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RESEARCH ARTICLE

Hemp sesbania (*Sesbania exaltata*) control in rice (*Oryza sativa*) with the bioherbicidal fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene* formulated in an invert emulsion

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In greenhouse and field experiments, an invert emulsion (MSG 8.25) was tested with dried, formulated spores of the bioherbicidal fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, a highly virulent pathogen of the leguminous weed *Aeschynomene virginica* (northern jointvetch), but considered 'immune' against another more serious leguminous weed, *Sesbania exaltata* (hemp sesbania). A 1:1 (v/v) fungus/invert emulsion mixture resulted in 100% infection and mortality of inoculated hemp sesbania seedlings over a 21-day period under greenhouse conditions. In replicated field tests of the fungus/invert formulation conducted in Stuttgart, AR, and Stoneville, MS, hemp sesbania was controlled 85 and 90%, respectively. These results suggest that this invert emulsion expands the host range of *C. gloeosporioides* f. sp. *aeschynomene*, with a concomitant improvement of the bioherbicidal potential of this pathogen.

Keywords: bioherbicide; biocontrol; hemp sesbania; *Colletotrichum gloeosporioides* f. sp. *aeschynomene*; invert emulsion

1. Introduction

Hemp sesbania [*Sesbania exaltata* (Rydb.) ex. A.W. Hill] is a vigorous, nodulating leguminous weed in rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.], and cotton (*Gossypium hirsutum* L.) capable of reaching heights of 3 m at maturity (Lorenzi and Jeffery 1987). Hemp sesbania is rated as one of the 10 most troublesome weeds in the three southern US states of Arkansas, Louisiana, and Mississippi (Dowler 1992), reducing crop seed yield by shading and competition (King and Purcell 1997; Norsworthy and Oliver 2000), and is a prolific seed producer, yielding up to 21,000 seeds per plant[1] (Lovelace and Oliver 2000). Weed infestations in rice can also interfere with combine operation at harvest by the fibrous stems twining around combine blades, resulting in extended time of harvest and equipment breakdown, thereby significantly increasing harvesting and drying costs. Weed seed

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contamination of rice grain also lowers grain quality and may lower the cash value of the crop (Lovelace and Oliver 2000).

The use of fungi and bacteria as inundative biological control agents (bioherbicides) has been recognized as a significant technological alternative to chemical herbicides (e.g., Hoagland 1990; Rosskopf, Charudattan, and Kadir 1999; Charudattan 2005; Hallett 2005; Weaver, Lyn, Boyette, and Hoagland 2007). In order to be even more predictable and acceptable, it is necessary to achieve the maximum capability of a bioherbicidal pathogen to infect, kill, or reduce the competitiveness of the weed host. Research has indicated that the fungus *Colletotrichum truncatum* has promise as a bioherbicide for controlling hemp sesbania (Boyette 1991; Boyette, Quimby, Bryson, Egley and Fulgham 1993; Abbas and Boyette 2000). However, as is the case with most foliar pathogens, spores (conidia) of this fungus require a dew period in order to germinate, establish infection, and cause disease (Boyette 1991; Boyette et al. 1993).

Previous research here and elsewhere has shown that invert (water-in-oil) emulsions and various vegetable oil-in-water emulsions provide a method to retard evaporation and trap water in the spray mixture, thereby decreasing the amount of additional free-moisture required for spore germination and infection to occur (e.g., Quimby, Fulgham, Boyette, and Connick 1989; Auld 1993; Boyette 1994; Sandrin, TeBeest, and Weidemann 2003; Boyette, Hoagland, and Weaver 2007a). For example, greenhouse and field results indicated that excellent control (>95%) of sicklepod (*Senna obtusifolia* L.) with the fungus *Alternaria cassiae* Jurair & Khan could be achieved with little or no dew (Quimby et al. 1989). In those studies, lecithin was used as an emulsifying agent, and paraffin oil and wax were used to further retard water evaporation and help retain droplet size. Similarly, it was shown that hemp sesbania could be effectively controlled in soybean by *C. truncatum* spores formulated in a water-in-oil invert emulsion (Boyette et al. 1993).

A formulation of a strain of the fungus, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Penz) Sacc. (CGA) (ATCC No. 20358), for controlling the weed (northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.]) received U.S. Environmental Protection Agency (US-EPA) registration in 1982 as a commercial biological herbicide under the trade name Collego® (Templeton, Smith, and TeBeest 1989). The fungus induces anthracnose lesions on northern jointvetch that increase in severity over a several week period under field conditions, eventually killing infected weeds as the lesions formed by the fungus girdle the stem (Sandrin et al. 2003).

The fungus effectively (and selectively) controls northern jointvetch in rice and irrigated soybean fields (TeBeest 1985; Templeton et al. 1989). Host range tests originally indicated that this strain of CGA (ATTCC No. 56897) was highly virulent against northern jointvetch, while several other crop and weed species, including hemp sesbania, were considered 'immune' to infection by CGA (Daniel, Templeton, Smith, and Fox 1973). However, TeBeest (1988) later found that CGA was also pathogenic, with varying degrees of virulence, to seven of 13 *Aeschynomene* spp., as well as several other leguminous spp., such as some cvs. of *Lathyrus*, *Lupinus*, *Pisum*, and *Vicia*. No weed species other than *Aeschynomene* spp. were included in that report.

Although narrow host specificity of a bioherbicidal fungus may be beneficial from both biological and perhaps US-EPA registration perspectives, this trait may restrict bioherbicidal agents from practical and commercial standpoints (Cartwright

2008, personal communication). Research has shown that it is possible to expand the host ranges of some fungal pathogens through formulation-based approaches using an invert emulsion formulation (Amsellem, Sharon, and Gressel 1991; Boyette, Abbas, and Smith 1991; Boyette and Abbas 1994). The purpose of the present research was to determine if the host range of CGA could be expanded to control hemp sesbania in rice under field conditions through a formulation-based approach.

2. Materials and methods

2.1 Accession of the fungus

A dried, formulated spore and a fungal spore-rehydrating agent (a sugar solution) (COLLEGO™) were obtained from Encore Technologies, Inc., Minnkota, MN, USA, and was used in all greenhouse and field testing.

2.2 Greenhouse experiments

Hemp sesbania seeds (Azlin Seed Co., Leland, MS, USA) were surface-sterilized in 0.05% NaOCl for 5 min, rinsed with sterile distilled water, and germinated on moistened filter paper in Petri dishes. After the seeds germinated (~ 48 h) they were planted in a commercial potting mix (Jiffy-mix; Jiffy Products of America, Batavia, IL, USA) contained in peat strips. Each strip contained 12 plants. The potting mix was supplemented with a controlled-release (14:14:14, N:P:K) fertilizer (Osmocote; Grace Sierra Horticultural Products, Milpitas, CA, USA). The plants were placed in subirrigated trays that were mounted on greenhouse benches. Greenhouse temperatures ranged from 25 to 30°C with 40–90% relative humidity (RH). The photoperiod was approximately 14 h L:10 h D, with 1800 photosynthetically active radiation (PAR) as measured at midday with a light meter.

The treatments utilized were as follows: (1) Collego in water suspension; (2) a 1:1 (v/v) aqueous Collego suspension/invert emulsion; (3) an invert emulsion control; and (4) a water control. The composition of the invert emulsion was identical to that used previously to investigate control of hemp sesbania with the bioherbicidal fungus *Colletotrichum truncatum* (Schw.) Andrus & Moore (Boyette et al. 1993). The oil phase of the invert emulsion consisted of a paraffinic oil (Orchex 797; Exxon Corp., Baytown, TX, USA) (777.5 g L^{-1}), a monoglyceride emulsifier (Myverol 18–99; Eastman Chem. Prod., Inc., Kingsport, TN, USA) (14.5 g L^{-1}), paraffin wax (Strohmeyer & Arpe Co., Inc., Short Hills, NJ, USA) (74.25 g L^{-1}), and lanolin (93g L^{-1}). A stable invert emulsion was formed when equal parts of the oil phase and water phase were combined and stirred briskly by hand for 2–3 min. Inoculum densities for all treatments containing the fungal component were adjusted to 2.0×10^6 spores mL^{-1} based on pre-determined assays of viable CGA spores in the formulated product. Spray application rates were approximately 100 L ha^{-1}, and were made with a pressurized backpack sprayer (Spray doc, Model 101P; Gilmour Mfg., Somerset, PA, USA). Following treatments, seedlings were placed in darkened dew chambers (Model I-36 DL; Percival Sci. Ind., Perry, IA, USA) at 28°C, 100 RH for 12 h, and then placed on greenhouse benches. Plants were monitored at 3-day intervals for disease kinetic studies over a 21-day period after treatment. A subjective visual disease severity rating scale (per plant basis) (Sandrin et al. 2003) was used to

estimate disease progression where 0 = no disease, 1 = 1–25% disease, 2 = 26–50% disease, 3 = 51–75% disease, 4 = 76–99% disease, and 5 = complete plant death. Percent control, plant height, and biomass reductions were determined after 21 days. Surviving plants were excised at the soil line, oven-dried for 48 h at 85°C, weighed, and the percent biomass reduction was determined. Treatments were replicated four times, for a total of 48 individual plants per treatment. The experiment was repeated over time, and data were averaged following Bartlett's test for homogeneity of variance (Steel, Torrey, and Dickeys 1997). A randomized complete block experimental design was utilized. The mean percentage of plant mortalities, plant height reductions, and biomass reductions were calculated for each treatment, and were subjected to arcsin transformation. The transformed data were statistically compared using analysis of variance (ANOVA) at the 5% probability level. Values are presented as the means of replicated experiments. When significant differences were detected by the *F*-test, means were separated with Fisher's protected LSD test at the 0.05 level of probability. In the disease kinetic studies, data were analyzed using standard mean errors and best-fit regression analysis.

2.3 *Field experiments*

Field experiments were conducted in flooded rice field test plots at the Rice Research and Extension Center, Stuttgart, AR, USA, in 1996–1997, and at the Southern Weed Science Research Unit Experimental Farm, Stoneville, MS, USA, in 1998–1999. In the experiments conducted in Stuttgart, rice (Cypress cv.) was planted in mid-April of each year that the experiments were conducted. In mid-May of each year, hemp sesbania seedlings (2–4 leaf stage of growth, ca. 7 cm) that had been grown in a greenhouse were transplanted into flooded rice test plots (3 × 9 m) at a rate of 20 seedlings plot^{-1}. When the hemp sesbania seedlings acclimated to the flooded field conditions and were ca. 15–20 cm in height, treatments were applied. Treatments consisted of: (1) Collego in water suspension; (2) a 1:1 (v/v) aqueous Collego suspension/invert emulsion; (3) an invert emulsion control; and (4) a water control. Hemp sesbania control was determined 21 days after treatment, and rice yields were recorded at seasons end in September. In the experiments conducted at Stoneville, Cypress cv. rice was planted in mid-April, with hemp sesbania seeded simultaneously at a rate of ca. 20 seeds per meter of row. Plot sizes were as described previously. Following flooding and when hemp sesbania plants were ca. 15–20 cm in height, treatments were applied. Twenty hemp sesbania plants were randomly selected in each plot and loosely tagged with plastic tape, and disease monitoring and weed control percentages were based upon these tagged plants. Disease progression was monitored at 3-day intervals for 21 days. The extent of disease progression was based on a modified Horsfall and Barratt (1945) rating scale, assigning symptom expression from 0 to 1.0, with 0 being unaffected, and 0.2, 0.4, 0.6, 0.8 = 20, 40, 60, and 80% leaf and stem lesion coverage/injury, respectively, and 1.0 = plant mortality. Symptomatology was considered 'severe' at ratings of 0.8–1.0. In both locations (Stuttgart and Stoneville), randomized complete block experimental designs were utilized. Data over the 2 years were averaged, followed by subjection to Bartlett's test for homogeneity of variance (Steel et al. 1997). The data were analyzed using ANOVA. The percentage data of the hemp sesbania injury/control

and of the biomass reductions were subjected to arcsin transformation prior to analysis. The treatment means and standard errors of the mean are presented.

3. Results and discussion

In greenhouse experiments, hemp sesbania seedlings were controlled 100% 21 days after treatment (DAT) when spores were formulated in the invert emulsion (Table 1). Plant biomass (dry weights) and plant heights were also greatly reduced by the CGA/invert emulsion treatments, with only slight reductions occurring with the invert emulsion alone treatment (Table 1). No mortality, biomass, or plant height reduction occurred on hemp sesbania seedlings that were inoculated with the fungus in water (Table 1). In the disease kinetic studies, a polynomial regression curve provided the best fit, with an R^2 value of 0.98. Hemp sesbania seedlings treated with CGA/invert emulsion were severely injured 9 DAT (≥ 3.5 disease rating), and the disease continued to progress until complete mortality (5.0 disease rating) occurred in all plants inoculated with the CGA/invert emulsion treatment 21 DAT (Figure 1).

In the field experiments conducted in Stuttgart, AR, hemp sesbania was controlled 85% compared to 0% control for Collego/H_2O, and H_2O alone treatments (Table 2). The invert alone resulted in 30% control of hemp sesbania, which was unusually high based on previous observations of the invert alone effects on sicklepod under field conditions where only minor injury was noted (Boyette, Hoagland, and Weaver 2007b). We have observed that the invert can cause plant growth effects and/or injury depending on the species, temperature, amount applied, etc., but generally these effects range from 0 to ~ 15–20%, and the plants usually recover (Boyette et al. 1993). It is also possible that the invert emulsion could alter the populations of naturally occurring microbes (pathogens) found in nature on plant surfaces. However, generally these organisms would occur only in very low concentrations, so that even if there was a promotive effect, it would not translate to a meaning degree of injury/phytotoxicity. Alternatively, the invert emulsion does not promote bioherbicidal activity of some microorganisms.

In the field experiments conducted at Stoneville, similar results with all treatments occurred, with an average control of 90% of hemp sesbania (Figure 2). In all experiments at both locations, we speculate that some drift of CGA spores occurred, and caused injury to hemp sesbania plants treated with invert only. Other

Table 1. Formulation effects on biocontrol of hemp sesbania with *Colletotrichum gloeosporioides* f. sp. *Aeschynomene*.

Treatment[a]	Mortality (%)[b]	Plant Height Reduction (%)	Dry Weight Reduction (%)
GGA/INV	100a	100a	100a
CGA/H_2O	0b	0c	0c
INV	0b	5bc	8b
H_2O	0b	0c	0c

[a]Data followed by the same letter within columns are not significantly different at p = 0.05 according to Fisher's least significant difference.
[b]Hemp sesbania plants averaged 10 cm in height at time of treatment; plant mortality, plant height reduction, and dry weight reduction were measured at 21 days after treatment.
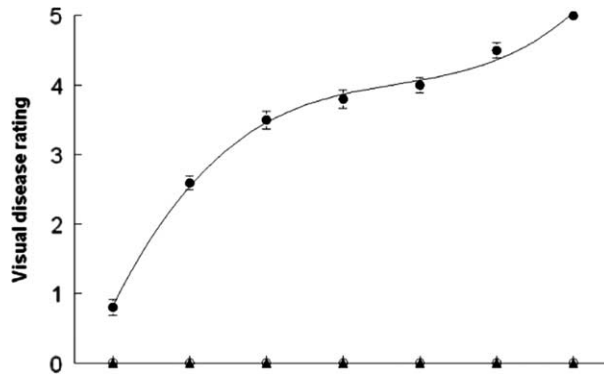
Figure 1. Disease progression of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* infecting hemp sesbania in the greenhouse. A subjective visual disease severity rating scale (Sandrin et al. 2003) was used to estimate disease progression where: $0 =$ no disease, $1 = 1$–25% disease, $2 = 26$–50% disease, $3 = 51$–75% disease, $4 = 76$–99% disease, and $5 =$ complete plant death. Symptomatology was considered 'severe' at ratings of 3.5–5.0. Inoculum densities for all treatments containing the fungal component were adjusted to 2.0×10^6 spores mL^{-1} using a hemacytometer. The relationship for Collego/invert [solid spheres (●)] is best described by the equation $Y = -1.99 + 3.47 \ X - 0.700 \ X^2 + 0.050 \ X^3$, $R^2 = 0.98$. Open spheres (○), solid triangles (▲) and inverted open triangles represent invert only, water control and Collego/water, respectively. Error bars represent ± 1 SEM.

research has shown that drift and spread of CGA spores to non-targeted northern jointvetch plants (Yang and TeBeest 1993).

In the disease kinetic studies conducted under field conditions at Stoneville, Collego/H_2O and H_2O treatments exhibited little or no symptomatology on hemp sesbania when monitored over a 21-day period (Figure 3). Disease progressed rapidly on hemp sesbania treated with Collego/invert, and was rated as 'severe' as early as 6 days after application. Some 'moderate' injury occurred with the invert only treatment, possibly as result of drift from the fungus treated plots (Figure 3).

The Clearfield system has become the predominant rice production system in the mid-south rice producing states of Arkansas, Louisiana, Mississippi, and Missouri (Shivrain, Burgos, Moldenhauer, McNew, and Baldwin 2006). The rice varieties utilized in this system are not genetically modified organisms (GMOs), but are natural mutants with tolerance to imazethapyr [2-(4,5-dihydro-4-methyl-4-(1-methy-lethyl)-5-oxo-1H-imidazol-2-yl)-5-ethyl-3-pyridine-carboxylic acid] (Newpath™). This

Table 2. Biological control of hemp sesbania in rice, Stuttgart, AR.

Treatment	Hemp sesbania Control (%)	Rice Yield (Kg ha^{-1})
H_2O Control	0c	1976c
Collego/H_2O	0c	1988c
Invert control	30b	2060b
Collego/Invert	85a	2222a

[a]Data followed by the same letter within columns are not significantly different at p = 0.05 according to Fisher's least significant difference.
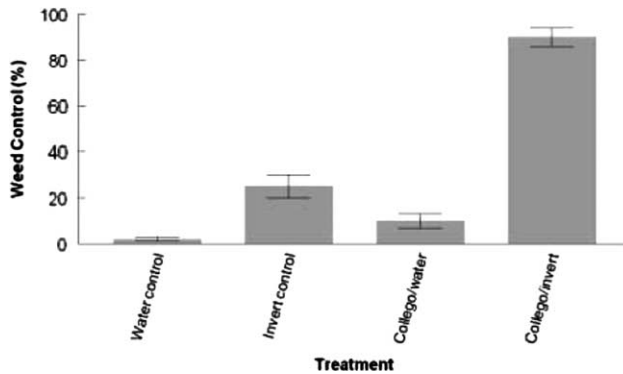[b]Hemp sesbania plants averaged 10 cm in height at time of treatment.

Figure 2. Biological control of hemp sesbania using *Colletotrichum gloeosporioides* f. sp. *aeschynomene* under field conditions at Stoneville, MS.

herbicide controls many broadleaf and grassy weeds, including red rice, but has no activity on hemp sesbania or northern jointvetch, which can result in tremendous infestations of these weeds if other weed control measures are not utilized (Scott, Meins, and Smith 2005). This complete lack of control of hemp sesbania and northern jointvetch creates a significant need for an effective weed control agent, such as CGA, for these troublesome weeds.

Although the registration of this effective bioherbicide (Collego™) was allowed to expire, it was re-registered with US-EPA in 1997, and more recently, this fungus was newly registered under the trade-name Lockdown™ and Lockdown Retro™ for the control of northern jointvetch (Cartwright et al. 2008; Cartwright, Boyette, and
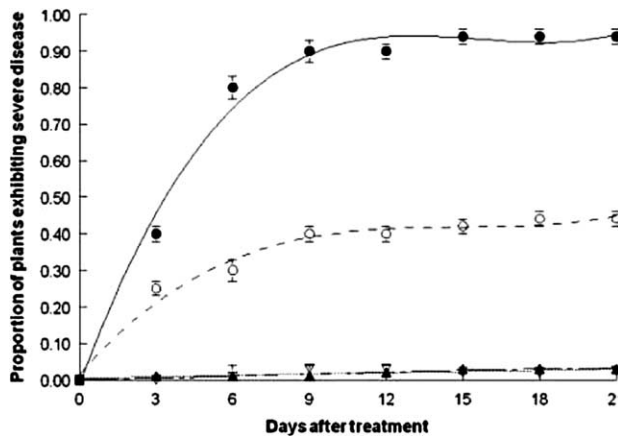

Figure 3. Disease progression of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* infecting hemp sesbania under field conditions at Stoneville, MS. Diseased plant ratings were based on a modified Horsfall–Barratt rating scale as described in section 2. Symptomatology was considered 'severe' at ratings of 0.8–1.0. The relationship for Collego/invert is best described by the equation $Y = -0.02 + 0.1947\,X - 0.01\,X^2 + 0.050\,X^3$, $R^2 = 0.99$, represented by solid spheres (●); for invert emulsion alone by $Y = 0.01 + 0.08\,X - 0.01\,X^2$, $R^2 = 0.98$, represented by open spheres (○). Error bars represent ± 1 SEM. Solid triangles (▲) represent the water control and open inverted triangles (▽) are Collego/water.

Roberts 2010). Because Collego provides excellent control of northern jointvetch (Sandrin et al. 2003; Yang and TeBeest 1993), the results from our research findings in this report indicate that it is also possible to control hemp sesbania with this fungus. Since this formulation would allow control of both of these serious weeds this should make this product more economically acceptable to rice producers.

Similar phenomenology has been reported with other bioherbicidal fungi formulated in invert emulsions. For example, Amsellem et al. (1991) reported that the host specificities of *A. cassiae* Juriar. & Khan (a pathogen of sicklepod) and *A. crassa* Sacc. Rands (a pathogen of jimsonweed (*Datura stramonium* L.) were expanded, and that saprophytic fungi (*Aspergillus nidulans* G. Winter and *Tricoderma harzianum* Rifai) became pathogenic when formulated in an invert emulsion (Amsellem et al. 1991). More recently, greenhouse studies revealed that freshly produced CGA spores caused lesions and infection structures (acervuli) to occur on hemp sesbania stems, similar to those that occur on northern jointvetch infected by this fungus (Boyette, Bowling, Vaughn, Hoagland, and Stetina 2010). Further research will focus on applications of the current Lockdown products to commercial rice fields.

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