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SHORT COMMUNICATION

Bioherbicidal enhancement and host range expansion of a mycoherbicidal fungus via formulation approaches

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ABSTRACT

Eastern black nightshade (*Solanum ptycanthum*; EBN) is a problematic weed partly due to its tolerance or resistance to certain herbicides. We examined the effects of an invert emulsion (IE) on the host range and weed control efficacy of the fungus *Colletotrichum coccodes* (NRRL strain 15547) for biocontrol of EBN. Greenhouse tests demonstrated that several other solanaceous weeds were also infected and killed, and field tests revealed >90% EBN control and dry weight reduction in plants treated with the fungus-IE formulation. These results demonstrate that this IE formulation can promote the efficacy of this bioherbicidal pathogen.

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Eastern black nightshade (*Solanum ptycanthum* Dun.) (EBN), native to the Americas and commonly found throughout the U.S. east of the Rocky Mountains, is an erect, branching annual or short-lived perennial herb (Bryson & DeFelice, 2010). One plant may produce up to 1000 berries containing 50 to 100 seeds each and seeds can germinate soon after anthesis and can retain viability in soil for many years (Bassett & Munro, 1985; Ogg, Rogers, & Schilling, 1981; Werner, Curran, & Lingenfelter, 1998; Zhou, Deckard, & Messersmith, 2005). EBN can lower yields up to 40% at a density of 5 plants m^{-2} in soybean (*Glycine max* [L.] Merr.) and 7% at 5 plants m^{-2} in corn (*Zea mays* L.) (Anonymous, 2012). The weed and its fruit not only reduce crop yields, but also interfere with harvest quality and lower crop value (McGiffen, Masiunas, & Hesketh, 1992; Quakenbush & Andersen, 1984; Werner et al., 1998).

One option for post-emergence control of EBN (primarily in soybean) is the acetolactate synthase (ALS) inhibiting class of herbicides that block the enzyme ALS (LaRossa & Schloss, 1984). Frequent use of imidazolinone herbicides has led to resistant populations of EBN in several areas of the U.S. (Heap, 2017; Milliman, Riechers, Wax, & Simmons, 2003; Volenberg, Stoltenberg, & Boerboom, 2000). Furthermore, reliance on glyphosate-tolerant and sulfonylurea-tolerant (STS) crops has resulted in heavy use of the herbicides chlorimuron and thifensulfuron, and inadequate control has been reported using these herbicides and other compounds of the sulfonylurea family of ALS-inhibiting herbicides. The efficacy of ALS-inhibiting herbicides can vary among different nightshade species,

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due to differential absorption, translocation, and metabolic detoxification in these plants (Ackley, Hatzios, & Wilson, 1999). In a molecular-based study of 12 herbicide-resistant (imazethapyr) populations of EBN from Ontario, Canada, the molecular basis of resistance was found to be target-site modification (Ashigh & Tardif, 2007).

Because control of EBN is difficult due to its tolerance or resistance to several commonly used herbicides, its prolific growth habit, and continuous emergence throughout the growing season, alternative controls are needed to replace/supplement existing methods. The technical and biological feasibility of mycoherbicides for controlling various weeds have been established, as summarised in various reviews (Duke, Scheffler, Boyette, & Dayan, 2015; Hoagland & Boyette, 2016; Weaver, Lyn, Boyette, & Hoagland, 2007), and this method warrants consideration for controlling EBN.

Previous reports in our laboratory showed that artificially induced dew period durations of 4, 8, or 12 h provided 10%, 25%, and 40% control, respectively, of EBN plants in the 2–5 leaf stage when *Colletotrichum coccodes* (Wallr.) S. Hughes spores were applied in water + Tween-80 surfactant (T-80) (Sigma Chemical Co., St. Louis, MO), 12 days after inoculation (Boyette, Hoagland, & Stetina, 2016). However, a minimum of 16 h of dew was required to achieve ~ 95% plant mortality. In contrast, at these same intervals of dew, 95%, 100%, and 100% mortality occurred, respectively, when spores were formulated in the IE. Even in the absence of dew, 60% mortality and 70% plant dry weight reductions were achieved with the fungus/IE formulation. Delaying dew by 2 h after inoculation did not significantly reduce weed control or plant dry weight when plants were inoculated with the fungus in T-80 or in the IE formulation. However, when dew was delayed for 4, 8, or 12 h, only 60%, 50%, and 25% mortality occurred, respectively, of plants receiving the T-80 spore formulation. In contrast, 95%, 90%, and 90% mortality occurred after the same dew delays of plants receiving the fungus-IE formulation (Boyette et al., 2016).

C. coccodes NRRL strain 15547 was highly virulent against EBN, but much less so against several other solanaceous weed species when fungal spores were formulated in the T-80 surfactant (Andersen and Walker (1985). These authors also found that infection and weed control under field conditions were unsatisfactory, likely due to inadequate dewfall. Although a narrow host range specificity of bioherbicidal plant pathogens may be beneficial from the biological and United States Environmental Protection Agency (US-EPA) registration perspectives, this trait can eliminate certain bioherbicidal agents from practical and commercial considerations (Hoagland, 2001). Research has shown that the host ranges of some fungal pathogens can be modified through formulation-based approaches using an invert emulsion (IE) formulation (Amsellem, Sharon, & Gressel, 1991; Bowling, Vaughn, Hoagland, Stetina, & Boyette, 2010; Boyette, Bowling, Vaughn, Hoagland, & Stetina, 2010; Boyette, Gealy, Hoagland, Vaughn, & Bowling, 2011; Boyette, Hoagland, & Weaver, 2007). Our objectives were to determine if the host range of *C. coccodes* could be expanded through a formulation-based approach using an IE formulation (Boyette, Quimby, Bryson, Egley, & Fulgham, 1993; Quimby, Fulgham, Boyette, & Connick, 1989), and to evaluate efficacy on EBN using this IE formulation of *C. coccodes* under field conditions.

Cultures of *C. coccodes* (NRRL 15547) were grown on half-strength Emerson's yeast-starch agar (Andersen & Walker, 1985) in petri dishes inverted on open-mesh wire shelves in an incubator (Precision Scientific Inc., Chicago, IL) at 25°C. Photoperiods

(12 h) were provided by 20-W cool white fluorescent lamps positioned 12 cm above each shelf to provide 200 μE m^{-2} s^{-1}. Spores from 10- to 12-day-old cultures were harvested by rinsing spore lawns with distilled water and filtering debris through 4 layers of cheesecloth. Freshly harvested spores were used as the inoculum for all experiments. Spore concentrations were estimated using haemacytometers. Long-term storage of the fungus was achieved in screw-capped tubes containing sterilised soil, maintained at 4°C (Bakerspigel, 1953).

To evaluate the virulence and plant growth effects (mortality, dry weight reductions, and relative virulence of *C. coccodes* to closely related solanaceous plants on plants closely related to EBN under greenhouse conditions, seeds of various Solanaceae species were obtained from C.T. Bryson (USDA-ARS, Stoneville, MS), all seeds were surface-sterilised in 0.05% NaOCl for 5 min, rinsed with sterile distilled water, and germinated in darkness on moistened filter paper in petri dishes in a growth chamber (25°C). After the seeds germinated (~48 h), they were planted in a commercial potting mix (Jiffy-mix; Jiffy Products of America, Batavia, IL) contained in peat strips. Each strip contained 12 plants. The potting mix was supplemented with a controlled-release (14:14:14, NPK) fertiliser (Osmocote; Grace-Sierra Horticultural Products, Milpitas, CA). The plants were placed in sub-irrigated trays on greenhouse benches at 25–30°C, and 40–90% relative humidity (RH). The photoperiod was approximately 14 h, with 1800 μE m^{-2} s^{-1} (PAR) as measured with a light meter (LI-COR, Inc., Lincoln, NE) at midday. Seedlings at the 3–4 leaf stage were used in all experiments.

Seedlings were inoculated with *C. coccodes* spores formulated in either T-80 surfactant or in an IE. The composition of the IE was as described previously (Boyette et al., 1993, 2011). Inoculum densities of the spores were adjusted to 1.0×10^6 spores ml^{-1}. Spray application rates were ~200 L ha^{-1}, made with a backpack sprayer (Spray Doc, Model 101P; Gilmour Mfg., Somerset, PA). Treatments were: (1) untreated (UNT); (2) water control (H_2O); (3) T-80 surfactant (T-80); (4) IE; (5) *C. coccodes* spores in water (CCOC/H_2O); (6) *C. coccodes* spores in T-80 surfactant (CCOC/T-80); and (7) *C. coccodes* spores in IE (CCOC/IE). Following treatments, seedlings were placed in darkened dew chambers (Model I-36 DL; Percival Sci. Ind., Perry, IA) at 28°C, 100 RH for 12 h, and then placed on greenhouse benches under conditions described above. Percent control, biomass reductions, and disease ratings were determined after 15 days. A visual disease severity rating scale (per plant basis) (Sandrin, TeBeest, & Weidemann, 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 = plant death. Disease ratings ≤2.0 were considered 'slight', 2.1–3.9 were considered 'moderate', and ≥4.0 were considered 'severe'. Percent control [based on the number of severely infected (disease ratings of 4–4.9) and dead seedlings], and biomass reductions were determined after 15 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. In all greenhouse experiments, treatments were replicated 4 times, for a total of 48 individual plants per treatment. The experiments were repeated over time, and data were averaged following Bartlett's test for homogeneity of variance (Steel, Torrey, & Dickeys, 1997). A randomised complete block experimental design was utilised. The mean percentage of weed control, and biomass reductions were calculated for each treatment, and were subjected to Arcsin transformation. The transformed data were compared using ANOVA ($P = .05$). 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 ($P = .05$).

Field experiments were conducted at USDA-ARS, Stoneville, MS, in test plots [1.0×1.0 m micro-plots (1.0×10^{-5} ha)] seeded with pre-germinated EBN (75 seedlings per plot) in the cotyledonary to first leaf growth stages. Within each plot, 20 plants were randomly selected and tagged, using 7.6 cm plastic pot markers. Applications were made at dusk to all plants in each plot 10 d after transplanting when EBN seedlings were at the 2–4 leaf growth stage. Treatments were: (1) untreated (UNT); (2) water control (H_2O); (3) T-80; (4) IE; (5) *C. coccodes* spores in water (CCOC/H_2O); (6) *C. coccodes* spores in T-80 (CCOC/T-80); and (7) *C. coccodes* spores in IE (CCOC/IE). Spores (1.5×10^6 spores ml^{-1}) were applied with a pressurised backpack sprayer as described above. In each plot, the 20 tagged plants were monitored for disease development at 3-day intervals over a 15-day period, and at the termination of the experiments (15 days after inoculation), percentages of weed control and dry weight determinations were ascertained as described above in the greenhouse experiments. Daily observations were also performed in the morning to observe the presence of dew. No attempts were made to measure the length of dew periods or the quantity of dew formed on plants. Dew that formed on inoculated plants were recorded as either 'light', 'moderate', or 'heavy' by observations. In these field tests, a randomised complete block design with four replications was utilised and the experiment was repeated in successive years. The percent weed control, biomass reduction and disease kinetic data were subjected to Arcsin transformation. The transformed data were statistically compared using analysis of variance (ANOVA) at the 5% probability level. Results were back-transformed to the original measurements (percentages) for presentation. 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 ($P = .05$). In all experiments, data were analysed via the PROC MIXED function of SAS v9.3 (SAS Institute, Cary, NC) using a least significant difference of 0.05.

In greenhouse tests of 9 solanaceous weed species, EBN was the most susceptible when *C. coccodes* was formulated in the IE or in T-80 (95% and 80% control, respectively) 15 days after treatment (Table 1). The least susceptible weed was jimsonweed (*Datura stramonium*), i.e. 65% and 5% control in the IE and T-80 formulations, respectively. Other solanaceous weeds [hairy nightshade (*Solanum sarrachoides*), American nightshade

Table 1. Effect of *C. coccodes* in various formulations on mortality of several solanaceous weeds in the 3–4 leaf stage under greenhouse conditions.

	Treatment						
	UNT	H_2O	T-80	IE	CCOC-H_2O	CCOC-T-80	CCOC-IE
Species	Weed control (%)[a]						
EBN (*Solanum ptycanthum* Dun.)	0 j	0 j	0 j	5 hi	30 f	80 bc	95 a
Hairy nightshade (*S. sarrachoides* Sendtner)	0 j	0 j	0 j	5 hi	15 gh	20 gh	80 bc
American nightshade (*S. americanum* L.)	0 j	0 j	0 j	7 hi	10 h	17 gh	85 b
Black nightshade (*S. nigrum* L.)	0 j	0 j	0 j	4 hi	10 h	15 gh	78 c
Cutleaf nightshade (*S. triflorum* Nutt.)	0 j	0 j	0 j	5 hi	10 h	15 gh	75 cd
Sticky nightshade (*S. sisymbriifolium* Lam.)	0 j	0 j	0 j	7 hi	5 hi	10 h	70 d
Wetland nightshade (*S. tampicense* Dun.)	0 j	0 j	0 j	5 hi	5 hi	8 hi	75 cd
Tropical soda apple (*S. viarum* Dun.)	0 j	0 j	0 j	6 hi	5 j	5 hi	75 cd
Jimsonweed (*D. stramonium* L.)	0 j	0 j	0 j	5 hi	0 j	5 hi	65 e

[a]Values followed by the same letter do not differ significantly at $P = .05$.

(*Solanum americanum*), black nightshade (*Solanum nigrum*), cutleaf nightshade (*Solanum triflorum*), sticky nightshade (*Solanumsisymbriifolium*), wetland nightshade (*Solanum tampicense*), tropical soda apple (*Solanum viarum*), and jimsonweed] were controlled at a significantly greater level (65–85% control) by the fungus in the IE, when compared to the fungus-T-80 formulation (5–20% control). Similar trends were observed when dry weight reduction measurements were analysed (Table 2). Disease levels caused by the fungus in these weeds generally followed the trends observed for the weed control tests, with some exceptions. For example, jimsonweed exhibited the lowest susceptibility (65% control), with a 3.0 disease rating, while sticky nightshade exhibited the lowest disease rating (2.4) but had higher susceptibility (70% control) (Table 1; Figure 1).

In field experiments, EBN control achieved in the fungus-IE formulation was significantly greater (95%) than control provided by the fungus in water (15%) or the T-80-fungus-formulation (25%), 15 days after inoculation (Table 3). Similar trends caused by these two formulations were reflected in dry weight reduction analyses (Table 3). The disease progression of this fungus on EBN under field conditions was slower than as reported in greenhouse experiments (Boyette et al., 2016). In those results, 3 days after inoculation, plants receiving the T-80 fungal spore treatment were only slightly infected (disease rating 2.0), while plants treated with the fungal-IE treatment were severely infected (disease rating 4.5). After 6 days, the T-80 fungal treatment caused only moderate infectivity (disease rating 2.9), while 100% mortality occurred in plants treated with the fungus-IE formulation. A period of 9 days after inoculation was required before severe disease (disease rating 4.8) levels were achieved on plants treated with the T-80 fungal formulation under greenhouse conditions (Boyette et al., 2016).

In the present studies under field conditions, a maximum disease rating of 0.5 (slight infection) was recorded on plants receiving the T-80 fungal treatment at 12–15 days after inoculation, while severe infection (disease rating of 4.8) was caused by the fungus-IE formulation after this same time period (Figure 2). It is important to point out that the IE alone caused slight mortality (5–7%) and dry weight reductions (6–8%) of the weeds that were examined (Tables 1 and 2; Figure 1). Observations from our laboratory and others have also reported this phenomenon in various other pathogen-

Table 2. Effect of *C. coccodes* in various formulations on dry weight reduction of several weeds at the 3–4 leaf stage in the greenhouse.

	Treatment						
	UNT	H_2O	T-80	IE	CCOC-H_2O	CCOC-T-80	CCOC-IE
Species	Dry weight reduction (%)[a]						
EBN (*Solanum ptycanthum* Dun.)	0 j	0 j	0 j	8 hi	35 f	84 bc	98 a
Hairy nightshade (*S. sarrachoides* Sendtner)	0 j	0 j	0 j	7 hi	15 gh	20 gh	85 bc
American nightshade (*S. americanum* L.)	0 j	0 j	0 j	8 hi	13 h	20 gh	90 b
Black nightshade (*S. nigrum* L.)	0 j	0 j	0 j	6 hi	13 h	17 gh	85 bc
Cutleaf nightshade (*S. triflorum* Nutt.)	0 j	0 j	0 j	8 hi	12 h	18 gh	80 cd
Sticky nightshade (*S. sisymbriifolium* Lam.)	0 j	0 j	0 j	7 hi	10 hi	12 h	80 cd
Wetland nightshade (*S. tampicense* Dun.)	0 j	0 j	0 j	8 hi	8 hi	10 hi	80 cd
Tropical soda apple (*S. viarum* Dun.)	0 j	0 j	0 j	6 hi	5 j	8 hi	78 cd
Jimsonweed (*D. stramonium* L.)	0 j	0 j	0 j	7 hi	5 j	7 hi	75 e

[a]Values followed by the same letter do not differ significantly at $P = .05$.

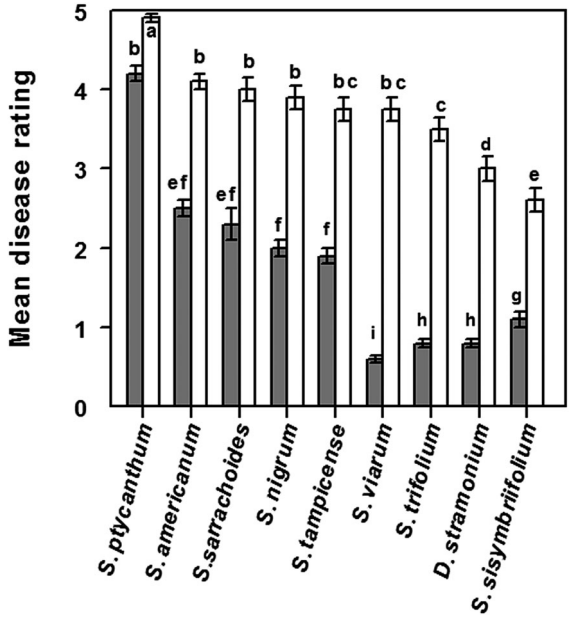

Figure 1. Effect of T-80 surfactant (white histogram bars) and IE (grey histogram bars) of *C. coccodes* spore formulations (1.0×10^6 spores ml^{-1}) on infection of various solanaceous plants (3–4 leaf stage), 15 days after inoculation under greenhouse conditions. Disease rating data are presented in order of descending of these plants susceptibility based on fungus-IE treatment. Disease rating scale: 0 = healthy to 5 = dead. Error bars = ±1 SD. Histogram bars with the same letter are not different at $P = 0.05$.

weed interactions, e.g.: *Colletotrichumtruncatum* (Schw.) Andrus & Moore and hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. Ex A.W. Hill] (Boyette et al., 1993); *Colletotrichumgloeosporioides* (Penz.) Penz. & Sacc. and sicklepod [*Senna obtusifolia* (L.) H.S. Irwin & Barneby] (Boyette et al., 2007); *Myrothecium verrucaria* (Alb. and Schwein.) Ditmar: Fr. and morning glories (*Ipomoea* spp.) (Hoagland, McCallister, Boyette, Weaver, & Beecham, 2011; *Phomopsis amaranthicola* Rosskopf, Charudattan, Shabana, & Benny and smooth pigweed (*Amaranthus hybridus* L.) (Rosskopf & Yandoc, 2005); and *Ascochyta pteridis* Bres. and bracken fern [*Pteridium aqualinum* (L.) Kuhn] (Womack & Burge, 1993). In addition to 'trapping' water by the invert formulation (thus providing

Table 3. Effects of *C. coccodes* (1.0×10^6 spores ml^{-1}) in various formulations on mortality and dry weight reduction of *S. ptycanthum* (cotyledonary to first leaf growth stages) conducted under field conditions in Stoneville, MS, U.S.A.

Treatment	Weed control (%)[a]	Dry weight reduction (%)
UNT	0 e	0 e
H_2O	0 e	0 e
T-80	0 e	0 e
IE	8 d	10 d
CCOC-H_2O	15 c	20 c
CCOC-T-80	25 b	30 b
CCOC-IE	95 a	98 a

[a]Values followed by the same letter do not differ significantly at $P = .05$.

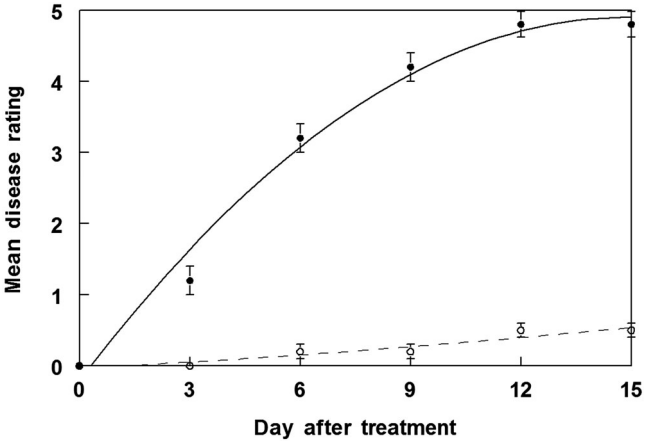

Figure 2. Disease progression of *C. coccodes* (1.0×10^6 spores ml^{-1}) formulated in T-80 (open circles, dashed line) or IE (closed circles, solid line) infecting eastern black nightshade (cotyledonary – first leaf stage) under field conditions at Stoneville, MS, U.S.A. The relationships for disease progressions are best described by the equations: T-80 + *C. coccodes*; $Y = -0.03 + 0.03X$, $R^2 = 0.95$; IE + *C. coccodes*; $Y = -0.21 + 0.69X - 0.02X^2$, $R^2 = 0.98$. Error bars represent ± 1 SD.

a favourable environment for the infection process), the IE may also cause plant cuticle and tissue damage, thereby facilitating entry of the pathogen.

Although light to moderate dew occurred on several occasions during the testing periods (dew not measured; observational data, not shown), only slight infection and control of EBN occurred with the fungus-water or the fungus/T-80 formulations (Table 3). Prolonged free-moisture requirements and narrow host ranges are major factors limiting the practicality of many bioherbicidal fungi (Weaver et al., 2007). Previously, we reported that the *C. coccodes* – IE formulation could significantly reduce the dew duration requirement and increase the dew onset time following inoculation in greenhouse studies (Boyette et al., 2016). In the findings reported herein, the *C. coccodes* – IE formulation effectively controlled EBN under field conditions, and extended the host range of this bioherbicidal fungus to several other solanaceous weed species. This latter point is particularly important since some of these weeds (wetland nightshade, sticky nightshade, and tropical soda apple) are considered to be exotic, invasive weeds in several U.S. states (Bryson, Reddy, & Byrd, 2012). Early reports demonstrated that several economically important Solanaceae crop species [e.g. eggplant (*Solanum melongena* L.), potato (*Solanum. tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), and pepper (*Capsicum annuum* L.)] were unaffected by this strain of *C. coccodes* formulated in T-80 surfactant (Andersen & Walker, 1985). Because all of the solanaceous weeds examined exhibited various degrees of susceptibility to this fungus formulated in the IE, future work should include evaluations of the fungus-formulation effects on Solanaceae crops.

The ability to mitigate environmental factors, such as requirements for lengthy dew periods that diminish a bioherbicidal microorganism's efficacy, and restrictively narrow host ranges that limit their practicality, may significantly improve their bioherbicidal potential. These results further demonstrate that formulating *C. coccodes* spores in an

IE greatly improves its bioherbicidal potential for controlling Eastern black nightshade, which is becoming an increasingly important issue, due in large part to the development of herbicide resistance. Moreover, the use of this IE formulation that lowers the amount or duration of free-moisture required, improves efficacy, and alters or broadens the host range of *C. coccodes* may also improve the effectiveness of certain other fungi that have previously been considered impractical for use as bioherbicides due to low efficacy and/or fastidiousness.

Disclosure statement

No potential conflict of interest was reported by the authors.

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