

Abolition of Selectivity of Two Mycoherbicidal Organisms and Enhanced Virulence of Avirulent Fungi by an Invert Emulsion

Z. Amsellem, A. Sharon, and J. Gressel

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot IL 76100, Israel.

The research was supported in part by a grant from the National Council for Research and Development, Jerusalem, and by the Gilbert de Botton endowed professorial chair in plant sciences to J. Gressel.

Accepted for publication 26 April 1991 (submitted for electronic processing).

ABSTRACT

Amsellem, Z., Sharon, A., and Gressel, J. 1991. Abolition of selectivity of two mycoherbicidal organisms and enhanced virulence of avirulent fungi by an invert emulsion. *Phytopathology* 81:985-988.

Mycoherbicidal preparations to selectively control weeds typically require high inoculum levels and long dew periods for establishment. The dew requirement was obviated and the inoculum threshold was reduced to one conidium per droplet when an invert emulsion was used as an adjuvant. Invert emulsion also abolished the selectivity of *Alternaria cassiae* and *A. crassa*. *A. cassiae* attacked eight plant species, including soybean and members of other families. The emulsion also facilitated

cross-infectivity by *A. crassa*, a mycoherbicide specific to *Datura* spp., *Cassia obtusifolia*, and other species. Nonpathogenic fungi, such as *Aspergillus nidulans* and *Trichoderma harzianum*, colonized soybean when applied in the emulsion. Besides retaining water for spore germination, the invert emulsion may cause cuticular damage, allowing leaf penetration by the fungi. The emulsion could also suppress the plants' elicited responses to infection.

Additional keywords: radioimmunoassay, immunoautoradiography.

The use of mycoherbicidal preparations of some fungi in the field has been limited because of inadequate formulations to overcome the partially interrelated requirements for a high inoculum concentration (3,5,6,21,22) and a long dew period (6–10 h) needed for germination and establishment of the pathogen (6,15). The first highly successful formulation tested in the field that overcame these impediments was an invert emulsion having water around the spore and a mixture of oils and waxes on the outside of the spray droplet (7,16). Indeed, only one conidium of *Alternaria cassiae* A. M. M. Jurair & A. Khan per 2- μ l droplet was needed to infect leaves of *Cassia obtusifolia* L. (sicklepod) or for *A. crassa* (Sacc.) Rands to infect *Datura stramonium* L. (jimsonweed), even in the greenhouse under dry conditions (1).

Single conidia of the pathogenic strain of *A. cassiae* were significantly more virulent in the invert emulsion than were higher concentrations of conidia in water (1). Additionally, we observed that a strain of *A. cassiae* that had lost pathogenicity under normal conditions was virulent when applied in the invert emulsion (1). Moreover, cultures reisolated from spots infected with this isolate in emulsion failed to infect when applied in water; that is, the virulence was not inherited. Such results indicate that the invert emulsion may affect more than water retention around the

inoculum.

It is well known that oils used as adjuvants affect the leaf cuticle (2,13) and can be synergistic in enhancing herbicidal effects (4,10). Damage caused by oils, which is readily visible by scanning electron microscopy (13), could possibly facilitate fungal penetration and establishment. Herbicides and plant growth regulators are known to enhance virulence. This enhanced virulence has been shown with phenoxy herbicides and root pathogens (9), with atrazine and *Curvularia lunata* (Wakk.) Boedijn on *Echinochloa crusgalli* (L.) P. Beauv. (barnyard grass) (17), and with thidiazuron and *Colletotrichum coccodes* (Wallr.) S. J. Hughes on *Abutilon theophrastii* Medik. (velvetleaf) (11,23). This enhanced virulence may be due to tissue damage by the herbicides. It is long known that many nonpathogenic fungi can invade physically damaged plants (24). Our objective for the study reported herein was to determine whether the effect of invert emulsion on host selectivity of two mycoherbicides and two nonpathogenic fungi was wholly due to water retention.

MATERIALS AND METHODS

Fungal isolates. An infective isolate of *A. cassiae* was obtained from a soil sample provided by C. D. Boyette, U.S. Department of Agriculture, ARS, Stoneville, MS. *A. crassa* No. 103.18 was

obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The virulent isolates of *A. cassiae* and *A. crassa* were reisolated from inoculated plants at monthly intervals to prevent loss of infectivity. *Trichoderma harzianum* Rifai (strain T-35) and *Aspergillus nidulans* (Eidam) G. Wint. were obtained from Miriam Abramsky, Department of Phytopathology, the Hebrew University of Jerusalem, Rehovot.

The two *Alternaria* spp. were cultured on V8 juice agar (14) in plastic petri dishes at 25 C, with 12-h photoperiods (30- μ mol photon $m^{-2} s^{-1}$ at plate level). Seven-day-old cultures were induced to sporulate by a 40-min exposure to 4- μ mol photon $m^{-2} s^{-1}$, 300-nm ultraviolet (UV) light. One additional 30-min UV exposure was given 24 h after the first exposure in cases of poor sporulation. Conidia were removed by washing the plates with sterile distilled water.

T. harzianum and *A. nidulans* were cultured on potato-glucose agar (Difco Laboratories, Detroit, MI) at 25 C with a 12-h photoperiod, where they sporulated without special treatment. Conidia of all species were separated from hyphal fragments by filtration through 300- μ m-mesh monofilament nylon bolting cloth.

Plant material. The sources, germination, and cultivation of *C. obtusifolia* and *D. stramonium* were described previously (1). Seeds of cotton (*Gossypium hirsutum* L. 'Acala 59-2') and sunflower (*Helianthus annuus* L.) were from the Department of Plant Introduction, Volcani Center, Bet Dagan, Israel. Seeds of bean (*Phaseolus vulgaris* L. 'Bulgari') were from Zerain, Gedera, Israel, whereas seeds of soybean (*Glycine max* (L.) Merr. 'Centennial,' 'Hill,' and 'Williams 82') were from P. C. Quimby, Jr., Stoneville, MS. Weed seeds of black nightshade (*Solanum nigrum* L.) were from J. Gasquez, Dijon, France, and seeds of *A. theophrastii* were from local collections. All species were germinated in the laboratory and grown in a 25-C growth chamber with 14 h of light (30 μ mol photon $m^{-2} s^{-1}$ at plant level) supplied by cool white fluorescent tubes.

Invert emulsion and application. The emulsion, composed of water, lecithin, and oils, was modified from Quimby et al (16) as previously described (1). Conidial suspensions of various concentrations were prepared from a standard solution of 5×10^5 conidia per milliliter determined with the aid of a hemacytometer. For the water formulation, the standard solution was diluted with sterile distilled water containing 0.02% Tween-80 (Sigma, St. Louis, MO) (for conidial dispersion) to give the required concentration of conidia. For the emulsion formulation, conidia were diluted serially in the water-alginate solution to give a concentration double that of the required final concentration and then mixed with an equal volume of the oil phase.

Inoculation with *A. cassiae*. Four leaflets of plants of *C. obtusifolia* at the first-leaf stage were treated. Other species were treated when the first pair of true leaves had developed. Three 2- μ l droplets were used for each treatment. The four treatments were: water or emulsion, with or without conidia. Each treatment was randomly placed on one leaflet of *C. obtusifolia* or randomly placed on half leaves of two leaves of other species (all four treatments were always given on one plant). Four plants were treated of each species or cultivar. Thus, there were four plants, each with three droplets per treatment, that is, twelve replicated droplets per treatment.

Inoculation of *C. obtusifolia* with other fungal species. Three of the four leaflets were excised and the outer leaflet was treated. One 2- μ l droplet of water or droplet of emulsion, with or without conidia, was placed on each half leaf (one treatment per plant), with eight replicates per treatment in a randomized complete block design.

In both types of experiments, water droplets contained 200 conidia each, whereas emulsion droplets contained 20 conidia. Application of droplets was as previously described (1). The treated plants were placed in a closed dew chamber at 100% relative humidity within a growth chamber for 20 h and then transferred to open shelves. All experiments were repeated at least three times.

Virulence. Virulence of *A. cassiae* was visually estimated (1) and, where appropriate, was immunologically quantified by radioimmunoassay and immunautoradiography as developed in our

laboratory. Briefly, a polyclonal antibody was produced in rabbits with ground hyphae without conidia. The antiserum, used at a 1:200 dilution, recognized 1.6–300 ng/ml hyphae in a log linear manner, as determined by a standard radioimmunosorbent assay (8,12). Leaf tissue disks (4 mm in diameter) were ground in standard phosphate-buffered saline (PBS; 10 mM NaH_2PO_4 , 150 mM NaCl, 3 mM KCl, pH 7.4), centrifuged, washed, incubated with albumin in PBS, and reacted with 1:200 antiserum in microtiter dishes. The wells were washed, reacted with ^{125}I protein A (Amersham, Buckinghamshire, England), incubated, washed again, and counted as described by Ghabrial and Shepherd (8). Results of the radioimmunoassay experiments were analyzed by Student's *t* test (18).

Detection of fungal mycelium on leaves was done by modification of the western blot analysis (19). Inoculated leaves were incubated overnight with 5% w/v reconstituted powdered skim milk in PBS containing 0.1% Triton X100 (Sigma, St. Louis, MO) (blocking buffer). Each leaf was washed twice with PBS containing 0.1% Triton X100 (washing buffer). Diluted antiserum (1:200) was added for a 2-h incubation at room temperature. Leaves were then washed in washing buffer, ^{125}I protein A was added, and the leaves were incubated for an additional 2 h. Leaves were then washed, dried, and exposed for autoradiography.

RESULTS

A. cassiae was developed for control of *C. obtusifolia* in soybeans (21), and its activity was enhanced by an invert emulsion (2,7). It was imperative to know whether *A. cassiae* in emulsion had the potential of infecting soybeans. Various permutations of water versus emulsion were tested (Fig. 1). No infection was visible 5 days after inoculation with the water formulation, whereas conidia in the emulsion caused lesions with necrotic centers (Fig. 1A). This infection of soybeans was clearly seen microscopically (Fig. 1B), and the extent of fungal infection was visualized by immunoradioautography of the leaves (Fig. 1B, insert). Because the antibody reacts specifically with *A. cassiae* and the invert emulsion treatment was not infective without inoculum, it is unlikely that the induced lesions were due to an airborne pathogen of soybean.

A quantitative determination of the extent of the infection was made with leaf disks that were removed for radioimmunosorbent assay 5 days after inoculation (Table 1). A considerable amount of fungus was detected in soybean leaves with the assay, but only when invert emulsion was used. The small amount of hyphal material found in the treatment of conidia in water probably was due to some adherent germinated sporelings that did not penetrate the leaf. As expected, *C. obtusifolia* was infected by conidia of *A. cassiae* in water. The other species, from three botanical families, were infected by *A. cassiae* when conidia were applied in the invert emulsion. The infection in water varied, but it was negligible compared with that in emulsion (Table 1).

Infected cotton and sunflower lesions were very light brown in color, whereas the invert emulsion control spots (uninoculated) were slightly translucent. Lesions on inoculated *D. stramonium*, bean, and *S. nigrum* were darker in color, and no effect was visible when the invert emulsion was used alone. Lesions on infected *A. theophrastii* were slightly darker than background, and the controls treated with invert emulsion showed some translucency. Microscopic examination of leaves treated with conidia in water showed adherent conidia of the initial inoculum, along with small, germinated sporelings that did not penetrate the leaf.

Other fungi were formulated in the invert emulsion to ascertain whether this abolition of specificity of infectivity by the invert emulsion was unique to *A. cassiae* or whether the emulsion would induce virulence in avirulent fungi. We presumed the latter, as infection of *C. obtusifolia* had been seen sporadically in a contaminated growth chamber when drops of invert emulsion without conidia were placed on leaves. Microscopic examination in those cases precluded the infections being due to *A. cassiae*.

When leaves of *C. obtusifolia* were inoculated with *A. crassa*, *T. harzianum*, and *A. nidulans*, infectivity could not be immuno-

logically determined because of the lack of antibodies to these three organisms. These species were only slightly cross-reactive with the antibody prepared against *A. cassiae*. None of these

TABLE 1. Infectivity of *Alternaria cassiae* in the presence of water or invert emulsion

Species ^a	Infectivity (μg hyphae/site \pm S.E.) ^b	
	Water	Invert emulsion
Soybean		
cv. Centennial	1.1 \pm 0.4	8.1 \pm 0.9
cv. Hill	0.4 \pm 0.3	6.3 \pm 0.6
cv. Williamson 82	0.09 \pm 0.08	8.4 \pm 2.1
<i>Cassia obtusifolia</i>	7.9 \pm 0.6	12.0 \pm 0.3
<i>Solanum nigrum</i>	2.1 \pm 0.7	14.5 \pm 1.8
<i>Abutilon theophrasti</i>	1.9 \pm 0.7	7.1 \pm 1.0
<i>Datura innoxia</i>	0	15.4 \pm 1.3
Cotton	0.4 \pm 0.3	4.4 \pm 0.6
Bean (<i>Phaseolus</i>)	0.7 \pm 0.2	12.8 \pm 0.3
Sunflower	0.3 \pm 0.2	8.4 \pm 1.1

^a Soybeans were tested in a separate experiment.

^b Infectivity was measured 5 days after inoculation by radioimmunosorbent assay with antiserum against *A. cassiae*. The counts were translated to fungal fresh weight (after subtracting the background from uninoculated leaves) from a standard curve with fungal hyphae (0.05 μg hyphae = 1,900 cpm). There were 12 replicates per point; S.E. = standard error. Differences between all water treatments versus invert emulsion treatments were significant at $P < 0.01$ with Student's *t* test.

organisms were pathogenic in water, as determined visually and microscopically (Fig. 2). Necrosis was apparent with all these organisms 3 days after inoculation of conidia in the invert emulsion. Lesions expanded beyond the diameter of the droplet, but it was hard to see the hyphae on the leaflets. The hyphae broke the leaf surface of the invert emulsion treatments and were visible on the leaf surface when the leaflets were incubated 2 additional days on damp filter paper in closed petri dishes (Fig. 2). We determined that the hyphae on the leaves matched the inoculum by surface-disinfestation of each lesion and plating reisolated hyphae or conidia on nutrient agar for comparison with the initial cultures; they were identical with the inoculum applied in each case.

The utility of this invert emulsion could be enhanced if the factor causing the loss of specificity could be isolated and then removed from the mixture. Emulsions lacking each of the components were tested in the *A. cassiae*/*C. obtusifolia* system. Each of the two oils (soybean and paraffin), the wax, and the emulsifier (lecithin) strongly contributed to the response of the invert emulsion; that is, there was a coalitive synergism (a synergism where no component shows activity by itself) between all four components (data not shown).

DISCUSSION

A. cassiae in water is a highly specific pathogen of *C. obtusifolia* and not to other species (20) (Table 1); this specificity was

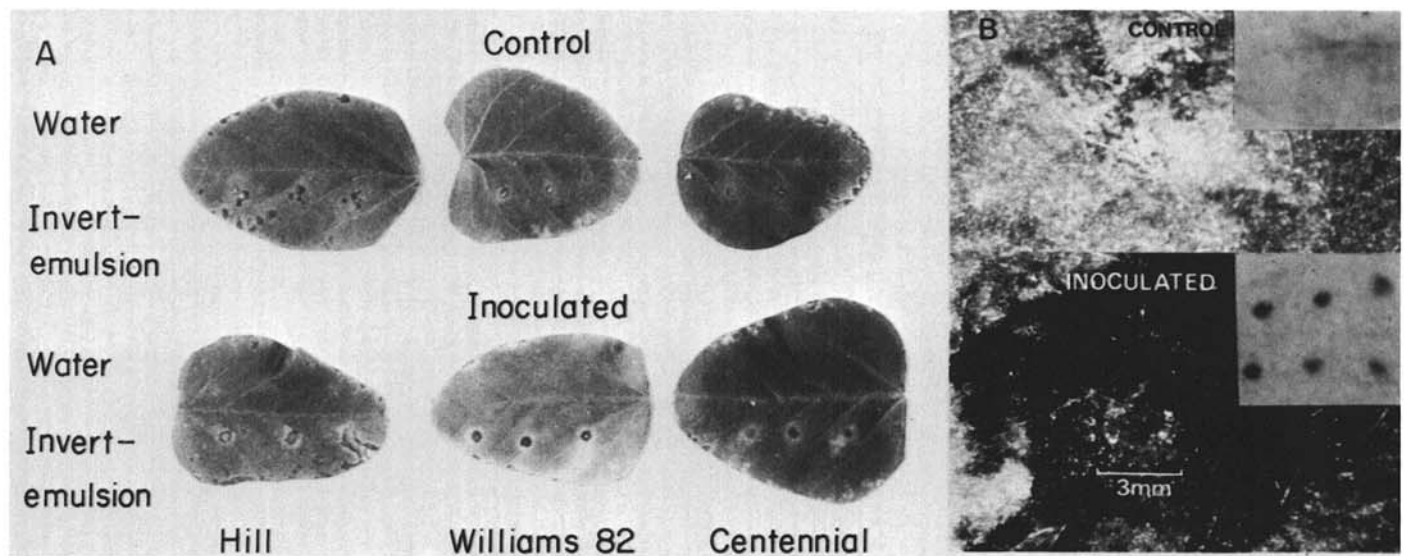


Fig. 1. Infection of soybeans by *Alternaria cassiae* when applied in invert emulsion. A, Infection of three soybean cultivars. B, Microscopic appearance of infection site (cultivar Williams 82); insert, immunautoradiography of leaf showing specificity. The 2- μl droplets on half leaves contained 200 conidia in water application and 20 conidia in invert emulsion.

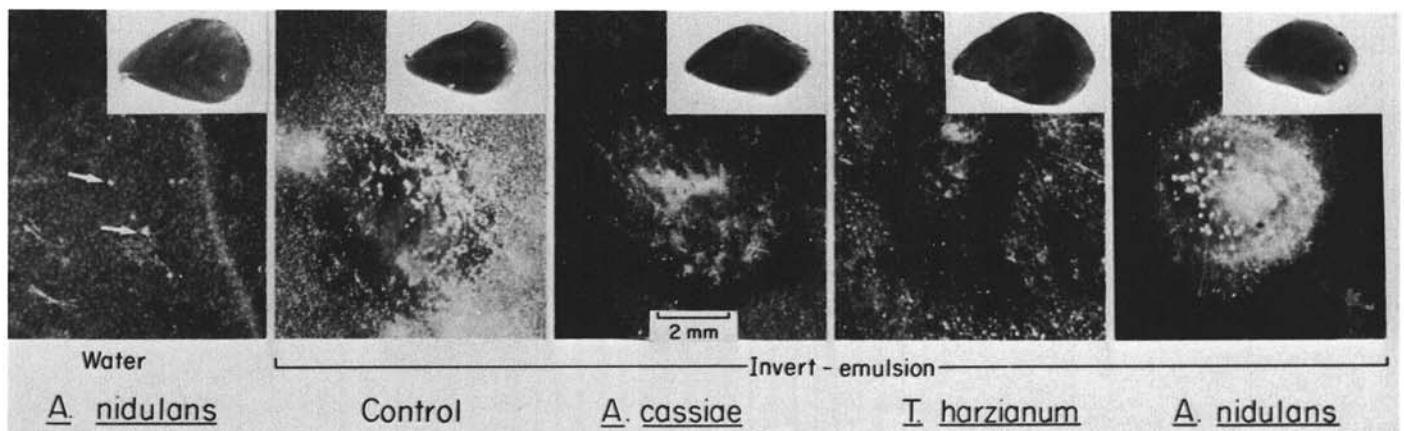


Fig. 2. Infectivity of *Alternaria crassa*, *Trichoderma harzianum*, and *Aspergillus nidulans* induced by invert emulsion on *Cassia obtusifolia*. Microscopic view and (insert) whole leaves. The arrows on the water control point to germinated conidia whose hyphae did not penetrate the cuticle.

abolished when conidia were applied in invert emulsion.

The field significance of the potential crop damage by mycoherbicides in invert emulsion is not clear. *A. cassiae* did not visibly damage soybeans when used in a similar invert emulsion as a directed spray to control *C. obtusifolia* (7,16). Possibly, soybean plants can overcome the infection, or the infection cannot spread without the invert emulsion. An additional factor to consider is the crude nature of the components of the invert emulsion, especially the soybean oil and the crude lecithin. The latter is a byproduct from oil pressing.

The invert emulsion abolished host specificity of *A. cassiae* and *A. crassa* and allowed nonpathogenic fungi to infect a variety of plants. It is not clear whether the invert emulsion acts by physically damaging the cuticle or the subtending tissue or by suppressing the plants' natural defenses.

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. Baker, J. M. 1970. The effects of oils on plants. *Environ. Pollut.* 1:27-44.
3. Baker, R. 1968. Mechanisms of biological control of soilborne pathogens. *Annu. Rev. Phytopathol.* 6:263-294.
4. Barrentine, W. L., and McWhorter, C. G. 1988. Johnsongrass (*Sorghum halepense*) control with herbicides in oil diluents. *Weed Sci.* 36:102-110.
5. Boyette, C. D. 1986. Evaluation of *Alternaria crassa* for biological control of jimsonweed: Host range and virulence. *Plant Sci.* 45:223-228.
6. Boyette, C. D., and Turfitt, L. B. 1988. Factors influencing biocontrol of jimsonweed (*Datura stramonium* L.) with the leaf-spotting fungus *Alternaria crassa*. *Plant Sci.* 56:261-264.
7. 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.
8. Ghabrial, S. A., and Shepherd, R. J. 1980. A sensitive radioimmunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 48:311-317.
9. Greaves, M. P., and Sargent, J. A. 1986. Herbicide-induced microbial invasion of plant roots. *Weed Sci.* 34:50-53.
10. Gressel, J. 1990. Synergizing herbicides. *Rev. Weed Sci.* 5:49-82.
11. Hodgson, R. J., Wymore, L. A., Watson, A. K., Snyder, R. H., and Collette, A. 1988. Efficacy of *Colletotrichum coccodes* and thidiazuron for velvetleaf (*Abutilon theophrasti*) control in soybean (*Glycine max*). *Weed Technol.* 2:473-480.
12. Lommel, S. A., McCain, A. H., and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. *Phytopathology* 72:1018-1022.
13. McWhorter, C. G., and Barrentine, W. L. 1988. Spread of paraffinic oil on leaf surfaces of johnsongrass (*Sorghum halepense*). *Weed Sci.* 36:111-117.
14. Miller, P. M. 1955. V-8 juice agar as a general-purpose medium for fungi and bacteria. *Phytopathology* 45:461-462.
15. Philipp, W.-D., and Hellstern, A. 1986. Biological control of powdery mildew *Ampelomyces quisqualis* under reduced air humidity conditions. *Z. Pflanzenkrankh. Pflanzenschutz* 93:384-391.
16. Quimby, P. C., Jr., Fulgham, F. E., Boyette, C. D., and Connick, W. J. 1990. An invert emulsion replaces dew in biocontrol of sicklepod—A preliminary study. Pages 264-270 in: *Pesticide Formulations and Application Systems*, Vol. 8. D. A. Hoved and G. B. Beestman, eds. American Society for Testing and Materials, Philadelphia, PA.
17. Scheepens, P. C. 1979. The use of pathogenic mildews to control problem weeds. *Gewasbescherming* 10:31.
18. Snedecor, G. W., and Cochran, W. G. 1967. *Statistical Methods*. Iowa State University Press, Ames.
19. Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76:4340-4354.
20. Walker, H. L. 1982. Seedling blight of sicklepod caused by *Alternaria cassiae*. *Plant Dis.* 66:426-428.
21. Walker, H. L., and Boyette, C. D. 1985. Biocontrol of sicklepod (*Cassia obtusifolia*) in soybeans (*Glycine max*) with *Alternaria cassiae*. *Weed Sci.* 33:212-215.
22. Walker, H. L., and Riley, J. A. 1982. Evaluation of *Alternaria cassiae* for the biocontrol of sicklepod (*Cassia obtusifolia*). *Weed Sci.* 30:651-654.
23. Wymore, L. A., and Watson, A. K. 1989. Interaction between a velvetleaf isolate of *Colletotrichum coccodes* and thidiazuron for velvetleaf (*Abutilon theophrasti*) control in the field. *Weed Sci.* 37:478-483.
24. Young, P. A. 1926. Facultative parasitism and host ranges of fungi. *Am. J. Bot.* 131:502-521.