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Adjuvant and Refined Corn Oil Formulation Effects on Conidial Germination, Appressorial Formation and Virulence of the Bioherbicide, *Colletotrichum truncatum*

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Abstract: Several surfactants, plant extracts and fatty acids were tested for stimulation of conidial germination, appressorial formation and virulence of *Colletotrichum truncatum*, a bioherbicide of the weed, hemp sesbania (*Sesbania exaltata*). The commercial surfactants (Tweens® 40, 60, 80, 85 and Myvatex® 60) at concentrations of 0.25 to 1.0% (v/v), stimulated germination, but these effects were not exclusively related to their hydrophylic-lipophylic balance values. The stimulatory surfactants were also tested for germination and virulence of *C. truncatum* when formulated with the conidia and applied to hemp sesbania seedlings. Conidia formulated in either water, a surfactant or, an emulsion of refined corn oil were ineffective on the plant in the absence of dew or when dew was delayed. However, formulations of conidia combined with surfactant in emulsified refined corn oil did exhibit bioherbicidal activity when adequate dew or free-moisture was unavailable. This is important since previous reports indicated that refined corn oil did not enhance bioherbicidal activity, whereas unrefined corn oil promoted pathogen germination and efficacy of *C. truncatum*. The fatty acids tested had little or no effect on conidial germination but, aqueous extracts of several plant species including pathogen hosts and non-hosts, stimulated germination. Appressorial formation influenced by the surfactants did not necessarily reflect the disease rating on hemp sesbania seedlings. Overall, results show that formulations containing an emulsion of conidia, a surfactant and refined corn oil have potential for enhancement of the efficacy of *C. truncatum*.

Key words: Surfactants, spore germination, appressorial formation, pathogenicity, bioherbicide, *Colletotrichum truncatum*

INTRODUCTION

Previous reports indicate that unrefined, but not refined corn oil, stimulated germination of *Colletotrichum truncatum* conidia (Egley and Boyette, 1995). Other refined oils were likewise non-stimulatory (Egley and Boyette, 1995). The virulence of *C. truncatum* on hemp sesbania (*Sesbania exaltata*) increased when conidia of the pathogen were applied to the plant in a water-unrefined corn oil emulsion (Boyette, 1994). This result is important because *C. truncatum* has potential for the biological control of hemp sesbania, a serious weedy pest in several crops including soybean, cotton and rice (Boyette, 1991, 1994; Boyette *et al*., 1993, 2007b). Factors that increase the germination and virulence of this bioherbicide may also increase efficacy (Egley and Boyette, 1995; Boyette *et al*., 2007b; Boyette and Hoagland, 2012).

One method to improve pathogen efficacy is the use of selected adjuvants. Adjuvants include surfactants, stickers, sun screen agents, humectants, anti-evaporation agents and micro-nutrients (Prasad, 1993) that may improve bioherbicidal efficacy through various mechanisms. Adjuvant:bioherbicide interaction studies have primarily focused on their influence on spore germination, appressorial formation and mycelial growth (Prasad, 1994; Zhang *et al*., 2003; Bailey *et al*., 2004). Appressoria formation by germinated conidia of *C. truncatum* and some other *Colletotrichum* spp. is crucial to establishment of infection by this pathogen (Emmett and Parbery, 1975). Many studies have reported improved bioherbicidal potential of various bioherbicidal pathogens using propagule formulations applied in oil and water emulsions (Boyette, 2006; Boyette *et al*., 2007a, b; Quimby Jr *et al*., 1988; Sandrin *et al*., 2003; Shabana, 2005). Other studies have improved efficacy through addition of surfactants (Wyss *et al*., 2004; Zhang *et al*., 2003).

Surfactants (surface active agents) act as wetting agents to lower the surface tension of a liquid and allowing for increased spreadability. Non-ionic surfactant molecules contain both hydrophilic and lipophilic groups

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(polar and non-polar groups) and the size and strength of these two groups is termed the hydrophilic-lipophilic balance number (HLB) (Griffin, 1949, 1954). The computation of the HLB value for a non-ionic surfactant is based on the assignment of a low number for a compound of hydrophobic character and a high number for a surfactant with hydrophilic character (Griffin, 1954). Griffin's method has been applied to various non-ionic surfactants and used as a guideline to optimize a non-ionic surfactant in the formulation development of a hydrophobic conidia-based mycoinsecticide (Jin *et al*., 2008). In bioherbicidal studies, several commercial adjuvants and the detergent polyoxyethylene tridecyl ether (TDA) formulations improved the activity of the bioherbicidal fungus *Myrothecium verrucaria* against sicklepod (Weaver *et al*., 2009). TDA formulations with a HLB number of 8 or 10 had the highest activity.

Formulations that promote germination within the formulation matrix, or on host plant surfaces, could greatly improve the bioherbicidal potential of a candidate pathogen. Some research has focused on the use of natural product adjuvants for control of various plant disease organisms (Askarne *et al*., 2012; Bussaman *et al*., 2012; Arzoo *et al*., 2012). Germination and appressorium formation by *C. gloeosporioides* conidia were induced by the surface wax of its host, avocado, but not by surface waxes of other plants (Podila *et al*., 1993). In contrast to this specific signaling effect, only a hydrophobic material was needed to stimulate germination and appressorium formation by *Magnaporthe grisea* (Hegde and Kolattukudy, 1997). In other cases, both hydrophobic and hydrophilic surfaces imparted appressoria formation equally well (Lee and Dean, 1994).

Many plant pathogens including bioherbicides generally require a free-moisture period to achieve propagule germination and infectivity of their target weed host(s) (Weaver *et al*., 2007). Recently, several adjuvants were tested for stimulation of *C. truncatum* conidial germination that could enhance activity of this bioherbicide when applied to hemp sesbania under optimal free- moisture (dew) conditions (Boyette and Hoagland, 2012), but the effects of delayed dew was not examined. Although, previous studies have shown that unrefined corn oil can reduce the requirement of a lengthy dew period for *C. truncatum* infectivity and virulence (Boyette *et al*., 2007b; Egley and Boyette, 1995; Boyette and Hoagland, 2012), the effects of refined corn oil have heretofore not been reported.

Due to the relative expense and lack of availability of unrefined corn oil we attempted to find a suitable alternative that would provide the promotive qualities imparted by unrefined corn oil. Here we report that several adjuvants and surfactants can stimulate conidial germination and enhance virulence of *C. truncatum* when applied to hemp sesbania seedlings in emulsified, refined corn oil under optimal and sub-optimal environmental conditions, e.g., free-moisture and temperature (Boyette, 1991).

MATERIALS AND METHODS

Collection of conidia: *C. truncatum* conidia were harvested from petri dishes containing potato dextrose agar (Difco, Detroit, MI) upon which the fungus was cultured, as previously described (Boyette, 1991). The conidia were strained through two layers of cheese cloth and rinsed with distilled water. The conidial concentration was determined using a hemocytometer and dilutions were made with distilled-deionized water or adjuvants to give the desired concentrations.

Conidial germination and appressorial formation of conidia suspended in glass vials: *C. truncatum* conidia were suspended in sterile deionized water and used in the experiments within 4 h after collection as described previously (Egley *et al*., 1993). Conidia were then transferred via pipettes to 15 mL glass vials and test solutions were added to give a total volume of 8 mL (5.0×10^5 conidia mL^{-1}). Suspension of conidia in the solution was maintained with periodic gentle agitation. The vials were incubated in the light (165 $\mu E\ m^{-2}\ s^{-1}$) at 28°C. After 5 and 24 h incubation, three 25-µL drops from each vial were transferred onto microscope slides. A drop of lactophenol cotton blue was added to each 25-µL drop and the first 200 conidia observed under 400x magnification were scored for germination. The criterion for germination was visible protrusion of a germ tube from the conidium. Each germination test was conducted at least three times and results averaged.

Sources of chemicals: The fatty acids (linoleic, linolenic, oleic, palmitic, stearic) were obtained from ICN Biochemicals, Cleveland, OH. The various Tween®s were obtained from Sigma Chemical, St Louis, MO. Alfonic® ethoxylates were from Vista Chemical Co., Houston, TX and Myvatex®-60, Myvaet®-9-45K and Myverol®-1899 were products of Eastman Chemical Co., Kingsport, TN. Surfactants (Table 1) were tested at concentrations of 0.025, 0.25, 0.5 and 1.0% (v/v). Unrefined and refined corn oil were products of Spectrum Marketing, Inc., Petaluma, CA and ACH Food Companies, Inc., Memphis, TN, respectively.

Table 1: Composition of nonionic surfactants used in these experiments

Trade name	Chemical composition
Tween® 20	Polyoxyethylene sorbitan monolaurate
Tween 40	Polyoxyethylene sorbitan monopalmitate
Tween 60	Polyoxyethylene sorbitan monostearate
Tween 80	Polyoxyethylene sorbitan monooleate
Tween 85	Polyoxyethylene sorbitan trioleate
Alfonic® 810-60 ethoxylate	R = 4 to 8, n = 4.8[a]
Alfonic 1012-60 ethoxylate	R = 8 to 10, n = 5.7 avg
Alfonic 1412-60 ethoxylate	R = 10 to 12, n = 7.0 avg
Alfonic 610-50R ethoxylate	R = 4 to 8, n = 3.0 avg
Alfonic 810-40 ethoxylate	R = 4 to 8, n = 2.2 avg
Alfonic 1012-40 ethoxylate	R = 8 to 10, n = 2.5 avg
Alfonic 1412-40 ethoxylate	R = 10 to 12, n = 3.0 avg
Myvatex® 60	50% Polysorbate 60 (Tween 60®), <50% acetylated monoglycerides, <0.1% propylene, <0.1% propylene glycol, <0.1% propyl gallate, <0.1% citric acid.
Myvacet®-9-45K	>99% distilled, acetylated monoglycerides, <0.1% propylene glycerol, <0.1% propyl gallate, <0.1% citric acid.
Myverol®-18-99	>99% distilled monoglycerides, <0.1% antioxidants (ascorbic acid, citric acid)

Alfonic® Ethoxylates are derived from primary alcohol blends of various molecular weights and the general structural formula: $R(OCH_2CH_2)_nOH$, where R = a long chain alkyl group or mixture of alkyl groups

Aqueous extracts of plants: To determine the effects of plant constituents on conidial germination, appressorial formation and virulence, the extracts of hemp sesbania, sicklepod (*Cassiae obtusifolia*) soybean (*Glycine max*) corn (*Zea mays*), wheat (*Triticum aestirum*) ryegrass (*Secale cereale*), bermudagrass (*Cynodon dactylon*) and johnsongrass (*Sorghum halepense*) were used. The leaves and stems of two to four-week-old plants grown under greenhouse conditions were oven-dried for 48 h (30 to 35°C) and ground to a powder using a mill grinder (Stein Mill, Model M-2; F. Stein Laboratories, Inc., Atchison, KS) housed in a biosafety cabinet. Samples were stored frozen until use. The dried plant material was homogenized in deionized water, filtered and the filtrates were tested at concentrations of 4, 8 and 80 mg mL^{-1} (dry weight basis). Because conidia also germinate when incubated on a firm or solid surface without chemical additions (Egley, 1994), tests for effects of additives on germination were conducted on conidia incubated while suspended in water. Controls consisted of conidia suspended in deionized water and in emulsions of unrefined corn oil (water:oil, 1:1, v/v). *C. truncatum* conidia germinate poorly or not at all when suspended in water, but germination is induced when suspended in an emulsion of unrefined corn oil (Egley and Boyette, 1995). Conidial germination and appressorial formation were determined as described above.

***C. truncatum* germination and appressoria formation on hemp sesbania leaves:** Various surfactants that stimulated conidial germination were also sprayed (Laboratory Aerosol Sprayer, Spra-Tool®, Crown Industrial Products, Co., Hebron, IL) along with conidia onto hemp sesbania leaves. The aqueous test solutions were sprayed either directly onto the plants or were emulsified with refined corn oil (water: oil, 1:1, v/v) and then sprayed. Each treatment contained 2.0×10^6 conidia mL^{-1} and was sprayed to runoff onto thirty, 10 to 14-day-old seedlings (10 seedlings per replication, three replications per treatment). Controls consisted of conidia sprayed in deionized water alone or in the water-unrefined corn oil emulsion. Each experiment was conducted twice. The treated seedlings were then placed under various environmental conditions as outlined below.

Growth chamber experiments

Effects of immediate dew in growth chamber experiments: Treated seedlings were incubated in the light (165 $\mu E\ m^{-2}\ s^{-1}$) for up to 24 h at 20, 30 or 35°C. The Relative Humidity (RH) at each temperature was 21, 14 and 9%, respectively. No dew occurred under these conditions. After 1, 3, 5 and 24 h of incubation, eight leaflets (approximate leaflet size 20×8 mm) were randomly collected from different seedlings in each replication of each treatment and placed on a strip of wet filter paper on a glass microscope slide. The conidia were stained by lactophenol cotton blue on the leaf surface. The first 200 conidia on the leaves in each replication that were observed under 400x magnification were scored for germination and appressoria formation. The data on appressoria formation (expressed as percent of germinated conidia) were examined to determine the effects of the treatments upon *C. truncatum* virulence.

Effects of delayed dew: In another experiment, the spray-treated hemp sesbania seedlings were incubated in the dark at 20, 25, or 35°C for 24 h and then transferred to a 25°C dew chamber (Model I-36 DL; Percival Sci., Ind., Perry, IA, USA). Leaflets were collected from seedlings after 1, 3 and 5 h in the dew chamber and observed as described above for conidia germination and appressoria formation.

Greenhouse experiments

Effects of delayed dew: Hemp sesbania seedlings spray-treated with various adjuvants;refined corn oil were placed in the greenhouse (28 to 33°C, 42 to 80% RH; ~12 h day length) for 24 h before transfer to a dew chamber (25°C). Disease ratings were determined 14 days after treatment using a modified (Horsfall and Barratt, 1945) method, where 0 = no infection, 1 to 3 = slight

infection, 4 to 6 = moderate infection, 7 to 9 = severe infection and 10 = seedling death. After 1, 3, 5 and 24 h in the dew chamber, leaflets were collected and observed for *C. truncatum* conidia germination and appressoria formation as described above. After 24 h of dew, the seedlings were returned to the greenhouse and observed for disease development during the subsequent 14 days. The effects of the treatments on *C. truncatum* germination and appressoria formation were compared with the effects on virulence of this bioherbicide.

Statistical analyses: Treatments (in triplicate) were arranged in a randomized complete block design and all experiments were repeated in time. Means were subjected to analysis of variance and were compared with Fisher's LSD (p = 0.05) only when the F-test from the analysis indicated significance. All data were analyzed using statistical software (SAS, 1999).

RESULTS

Germination of suspended conidia in glass vials: Thirty five percent of the conidia germinated in the emulsified unrefined corn oil, while only 2% germinated in refined corn oil and none germinated in either water, or oleic, linoleic, palmitic and stearic acids (Table 2). Results with corn oil and water were typical as reported previously (Egley and Boyette, 1995; Boyette and Hoagland, 2012). Only 5% of the conidia germinated in linolenic acid, which was the only fatty acid exhibiting stimulatory activity. The extracts of several plants, including the host (hemp sesbania), two other legumes (sicklepod, soybean) and four grasses (corn, wheat, ryegrass and bermudagrass) were very active germination stimulants and germination was generally directly proportional to the extract concentration (Table 3). Johnsongrass was the exception and caused inhibition of conidial germination at the highest extract concentration.

Tween®s 40 and 85 and Myvatex® 60 caused the greatest stimulation of germination at both the 0.5 and 1.0% concentration, while Tweens 60 and 80 were only stimulatory at 1.0% (Table 4). The other compounds were not effective. Tweens® with a hydrophilic-lipophylic balance (HLB) of 11 to 16 were very effective conidial germination stimulants (Table 4). None of the Alfonic® surfactants (HLB values of 8 to 12) stimulated germination. Overall, Myvatex 60 with an HLB value of 8 exhibited the greatest increase of conidial germination.

Conidial germination and appressorial formation of conidia on hemp sesbania leaves

Immediate dew: Tween 40® alone did not stimulate conidial germination on leaves incubated for 24 h without dew (Table 5). At 24 h incubation, the addition of 0.25 and 0.5% Tween 40® to the refined corn oil emulsion enhanced conidial germination on leaves irrespective of the incubation temperature (Table 5). Regardless of

Table 2: Effect of fatty acids on *C. truncatum* conidial germination in glass vials

	Conidial germination (%)			
	Treatment concentration (%)			
Treatment	0	0.25	0.50	1.0
H_2O	0	-	-	-
Linolenic acid	-	1.6	3.5	5.0
Oleic acid	-	0	0	0
Linoleic acid	-	0	0	0
Palmitic acid	-	0	0	0[b]
Stearic acid	-	0	0	0[b]
Unrefined corn oil[a]	35.0	-	-	-
Refined corn oil[a]	2.0	-	-	-
LSD (0.05)		2.1		

[a]Water:corn oil emulsion (1:1, v/v), [b]Less than a 1.0% solution was obtained

Table 3: Effect of aqueous plant extracts on *C. truncatum* conidial germination in glass vials

	Conidial germination (%)[a]			
	Plant extract concentration (mg mL^{-1})			
Plant species	0	4	20	4 0
H_2O alone	0	-	-	-
Unrefined corn oil	35	-	-	-
Hemp sesbania	-	22	51	98
Sicklepod	-	2	20	98
Soybean	-	4	91	97
Corn	-	3	21	89
Wheat	-	1	42	92
Ryegrass	-	0	17	47
Bermudagrass	-	0	26	97
Johnsongrass	-	1	38	13
LSD (0.05)		9.0		

[a]Conidia (5.0×10^5 mL^{-1}) were suspended in droplets in all treatments, Germination in H_2O = 0%

Table 4: Effects of surfactants on germination of *C. truncatum* conidia in glass vials

		Conidial germination in treatment concentration[a]			
Treatment	HLB[b] value	0.025%	0.25%	0.5%	1.0%
Tween 20	17	0	<1	0	0
Tween 40	16	0	1	38	74
Tween 60	15	0	1	2	36
Tween 80	15	0	1	9	31
Tween 85	11	0	<1	51	60
Alfonic 810-60	12	0	0	0	0
Alfonic 1012-60	12	0	0	0	0
Alfonic 1412-60	12	0	0	0	0
Alfonic 610-50	10	0	0	0	0
Alfonic 810-40	8	0	0	0	0
Alfonic 1012- 40	8	0	0	0	0
Alfonic 1412- 40	8	0	0	0	0
Myvatex -60	8	0	<1	76	86
Myvacet -9.45	4	0	2	<1	2
Myverol -18-99	4	0	3	3	1
LSD (0.05)			7.0		

[a]Conidia (5.0×10^5 mL^{-1}) were suspended in droplets in all treatments. Germination in H_2O = 0%, in refined corn oil:H_2O = 0% and unrefined corn oil: H_2O (1:1, v/v) = 28%; [b]Hydrophylic-Lipophylic Balance (Griffin, 1954)

Table 5: Effects of incubation temperature and Tween®-40 on germination and appressoria formation of *C. truncatum* on hemp sesbania leaves without dew

		Temperature (°C)					
		Germination (%)			Appressoria (%)		
Conidia formulation[a]	Incubation time (h)	20	30	35	20	30	35
H_2O	1	0	0	0	0	0	0
	3	0	0	0	0	0	0
	5	0	0	0	0	0	0
	24	1	0	<1	0	0	0
0.25% Tween® 40	1	0	0	0	0	0	0
	3	0	0	0	0	0	0
	5	0	0	0	0	0	0
	24	1	0	<1	0	0	0
0.5% Tween® 40	1	0	0	0	0	0	0
	3	0	0	0	0	0	0
	5	0	0	0	0	0	0
	24	1	0	<1	0	0	0
H_2O: refined corn oil	1	0	0	0	0	0	0
	3	0	0	0	0	0	0
	5	0	0	0	0	0	0
	24	1	0	<1	0	0	0
0.25% Tween®40:	1	0	0	0	0	0	0
refined corn oil	3	4	5	0	0	0	0
	5	7	25	6	0	3	0
	24	20	12	30	9	16	18
0.5% Tween®40:	1	0	0	0	0	0	0
refined corn oil	3	0	14	<1	0	7	0
	5	0	31	47	0	0	5
	24	40	50	19	35	30	5
LSD (0.05)			9.0			11.0	

[a]Conidial (2.0×10^6 mL^{-1}) treatments were sprayed onto hemp sesbania seedlings and incubated 1 to 24 h at 20°, 30° and 35°C. Refined corn oil emulsified in water (water: oil, l:l, v/v)

Table 6: Effects of incubation temperature, dew and Tween® -40 on germination and appressoria formation of *C. truncatum* on hemp sesbania leaves

		Incubation temperature (°C)					
		Germination (%)			Appressoria (%)		
Treatment[a]	Dew duration (h)	20°	30°	35°	20°	30°	35°
H_2O	1	0	0	0	0	0	0
	3	1	2	0	0	0	0
	5	6	0	0	51	0	0
H_2O: refined corn oil	1	<1	2	1	0	0	0
	3	<1	2	1	0	0	0
	5	3	20	7	2	0	20
0.25% Tween® 40:	1	8	26	46	0	16	38
refined corn oil	3	39	45	46	18	27	23
	5	42	61	39	2	28	40
0.5% Tween® 40:	1	11	28	27	22	29	7
refined corn oil	3	31	54	60	8	27	23
	5	58	57	75	4	44	36
LSD (0.05)		17				19	

[a]Conidia (2.0×10^6 mL^{-1}) in all treatments were sprayed onto hemp sesbania seedlings and then incubated at 20°, 30° and 35° C for 24 h before treatment with 1, 3 and 5 h of dew at 25°C. Refined corn oil emulsified in water (water: oil, l:l, v/v)

incubation temperature, individual applications of conidia to the seedlings in water, Tween® 40 or the refined corn oil emulsion, resulted in <3% germination. The addition of 0.5% Tween 40 to the refined corn oil emulsion enhanced germination to a maximum of 50% at 30°C and 24 h. Germination was greater at 30 and 35°C than at 20°C. Appressoria formed on 30 to 35% of the germinated conidia by 24 h at 20 and 30°C. These results indicate that both the surfactant and the refined corn oil emulsion were necessary for stimulation of conidial germination on plant leaves.

Delayed dew: The surfactant-refined corn oil treatment also enhanced germination on the hemp sesbania leaves when one to 5 h of dew followed a dew-free period of 24 h (Table 6). When conidia were applied in water alone, germination was <6% or less even with 5 h of dew. Germination was less than 20% when conidia were applied

in only the refined corn oil emulsion. The addition of 0.25 or 0.5% Tween® 40 to the emulsion enhanced germination at all temperatures. Germination reached a maximum of 75% at 35°C with 0.5% Tween 40®. However, 0.25 and 0.5% Tween® 40 were of nearly equal activity (Table 6). The dew periods most notably benefited the germination of conidia incubated at 20 and 35°C as compared to conidia incubated at the same temperature but without a dew period (Table 5, 6). The dew periods also enhanced the number of germinated conidia that formed appressoria.

Effects of surfactants upon conidial germination and virulence: The additions of Tween® 40, 60, 80, 85 and Myvatex®-60 to the refined corn oil emulsions also enhanced conidial germination on hemp sesbania in the greenhouse when the plants received dew after a dew delay of 24 h (Fig. 1). Even with the dew period, only 2 and 4% of the conidia germinated when applied to the plant in either water or the refined corn oil emulsion alone. Regardless of the surfactant addition, germination was no greater than 2% without a dew period.

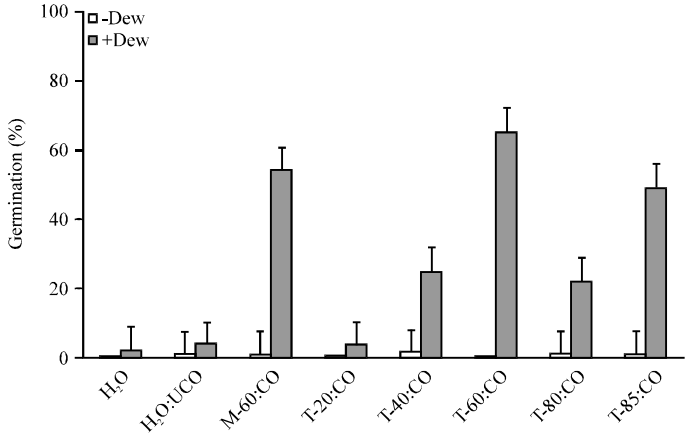

Fig. 1: Effects of various adjuvants:refined corn oil emulsions on germination of *C. truncatum* conidia (2.0×10^6 mL^{-1}) on hemp sesbania seedlings in the greenhouse with delayed or immediate dew events, 14 days after treatment. Seedlings were sprayed with conidia:adjuvant oil emulsions followed by delayed dew or immediate dew events as described in the Materials and Methods. Error bars represent Fisher's $LSD_{0.05}$ values (17.0)

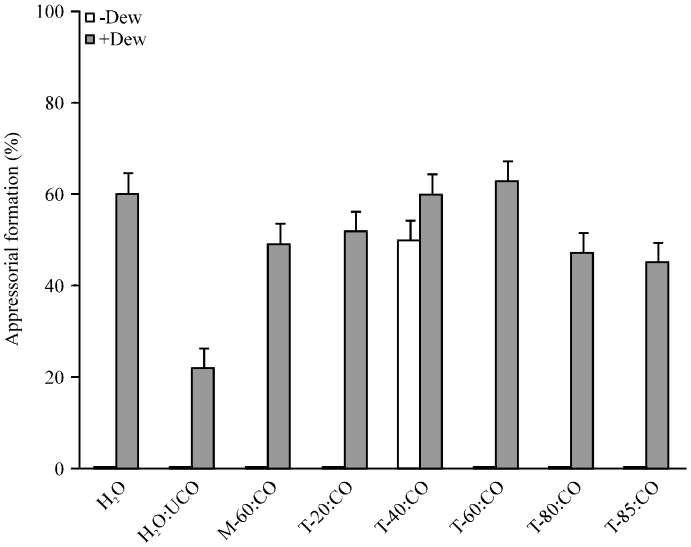

Fig. 2: Effects of various adjuvants:refined corn oil emulsions on appressorial formation of germinated *C. truncatum* conidia (2.0×10^6 mL^{-1}) on hemp sesbania seedlings in the greenhouse with delayed or immediate dew events. Seedlings were sprayed with conidia:adjuvant oil emulsions followed by delayed dew or immediate dew events as described in the Materials and Methods. Error bars represent Fisher's $LSD_{0.05}$ values (5.3)

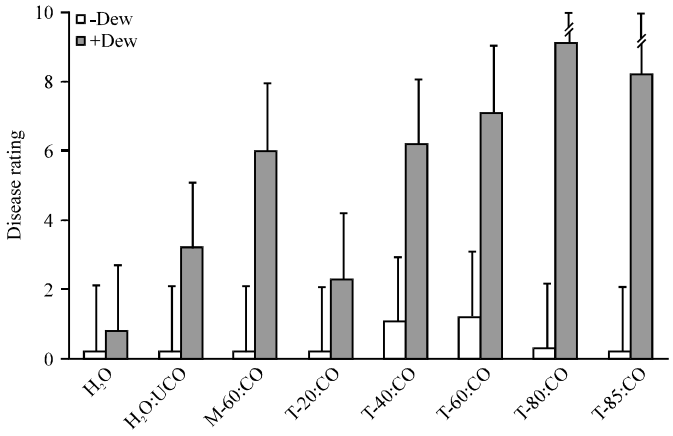

Fig. 3: Effects of various adjuvants:refined corn oil emulsions on disease of hemp sesbania seedlings incited by *C. truncatum* conidia (2.0×10^6 mL^{-1}) in the greenhouse with delayed or immediate dew events. Seedlings were sprayed with conidia:adjuvant oil emulsions followed by delayed dew or immediate dew events as described in the Materials and Methods. Disease ratings were determined 14 days after treatment using a modified (Horsfall and Barratt, 1945) method, where 0 = no infection, 1 to 3 = slight infection, 4 to 6 = moderate infection, 7 to 9 severe infection and 10 = seedling death. Error bars represent Fisher's $LSD_{0.05}$ values (2.0)

The percentage of germinated conidia that formed appressoria also varied among the treatments, but none were better than the control (H_2O alone) when sprayed seedlings were subjected to an immediate dew event (Fig. 2). When plants were subjected to a delayed dew event after conidial application, the only treatment resulting in significant appressorial formation was the Tween® 40 plus refined corn oil (Fig. 2).

The surfactant additions (except Tween® 20) to the emulsions incited infection of hemp sesbania that was greater than that incited by the pathogen applied in either water, or in the refined corn oil emulsion alone. Tween® 80 and 85 additions to the pathogen-corn oil emulsion were significantly greater than the levels induced by the pathogen-corn oil emulsion without addition of surfactant (Fig. 3).

The disease level was not commensurate with the level of conidia germination in this greenhouse experiment. For example, the corn oil emulsion alone produced only 4% germination and an infection that resulted in a disease rating of 3.2. Also, the corn oil emulsions amended with Tween® 80 resulted in 22% germination and a disease rating of 9.1 whereas the Tween® 60 addition resulted in 65% germination and a disease rating of 7.1 (Fig. 3). However, the germination and disease levels attained with the water-conidia controls were both low; 2% and 0.8, respectively.

DISCUSSION

It was previously shown that conidia of *C. truncatum* germinated very poorly or not at all while suspended in water (Egley and Boyette, 1995). However, the conidia germinated well when incubated on certain firm substrates or surfaces, which indicated a germination response to contact with surfaces (Egley, 1994) suggesting a thigmotrophic response. Surface hydrophobicity and surface rigidity has been shown to induce spore germination in *Colletotrichum graminicola* (Chaky *et al*., 2001). Furthermre, in the bioherbicidal fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, plant surface signals were found to induce pathogenic-specific spore germination (Barhoom and Sharon, 2004).

Thigmotropism involves the sensing of surface topography and has been studied primarily in biotrophic fungi. Appressorium formation can be influenced by physical and chemical cues or by hydrophobic characteristics of the contact surface (Staples and Hoch, 1995; Terhune and Hoch, 1993). Phytopathogenic fungi can respond to contact stimuli through a directional change, re-orientation of the direction of germ tube growth, or by direct differentiation of appressoria (Staples and Hoch, 1995). For example, the directional growth of rust germ tubes was attributed to physical features of the leaf surface (Wynn, 1976). Using plastic leaf surface replicas, it was confirmed that no chemical stimulus was involved in the surface sensing process of rust fungi.

It was also previously shown that the unrefined corn oil water emulsion stimulated germination of suspended *C. truncatum* conidia whereas a refined corn oil-water emulsion did not (Egley and Boyette, 1995). From that report it was concluded that the unrefined corn oil helped to maintain virulence of the conidia on the plant surface during a dew-free period and may have also protected the conidia from desiccation. The unpredictability of dew or

free-moisture events and the resulting inconsistent level of weed control are major factors limiting the use of mycoherbicides in agriculture (Weaver *et al*., 2007). Although the role of unrefined corn oil in the stimulation of conidia germination is unknown, the fact that addition of a surfactant to refined corn oil stimulated germination suggests that the refined oil may lack a stimulant (perhaps a natural surfactant) that was removed during refinement. The unrefined corn oil is the result of the first pressing of corn kernels, thus the product contains a multitude of materials in addition to fatty acids. The predominant fatty acids in corn oil are oleic, linoleic, palmitic and stearic, however, none of these compounds individually stimulated germination of *C. truncatum* conidia (Table 2). Only linolenic acid was slightly active. Previously xylose, galactose, glucose, glycine, alanine, phenylalanine and tryplophan were found to enhance germination of *C. truncatum* conidia when incubated on a surface (2% water agar) (Boyette and Hoagland, 2012). Apparently the sugars and amino acids were more effective on conidia that were on a surface. Thus substances capable of stimulating germination of suspended conidia may not necessarily be able to enhance germination on a firm surface.

The stimulatory activity of the plant extracts was not due to a property solely of the host as several non-host extracts also stimulated germination (Table 3). The crude extracts may have contained nutrients and/or natural surface-active agents e.g., some plant phospholipids have surfactant activity. Contrary to this, several plant extracts exhibited antifungal activity against *C. lagenarium* which causes anthracnose in cucumber (Chen and Dai, 2012) and *Drechslera heveae*, the causal organism of bird's eye spot disease of rubber (*Hevea brasiliensis*) (Ogbehor and Adekunle, 2008). Furthermore, many plants produce natural soaps (called saponins i.e., steroid or triterpenoid glycosides) which have surfactant properties (Osbourn, 1996). Some of these chemicals are toxic to bacteria and fungi (Zablotowicz *et al*., 1996) and/or act as protection against disease in plants (Osbourn, 1996), but some saponins have phytotoxic properties (Hoagland *et al*., 1996). Certain microbes can successfully infect saponin-containing plants by producing enzymes that degrade the saponins into less toxic chemicals thus, enabling invasion and infection (Osbourn, 1996).

Although, Tween® 40 stimulated germination of suspended conidia, the surfactant did not affect conidia on plant surfaces without dew (Table 5). However when Tween® 40 was added to the refined corn oil emulsion, 50% of the conidia germinated after 24 h at 35°C on the host plant leaves (Table 5). Germination was generally greater when the treated plants received 5 h dew (Table 6). These results support the idea that corn oil (unrefined or refined plus certain adjuvants) can protect conidia if exposed to sub-optimal moisture conditions.

It has been known for sometime that surfactants can affect growth and physiological processes of higher plants, fungi and bacteria (Millar and Hancock, 1963; Knypl, 1977; Parr, 1982; McWhorter, 1985). These effects generally were related to surfactant chemistry and concentration, the species tested and ranged from none to stimulation or inhibition. For example, Tween® 20 at concentrations of 0.0083 to 0.083% stimulated germination of *Collectotrichum trifolii,* but inhibited germination at 0.83% (Miehle and Lukezic, 1972). In our tests, Tween® 20 at 0.025 to 1.0% was inactive, whereas Tweens® 40, 60, 80 and 85 stimulated *C. truncatum* conidial germination at 0.25 to 1.0% (Table 4).

Surfactants can also alter characteristics of biological membranes, often increasing permeability (Helenius and Simons, 1975; John *et al*., 1974). Such effects can occur in membranes of a pathogen and/or its host. This has been shown to occur when 2-(dec-2-enyl)succinic acid (an unsaturated fatty acid) was applied to broad bean (*Vicia faba*) tissues, resulting in elevated sugar and amino acid levels in the leaf leachate (Blakeman, 1973). This nutrient-rich leachate enhanced *Botrytis fabae* spore germination and infection when spores were applied to these leaves. Contrary to this, treatment of beet (*Beta vulgaris*) leaves with this succinic acid derivative reduced germination of *B. cinerea* spores on leaves even though carbohydrates in leaf leachates were elevated (Blakeman, 1973).

Certain fatty acids can also suppress mycelial growth and inhibit spore germination and depending on the type of fatty acid and the fungi tested (Liu *et al*., 2008). Surfactant concentration is also critical because high concentrations can cause loss of physiological activity and cell death, but increased permeability may also alter enzyme activity, growth and germination. Biological effects other than those on membranes may play critical roles in pathogen germination and virulence (Norris, 1982). The fatty acids tested in this report had little or no effect on conidial germination of *C. truncatum*, but were not examined for effects with this bioherbicide on hemp sesbania membrane permeability or virulence. This would be an interesting subject for future studies.

Because water is one of the most important factors in conidial germination, water uptake and permeability of biological structures is important. For example, fungal spore wall impermeability to water may play a role in spore dormancy, since an increase of water influx into the spore may provide the turgor force necessary to initiate expansion and protrusion of the germ tube (Griffin, 1994).

The specific requirements for stimulation of *C. truncatum* conidia germination by surfactants are not clearly defined. The HLB values of several active Tween® surfactants in this study classified them as detergents and solubilizers (Becher, 1965). Although detergents can solubilize membranes (Helenius and Simons, 1975), HLB may not be the sole criterion for this activity. For example in our studies, Alfonic ethoxylates (HLB values of 6, 8, 10 and 12) did not stimulate conidial germination, while Myvatex 60 (HLB of 8), exhibited conidial germination stimulator activity (Table 4). Overall, activity of these commercial surfactants was not solely linked to their HLB values, indicating that factors other than hydrophobic/lipophobic interactions are operative.

These surfactants are not precisely defined chemicals (Parr, 1982). The length or weight of side groups on a molecule may be expressed as an average. Thus it is not possible to precisely relate molecular structure to activity. Furthermore, some commercial formulations are mixtures of surfactants (Parr, 1982). The activity of Myvatex®-60 may have been due to the polysorbate 60 (Tween®60) component of the mixture. Thus, the Tween®s 40, 60, 80 and 85 may contain specific factors that stimulate conidia germination. It is possible that Tween® 20 either lacked such factors or contained an inhibitor.

CONCLUSION

Data from the above experiments indicate that certain adjuvants enhanced *C. truncatum* conidial germination and appressorial formation. Furthermore, refined corn oil formulated with certain surface-active agents enabled *C. truncatum* to retain viability on the weed in the absence of dew and germinate and infect when free-moisture becomes available. This suggests that unrefined corn oil may contain a natural surfactant(s) removed during the refining process. More research is necessary to determine the agent(s) and/or characteristics of unrefined corn oil responsible for the protective and promotive actions on *C. truncatum* conidia.

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