stomata, whereas a high frequency of entrance through stomata was observed for *A. solani* and *A. alternata* in safflower leaves. *S. vesicarium* did not penetrate directly through the epidermis of safflower leaves. Similarly, Diener (4) found that stomatal entrance was the dominant mode of penetration by *S. solani* on tomatoes.

Resistance in safflower to *A. solani*, *A. alternata*, and *S. vesicarium* is apparently caused by a mechanism that restricts mycelial growth in the host leaf tissue until onset of leaf senescence.

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