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Surface Charge Properties and Soil Mobilities of Mycoherbicidal Spores

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The contamination of public drinking water supplies from agricultural pesticides continues to be a major concern of both public and government organizations. These concerns, combined with a desire to enhance the long-term sustainability of current agricultural practices, have led to a re-evaluation of alternative pest control strategies, such as biological control (Boyette et al. 1996).

Biological herbicides are indigenous weed pathogens, usually fungi (mycoherbicides). The target zone of many soil-applied mycoherbicides is the rhizosphere. This is particularly true for *Fusarium* sp. because they are active root pathogens. Thus, there is not only a need to deliver the agents to the root zone, but also to prevent transport out of this region. The transport of the applied biocontrol agents will be governed by a complex interaction of soil physical and chemical properties, in addition to properties of the fungi (Gullino et al. 1995).

This transport is closely related to the surface charge properties of the microorganism itself. Clearly the mobility of microorganisms will be influenced by complex interactions between soil properties and chemical and physical properties of the organism itself. This research investigates 1) the surface charge properties of selected fungal spores used for biological control; 2) the influence of chemical surfactants on the surface charge properties of biocontrol spores; and 3) the soil mobility of several sustained release spore formulations in an unsaturated soils.

MATERIALS AND METHODS

Alternaria cassiae Jurair and Khan, a pathogen for control of sicklepod (*Cassiae obtusifolia L.*) was obtained as dried spores (Mycogen, San Diego, CA). Spore size averaged 20 x 90 μm for the spore body with an overall length of 150 μm (Jurair & Khan 1960). *Colletotrichum truncatum* (Schw.) Andrus & Moore, a pathogen of hemp sesbania (*Sesbania exaltata* [Rydb.] ex. A. W. Hill), was obtained from C. D. Boyette (USDA, ARS, Southern Weed Science Laboratory, Stoneville, MS) and grown on acidified potato dextrose agar. The size of the spores averaged 16 x 2.3 μm (Schisler *et al.* 1991). *Fusarium oxysporum f. sp. papaver*, a pathogen of poppy (*Papaver somniferum),* from our collection was grown on acidified potato dextrose agar. Microconidia of *F. oxysporum* ranged in size from 5-12 x 2.3-3.5 μm (Domsch et al. 1993).

The soil used was a Gilman sandy loam (coarse-loamy, mixed, hyperthermic Typic Torrifluvent, 58% sand, 24% silt, 18% clay, pH = 5.2). The soil was air-dried and sieved

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to < 2-mm prior to use in column studies. It was not autoclaved to avoid changes in soil chemical and physical properties. Sand was autoclaved at 121^0 C, 15 psi for 1 h on 3 successive days.

Hydrophobicity was determined by hydrophobic-interaction chromatography (HIC) (Gannon et al. 1991). An octyl-Sepharose gel (Sigma Chemical Company, St. Louis, MO) was washed with either 4 M NaCl or de-ionized water (6 x 1 mL) after packing in triplicate glass wool-plugged Pasteur pipettes to a final bed volume of 2 mL. The fungal spore suspension (1 mL) was applied to the gel bed and eluted with six 1 mL aliquots of 4 M NaCl or de-ionized water. Because solutions of low ionic strength are unfavorable for hydrophobic binding, the possibility that retention of the cells by the HIC column resulted merely from filtration was tested by elution of the columns with de-ionized water. The percentage of hydrophobicity is expressed as follows: # cfu (colony forming units) in the eluate / # cfu in 1 mL of the original fungal suspension.

Net surface electrostatic charge was determined by electrostatic interaction chromatography (ESIC) as a measure of the affinity of fungi for cation- or anion-exchange resins (Pederson 1981). Pasteur pipettes plugged with glass wool were packed with either CM- Sepharose CL-6 B cation exchanger or DEAE-Sepharose CL-6 B anion exchanger (Sigma) to a final bed volume of 2 mL. Triplicate columns of the two resins were washed with 0.9% NaCl (6 x 1 mL). Fungal suspension (1 mL) was added to the columns, and the spores were eluted with six 1 mL aliquots of 0.9% NaCl.

The influence of surfactants on spore mobility in the hydrophobic resin was studied by adding Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma Chemical Co.) at a rate of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0% (w/w).

Granules containing *F. oxysporum* inoculum were prepared by modifications of the Pesta method of Connick *et al.* (1996). Formulation A consisted of 32 g of a durum wheat flour (semolina), 5 g kaolin clay and 22 g of an aqueous suspension containing 3.2×10^8 cfu g^{-1} of *F. oxysporum*. Formulation B contained 32 g durum wheat flour, 2 g kaolin, 3g activated carbon (decolorizing, neutral, Fisher Scientific, Pittsburgh, PA), and 22g of *F. oxysporum* suspension. Formulation A and B granules contained 3.7×10^7 cfu g^{-1}.

The extractability of spores of *F. oxysporum* was determined from sand and soil samples and control de-ionized water solutions. Spores were grown in 12-well plates. Pesta granules (0.2 g) were placed on top of 2 g of sand and 0.5 mL water in each well. Five wells were used with both formulations of Pesta granules and two wells were left empty. The plates were covered but not sealed. After a 7-d period of incubation under diurnal fluorescent light at 25°C the five wells containing the samples were combined and washed with 100 mL water. From this suspension, containing some sand, an uncontaminated stock solution was prepared. The number of cfu was determined in the stock solution by dilution plating on acidifed potato dextrose agar (Daigle et al. 1997). Sand (50 g) was placed in a 250 mL Erlenmeyer flask with 100 mL water and 10 mL of the stock spore solution. The flasks were shaken at 200 rpm at room temperature for 2 hours. This procedure was triplicated and the number of spores in suspension was determined by dilution plating (3 plates per sample for a total of 9 determinations). The same procedure was followed with soil. The control consisted of the stock solution and 150 mL water. The number of cfu retained by either the sand or soil was calculated by dividing the cfu extracted from the sand/soil suspension by the number calculated to be present in the 110 mL solution.

Duplicate columns of clear plexiglas pipe, 5-cm ID x 30-cm long, were split longitudinally and resealed with silicone sealant and duct tape to allow disassembly and sampling of the column after leaching. The bottom of each column was covered with Whatman #4 filter paper, supported by 200-mesh stainless steel wire cloth. Each column was packed to a depth of 25 cm with either sterile sand or a single soil horizon with bulk densities of 1.5 g cm^{-3}. This gave pore volumes of approximately 110 mL for both the sand and the Gilman soil. The pore volume approximates the total void volume present in the soil after correction for moisture content. Prior to application of the granules, the columns were saturated with tap water via capillary action and allowed to drain for 24-h.

Granules (1 g) containing *F.* oxysporum were evenly distributed on the soil surface avoiding direct contact between granules and with the column wall to prevent bypass flow problems. A disk of wet Whatman #4 filter paper covered the granules to prevent erosion of the soil surface. The column was covered with a Petri plate and incubated for 7 d at 25°C prior to leaching. A lush growth of fungus covered each granule.

Columns were leached with 0.01 M $CaCl_2$ to help simulate the soil solution and prevent dispersion of the soil clays during the leaching procedure. The sterile sand was leached with 600 mL solution (30 cm) over a period of 5 days (6-cm day^{-1}). The Gilman soil was leached with 1200 mL of solution (60 cm) over a period of 10 days (6-cm day^{-1}). All treatments for a given soil were leached simultaneously, with the time and volume of each leaching being recorded. An aliquot (0.1 mL) was then taken at the end of the leaching period and the cfu mL^{-1} of *F. oxysporum* was determined. After leaching, the columns were allowed to drain for 72 h. The columns were then split vertically, and the soil was removed in the following sections (0-2.5, 2.5-5, 5-10, 10-15, 15-20, 20-25-cm). Each section of soil was then extracted with de-ionized water (110 mL) and the cfu mL^{-1} of *F. oxysporum* determined as described above.

Data from EIC and HIC determinations and soil recovery experiments were subjected to analysis of variance. Means were separated with Fisher's Least Significant Difference Test for recovery experiments and the LSMEANS procedure for EIC and HIC data (SAS, 1990). Data on spore distribution within, and between, each column were analyzed with a repeated measures analysis of variance, with formulation as the subject effect and depth as the repeated measure.

RESULTS AND DISCUSSION

Significant differences in spore mobility existed between the three fungal species investigated. Spores from *A. cassiae* were strongly retained (100%) by all the resins investigated, including the de-ionized water HIC test. These spores were highly hydrophobic, not readily wettable by de-ionized water, and preparation of spore suspensions was slow. Due to the high retention by all resins, significant differences were not observed between treatments and it was not possible to comment on the spore's surface properties. In addition, these spores were the largest of the three species investigated.

C. truncatum spores were most strongly retained by the anion exchange resin, followed by the hydrophobic resin (4M NaCl), the hydrophobic resin (water) and the cation resin,

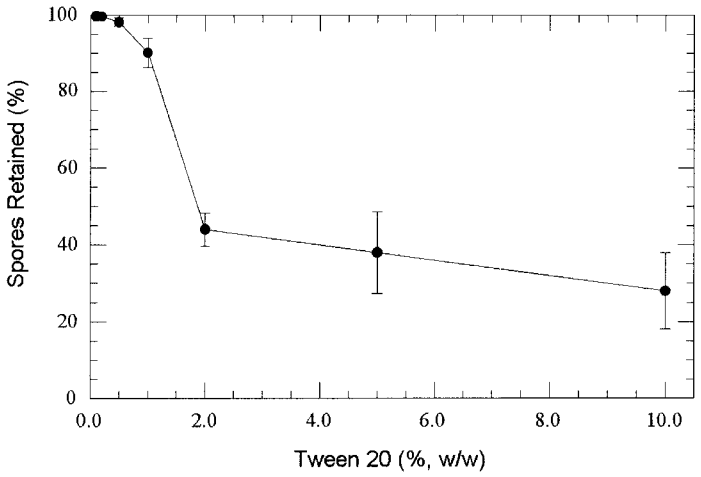

Figure 1. Effect of Tween 20 on Percent *F. Oxysporum* spores retained by hydrophobic resin.

with 100, 96.5, 84, and 17.9% retained, respectively. The strong retention by the anion resin indicates a negative surface charge. This conclusion is supported by the very weak retention by the cation exchange resin. The strong retention by the hydrophobic resin (4M NaCl) indicates that the spore surfaces also are hydrophobic in nature. However, a filtration effect was also noted as indicated by the retention noted when the hydrophobic resin was leached with de-ionized water.

All of the applied *F. oxysporum* was retained by the anion resin but none were retained by the cation exchange resin. This suggests a strong negative surface charge on the spores. The spores also were hydrophobic, although less than that of *C. truncatum* and exhibited a smaller filtration effect. The amounts retained were 100, 0, 63 and 30% for the anion, cation, hydrophobic (NaCl) and hydrophobic (water), respectively.

Addition of a surfactant to the spore suspensions dramatically altered the mobility of *F. oxysporum* spores in the hydrophobic resins (Figure 1). Tween 20 did not significantly influence mobility at levels up to 1%(w/w), with retention of *F. oxysporum* spores ≥ 90%. However, from 1.0% to 2% (w/w) *F. oxysporum* spores mobility increased very rapidly from 90% retention to 45% retention respectively. Increasing the level to 10% lowered spore retention to 28%.

Recovery of *F. oxysporum* spores from amended samples by water was dependent on both formulation and mineralogy. A significantly greater percentage of spores was recovered from amended sand compared to the Gilman soil. Averaged across formulation, the recovery from sand was 31.4% compared to 2.5% for the Gilman soil. There was also a significant difference in recovery observed between formulations. For both the sand and soil, formulation B (no carbon) resulted in a greater retention of the spores that were grown from inoculum in Pesta granules. Note that all spores were

recovered from the control sample (de-ionized water). The low recovery of the spores from soil indicates a strong retention mechanism that should result in decreased leaching of the spores. Minimally, one order of magnitude of spores was adsorbed by the soil upon mixing.

Although *F. oxysporum* spores were distributed throughout the 25 cm columns after leaching, most were recovered from the surface layer (0-2.5 cm) for both formulations (one with [A] and one without carbon [B]) (Table1). Although any difference between formulations was not significant ($P = 0.05$), a trend ($P = 0.10$) indicating greater recovery from formulation A compared to B was observed. This is perhaps not surprising because formulation A, which contained supplemental carbon, also exhibited the highest recoveries from amended soil. There was a steady decrease in spore numbers as soil depth increased.

Table 1. *Fusarium oxysporum* spores (cfu) recovered from sand columns leached with 600 mL 0.01 M $CaCl_2$.

Depth (cm)	CFU Grown from Formulation	
Formulation	A†	B
0-2.5	1.32 x 10^7a	8.8 x 10^6a
2.5-5	1.02 c 10^6a	2.88 x 10^5b
5-10	1.15 x 10^6a	2.60 x 10^5b
10-15	2.34 x 10^5a	4.52 x 10^4a
15-20	1.03 x 10^5a	1.35 x 10^4a
20-25	9.53 x 10^4a	2.72 x 10^4a

† - Means within a row, followed by the same letter are not significantly different at $P = 0.05$.

Significantly greater numbers of spores were recovered from the second and third layers (2.5-5, .0-10 cm) columns inoculated with formulation A, as compared to formulation B. For the remaining layers significant differences were not noted between formulations.

Breakthrough of the *F. oxysporum* spores appeared in the leachate from the sand columns only on the second day after the passage of approximately 300 mL of solution (Fig. 2). The number of spores found in the leachate was very small compared to the total number of spores recovered from the columns forming only 0.38, and 0.13 % for formulation A and B, respectively. Spore distribution in columns of Gilman soil was similar to that of sand columns (Fig. 2), although the initial breakthrough occurred at twice the leachate volume (~ 600 mL), indicating greater spore retention. Formulation A spores leached to a greater extent (0.41%) compared to formulation B (0.01%).

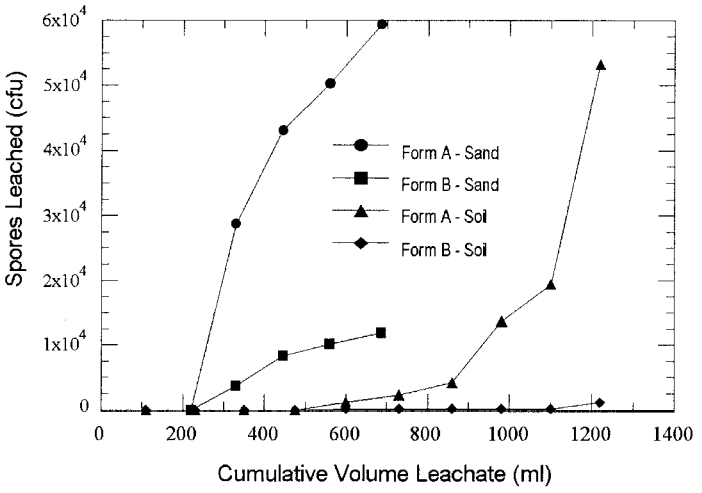

Figure 2. Breakthrough of Spores from Two Formulations of *F. Oxysporum* from Sand and Soil Columns Leached with 0.01 M $CaCl_2$.

Table 2. *Fusarium oxysporum* spores recovered from Gilman sandy loam soil column leached with 1200 ml 0.01 M $CaCl_2$.

Depth (cm)	CFU Grown from Formulation	
Formulation	A†	B
0-2.5	1.3 x 10^7a	8.8 x 10^6a
2.5-5	1.4 x 10^4a	4 x 10^3a
5-10	3.2 x 10^4a	1.5 x 10^3a
10-15	4 x 10^3a	1.8 x 10^3a
15-20	3 x 10^3a	0.8 x 10^3a
20-25	4 x 10^3a	3.6 x 10^3a

† - Means, within a row, followed by the same letter are not significantly different at $P = 0.05$difference between formulations

Results from soil column mobility studies are presented in Table 2. For both

formulations the majority of the spores produced from the Pesta granules were retained in the surface layer (0-2.5 cm) of the soil columns. There was not a significant, although a trend was observed. The formulations containing supplemental carbon (formulation A), retained a slightly higher percentage of the spores in the top three column sections.

A. cassiae spores may be poorly mobile for several reasons. These spores are large, elongated, and carry appendages. In addition, the spores are highly hydrophobic and have non-wetting waxy coats. This is typical of surface living species, whereas those at greater depths in the soil have wettable spores. These combined factors suggest that filtration and sorption effects may be responsible for the lack of mobility of these spores through the resins.

C. truncatum's size and hydrophobicity may also inhibit its mobility. Although less hydrophobic and smaller than *C. truncatum* spores, *F. oxysporum* spores had restricted mobility in Gilman sandy loam soil columns as a result of adsorptive forces. Both sand and more significantly, soil adsorbed this fungus from suspensions in water. The spores' adhesion to sand grains may be due to the spore's electrical charge.

Although in these experiments *F. oxysporum* spores moved throughout the depth of the 25 cm columns, the vertical movement in field soils is likely short distances. Rain will lead to short periods of waterlogging and subsequent drainage would carry spores a short distance before being re-deposited. Formulation adjuvants may weaken adhesion of the spores to soil and facilitate movement of spores through soil. This effect was demonstrated clearly by the effect of Tween 20 on mobility of *F. Oxysporum* spores in the HIC resin. Further research is needed to understand how mycoherbicide agents may be optimally transported through soil by formulation technology.

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