Infection of Leafy Spurge by *Alternaria alternata* and *A. angustiovoidea* in the Absence of Dew

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ABSTRACT

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An invert emulsion carrier (IEC, water-in-oil type) was developed that could be applied easily with a garden sprayer (3.785 L with T-Jet 8002 nozzle) and that showed negligible phytotoxicity to leafy spurge. The IEC contained an oil phase and a water phase (1:1, v/v). The oil phase contained 20 ml of mineral oil, 2 ml of Myverol 18-99, 80 ml of Orchex 796, and 6 g of paraffin wax. The water phase contained 0.5 g of sucrose, 0.1 ml of Tween 20, and 100 ml of tap water. Eighty-five percent of conidia of *Alternaria alternata* and *A. angustiovoidea* germinated in the IEC in uncovered petri dishes in the greenhouse in the absence of dew at 21–25 C. However, less than 10% of conidia of *A. alternata* and 0% of that of *A. angustiovoidea* germinated in the aqueous sucrose solution under the same greenhouse conditions. When conidia of both *Alternaria* species were suspended in the IEC and sprayed onto leafy spurge, the plants were infected and killed in the absence of dew in growth chambers, greenhouse, and field plots. On the other hand, leafy spurge plants remained healthy under the same conditions when conidia of both *Alternaria* species were suspended in the aqueous sucrose solution and sprayed onto the plants.

Additional keywords: biocontrol, invert emulsion.

Alternaria angustiovoidea E. Simmons (9) and *A. alternata* (Fr.:Fr.) Keissl. require at least 48 h dew at 20–25 C to cause severe infection of leafy spurge (S. M. Yang, *unpublished data*). If the two pathogens are to be considered as potential mycoherbicides for controlling leafy spurge (*Euphorbia esula* L.), an emulsion or a carrier for effective delivery of the fungal pathogens is needed that has negligible phytotoxicity and that provides moisture and nutrients for the pathogens to germinate and infect the leafy spurge. A phytotoxic reaction to the emulsion could limit the evaluation of the efficacy of the pathogens for killing the leafy spurge.

Previous reports have demonstrated that an invert (water-in-oil) emulsion provided moisture for germination of conidia of *A. cassiae* Jurair & A. Khan and infection of sicklepod (*Cassia obtusifolia* L.) (4,6) and also improved the efficacy of *Colletotrichum truncatum* (Schwein) Andrus & W. D. Moore for control of *Sesbania exaltata* (Raf.) Cory. (hemp sesbania) (2) in absence of dew in greenhouses or fields. The invert emulsion plus conidia could not be applied using an atomizer or a garden sprayer.

Connick et al (3) developed an invert emulsion containing an oil phase, designated as CDQ-l, which had low viscosity and excellent water-holding properties for delivery of fungal weed pathogens. However, this emulsion was phytotoxic to leafy spurge and could not be readily sprayed using a garden sprayer or atomizer (S. M. Yang, *unpublished data*).

This study was conducted to develop and test an invert emulsion carrier (IEC) that had little phytotoxicity to leafy spurge and could be applied easily with a garden sprayer; to demonstrate the germination of conidia of *A. alternata* and *A. angustiovoideae* and the infection of leafy spurge in absence of dew; and to determine the infection of rangeland grasses by the two *Alternaria species* in absence of dew.

MATERIALS AND METHODS

Growth of leafy spurge. Leafy spurge used in this study was propagated from root buds in greenhouse-mixed soil in 15-cm diameter clay pots or square plastic pots (9.5 cm × 9.5 cm).

The greenhouse soil was prepared as previously described (9). Three- to 4-wk-old leafy spurge plants were used for the spray and inoculation studies.

Development of IECs applicable by a garden sprayer. The IEC is an invert emulsion containing oil and water phases. The oil phase, a modification of CDQ-1 (3), contained 0–30 ml of mineral oil (Plastodent Inc., Bronx, NY), 70–80 ml of Orchex 796 refined paraffin oil (density 0.85) (Gil Chambers, Exxon Research and Engineering Co., Baytown, TX), 1–8 ml of Myverol 18-99, a distilled monoglyceride emulsifier (M. Edwards, Eastman Chemical Products, Inc., Kingsport, TN) and 6 g of paraffin wax. The water phase consisted of 100 ml of tap water, 0.1 ml of Tween 20, and 1 g of dextrose or 0.5 g of sucrose with or without 0.5 g of gelatin, unless otherwise stated. The addition of dextrose or sucrose was to facilitate the uniform and rapid germination of conidia of *Alternaria spp.* on plant surfaces after inoculation.

The IECs were prepared by shaking 50 ml each of the oil and water phases (1:1, v/v) in a capped flask (250 ml). The IEC was applied with a 3.785-L (gallon) garden sprayer with a T-Jet 8002 nozzle. Both IECs (100 ml of each) were applied to a 1-m^2 area (= 10^3 L/ha = 106 gallons/acre) in which 5–25 pots (5–10 plants per pot) of 3- to 4-wk-old leafy spurge were randomly placed. Leaf wetness on leafy spurge was recorded by examining the sprayed plants every day for 5 days. The amount of phytotoxicity was measured 2–4 wk after application of an IEC. A 0–4 numerical scale was used to rate the severity of injury where 0 = no injury; 1 = tip of leaves brown or brown-black or up to 25% of leaves yellowed; 2 = 26–50% of leaves yellowed or brown; 3 = top of plants brown and dead but lower leaves still green or 51–75% of leaves brown, easily detached or defoliated; and 4 = more than 75% of leaves brown or defoliated or plants dead. The phytotoxicity index was then calculated by (summation of [severity ratings × number of plants in that class])/total number of plants. An index rating at or below 1.5 indicates slight injury, 1.6–2.5 indicates moderate injury, and above 2.6 indicates severe injury by the IEC.

Germination of conidia of *A. alternata* or *A. angustiovoidea* in two IECs in the absence of dew. *A. alternata* and *A. angustiovoidea* were grown on sterile potato-carrot agar (PCA) (8) and incubated at 20–23 C (12 h light 40 $\mu E \cdot s^{-1} \cdot m^{-2}$). Conidia of *A. alternata* and *A. angustiovoidea* were collected from 2- to 5-wk-old cultures by flooding cultures with an aqueous dextrose solution (1 g of dextrose, 0.5 g of gelatin, 100 ml of tap water, and 0.1 ml of Tween 20) or an aqueous sucrose solution (0.5 g of sucrose, 100 ml of tap water, and 0.1 ml of Tween 20) and rubbing the surface with a rubber spatula. The combined conidial suspension was filtered through two layers of cheesecloth and adjusted to 1–2 × 10^5 conidia per milliliter with the aqueous dextrose or sucrose solution.

Two IECs, 12 and 14, were used for germination studies. IEC 12 was prepared by mixing equal amounts of oil phase (20 ml of mineral oil, 80 ml of Orchex, 2 ml of Myverol, and 6 g of paraffin wax) and water phase (the previously mentioned aqueous dextrose solution). IEC 14 was prepared by mixing equal amounts of the previously mentioned oil phase and the aqueous sucrose solution. For preparation of IECs plus conidia, conidia of *A. alternata* or *A. angustiovoidea* were suspended in the aqueous dextrose or sucrose solution before mixing with the oil phase.

Two drops (0.1 ml/drop) of the IEC-containing conidia were placed separately into petri dishes. The dishes were uncovered. Two drops of aqueous conidial suspension of *A. alternata* or *A. angustiovoidea* in covered and uncovered petri dishes served as controls (Table 1). There were five dishes (2 drops/dish) for each treatment in each test, and the test was repeated once. The dishes containing IEC 12 plus conidia or conidia suspended in aqueous dextrose solution were then maintained, respectively, in four different growth chambers ranging from 15–30 C at 5 C intervals and in a greenhouse (Table 1). Dishes containing IEC 14 plus conidia or conidia suspended in aqueous sucrose solution were maintained only in the greenhouse. Relative humidity (RH) of the growth chambers and greenhouse during the tests was 35–50% as recorded by the hygrothermograph. Conidia were killed with cotton-blue in lactophenol 16–17 h after initiation of incubation. Percentage of germination was determined from 100 spores counted from each drop. Conidia were counted as germinated when the germ tube was longer than half of the length of the conidia. A Student's *t*-test was computed for comparing percentage of germination between *A. alternata* and *A. angustiovoidea* in each treatment at each temperature. However, Waller-Duncan's Bayesian *k*-ratio test (10) was used to compare the means among the treatments within each pathogen.

Inoculation of leafy spurge with the IEC 14 plus conidia of *A. alternata* or *A. angustiovoidea* in the absence of dew in growth chambers and greenhouse. Since sucrose is readily available and cheaper than dextrose, conidia were suspended in IEC 14 for inoculation studies. One hundred milliliters of the IEC 14 plus conidia were sprayed with a garden sprayer to a meter square area in which 25 pots of 3- to 4-wk-old leafy spurge (5–10 plants per pot) were randomly placed. Five pots of the inoculated plants were incubated in absence of dew (RH 30–50%) in four different growth chambers ranging from 15–30 C at 5 C intervals and in a greenhouse. Plants sprayed with the IEC 14 alone, aqueous sucrose solution alone, and aqueous conidial suspensions served as controls. The conidial concentrations in the IEC 14 or suspensions were 1–2 × 10^6 conidia per milliliter for *A. alternata* and 1–2 × 10^5 conidia per milliliter for *A. angustiovoidea*. The reason for using different conidial concentrations is that conidial concentration of *A. angustiovoidea* at 10^6 could not be sprayed using the garden sprayer as indicated later. This experiment was repeated once.

Disease severity on inoculated plants was rated 4 wk after inoculation. A 0–4 numerical system was used to rate the severity where 0 = no infection, no lesions on leaves; 1 = tip of leaves curled or up to 25% of leaves brown; 2 = 26–50% of leaves brown; 3 = 51–75% of leaves brown and defoliated or only the upper parts black and dead but the lower portion of plants still green; and 4 = more than 75% of leaves brown or defoliated or the plants dead. A disease index score was then calculated by (summation of [severity rating × number of plants in that class])/total number of plants. An index below 3.0 indicated slight-to-moderate infection, but at or above 3.0 indicated severe infection. Severity of injuries by the IEC alone was also taken 4 wk after inoculation using the previously mentioned phytotoxicity rating system. A Student's *t*-test was used to compare the infection of leafy spurge between *A. alternata* and *A. angustiovoidea* in IEC at each temperature.

Effect of conidial concentrations on application and infection of leafy spurge. Plants (3–4 wk old) were sprayed with conidia of both *Alternaria* suspended in IEC 14 or aqueous sucrose solution. Conidial concentrations in the IEC 14 or in the aqueous sucrose solution ranging from (1.4–2.0) × 10^1 to (1.4–2.0) × 10^6 conidia per milliliter were used in this test (Table 3). Leafy spurge plants sprayed with IEC alone or aqueous sucrose alone served as controls. Leafy spurge plants sprayed with IEC alone or IEC 14 plus conidia of *A. alternata* or *A. angustiovoidea* were directly maintained in the greenhouse after inoculation, while the plants sprayed with aqueous sucrose solution alone or conidia suspended in the aqueous sucrose solution were first incubated in the dew chamber for 48 h before placing in the same greenhouse. The greenhouse temperatures and relative humidities during the test were 21–28 C and 40–50%, respectively. Each treatment had five pots of plants (two plants/pot), and the test was repeated once. A Student's *t*-test was used to compare the infection of leafy spurge by *A. alternata* or *A. angustiovoidea* in IEC and aqueous sucrose solution at each conidial concentration.

Inoculation of leafy spurge in field pots with *A. alternata* or *A. angustiovoidea*. Leafy spurge plants (3–4 wk old) growing in field plots were sprayed with IEC 14 alone and IEC 14 plus conidia of *A. alternata* or *A. angustiovoidea*. Each treatment had four replicated plots, and each plot consisted of a single row 1 m long unless stated otherwise. One hundred milliliters of the IEC alone or the IEC plus conidia were sprayed onto each plot. Conidial concentrations were 1.5 × 10^6 conidia per milliliter for *A. alternata* and 1.5 × 10^5 conidia per milliliter

for *A. angustiovoidea*. Different leafy spurge plots were sprayed in May, June, and July 1991. The sprayed plants were either slightly or severely injured (dead); therefore, average percentage of the dead plants per plot was used to indicate the efficacy of *Alternaria* spp. on control of leafy spurge. Number of dead and total plants in each plot was counted 4 wk after inoculation.

Inoculation of rangeland grasses with *A. alternata* or *A. angustiovoidea* in the absence of dew in the greenhouse. The following rangeland grasses at 3- to 4-wk old were tested for susceptibility to *A. alternata* and *A. angustiovoidea*: *Agrostis gigantea* Roth (red top), *Andropogon hallii* Hack. (sand bluestem), *Andropogon scoparius* Michx. (little bluestem), *Bouteloua gracilis* (H. B. K.) Lag. ex Steud. (blue grama), *Bromus inermis* Leyss. (smooth brome), *Calamovilfa longifolia* (Hook) Hack. (prairie sandreed), *Dactylis glomerata* L. (orchard grass), *Eragrostis trichodes* (Nutt.) Wood (sand lovegrass), *Festuca* sp. (fescue), *Panicum virgatum* L. (switchgrass), *Phalaris arundinacea* L. (reed canarygrass), *Phleum pratense* L. (timothy grass), and *Sorghastrum nutans* (L.) Nash. (Indian grass). The grasses were grown in clay pots (10 cm diameter, 10 plants per pot) and inoculated with IEC 14 alone and IEC 14 plus conidia of *A. alternata* or *A. angustiovoidea*.

The inoculated plants were kept on greenhouse benches and rated 4 wk after inoculation. The test was repeated once. Leafy spurge was included in each experiment as a positive control. The greenhouse temperatures and relative humidities ranged from 20–27 C and 30–60%, respectively. A brown-to-black discoloration of the leaves indicated injury of the rangeland grasses by the IEC 14. Formation of reddish brown lesions on the grass leaves indicated infection of the leaves by *Alternaria*.

Isolation, reisolation, and identification of fungi from the sprayed plants and pathogenicity tests of the selected isolated fungi. Fungi were isolated from leaves injured by the IECs alone or reisolated from the infected leaves inoculated with *A. alternata* or *A. angustiovoidea*. Tissue pieces (0.3 × 0.8 cm) cut from the leaves and surface-disinfected in 5% sodium hypochlorite solution were placed on PDA or PCA plates amended with penicillin G (30 mg/L) and streptomycin sulfate (100 mg/L) for 4–5 days. Mycelium from each colony was transferred to new PDA or PCA plates and incubated at 20–25 C with a 12-h photoperiod (40 $\mu E \cdot s^{-1} \cdot m^{-2}$) for fungal identification. Pathogenicity of the selected fungi was determined by placing an agar block with mycelium on leaves of intact leafy spurge plants as previously described (8). An agar block was cut from the margin of colonies of 3- to 5-day-old cultures grown on PDA. The inoculated plants were incubated in a dew chamber at 20–25 C in the dark for 16–20 h and then moved to a greenhouse. Infection levels were recorded 2 wk after inoculation.

RESULTS

Development of IECs applicable by a garden sprayer. IECs 12 and 14, easily applied by means of the garden sprayer, maintained leaf wetness of 3–4 days and showed very little phytotoxicity (index of 0.6-1.2) to leafy spurge.

Occasionally, the IECs caused severe injury to some leafy spurge plants, especially when weak plants were not growing well for other reasons or when plants were infected with powdery mildew (*Sphaerotheca* sp.). Some plants sprayed with the IECs turned brown to black (whole leaves) when the greenhouse temperature was above 35 C. The amount of Myverol in the IECs also affected the severity of injury to leafy spurge. Four milliliters of Myverol in 100 ml of IEC caused severe injury to leafy spurge. Plants sprayed with Orchex or Myverol alone turned brown and defoliated within 2 wk.

Germination of conidia of *A. alternata* or *A. angustiovoidea* in two IECs in the absence of dew. When petri dishes containing IEC 12 plus conidia were left uncovered, conidia were still wet at the time of adding cotton-blue in lactophenol. When the petri dishes containing the conidia suspended in aqueous dextrose solution were left uncovered, conidia dried within 6 h after incubation in the growth chambers or in the greenhouse.

Conidia of both *A. alternata* and *A. angustiovoidea* germinated in the IEC 12 in the growth chambers between 15–30 C and in the greenhouse (21–25 C) where the relative humidity was 35–50%. Germination was not tested below 15 and above 30 C. The conidia of *A. alternata* that germinated in the IEC 12 in uncovered petri dishes also germinated in the aqueous dextrose

TABLE 1. Germination of conidia of *Alternaria alternata* and *A. angustiovoidea* in invert emulsion carrier (IEC) and aqueous dextrose solution in growth chambers at four different temperatures and in the greenhouse in absence of dew[a]

Location	Temperature (C)	Treatment[b]	Percent germination[c]	
			A. alternata	*A. angustiovoidea*
Growth chamber				
	15	IEC 12/uncovered	78.5*[d]	34.5
		Aqueous dextrose/uncovered	0	0
		Aqueous dextrose/covered	75.7*	20.2
	20	IEC 12/uncovered	78.7*	50.4
		Aqueous dextrose/uncovered	18.8	0
		Aqueous dextrose/covered	78.0*	33.6
	25	IEC 12/uncovered	77.7*	33.6
		Aqueous dextrose/uncovered	18.0	49.6
		Aqueous dextrose/covered	75.7*	0
	30	IEC 12/uncovered	49.5	48.8
		Aqueous dextrose/uncovered	2.9	0
		Aqueous dextrose/covered	44.6	37.7
Greenhouse				
	21–25	IEC 12/uncovered	69.7	58.7
		Aqueous dextrose/uncovered	9.9	0
		Aqueous dextrose/covered	67.2	42.4
WD-B (k = 100)[e]			26.5	9.9

[a] Relative humidities of growth chambers and greenhouse during the tests were 35–50%.
[b] Treatments were: conidia (0.5–1 × 10^5 conidia per milliliter) in IEC 12 in uncovered plates; conidia (0.5–1 × 10^5 conidia/per milliliter) in aqueous dextrose solution in uncovered plates; conidia (0.5–1 × 10^5 conidia per milliliter) in aqueous dextrose solution in covered plates.
[c] Average germination percentage of two tests. Percentage was calculated from 1,000 conidia counted in 10 drops (100 conidia from each drop) in 5 petri plates (2 drops per petri plate) in each test.
[d] Asterisk indicates significant differences in the percentage of germination between the two pathogens at each treatment according to the Student's *t* test ($P = 0.05$).
[e] WD-B (k = 100) LSD indicates Waller-Duncan Bayesian LSD.

TABLE 2. Injuries of leafy spurge by invert emulsion carrier (IEC) 14 or aqueous sucrose solution (ASS) alone and infection of leafy spurge by *Alternaria alternata* and *A. angustiovoidea* in IEC 14 or in ASS in growth chambers at four different temperatures and in the greenhouse in absence of dew[a]

Location	Temperature (C)	Pathogen	Phytotoxicity index[b]	Disease severity[c]	
				A. alternata	*A. angustiovoidea*
Growth chamber					
	15	IEC	1.2	4.0*[d]	1.8
		ASS	0	0	0
	20	IEC	0.2	4.0	3.8
		ASS	0	0	0
	25	IEC	0.8	3.9*	3.2
		ASS	0	0	0
	30	IEC	0.9	3.9*	2.0
		ASS	0	0	0
Greenhouse					
	21–27	IEC	0.3	4.0	3.4
		ASS	0	0	0

[a] Relative humidities in the growth chambers and greenhouse during the tests were 30–50%

[b] Plants inoculated with IEC 14 or ASS alone. Phytotoxicity was rated on a scale of 0 = no injury to 4 = severe injury.

[c] Plants inoculated with conidia of *A. alternata* or *A. angustiovoidea* suspended in IEC or ASS. Disease severity was rated on a scale of 0 = no infection to 4 = plants dead.

[d] Asterisk indicates significant differences in infection of leafy spurge between the two pathogens in IEC at each temperature according to the Student's *t* test ($P = 0.05$).

solution in uncovered petri dishes under the same conditions between 20 and 30 C but not at 15 C. However, the percentage of germination in aqueous dextrose solution in uncovered plates was significantly less than that in the IEC 12 in uncovered plates. The germ tube in the IEC 12 was long, branched, and difficult to measure under the microscope, but the germ tubes in the aqueous solution were short and not branched. On the contrary, conidia of *A. angustiovoidea* that germinated in the IEC 12 in uncovered plates did not germinate in the aqueous dextrose solution in uncovered plates under the same conditions.

When the aqueous conidial suspensions in petri dishes were covered, conidia of *A. alternata* or *A. angustiovoidea* in the aqueous dextrose solution also germinated in the growth chambers and in the greenhouse (Table 1). Percentage of germination of conidia of *A. alternata* in the aqueous dextrose solution in covered petri dishes was not significantly different from that in the IEC 12 in the uncovered petri dishes at the same temperature (Table 1). But percentage of germination of conidia of *A. angustiovoidea* in aqueous dextrose solution in covered plates was significantly less than that in the IEC 12 in uncovered plates under the same conditions (except at 25 C, Table 1).

The percentage of *A. alternata* was significantly greater than that of *A. angustiovoidea* in IEC in uncovered petri dishes or in aqueous dextrose solution in covered petri dishes at 15–25 C but not at 30 C in the growth chamber. However, the percentage of germination of the two pathogens in IEC or aqueous dextrose solution was not different significantly in the greenhouse (Table 1).

When germination was tested in IEC 14 and aqueous sucrose solution in uncovered petri dishes in the greenhouse (data not shown), the average percent germination of conidia of *A. alternata* and *A. angustiovoidea* in the IEC 14 was 85.6 and 85.9%, respectively, and in the aqueous sucrose soluton was 8.8 and 0%, respectively.

Inoculation of leafy spurge with the IEC 14 plus conidia of *A. alternata* or *A. angustiovoidea* in the absence of dew in growth chambers and greenhouse. When leafy spurge plants were inoculated with IEC 14 plus conidia of *A. alternata* or *A. angustiovoidea*, *A. alternata* killed leafy spurge in the absence of dew in growth chambers at 15–30 C, but *A. angustiovoidea* killed leafy spurge only at 20 and 25 C in growth chambers (Table 2). Both *A. alternata* and *A. angustiovoidea* also killed leafy spurge in the absence of dew in the greenhouse (21–27 C, Fig. 1, *A. angustiovoidea*) when leafy spurge plants were inoculated with IEC 14 plus conidia. The IEC 14 alone did not injure most of the plants under the same conditions (Fig. 1). When leafy spurge plants were inoculated with aqueous conidial suspensions and maintained in the same growth chambers and greenhouse, neither *A. alternata* nor *A. angustiovoidea* infected the plants. Plants inoculated with aqueous sucrose solution alone showed no injuries.

Fig. 1. Leafy spurge inoculated with *Alternaria angustiovoidea* in the absence of dew in the greenhouse: Left: control, sprayed with invert emulsion carrier (IEC) alone; Right: sprayed with IEC plus conidia of *A. angustiovoidea*. Photograph was taken 4 wk after inoculation.

Effect of conidial concentrations on application and infection of leafy spurge. When the conidial concentration of *A. angustiovoidea* was 1.4×10^6 conidia per milliliter, the IEC 14 plus conidia could not be sprayed, but it squirted from the garden sprayer. With conidial concentrations of 7×10^5 conidia per milliliter, the IEC 14 plus conidia could be sprayed by the garden sprayer, but not as easily as the IEC 14 plus conidia at 1.4×10^5 conidia per milliliter. However, *A. angustiovoidea* in aqueous sucrose solution and *A. alternata* in IEC 14 or in aqueous sucrose solution at concentrations of 10^6 conidia per milliliter could be sprayed easily with the garden sprayer.

Efficiency of *A. alternata* or *A. angustiovoidea* to kill leafy spurge was reduced with the reduction in conidial concentration (Table 3). When conidia per milliliter of both *Alternaria* were 10^5 or above in aqueous sucrose solution and sprayed onto leafy

spurge plants, the two pathogens infected and killed the plants. When conidia per milliliter of *A. angustiovoidea* were 10^4, this pathogen infected leafy spurge moderately to severely. However, when the conidia per milliliter were reduced to 10^4 for *A. alternata* and 10^3 for *A. angustiovoidea*, slight infection occurred and the pathogens did not kill leafy spurge. Severity of disease on plants inoculated with IEC 14 plus conidia and not given a dew period was the same as that on plants inoculated with aqueous conidial suspension and incubated in a dew chamber for 48 h when the conidial concentration was the same except conidia per milliliter of *A. angustiovoidea* were 10^3 (Table 3). Leafy spurge plants inoculated with IEC alone or aqueous sucrose solution alone showed no infection (*data not shown*).

TABLE 3. Effect of conidial concentration of *Alternaria alternata* and *A. angustiovoidea* in the invert emulsion carrier (IEC) 14 and in aqueous sucrose solution (ASS) on infection of leafy spurge[a]

Conidial concentration (per ml[b])	Disease index[c]			
	A. alternata		*A. angustiovoidea*	
	IEC	ASS	IEC	ASS
10^6	3.8	3.7	4.0	4.0
10^5	3.4	3.4	3.9	3.8
10^4	1.3	0.4	3.0	3.0
10^3	0.2	0	2.7*[d]	0.5
10^2	0.1	0	1.1	0.3
10^1	0.2	0	1.2	0
CK[e]	0	0	1.2	0

[a] Plants inoculated with IEC 14 alone or IEC 14 plus conidia where placed in the greenhouse directly after inoculation. However, plants inoculated with ASS alone or conidia suspended in ASS where incubated first in dew chamber at 20–25 C for 48 hr after inoculation and then moved to greenhouse benches where the plants inoculated with IEC 14 alone or IEC 14 plus conidia were maintained.

[b] Disease severity was rated on a scale of 0 = no infection to 4 = dead plants.

[c] Highest concentration of *A. alternata* was 2.0×10^6 conidia per milliliter and of *A. angustiovoidea* was 1.4×10^6 conidia per milliliter.

[d] Asterisk indicates infection of leafy spurge by *A. alternata* or *A. angustiovoidea* in IEC is significantly different from that in ASS at the same conidial concentrations.

[e] CK = plants sprayed with IEC or ASS alone.

Inoculation of leafy spurge in field plots with *A. alternata* or *A. angustiovoidea*. In the May inoculation studies, 77% of the plants (102 of 132) in the four plots (m^2/plot) inoculated with the IEC 14 plus conidia of *A. angustiovoidea* were severely infected and killed. The infected leafy spurge in one of the four inoculated plots is shown in Figure 2 (pointed by black arrows). The *A. angustiovoidea* did not kill the weeds of other species in the same plot, i.e., annual fleabane (*Erigeron annuus* (L) Pers.), *Aster* sp., horseweed (*Conyza canadensis* (L.) Cronq.), and wild garlic (*Allium vineale* L.). The IEC 14 alone caused negligible injury to leafy spurge, and plants were already flowering 4 wk after inoculation (Fig. 2).

In the June test with *A. angustiovoidea*, the average percentage of dead plants per plot row for IEC 14 alone, IEC 14 plus conidia, aqueous sucrose solution, aqueous sucrose solution plus conidia, and untreated control plots was 48 (35/73 plants), 53 (55/103), 0 (0/112), 0 (0/117), and 0 (0/101), respectively. The number in parentheses indicates the average number of dead plants over the average total plants per plot row. Similarly, in the July inoculations, the average percentage of dead plants per plot row was 40 (33/97), 95 (99/104), 0 (0/100), 0 (0/112), and 0 (0/98), respectively.

In the field-plot experiments with *A. alternata*, the average percentage of dead plants per plot row for untreated, IEC 14 alone and IEC 14 plus conidia was 0, 14, and 19, respectively, for the first inoculation in May, and 0, 16, and 71, respectively, for the second inoculation in July.

Inoculation of rangeland grasses with *A. alternata* or *A. an-*

Fig. 2. Leafy spurge inoculated with *Alternaria angustiovoidea* in the field plot. Left: control, sprayed with invert emulsion carrier (IEC) alone. Right: sprayed with IEC plus conidia of *A. angustiovoidea*. Black area in the center (indicated by arrows) shows dead leafy spurge plants killed by *A. angustiovoidea*. Photograph was taken 4 wk after inoculation.

***gustiovoidea* in the absence of dew in the greenhouse.** Small, reddish-brown, rectangular spots appeared on leaves of smooth brome inoculated with *A. angustiovoidea*, but not *A. alternata*, 1 wk after inoculation. The *A. angustiovoidea* also infected little bluestem and orchard grass slightly. Leafy spurge was severely infected by *A. angustiovoidea* and moderately to severely by *A. alternata*. Only one of 37 leafy spurge plants (2.7%) was injured by the IEC 14 alone.

Isolation, reisolation, and identification of fungi from the sprayed plants and pathogenicity tests of the selected isolated fungi. *Alternaria alternata, Aspergillus, Chaetomium*, and *Penicillium* spp. were isolated on PDA from the leaves of leafy spurge injured by the IECs alone. Twelve of 25 isolates of *A. alternata* from the leaves injured by the IECs alone were pathogenic to leafy spurge. None of the other three fungal isolates were pathogenic to leafy spurge. Both *A. alternata* and *A. angustiovoidea* were isolated on PCA and PDA from the leafy spurge moderately to severely infected by *A. alternata* and *A. angustiovoidea*, respectively. We reisolated *A. angustiovoidea* on PDA from the lesions collected from the infected smooth brome but not from little bluestem and orchard grass.

DISCUSSION

The amount of paraffin wax in the IECs was one of the main factors responsible for the viscosity of the emulsion, and the amount of Myverol (emulsifier) and Orchex appeared to be the cause of phytotoxicity. The IEC 12 or IEC 14 prepared from the modified CDQ-1 and aqueous dextrose or sucrose solution, respectively, could be sprayed easily by the garden sprayer and showed negligible phytotoxicity to leafy spurge. IECs 12 and 14 could also maintain moisture for 3–4 days on the surface of treated plants.

Phytotoxicity of IEC is of concern because it could affect the evaluation of the efficacy of the pathogens on control of leafy spurge in the absence of dew. The IEC used in this test showed negligible phytotoxicity to leafy spurge. *A. alternata* and *A. angustiovoidea* infected and killed leafy spurge in growth chambers, a greenhouse, and field plots at Frederick, MD, in absence of dew when the pathogens were suspended in IEC and sprayed onto plants. On the basis of these results, both *Alternaria* but not IEC alone killed the leafy spurge plants in absence of dew. Further studies are needed on the efficacy of *A. alternata* and *A. angustiovoidea* to control leafy spurge in small field plots in the areas where the two pathogens are endemic.

Conidia of *A. alternata* and *A. angustiovoidea* at 15–30 C could germinate in the IECs 12 and 14 in uncovered plates or in aqueous dextrose or sucrose solution in covered plates. However, conidia of *A. alternata* at 15 C and *A. angustiovoidea* at 15–30 C did not germinate in aqueous dextrose solution in uncovered plates. Presumably, rapid evaporation of the water in the aqueous dextrose solution in the uncovered plates before initiation of conidial germination accounted for failure of the conidia to germinate.

Conidial concentrations in the IEC 14 and aqueous sucrose solution affected the severity of disease caused by *A. alternata* and *A. angustiovoidea*. Severe infection of leafy spurge in absence or presence of dew required 10^5 conidia per milliliter of *A. alternata* and 10^4 conidia per milliliter of *A. angustiovoidea*. When the conidial concentrations of *A. alternata* and *A. angustiovoidea* in IEC 14 or aqueous sucrose solution were reduced to 10^4 and 10^3 conidia per milliliter, respectively, the two pathogens infected the inoculated leafy spurge slightly.

Conidial concentrations in the IEC 14 also affected application using the garden sprayer. When conidia per milliliter of *A. alternata* were 10^6, IEC 14 plus conidia could be sprayed easily with the garden sprayer. However, when conidia per milliliter of *A. angustiovoidea* were 10^6, IEC 14 plus conidia could not be sprayed readily. The difference in the application by garden sprayer may be due to the size of conidia: conidia of *A. angustiovoidea* (50–100 × 4–5 μm) (7) are much larger than those of *A. alternata* (20–63 μm long) (5).

Alternaria cassiae could infect sicklepod severely when 500–1,500 conidia per milliliter were applied to the leaves (1). Improvement of our IEC or selection of more virulent isolates of *A. alternata* or *A. angustiovoidea* may allow reduction in the amount of inoculum required for effective control of leafy spurge.

Alternaria alternata is extremely common on many plants (5), and was frequently isolated from the plants severely injured by IECs. Forty-eight percent of *A. alternata* isolated from the leafy spurge injured by only the IECs was also pathogenic to leafy spurge. The IECs provide moisture and nutrients for the virulent isolates of *A. alternata* already on leafy spurge to germinate, resulting in severe infection of leafy spurge, which may explain why the IECs occasionally caused severe injuries to leafy spurge.

A. angustiovoidea, but not *A. alternata*, was weakly virulent to smooth brome, bluestem, and orchard grass. Both species of *Alternaria* did not infect the rest of the nontarget rangeland grasses tested. These results indicate that these two pathogens are safe to be further tested to determine their efficacy for control of leafy spurge growing in the rangeland.

LITERATURE CITED

1. Amsellem, Z., Sharon, A., Gressel, J., and Quimby, P. C., Jr. 1990. Complete abolition of high inoculum threshold of two mycoherbicides (*Alternaria cassiae* and *A. crassa*) when applied in invert emulsion. Phytopathology 80:925-929.
2. Boyette, C. D., Quimby, P. C., Jr., Connick, W. J., Jr., Daigle, D. J., and Fulgham, F. E. 1990. The biocontrol efficacy of *Colletotrichum truncatum* (Schw.) Andrus & Moore for control of hemp sesbania [*Sesbania exaltata* (Raf.) Cory.] is improved with an emulsifiable invert formulation. (Abstr.) Weed Sci. Soc. Am., 30:50.
3. Connick, W. J., Jr., Daigle, D. J., and Quimby, P. C., Jr. 1991. An improved invert emulsion with high water retention for mycoherbicide delivery. Weed Technol. 5:442-444.
4. Daigle, D. J., Connick, W. J., Jr., Quimby, P. C., Jr., Evans, J., Trask-Morrell, B., and Fulgham, F. E. 1990. Invert emulsions: Carrier and water source for the mycoherbicide, *Alternaria cassiae*. Weed Technol. 4:327-331.
5. Ellis, M. B. 1971. *Dematiaceous hyphomycetes*. Commonwealth Mycological Institute, Kew, England. 608 pp.
6. Quimby, P. C., Jr., Fulgham, F. E., Boyette, C. D., and Connick, W. J., Jr. 1990. An invert emulsion replaces dew in biocontrol of sicklepod — A preliminary study. Pages 264-270 in: Pesticide Formulations and Application Systems. Vol. 8. ASTM. STP. 980 D. A. Hovde & G. B. Beestman, eds. American Society for Testing & Materials, Philadelphia.
7. Simmons, E. G. 1986. Alternaria themes and variations (14–16). Mycotaxon 25:195-202.
8. Yang, S. M., Dowler, W. M., and Johnson, D. R. 1991. Comparison of methods for selecting fungi pathogenic to leafy spurge. Plant Dis. 75:1201-1203.
9. Yang, S.-M., Johnson, D. R., and Dowler, W. M. 1990. Pathogenicity of *Alternaria angustiovoidea* on leafy spurge. Plant Dis. 74:601-604.
10. Waller, R. A., and Duncan, D. B. 1969. A Bayes rule for the symmetric multiple comparisons problem. J. Am. Stat. Assoc. 64:1484-1503.