Complete Abolition of High Inoculum Threshold of Two Mycoherbicides (*Alternaria cassiae* and *A. crassa*) When Applied in Invert Emulsion

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ABSTRACT

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Fungal spore infectivity on leaves is a function of environmental factors (duration of dew point humidity, temperature), the defense mechanisms of the host plant, and fungal pathogenicity. The inoculum threshold of thousands of spores per square centimeter of leaf surface or tens to hundreds of spores per droplet has been expected and accepted. Evidence is presented that the high threshold concept does not hold with *Alternaria cassaie* and *A. crassa*, as one spore per 2-μl droplet was sufficient to infect plants of *Cassia obtusifolia* and *Datura stramonium*, respectively, when the droplet was an invert emulsion containing a 1-μl mixture of oils and waxes on the outside and 1 μl of water, sodium alginate, and conidia on the inside. The intensity of infection always was enhanced by the emulsion. Even a culture of *A. cassiae* that lost its infectivity, giving only a hypersensitive response when applied in water, became infective in the invert emulsion.

Additional keywords: biocontrol, jimsonweed, microbial herbicides, sicklepod, weed biocontrol.

It commonly is accepted that a certain minimal but high threshold of fungal inoculum is required to establish an infection (7,8,16,17,21). A few cases have been reported where a single spore caused a lesion, but such lesions usually remained small. There are cases where 1,000 propagules per gram of soil are required for soilborne pathogens, and tens to hundreds of spores are required per droplet to establish infections on stems or leaves. Garrett (8) suggested that the main reasons for the ubiquitous lack of infectivity of single propagules may be that each spore

may not fall on a susceptible site, there may be possible synergistic interactions between spores, and/or an environmentally controlled inoculum threshold governed mainly by temperature and dew may exist. The observation that a few spores often only cause a lesion that does not develop supports the second explanation of synergy. If either the first or second explanation is correct, a high inoculum threshold becomes an obligate requirement; if the third explanation alone is correct, the environment could be modified to lower the threshold to a single propagule per infection. If the first and third or second and third are correct, then altering the environment would lower the threshold, but the number of propagules needed for infection still would be greater than one per site.

The use of host-specific pathogens as biocontrol agents against noxious weeds recently has received attention (4). The concept of a self-replicating herbicide is especially inviting, as very small amounts of inoculum theoretically should decimate the weed population. However, in practice, exceedingly high rates of inocula must be used to control weeds. With soilborne organisms, 500–4,000 propagules per gram of soil often are used (1); with foliar and stem pathogens, 10^5–10^6 spores per milliliter commonly are sprayed to runoff (21). These levels of spore inocula represent thousands of spores per square centimeter of leaf surface.

Two pathogen/weed systems are reported to have exacting environmental requirements for infection. A dew point humidity of 6–8 hr was reported as necessary for infection of *Cassia obtusifolia* L. (sicklepod) and *Datura stramonium* L. (jimsonweed) by *Alternaria cassiae* Jurair & Khan and *A. crassa* (Sacc.) Rands, respectively (2,20), even at high densities of conidia. These conditions only rarely occur under field conditions. Two to three hours are required for conidia of both organisms to germinate, and approximately six germ tubes per conidium of *A. cassiae* reached a length of 50–300 μm in 20 hr (18). Large amounts of inocula were needed even when the high dew point requirements were met. No weed control of *C. obtusifolia* and *D. stramonium* occurred below a threshold of 3×10^4 conidia per milliliter of *A. cassiae* and 10^5 conidia per milliliter of *A. crassa*, respectively (3,20).

Efforts have been made to use various antidesiccants and humectants to protect spores and supply the critical free water component of the environmental threshold (12,15). It recently was shown that the dew point requirement could be negated by applying conidia of *A. cassiae* in an invert emulsion (13). Whereas a water droplet evaporates in minutes at normal field humidities, only 20% of the water evaporated in the first 24 hr with the emulsion, and 50% still remained after 100 hr (13). We report herein that this invert emulsion reduces the required inoculum threshold needed for infection to one conidium per inoculated site. It is not clear that this is by solely abolishing the environmental component of the inoculum threshold.

MATERIALS AND METHODS

Weed hosts and mycoherbicides. We chose two pathogen-weed systems as models to study infection: *A. cassiae* controls the weedy legume *C. obtusifolia* without infecting the related crops, soybeans, and peanuts (20); and *A. crassa*, which controls *D. stramonium* without infecting major crops, except some tomato cultivars (2). Seeds of *C. obtusifolia* initially were provided by V. A. Musco, Rohm and Haas, Springhouse, PA, and, subsequently, were propagated and grown in a controlled temperature, high-humidity greenhouse (60–80% RH) at 24 C day (14 hr) and 22 C night (10 hr). Seeds of *D. stramonium* were gathered from local wild populations. Germination was induced by altering the temperature at 24-hr intervals between 10 and 30 C for 6 days. Plants were grown under similar conditions as *C. obtusifolia* at 28 C day and 26 C night.

Two isolates of *A. cassiae* were used. ATCC 46687 was obtained from the American Type Culture Collection; this isolate has lost virulence and could induce only a hypersensitive host response when applied in water. An infective isolate was obtained from a soil sample provided by Dr. C. D. Boyette, USDA, Stoneville, MS. The two isolates were identical according to the literature (19). *A. crassa* No. 103.18 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The virulent isolates of *A. cassiae* and *A. crassa* were reisolated from inoculated plants monthly to prevent loss of infectivity.

The fungi were cultured on V-8 juice agar (10) in plastic petri dishes incubated at 25 C with 12-hr photoperiods (30 μE m^{-2} sec^{-1} at plate level). Seven-day-old cultures were induced to sporulate by a 40-min exposure to 4 μE m^{-2} sec^{-1} UV light at plate level (1.2 μE m^{-2} sec^{-1} penetrating the petri dish cover) provided by a 300-nm UV lamp (No. RPR-3000A; New England Ultraviolet, Hamden, CT). One additional 30-min UV light exposure was given 24 hr after the first exposure in cases of poor sporulation. Conidia were removed by washing the plates with sterile distilled water and separated from fragments of hyphae by filtration through 300-μm-mesh nylon monofilament bolting cloth (Nytex, Zurich, Switzerland). Conidial suspensions were adjusted to a standard concentration, usually 5×10^5 per milliliter after counting samples with the aid of a hemacytometer.

Invert emulsion and application. The emulsion was composed of water and oils and was modified from Quimby et al (13). The water phase contained 0.5% sodium alginate that was dissolved by stirring for 15 min in a 55 C water bath. One liter of the oil phase contained 120 g of crude soy lecithin, 550 ml of commercial edible soybean oil, 280 ml of mineral oil, and 50 g of paraffin wax. The paraffin wax was melted by warming to 50 C and then mixed with the mineral oil. Lecithin was added to the soybean oil and homogenized with an Ultra Turrax (Janke and Kunkel, Staufen im Breisgau, West Germany) for 2 min at full speed. The two oil fractions were mixed with a magnetic stirrer, and the final emulsion was obtained by mixing the oil and water phases (1:1) on a vortex stirrer. Conidial concentrations were prepared from the standard solution of 5×10^5 conidia

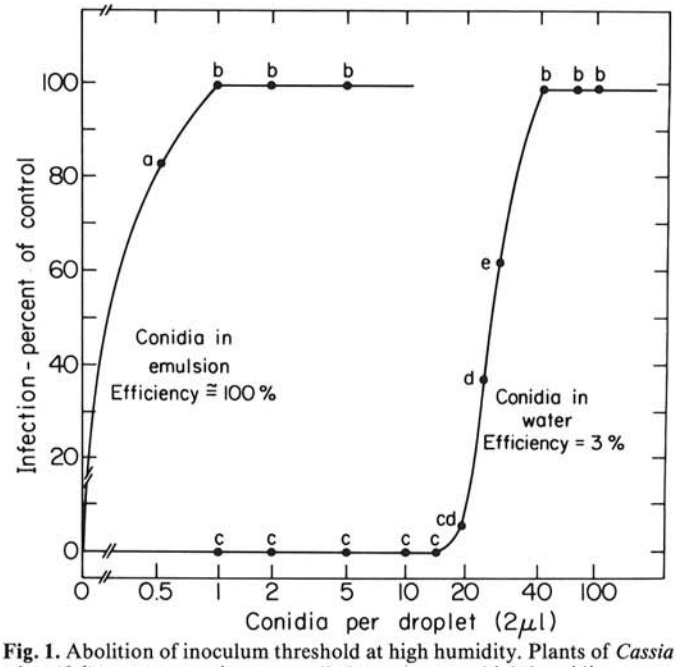

Fig. 1. Abolition of inoculum threshold at high humidity. Plants of *Cassia obtusifolia* were grown in a controlled temperature, high-humidity greenhouse (60–80% RH) at 24 C day (14 hr) and 22 C night (10 hr). Conidia were from an infective isolate of *Alternaria cassiae*. Low dilutions were checked by plating on V-8 medium, and efficiency was calculated based on colony formation on these plates. One outer leaflet was treated (after three of the four leaflets were removed) on 12-day-old plants bearing one leaf. One 2-μl droplet was applied to each opposite lobe of each leaflet, irrespective of position on the leaflet, with 20 replicates per data point. Plants were put in a closed dew chamber at 100% RH within the greenhouse for 16 hr and then transferred back to open shelves. Scoring was performed 5 days after treatment. Concentrations of conidia are presented in an exponential manner on this graph. Different letters denote a significant difference among treatments according to Duncan's multiple range test ($P \leq 0.05$).

per milliliter. For the water formulation, the standard solution was diluted with sterile distilled water containing 0.02% Tween 80 (Sigma, St. Louis, MO) (to keep the spores from coalescing and assist in dispensing) to give the required concentration of conidia. For the emulsion formulation, conidia were serially diluted in the water-alginate solution to give a concentration double that of the required final concentration, and then mixed 1:1 with the oil phase.

When dilutions containing an average of one (or fewer) conidium per droplet were used, the number of droplets containing a conidium was checked by plating parallel droplets on V-8 medium. Ten 2-μl droplets were applied separately and far from each other on a plastic petri dish containing V-8 medium, five plates per dilution. In a typical experiment, 66% of the droplets diluted to contain one conidium per droplet and 50% of the droplets diluted to contain one conidium in two droplets developed colonies. Such data were examined with a Poisson distribution equation $X = \ln[1 \div (1 - y)]$, where X was the calculated number of conidia per droplet from the dilution of a counted suspension, and y was the proportion of droplets expected to develop into colonies due to the presence of one or more conidia in a droplet. Suspensions diluted to contain one conidium per droplet were expected to have 37% of the droplets without conidia and 63% with one or more conidia. Suspensions diluted to contain one conidium per two droplets should give rise to 61% uninfective droplets and 39% with one or more conidia. Thus, the experimental and Poisson-calculated data were in close agreement. In practice, to calculate the efficiency of infection, we compared the percentage of droplets giving rise to colonies on plates to the percent causing infection of leaves.

Plants of *C. obtusifolia* at the first-leaf stage were selected randomly and one outer leaflet was treated after the other three of the four leaflets were excised. The first pair of true leaves of plants of *D. stramonium* were treated. Two 2-μl droplets of conidia in water or conidia in emulsion were placed on each half leaf, one near the petiole and the other closer to the far edge of the blade. Water droplets were applied with a 20-μl Pipetman (Gilson, Villiers Le Bel, France), whereas emulsion was applied with a 100-μl syringe in a repeating dispenser (Hamilton, Bonaduz, Switzerland). Water droplets covered approximately 1.8 mm^2, and emulsion droplets covered 3.1 mm^2.

Experiments were performed under high and low humidity. For high humidity, treated plants were placed in a dew chamber (100% RH) for 16 hr and then moved to the high humidity (60–80% RH) greenhouse. For low humidity, treated plants were kept in the greenhouse at 50% RH. Experiments with *C. obtusifolia* were repeated three or more times, with at least six replicates per treatment arranged in completely randomized designs.

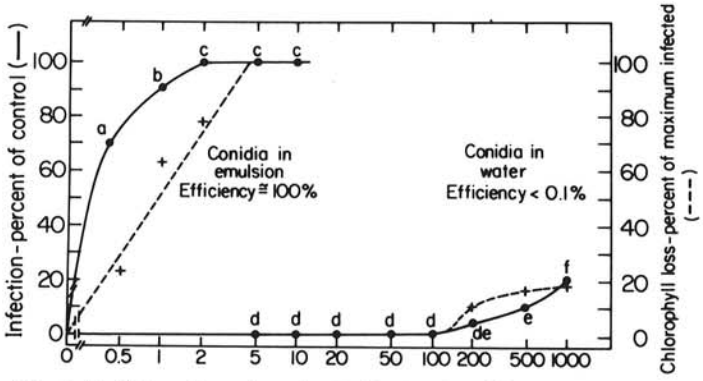

Fig. 2. Abolition of inoculum threshold at low humidity. Culture of plants of *Cassia obtusifolia* through conidial applications were as denoted for Figure 1. Plants were put on open shelves at low humidity (50% RH) immediately after inoculation. Scoring was performed 5 days after treatment. Both visual measurements of establishment/nonestablishment (solid line) and quantitative measurements of chlorophyll in the infected sites (dotted line) were made. Different letters denote a significant difference among treatments according to Duncan's multiple range test ($P \leq 0.05$).

Intensity of infection. Infection was evaluated 5 days after treatment, which was the most representative time based on preliminary observations. Intensity of infection was rated as follows: sites with no necrotic area were considered "not infected," and sites with hypersensitivelike light-brown necrotic lesions were considered to have an "initial infection." Sites with necrosis of <60% of the droplet area were considered not established, whereas sites with ≥60% of the droplet area necrotic were considered "established." Established spots always continued to grow and the fungus sporulated. Percentage of infection ("established" and "not established" sites) was corrected according to the number of colonies that grew on the plates. The I_{50} values are the extrapolated number of conidia per droplet required for infection (established and not established sites) of 50% of the treated sites based on the log/linear, dose/response measurements. Differences among mean treatment (number of conidia) values, to determine inoculum threshold of conidia in water and in emulsion, were separated with Duncan's multiple range test ($P \leq 0.05$).

The extent of plant tissue damage also was quantitatively estimated as the loss of chlorophyll as follows: 4-mm-diameter disks were excised with a cork borer at infection sites, placed in 0.5 ml *N,N*-dimethylformamide, and extracted overnight on an orbital shaker (Lab-line, Melrose, IL) operating at 40 rpm. Chlorophyll was determined according to Moran (11). The excised disks were all of greater area than the infection, leaving some green tissue, and the peripheral residual chlorophyll content of

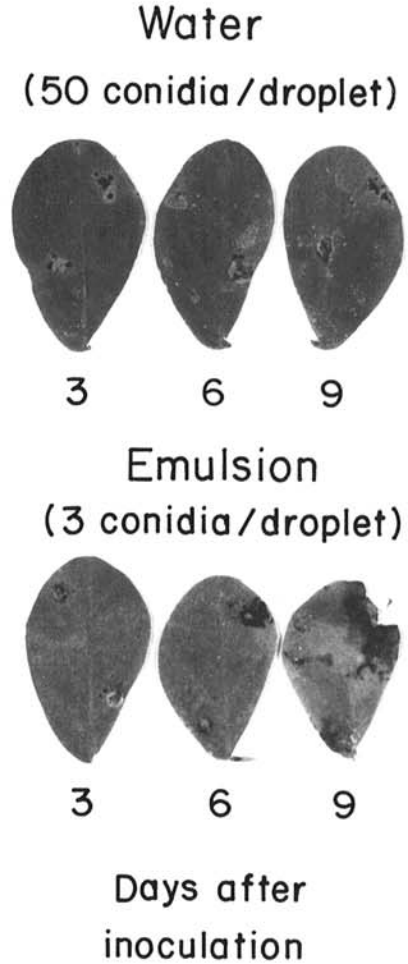

Fig. 3. Increase with time of intensity of infection by *Alternaria cassiae* (infective isolate) applied in water and emulsion. Leaves of *Cassia obtusifolia* shown at various times are from an experiment similar to that described in Figure 1, and the conditions of growth and application were the same. Infection whereby dilution to one conidium per droplet infected 60% of the sites (i.e., calculated as 100% infection by one conidium according to the Poisson distribution and from parallel plating experiments) was repeatedly found in three additional experiments.

the most necrotic disk was subtracted from each data point. Thus, a fully necrotic lesion was made equal to no chlorophyll.

RESULTS

Infection by *A. cassiae* at high relative humidity. Under high humidity (100% RH for 16 hr followed by 60–80% RH), a single viable conidium in a 2-μl emulsion droplet was sufficient to establish an infection (Fig. 1), irrespective of position on the leaflet of *C. obtusifolia*. Conversely, 40 conidia were needed in each 2-μl water droplet to establish 100% infection. The water droplets still had not evaporated when the plants were transferred back to the high humidity (60–80% RH) greenhouse. The use of the emulsion lowered the I_{50} for established infection to a value 100 times lower than that required with water (Fig. 1). After 5 more days all the "not established" sites still remained small, whereas the "established" infections continued to expand.

Infection by *A. cassiae* at low relative humidity. Under low humidity (50% RH), there was more than a 1,000-fold difference in I_{50} between application in water and in emulsion (Fig. 2). Only water droplets with 200 or more conidia gave even small hypersensitive-type lesions, but there was no establishment of infection even at 500 conidia per droplet. Some infections became established in water at 1,000 conidia per droplet, but 100% established infection was impossible to achieve, as it was not possible to suspend more conidia per droplet. There was no difference in the percent established infection between 5 days (Fig. 2) and later periods (data not shown). None of the small lesions developed into established infections after 5 days. Although the inoculum threshold at lower humidity was not completely suppressed when conidia were inoculated in emulsion, a vast majority of single-conidium emulsion droplets did develop a lesion. Thus, the threshold was reduced to near one conidium per droplet when the invert emulsion was used.

Intensity of infection. Data presented in the humidity experiments might suggest that the emulsion supplied only a superior environment and, thus, reduced the threshold to one conidium per infection site. The intensity of infection by 2-μl droplets bearing varied numbers of conidia was compared (Fig. 3). The area of lesions always was greater with conidia applied in emulsion than in water under both high and low humidity conditions. We could find no infection when the ATCC isolate of *A. cassiae* was inoculated on *C. obtusifolia* in 2-μl water droplets containing up to 500 conidia per droplet. At most, only a hypersensitive response was induced with this isolate. When the seemingly avirulent ATCC isolate was applied to leaves of *C. obtusifolia* in the invert emulsion, 100% infection was obtained with one conidium per droplet (Fig. 4). Thus, the emulsion not only abolished the threshold, it enhanced the intensity of infection and increased virulence.

Infection of *D. stramonium* by *A. crassa*. Plants of *D. stramonium* were inoculated under high humidity (100% RH for 16 hr followed by 60–80% RH) with various densities of conidia of *A. crassa* to ascertain whether the emulsion was as effective in lowering inoculum threshold of *A. crassa* as it was with *A. cassiae*. When conidia were applied in water, no droplets containing 10 conidia or fewer were infective. Initial infection was observed in about half of the sites inoculated with 20 conidia per water droplet. Eighty percent of these initial infections failed to become established. Only when there were 30 conidia per

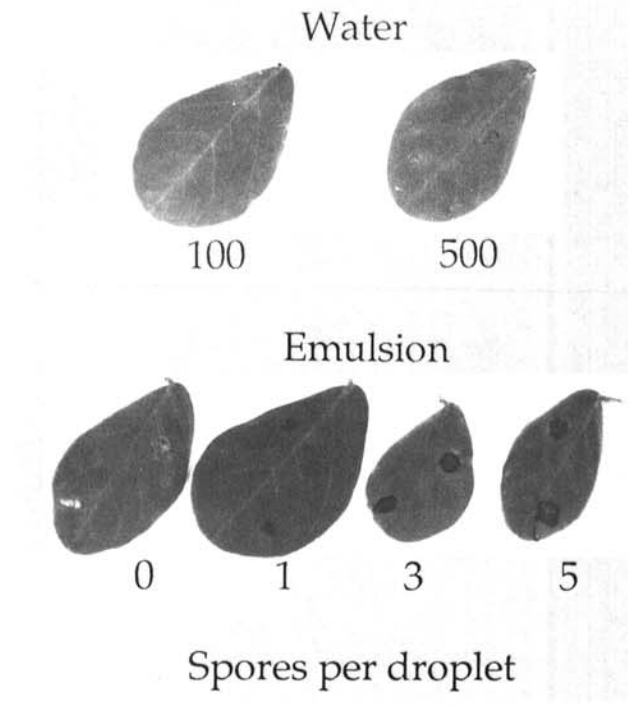

Fig. 4. Infectivity in emulsion of avirulent *Alternaria cassiae* inducing only a hypersensitive response in water. Plants of *Cassia obtusifolia* were grown as described in Figure 1. Conidial dilutions prepared of an isolate of *A. cassiae* were avirulent when applied in water. The first leaf was treated on 12-day-old plants bearing one leaf. One 2-μl droplet was applied to each lobe of each leaf, with eight replicates per data point. Plants were put in a closed dew chamber at 100% RH within the greenhouse for 16 hr and then transferred to open shelves.

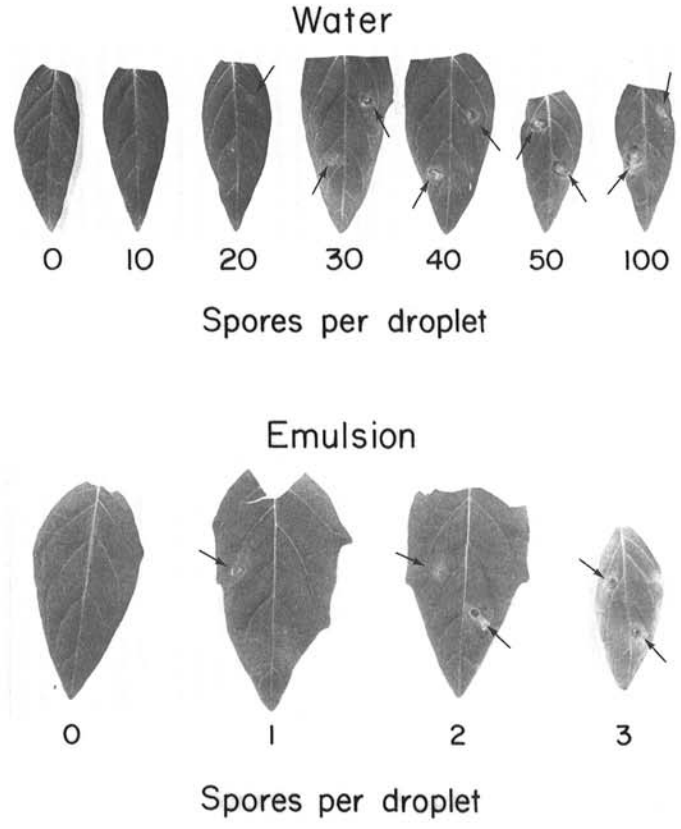

Fig. 5. Infectivity and intensity of infection of *Alternaria crassa* on *Datura stramonium*. **A,** In water; **B,** in emulsion. Plants of *D. stramonium* were grown in a controlled temperature, high-humidity greenhouse (60–80% RH) at 28 C day (14 hr) and 26 C (10 hr) night. Conidial dilutions were prepared from *A. crassa* and low dilutions were checked by plating on V-8 medium; efficiency was calculated based on colony formation on these plates. The first pair of true leaves was treated on 12-day-old plants. One 2-μl water or emulsion droplet with conidia was applied to each opposite lobe of each leaf, with four replicates per data point. Plants were put in a closed dew chamber at 100% RH within the greenhouse for 16 hr and then transferred to open shelves. Scoring was performed 5 days after treatment. Infection whereby one conidium per droplet infected 62% of the sites, the same as on plating (i.e., 100% infection by one conidium according to the Poisson distribution), was repeatedly found in three additional experiments.

droplet of water was there 100% infection and establishment (Fig. 5A). When *A. crassa* was applied in emulsion, a single conidium per droplet was sufficient to cause infection and establishment (Fig. 5B). After 10 days, all the infected sites that were defined "not established" still remained small, whereas infected sites defined as "established" continued to grow.

DISCUSSION

The lowest biologically possible inoculum threshold for *A. cassiae* on *C. obtusifolia* or *A. crassa* on *D. stramonium* was obtained when the conidia were applied in an invert emulsion; one conidium per droplet was sufficient to establish an infection under both high and low humidity. Thus, there is no basic need for a high inoculum threshold for infection with these organisms, if the threshold is determined under the ideal environmental conditions in the emulsion. The greatest differential between application in water and emulsion was found under the most severe conditions. The differential was 100-fold at high humidity and 1,000-fold at a lower humidity. For more than 30 yr, various oils have been tested and found to decrease partially, but not abolish the inoculum threshold (14). The importance of water for infection previously has been explored, including models to show its importance (5,6,9). The possibility of abolishing the inoculum threshold was not considered, but only the possibility of supplying the correct humidity so that infection could be established at high levels of inoculum.

The invert emulsion aided the growth of the pathogen even after the external humidity was no longer needed, suggesting additional roles for the emulsion beyond that of providing the necessary moisture for infection. The intensity of infection after establishment was greater with the emulsion (Figs. 3 and 5), and an isolate that had no virulence when applied in water was rendered virulent by application in the emulsion (Fig. 4). The nonvirulent isolate could only cause a hypersensitive response when applied in water, indicating that there had been germination and growth. The emulsion somehow affected establishment and infection. The emulsion, as modified from a published formulation (13), contains some relatively pure factors (paraffin wax and mineral oil), but it also contains crude biologicals (commercial edible soybean oil as well as soybean lecithin, a waste byproduct of food oil manufacture). These biologicals may contain components that affect infectivity, not just control free water around the conidia.

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