

Adjuvants enhance the biological control potential of an isolate of *Colletotrichum gloeosporioides* for biological control of sicklepod (*Senna obtusifolia*)

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Abstract

In greenhouse experiments, unrefined corn oil, Silwet L-77, and an invert emulsion were tested as adjuvants with the mycoherbicide fungus *Colletotrichum gloeosporioides*, a weakly virulent pathogen of sicklepod (*Senna obtusifolia*). A 1:1 (v/v) fungus/corn oil tank mixture containing 0.2% (v/v) Silwet L-77 surfactant reduced the dew period requirements for maximum weed infection and mortality from 16 to 8 h, and delayed the need for free moisture for greater than 48 h. This formulation also resulted in the ability of the pathogen to infect and kill weeds in larger (>5 leaf) growth stages. The invert emulsion resulted in similar effects upon these parameters. These results suggest that invert emulsions, unrefined corn oil and Silwet L-77 surfactant greatly improve the bioherbicide potential of this pathogen for control of sicklepod, a serious weed pest in the southeastern US.

Keywords: *Colletotrichum gloeosporioides*, bioherbicide, biocontrol agent, invert emulsion, unrefined corn oil, surfactant, *Senna obtusifolia*

Introduction

Sicklepod [*Senna obtusifolia* (L.) Irwin & Barnaby] is a non-nodulating legume that was originally introduced into the southeastern US from tropical America. Its center of origin is tropical, but the time and point of entry into the US is unknown (Brown & Bridges 1989). Teem et al. (1980) surveyed weed extension specialists in each of the 50 states to find that sicklepod was troublesome in 11 southern states and increasing in 12 other states. It has become an important weed pest in soybean [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.), and peanut (*Arachis hypogaea* L.) fields in much of the southeastern US (Buchanan et al. 1980; French 1980; Gossett 1981; Dowler 1992). It causes yield loss, seed quality degradation, and difficulty with harvest. Sicklepod is one of the most difficult weeds to control in soybeans. Although less competitive on a per-plant basis than many other common annual broadleaf weeds, heavy infestations can reduce soybean yields 60–70% or more (Brown & Bridges 1989). In addition, a price dockage may occur because of foreign matter (sicklepod seed) in the harvested product.

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Sicklepod is difficult to control because it is tolerant to many commonly used herbicides, has a prolific growth habit, copious seed production, and the capacity for emergence throughout the growing season (Brown & Bridges 1989; Elmore 1989). Several herbicides, such as imazaquin (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-quinolinecarboxylic) and metribuzin [4-amino-6 (1,1-dimethylethyl)-3-methylthio]1,2,4-triazin-5(4*H*)-one] are effective on sicklepod if used properly. In some situations, two herbicide applications (preplant incorporated or preemergence followed by postemergence) may be necessary for acceptable control of moderate to heavy infestations (Etheridge et al. 1996). Alternative controls are needed to replace or supplement existing methods. A fungal pathogen, *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. (deposited in the Northern Regional Research Laboratory Fungal Repository, No. 21046) isolated from coffee senna (*Senna occidentalis* L.) was found to be weakly pathogenic to sicklepod, a closely related species. However, when formulated in an invert emulsion, the pathogen was significantly more virulent against sicklepod (Boyette & McAlpine 1996).

Although this fungus showed promise as a biocontrol agent for coffee senna control when applied as an aqueous spore suspension, its relatively low virulence to sicklepod precluded it from serious consideration as a bioherbicide for that weed when applied as aqueous spore suspensions. In addition, it shared many of the same constraints exhibited by several other biocontrol pathogens, such as the necessity for a lengthy free-moisture (dew) period, the need for an immediate dew application, and the inability to satisfactorily infect and kill larger weeds larger than the cotyledonary to first-leaf stage of growth (Boyette & McAlpine 1996). Research with several pathogen–host systems has shown that some of these constraints can be overcome through formulation-based approaches using several weed pathogens (Boyette 1994; Weidemann et al. 1995). The use of various crop oils (Mintz et al. 1992; Auld et al. 1994; Boyette 1994; Egley & Boyette 1995; Ghorbani et al. 2000; Sandrin et al. 2003; Milhollon et al. 2005) and invert emulsions (Quimby et al. 1989; Amsellen et al. 1991; Boyette et al. 1993; Yang et al. 1996; Milhollon et al. 2005) have resulted in improved bioherbicide efficacy and performance of several biocontrol fungi. The research reported herein was conducted to determine if some of the constraints to disease expression, specifically lengthy dew period durations, the immediate necessity for a dew treatment, and the inability of this pathogen to effectively control plants larger than the cotyledonary to first-leaf stage of growth, could be overcome through the use of various adjuvants.

Materials and methods

Isolation and culture

The fungus was originally isolated from diseased coffee senna tissue by surface sterilizing sections of diseased tissue in 0.05% NaOCl for 1 min, then placing the sections on potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) amended with the antibiotics chloramphenicol (0.75 mg mL⁻¹) and streptomycin sulfate (0.125 mg mL⁻¹). The plates were incubated for 48 h at 25°C. Advancing edges of fungal colonies were transferred to PDA and incubated for 5 days at 25°C under alternating 12-h light/12-h dark regimens, provided by cool white fluorescent lights. The fungus was subcultured on PDA without antibiotics, and preserved at 4°C in sterilized sandy loam soil (25% water holding capacity), and on sterile silica gel

containing skim milk (Windels et al. 1988). Inoculum for all experiments was produced in modified Richards' medium containing vegetable juice (Daniel et al. 1972) either in 1-L Erlenmeyer flasks at 25°C and 125 rpm for 5 days, or in a laboratory fermenter under similar conditions. Conidia were separated from mycelium by filtering through double-layered cheesecloth and then removed from the spent medium by centrifugation at 500 rpm for 10 min at 16°C. Conidial concentrations were determined using a hemacytometer and were adjusted to the desired inoculum density by adding sterile, distilled water.

Adjuvants selected for study and spray mixture preparation

The adjuvants that were used in these studies [an invert emulsion designated MSG-8.25] (Quimby et al. 1989; Boyette et al. 1993); a silicone-polyether copolymer spray adjuvant [Silwet[®] L-77[™], Loveland Industries, Greeley, CO] (Boyette 1994); and unrefined corn oil [Spectrum Naturals, Inc., Petaluma, CA] (Boyette 1994), have all imparted increased biocontrol efficacy to various bioherbicidal pathogens.

Plants were sprayed using hand-held pump sprayers. Application rates were approximately 100 L ha⁻¹. The treatments utilized in these studies were as follows: (1) *C. gloeosporioides* conidia in water suspension; (2) 0.2% (v/v) aqueous conidial suspension/Silwet L-77 emulsion; (3) an oil-in-water (o/w) emulsion formed by combining 1:1 (v/v) aqueous conidial suspensions/unrefined corn oil; (3,4) a conidial suspension/Silwet L-77/unrefined corn oil emulsion (also an o/w emulsion); and (5) a 1:1 aqueous conidial suspension/MSG-8.25 invert emulsion [a water-in-oil (w/o) emulsion]. Inoculum densities for all treatments containing the fungal component were adjusted to 1 × 10⁷ conidia mL⁻¹. The respective controls consisted of sicklepod seedlings inoculated with distilled water or the previously listed adjuvants without the fungal component.

Test plant propagation

Sicklepod plants were grown from seed in a commercial potting mix (Jiffy Products of America, Inc., Batavia, IL) contained in peat strips. Each strip contained 12 plants. The potting mix was supplemented with a controlled-release (14:14:14, NPK) fertilizer (Grace Sierra Horticultural Products, Milpitas, CA). The plants were placed in sub-irrigated trays that were mounted on greenhouse benches. Greenhouse temperatures ranged from 25 to 30°C with 40–90% relative humidity (RH). The photoperiod was 12 h with 1650 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) measured at midday.

Effects of adjuvants on conidial germination

The effects of these adjuvants upon conidial germination was measured by placing 0.1-μL droplets of adjuvant suspensions that contained approximately 1 × 10⁶ conidia mL⁻¹ immediately after preparation on PDA plates (3 droplets/plate), and incubating them at 25°C in alternating 12-h light/12-h dark regimens. Light intensity at dish level was approximately 200 μmol m⁻² s⁻¹ PAR as measured with a light meter. Microscopic counts of germinated conidia were made after 8 h. At least 200 conidia (unstained) were counted per droplet and were scored for spore germination using a

light microscope at $\times 400$ magnification. The experiment was repeated over time and the results were pooled and averages of the means are presented.

Effect of dew period duration

Sicklepod seedlings in the cotyledonary-to-early first leaf stage of growth were sprayed until runoff occurred with a spray mix containing 1×10^7 conidia mL^{-1} in the previously listed adjuvants immediately following preparation of the spray mixes. The inoculated plants were then placed in darkened dew chambers at 25°C and 100% RH for periods ranging from 0 h (no dew) to 24 h in 4-h increments. Following the dew treatment, plants were immediately placed in sub-irrigated trays on greenhouse benches. Mortality was determined 14 days after inoculation. The experiment was conducted twice with three replications of 12 plants per replicate. Data from the replicate experiments were combined and averaged.

Effect of dew delays

Sicklepod seedlings in the cotyledonary to first-leaf growth stage were inoculated with the treatments as described previously. Following inoculation, the plants either were placed directly in dew chambers for 12 h at 25°C and 100% RH, or subjected to dew delay intervals of 1, 2, 4, 8, 12, or 24 h before being subjected to a 12-h dew treatment at 25°C . The plants were incubated on greenhouse benches and monitored as described previously. The experiment was conducted twice with three replications of 12 plants per replicate. Data from the replicate experiments were combined and averaged.

Effect of plant growth stage

Sicklepod plants in the cotyledonary to first true leaf, two to three true leaf, four to five true leaf and six to seven true leaf stages of growth were sprayed with conidial suspensions in the same adjuvants as previously described at an inoculum density of 1.0×10^7 conidia mL^{-1} and held in a dew chamber for 16 h at 25°C . Plants were then moved to the greenhouse as described, and mortality percentages were determined 14 days after inoculation. Experiments were conducted twice with three replications of 12 plants per replicate. Data from the replicate experiments were combined and averaged.

Statistical procedure

All experiments utilized a randomized complete block experimental design, with three replications of 12 plants per replicate. In all experiments, results were analyzed by ANOVA. All experiments were conducted twice and the results were pooled following subsection to Bartlett's test for homogeneity (Steele & Torrey 1980). Values from the conidial germination studies and in the plant growth stage studies were separated using Fisher's protected least significant difference at the 0.05 level of probability (FLSD). Nontransformed data for are presented throughout because arcsine square-root transformation did not alter interpretation of data. Values are presented as the means of replicated experiments. Regression analysis and confidence limits (95% level) were used to analyze the data in the dew duration and dew delay experiments.

Results and discussion

Isolation and culture

The fungus was readily isolated from diseased tissue and sporulated abundantly on PDA. When reinoculated onto healthy seedlings, typical anthracnose lesions formed on leaves and stems after 5 days, with acervuli scattered throughout the lesions. Conidia are elliptical, with rounded ends, and range in size from 15 to 19 μm by 4–6 μm and average 16.5 by 5.6 μm .

The fungus sporulated prolifically on PDA plates and under submerged culture in modified Richard's medium. Conidial yields averaged 3.0×10^8 conidia mL^{-1} after 7 days at 25°C and 250 rpm. This is comparable to conidial yields produced by other *C. gloeosporioides* formae speciales that have been evaluated as bioherbicides against other weeds (Daniel et al. 1969; Boyette et al. 1979).

Effect on conidial germination

Unrefined corn oil and Silwet L-77 promoted conidial germination slightly better than in water only (Table I). However, when unrefined corn oil and Silwet L-77 were combined, conidial germination was significantly greater. Conidial germination in MSG-8.25 invert emulsion was significantly less than that achieved in unrefined corn oil or Silwet L-77, either alone or in combination; however, conidial germination in the invert emulsion was not significantly reduced as compared to in distilled water (Table I). We have previously demonstrated that unrefined corn oil, but not refined corn oil, stimulated germination of *C. truncatum* spores (Egley & Boyette 1995). Other refined oils were likewise non-stimulatory (Egley & Boyette 1995). Research has also shown that the addition of the surfactant Silwet L-77 to an unrefined corn oil emulsion promoted germination and infectivity of *Alternaria helianthi* spores on common cocklebur (*Xanthium strumarium* L.) (Abbas & Egley 1996).

Effect of dew period duration

The duration of dew that was required to provide at least 60% control of sicklepod seedlings was reduced from 24 to 4 h when conidia were formulated either in the

Table I. The effect of unrefined corn oil, Silwet L-77 surfactant, and MSG-8.25 invert emulsion upon germination of *Colletotrichum gloeosporioides* conidia.^a

Germination (%) ^b	Treatment
Distilled water	79 c
Unrefined corn oil ^c	87 b
Silwet L-77 ^d	84 b
Unrefined corn oil + Silwet L-77 ^e	95 a
MSG-8.25 invert emulsion ^f	72 c

^aMeans followed by the same letter do not differ at $P=0.05$ using Fisher's protected least significant difference. ^bGerminated conidia were determined by microscopic count after 8 h incubation on PDA plates.

^cConidia were suspended in distilled water; unrefined corn oil was added to make a 1:1 (v/v) emulsion. ^dConidia were suspended in distilled water; Silwet L-77 was added to make a 0.2% solution.

^eSilwet L-77 was added to a 1:1 unrefined corn oil/aqueous conidia emulsion to make a 0.2% solution.

^fConidia were suspended in distilled water; MSG-8.25 invert was added to make a 1:1 emulsion.

invert emulsion or in the unrefined corn oil/Silwet L-77 emulsion (Figure 1). In addition, over 60% of seedlings that were treated with the conidia/invert emulsion were killed when subjected to only 4 h of dew, while no infection or mortality occurred to weeds treated with conidia in water at this dew period (Figure 1). Dew period durations from 4 to 8 h are not uncommon in the areas where sicklepod is problematic, thus the unrefined corn oil and unrefined corn oil with surfactant could possibly facilitate infection by the pathogen and promote disease spread under field conditions. Neither the conidia/water treatment nor the conidia/surfactant treatment provided more than 65% sicklepod mortality, even after 24 h of constant dew (Figure 1). The best-fit curves as indicated by R^2 values for all treatments were second-degree polynomials (Figure 1). Because no mortality occurred to sicklepod with any of the adjuvants alone or in the distilled water control, data are not presented in any of the weed control experiments.

Effect of dew delay

When aqueous sprays of conidia were applied to sicklepod seedlings, a dew treatment immediately following was required in order for the pathogen to provide 90% or

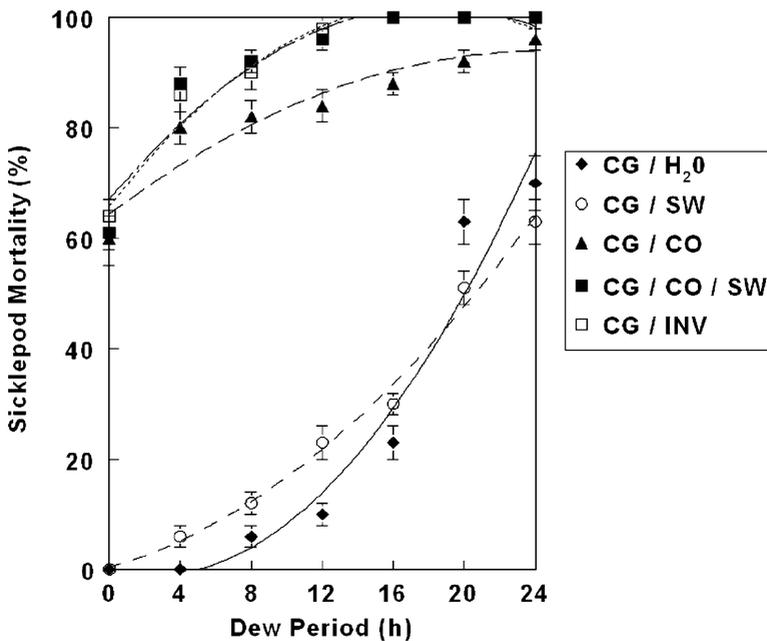


Figure 1. Effect of dew period duration on biocontrol of sicklepod with *Colletotrichum gloeosporioides* conidia formulated in: CG/H₂O, the relationship is best described by the equation: $Y = 6.00 - 8.63X + 2.65X^2$, $R^2 = 0.95$, where Y = weed mortality and X = dew period length; CG/SW, the relationship is best described by the equation: $Y = -2.14 + 1.37X + 1.15X^2$, $R^2 = 0.96$, where Y = weed mortality and X = dew period length; CG/CO, the relationship is best described by the equation: $Y = 54 + 11.21X - 0.79X^2$, $R^2 = 0.90$, where Y = weed mortality and X = dew period length; CG/CO/SW, the relationship is best described by the equation: $Y = 47.57 + 20.08X - 1.85X^2$, $R^2 = 0.99$, where Y = weed mortality and X = dew period length; CG/INV, the relationship is best described by the equation: $Y = 50.00 + 18.74X - 1.69X^2$, $R^2 = 0.90$, where Y = weed mortality and X = dew period length. Error bars represent 95% confidence limits.

greater mortality. If the onset of dew was delayed by only 1 h, sicklepod mortality was reduced by over 30%, and if the dew was delayed by 4 h or more, mortality was reduced by more than 80% (Figure 2). However, when conidia were formulated either with the invert emulsion or the unrefined corn oil/Silwet-L77 emulsion, mortality was 96 and 95%, respectively, after an 8-h dew delay. These results compare favorably to treatments subjected to an immediate dew treatment (Figure 2). Even after a 72-h delay of dew, 80% or greater mortality occurred with these treatments (Figure 2). Interestingly, the best-fit curves as indicated by R^2 values for the fungus in corn oil, fungus in corn oil with surfactant, and the fungus in invert treatments were linear, while the fungus in water and fungus in surfactant treatments were exponential (Figure 2). The results from this experiment suggest that the unrefined corn oil, unrefined corn oil with surfactant, and invert adjuvants may protect the fungus from desiccation following field applications, and could allow for applications to be made several hours before the onset of a dew event under field conditions.

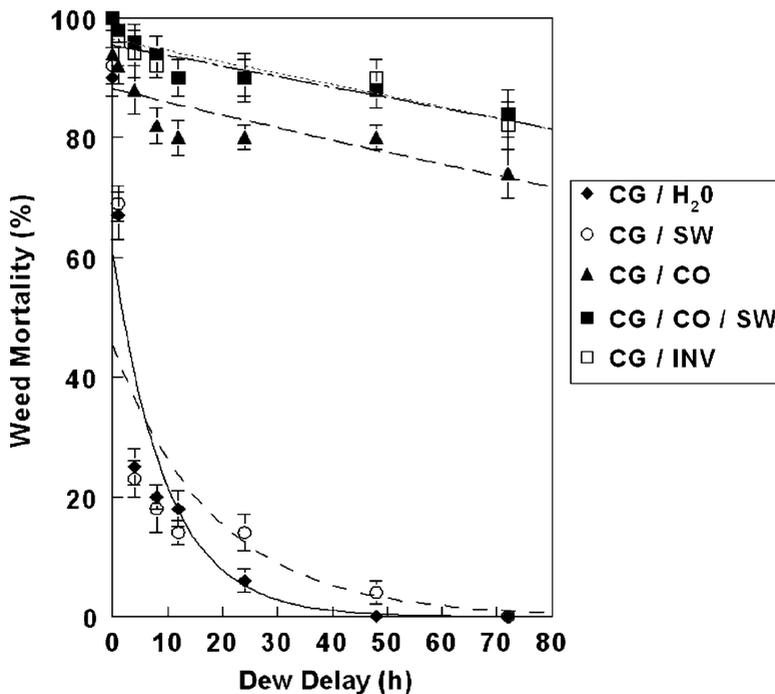


Figure 2. Effect of dew delay on biocontrol of sicklepod with conidia of *Colletotrichum gloeosporioides* formulated in: CG/H₂O, the relationship is best described by the equation: $Y = 60.90 - 0.10X$, where X = dew period delay and Y = weed mortality; CG/SW, the relationship is best described by the equation: $Y = 45.47 - 0.05X$, $R^2 = 0.92$, where Y = weed mortality and X = dew period delay; CG/CO, the relationship is best described by the equation: $Y = 88.17 - 0.20X$, $R^2 = 0.92$, where Y = weed mortality and X = dew period delay; CG/CO/SW, the relationship is best described by the equation: $Y = 96.49 - 0.18X$, $R^2 = 0.96$, where Y = weed mortality and X = dew period delay; and CG/INV, the relationship is best described by the equation: $Y = 95.48 - 0.18X$, $R^2 = 0.90$, where Y = weed mortality and X = dew period delay. Dew periods were 25°C for 16 h for those plants receiving a dew treatment. Data were collected 14 days after inoculation. Error bars represent 95% confidence limits.

Effect of plant growth stage

Conidia formulated in unrefined corn oil/Silwet L-77 and in the invert emulsion controlled sicklepod plants satisfactorily at all growth stages that were tested (Figure 3). The invert emulsion provided highest mortality overall. When inoculated in the cotyledonary to one-leaf growth stage, 100% mortality of sicklepod plants occurred with unrefined oil/Silwet L-77 and invert emulsion treatments (Figure 3). However, mortality of plants in the two- to three-leaf growth stage or larger was reduced by 50–60% in either the Silwet L-77 treatments or corn oil treatments. Aqueous conidia sprays were the least effective of all treatments that were tested (Figure 3). Although conidial germination in the invert emulsion was significantly reduced as compared to conidial germination in either the other treatments (except the conidia/distilled water treatment) (Table I), the effect on sicklepod mortality was not significantly different from the mortality rate achieved with the conidia/unrefined corn oil/Silwet L-77 treatment (Figure 3). A possible explanation is that conidial germination of this strain of *C. gloeosporioides* could be inhibited slightly on non-plant surfaces, such as PDA, but may be stimulated when in contact with plant surfaces, as was shown with *C. gloeosporioides* f. sp. *aeschyromene* on northern jointvetch (Sandrin et al. 2003). Although those investigations were conducted using rapeseed oil rather than an invert emulsion, it is possible that some of the findings may also be applicable in the present studies. Another possible explanation is that a component, or components, of the invert emulsion may have acted as a solvent to disrupt the cuticular waxes on the older sicklepod seedlings, allowing the fungus to penetrate and infect the older plants more effectively. Further investigations will be necessary to further explain these findings.

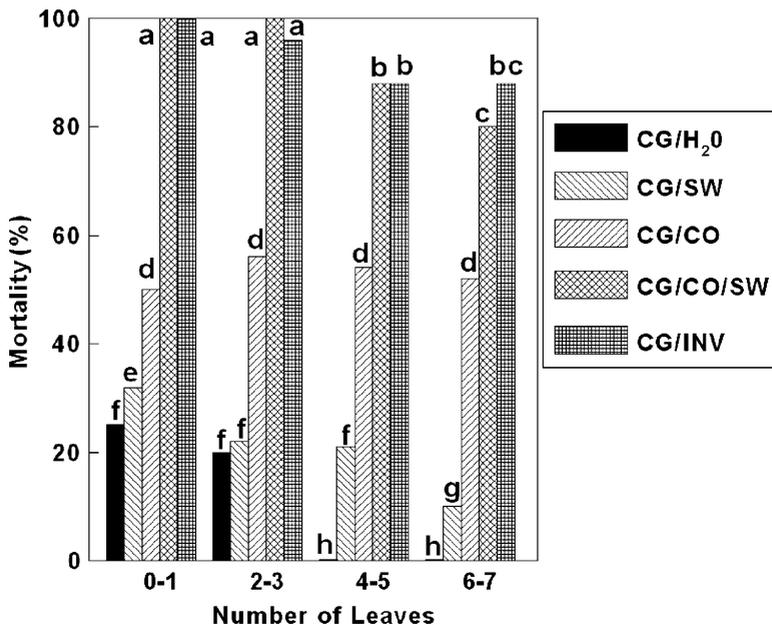


Figure 3. Effect of plant growth stage on biocontrol of sicklepod with conidia of *Colletotrichum gloeosporioides* formulated in: CG/H₂O, CG/SW, CG/CO, CG/CO/SW, and CG/INV. Plants were subjected to a 16 h dew period at 25°C. Data were collected 14 days after inoculation. Bars with the same letter do not differ significantly at $p=0.05$ according to FLSDD.

The ability to modify host ranges, significantly improve virulence, and overcome environmental dependencies through formulation-based approaches greatly improves the bioherbicidal potential of *C. gloeosporioides* and several other pathogens that are being evaluated as bioherbicides (Boyette & Abbas 1994a,b; Weidemann et al. 1995). These results in this report indicate that, when properly formulated, *C. gloeosporioides* NRRL No. 21046 can be a very effective bioherbicide for controlling sicklepod. Although none of the formulations entirely eliminated the requirement for free moisture, the dew period durations were reduced to levels that are common in the humid southeastern US during the growing season.

Of significant importance is the fact that the invert and unrefined corn oil/surfactant formulations extended the time in which the fungus remained infective between inoculation and the onset of dew. Previous research has shown that *Alternaria cassiae* Jurair and Khan could tolerate a dew delay of 2 days between inoculation and the onset of dew before the biocontrol efficacy of the pathogen was reduced for sicklepod control (Walker & Boyette 1986). Similar results have been reported on the biocontrol of hemp sesbania (*Sesbania exaltata* Rydb. ex A. W. Hill) by *Colletotrichum truncatum* (Schw.) Andrus and Moore in which a delay of 3 days was tolerated without affecting the biocontrol efficacy of the pathogen (Boyette 1994; Egley & Boyette 1995). The fact that these formulations also promote infection and control of larger sicklepod plants that cannot be controlled by aqueous fungal formulations alone is also of significance because it allows for a greater window of time in which weeds may be treated under field conditions.

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