Spatial dispersal of *Metarhizium anisopliae* and *Beauveria bassiana* in soybean fields

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ABSTRACT

The dispersal and persistence of *Beauveria bassiana* and *Metarhizium anisopliae* following application in soybean fields as kaolin dust and sprays of soybean oil formulations were investigated. Fungal dispersion was evaluated from a centrally-treated area by sampling leaflets on eight transects at 20-m intervals in concentric rings up to 100 m from the central area. Density of colony-forming units (CFU) per unit leaf area was determined by washing the soybean leaflets in water containing Tween 80, and plating the aqueous suspensions on an oatmeal-dodine semi-selective agar medium. Spatial dispersal of *B. bassiana* in soybean field was significantly higher when conidia were applied as an oil formulation compared with a kaolin dust formulation. No significant differences were observed in *M. anisopliae* CFU number between sprayed or dusted plots. Oil-based formulations favor the dispersal of *B. bassiana* in soybean crops, but not the dispersal of *M. anisopliae*. Kaolin-based formulations are not suitable for *B. bassiana* or *M. anisopliae* dispersal.
Key words: entomopathogens, epizootiology, fungi, insect, persistence.

RESUMO

Dispersão espacial de *Metarhizium anisopliae* e *Beauveria bassiana* em campos de soja

A dispersão e a persistência de *Beauveria bassiana* e *Metarhizium anisopliae* mediante aplicações por polvilhamento e pulverização em óleo de soja em campos de soja foram estudados. A dispersão fúngica foi avaliada a partir de uma área central mediante a amostragem de folíolos sobre oito transectos dispostos em anéis concêntricos em intervalos de 20 m até 100 m da área central. A densidade de unidades formadoras de colônias (UFC) por unidade de área foliar foi determinada mediante lavado dos folíolos de soja em água contendo Tween 80 e plaqueamento das suspensões em água em meio seletivo dodine-aveia-agar. A dispersão espacial de *B. bassiana* no campo de soja foi significativamente maior quando os conídios foram aplicados com óleo em comparação com a aplicação por polvilhamento. Não foram observadas diferenças significativas no número de UFC de *M. anisopliae* entre as parcelas pulverizadas ou polvilhadas. As aplicações em óleo favoreceram a dispersão de *B. bassiana* na cultura da soja, mas não a de *M. anisopliae*. As misturas em caulim não são apropriadas para a dispersão de *B. bassiana* ou de *M. anisopliae*.
Palavras-chave: entomopatógenos, epizootiologia, fungos, persistência, insetos.

INTRODUCTION

In Brazilian soybean fields, the entomopathogenic fungus *Beauveria bassiana* acts as a natural control agent for *Aracanthus mourei* (Col.: Curculionidae), *Cerotoma* sp., *Diabrotica speciosa*, and *Colaspis* sp. (Col.: Chrysomelidae), and interestingly, as an endophyte, this species also provides a protective effect against plant pathogens (Ownley et al., 2008). In addition, *Metarhizium anisopliae* plays an important role in controlling soil-inhabiting insects such as Scarabaeidae (Sosa-Gómez & Moscardi, 1994). The potential of entomopathogens as microbial control agents has been studied (Johnson & Goettel, 1993), but their dispersion patterns in crops following artificial application has received little attention. More emphasis has been placed on the dissemination of entomopathogens

in natural epizootics (Fuxa, 1984). The dispersal of some naturally occurring pathogens, such as *Nomuraea rileyi* and *Entomophthora maimaiga*, has been reported (Fuxa, 1984; Hajek et al., 1996). Improved knowledge of the spread of fungi following artificial applications would contribute to the understanding of factors regulating disease epidemics in field populations of insects and provide a basis, in a broad sense, for improving the technology for treating crops with pest and plant disease control agents.

Interest in oil-based formulations of entomopathogenic fungi has increased in recent years due to improved persistence (Inglis et al., 1995a), storage (Alves et al., 2002), protection from imbibitional damage (Faria et al., 2009), and increased mortality of target insects (Bateman et al., 1993). Little is known about the persistence and survival of the fungal inocula following application in crops

or how much secondary dispersal occurs after application. Our objective was to monitor the spatial dispersal of *M. anisopliae* and *B. bassiana* in soybean fields following application of conidia of the pathogens in oil-based and kaolin-based formulations.

MATERIALS AND METHODS

Fungal material

M. anisopliae sensu lato (CNPSo-Ma12 = ARSEF 5161) was isolated from soil and *B. bassiana* (CNPSo-Bb13) from *Diabrotica speciosa* (Col.: Chrysomelidae). The identity of both fungal species was confirmed by microscopic examination. Inoculum of the fungi was produced on rice grains according to Alves & Pereira (1989). After 15 days of growth, the conidia were separated from the rice medium using a standard soil sieve number 20 (50 mesh), and stored, unformulated, at -15°C for 10 days, before further use. Viability was determined by plating conidial suspensions on potato dextrose agar amended with 0.05% (w/v) streptomycin sulphate. After 24 h of incubation at 26 ± 1°C in the dark, conidia were stained with lactophenol and cotton blue (Sigma-Aldrich, São Paulo, SP, Brazil), and 100 conidia from each of the four replicate cultures were examined for germ tube formation. Conidial concentrations for field tests were estimated from aliquots with a hemocytometer, and dry conidia were mixed with kaolin (22 to 23 μm particle size; Mineração Hori, Mogi das Cruzes, SP, Brazil) or suspended

in commercially-refined edible soybean oil (Coamo, Campo Mourão, PR, Brazil) as required.

Field assays

Two 10,000-m^2 experimental areas were established with the soybean cultivar BR16. Experimental areas were located ~13 km due north of Londrina, Paraná State (W 23º 11' 37.0", S 51º 11' 5.0", Datum WGS84). In the summer, one area was treated with a dust formulation and the other with a soybean oil-conidia formulation, and the same procedure was used in the following soybean growing season. Immediately before application, dry *M. anisopliae* and *B. bassiana* conidia were suspended at 2% (w/v) in soybean oil or at 10% (w/v) in kaolin. A high dosage of $7.8 \pm 0.6 \times 10^{10}$ viable conidia was applied to a central 10-m^2 area to facilitate detection in the subsequent days after application. Conidia suspended in soybean oil were applied with a Backpack Sprayer (model X15, Jacto, Pompéia, SP, Brazil) using a hollow cone nozzle (300 L ha^{-1}). The dry formulation was spread by hand using a plastic dispenser with 0.6-mm diameter holes. At application, plants were 65 cm high and at the R2 stage (Fehr & Caviness, 1977). Immediately after application, samples of leaves were taken from each plot as defined by the intersection of concentric circles and transects laid out at 20-m intervals in eight opposing directions, arranged on north, northeast, east, southeast, south, southwest, west, and northwest axes (Figure 1). Samples were taken immediately before and after application and at 2-day intervals up to 11
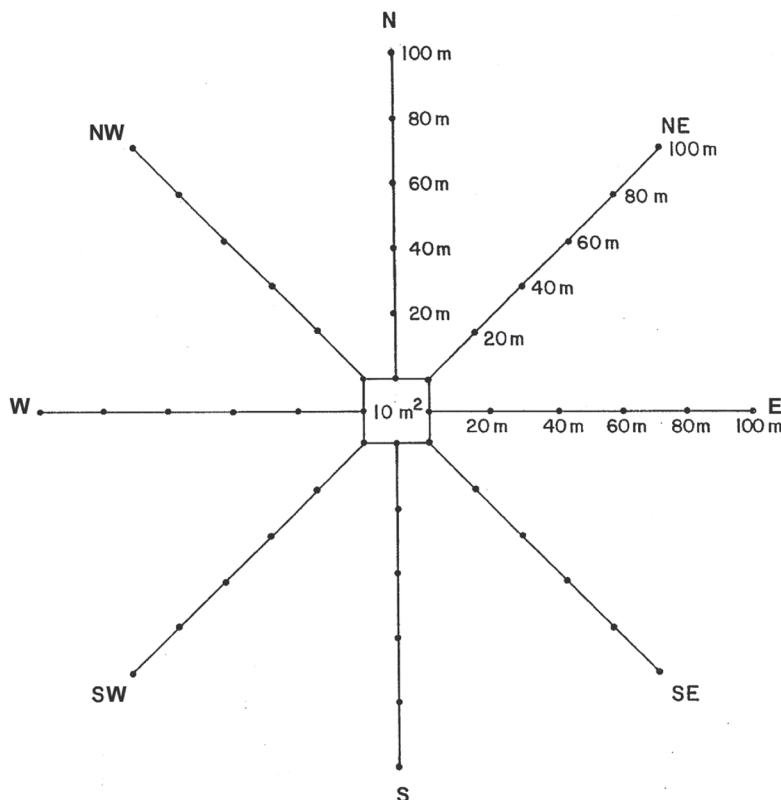

FIGURE 1 - Diagram of sample points used to evaluate dispersion of colony forming units of *Beauveria bassiana* and *Metarhizium anisopliae* after application in a central 10-m^2 square in soybean field plots.

or 12 days post-treatment. Previous studies had indicated that both fungi persist for 8-12 days in soybean fields (Sosa-Gómez & Moscardi, 1998). Mature soybean leaflets were sampled, dried with silica gel to preserve the inocula, and stored at - 20°C up to 4 months until assessed for colony

forming units (CFU) (Hedgecock et al. 1995). At each sampling date, we collected 41 leaflets [1165 ± 197 cm² (n ± SEM)] from the central plot and from the intersections of the concentric circles and transects (Figure 2). To recover propagules of *M. anisopliae* and *B. bassiana*, each
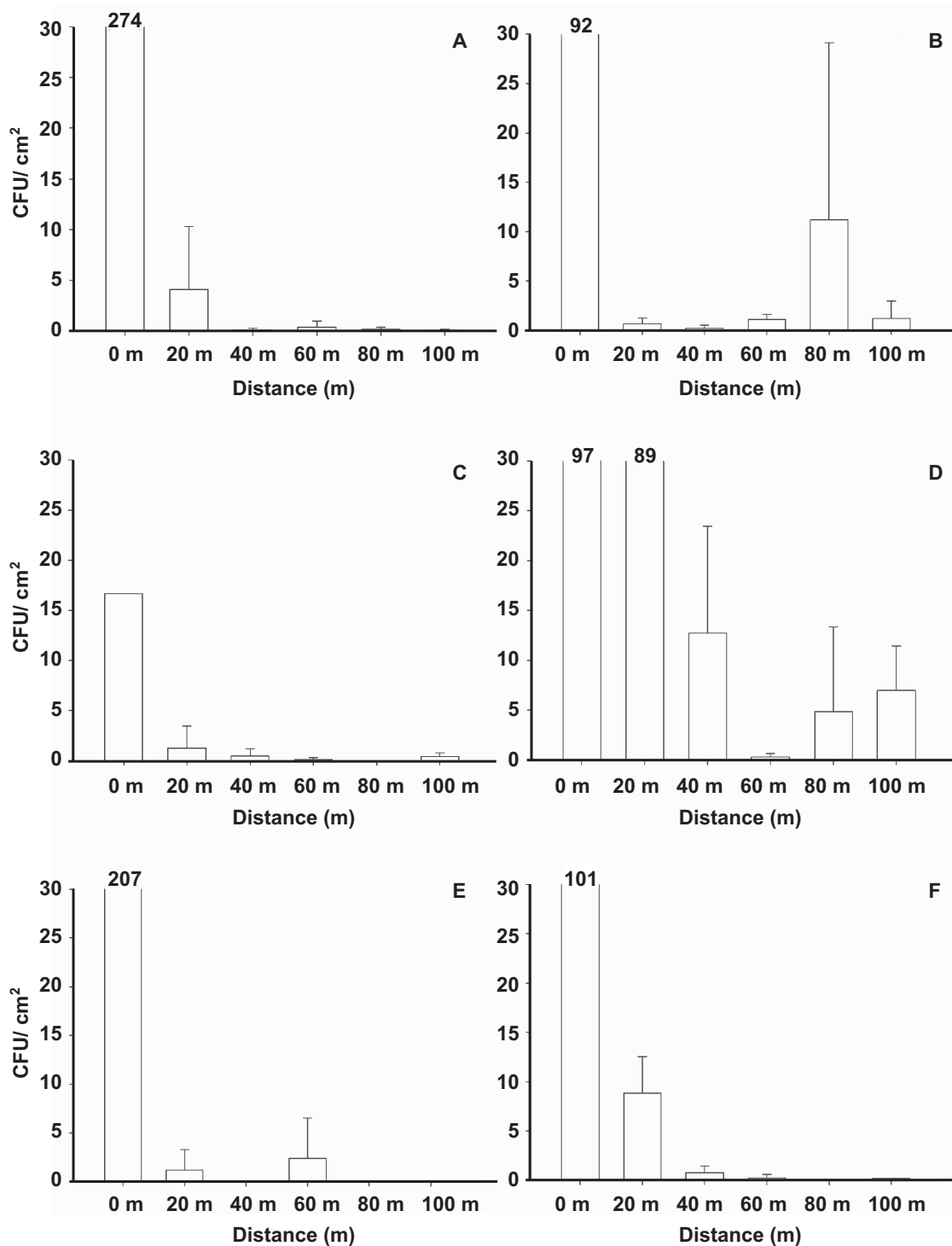

FIGURE 2 - **A.D.** Dispersion distance of colony-forming units (mean values) on soybean leaflets immediately after application of *Beauveria bassiana* and **E.F.** *Metarhizium anisopliae;* **A.C.E.** entomopathogenic fungi using kaolin as a carrier or **B.D.F.** soybean oil **A.B.** in the first and **C-F.** second growing seasons of the experiment. Error bars are 95% confidence intervals.

leaflet was vigorously vortexed for 1 min in 10 mL aqueous solution of 0.01% (v/v) Tween 80, and 0.2 mL aliquots of the suspension were serially diluted (10^{-1}, 10^{-2}, and 10^{-3}) and plated on oatmeal-dodine (Venturol, 650 g kg^{-1}, BASF, Germany) agar medium in Petri dishes (Chase et al., 1986). *B. bassiana*, *M. anisopliae* and *M. robertsii* are tolerant to the concentration of dodine used in this selective medium (Rangel et al., 2010). The washed leaflets were dried, and the area of each leaf was estimated with a leaf area meter (Model 3100, Li-Cor, Lincoln, NE, USA). Mean values were calculated after the number of CFU was quantified with a colony counter (Model EC 550A, Phoenix, São Paulo, SP, Brazil). Rain, wind speed, and temperature data were obtained from a weather station located 500 m from the field study.

Statistical analysis

The CFU per cm^2 of leaflets were analyzed by the non-parametric Mann-Whitney rank sum test with Sigmastat Software (Jandel Scientific, 1994) to determine differences among areas treated with kaolin-based and soybean oil-based formulations. Although the processed samples were numerous, insufficient data were obtained to allow a geostatistical study of surface distribution.

RESULTS

First growing season

One hour after application, there was a rain shower of 33.4 mm h^{-1}, the total rainfall on this day. Low-intensity showers (0.1, 0.9, and 1.2 mm) were registered at days 3, 4, and 5, respectively. *B. bassiana* occurred naturally in the dusted plot, where one CFU was recovered at 40 m northeast of the central area and one at 100 m on the east side, but none was found in the sprayed plot. The average CFU densities of *B. bassiana* on the foliage in the 10-m^2 central area immediately after application of the dust and oil formulations were 274 and 92 CFU cm^{-2}, respectively (Figs. 2A, 2B). Approximately 3 h after treatment, CFU were found at 47.5% of the sample sites in the plot treated with oil-formulated conidia, up to 100 m from the treated plot, whereas the area treated with powder formulation had low CFU counts at 20% of the sample points. Overall mean CFU counts were significantly greater for the oil than for the dust formulation (T = 1933, *P* = 0.032). Mean CFU counts at day 3 after application were significantly higher in the sprayed area (occurring at 37% of the sample points) compared to the dusted area (no CFU detected at the sample points) (T = 1983, *P* = 0.009). At day 5, the numbers of CFU in the two areas were not significantly different (T = 1660.5, *P* = 0.706); *B. bassiana* was present at seven and five points outside the central square in the dust- and oil-treated areas, respectively, with densities ranging between 1 and 5 CFU cm^{-2}.

Leaflets collected before fungal application in the dusted and sprayed plots did not contain detectable *M.*

anisopliae CFU. *M. anisopliae* deposits on soybean leaves immediately after application were 5 and 16 CFU cm^{-2} in the dusted and sprayed sites, respectively, and fungal dispersion was up to 60 m for both areas. The numbers of CFU on soybean leaves dusted with kaolin or sprayed with soybean oil were not significantly different at days 1, 3, 5, or 7 after application (T = 1660, *P* = 0.703; T = 1638, *P* = 0.557; T = 1758, *P* = 0.602; T = 1701, *P* = 1.000, respectively). The average number of CFU of *B. bassiana* and *M. anisopliae* in the entire experimental area was less than 1 CFU cm^{-2} (ranged from 0 to 1 CFU cm^{-2}) from day 6 to day 12 after application.

Second growing season

Estimated densities of naturally-occurring *B. bassiana* ranged up to 3 CFU cm^{-2} in pre-application samples. Foliar deposits of *B. bassiana* from kaolin and oil conidial formulations, respectively, were 17 and 97 CFU cm^{-2} (Figs. 2C, 2D). In both treatments, the number of CFU increased immediately after treatment, presenting higher density in the area sprayed with the oil formulation than in the area dusted with dry conidia in kaolin, occurring at 90% and 15% of the sample points, respectively (T = 2329, *P* < 0.001). These CFU counts were higher at 20, 40, 80, and 100 m from the central oil-treated plot than on the field treated with the dust formulation (Figs. 2C, 2D). After 24 h and over the following days, no significant differences were observed in the numbers of *B. bassiana* CFU in both areas.

The presence of 2 CFU cm^{-2} of *M. anisopliae* was detected before application at one sample point. As in the first trial, immediately after treatment, there was no significant difference between CFU cm^{-2} in plots that had been treated with *M. anisopliae* conidia in oil or dust (T = 1911.5, P = 0.051). *M. anisopliae* deposits on soybean leaves immediately after application were 207 and 101 CFU cm^{-2} in the dusted and sprayed plots, respectively, with fungal dispersion up to 100 m for oil treatment (Figs. 2E, 2F). *M. anisopliae* colonies were present at 32.5% and 5% of sample sites in the oil formulation and dust-treated areas, respectively. CFU counts ranged from 0 to 6 and from 1 to 2 counts in the oil and dust-treated areas, respectively. No significant differences were observed in CFU cm^{-2} between dusted and sprayed areas at days 3, 7, 9, and 11 after application T = 1661, *P* = 0.709; T = 1682, *P* = 0.859; T = 1662, *P* = 0.716; T = 1740, *P* = 0.720, respectively). At day 1 after application, the numbers of CFU remained high only in the central square plot that received the fungal application, with 118 and 372 CFU cm^{-2}, respectively, in the plots treated with the oil and dust formulations. In both areas, the highest CFU densities were observed only in the central plot until day 11 after application. There was no reduction in *M. anisopliae* CFU after day 2, when 9.1 mm rain fell in 5 h. The density of CFU was lower for *M. anisopliae* than for *B. bassiana*. The density of *M. anisopliae* remained below 2 CFU cm^{-2} until day 11, then increased to a mean of 6 and 2 CFU cm^{-2} for the kaolin and oil-treated plots, respectively.

DISCUSSION

The application of conidia suspended in soybean oil favored the dissemination of *B. bassiana*. *M. anisopliae* conidia suspended in oil or in kaolin formulations did not spread as well as *B. bassiana* outside the treated central square during the first 2-3 h after application. Spread of *B. bassiana* after application was greater than that of *M. anisopliae*. The smaller size of the *B. bassiana* conidia (2.1-2.6 µm) compared to *M. anisopliae* (5.0-7.0 × 2.0-3.5 µm) may favor its airborne dispersal and spread. Also, differences of conidial attachment to soybean surfaces could be another possible factor, considering that *M. anisopliae* conidia have a bigger surface compared to *B. bassiana*.

Numbers of recovered CFU were high for the treated central square areas at days 2 or 3 after application, but low outside these areas. Using a semi-selective dodine-based medium, Sosa-Gómez & Moscardi (1998) reported that the half-life of *M. anisopliae* and *B. bassiana* was between 1.2 and 2.9 days on soybean leaves, and this was confirmed using the same method in the present trials.

Artificial applications of *Metarhizium* conidia in the present studies increased the inocula level above naturally occurring densities reported by Sosa-Gómez et al. (2001), however, *Beauveria* increased but was in the range of natural occurrence reported previously. Inglis et al. (1995b) reported that 26.7 mm h^{-1} of rain, less than that observed in this study, could reduce *B. bassiana* conidial populations on leaves. Therefore, the 34.8 mm of rain 1 h after application may have affected leaf retention of both fungal species in the first field season.

According to Garcia & Ignoffo (1977), the minimum wind speed to dislodge conidia of *Nomuraea rileyi* (another clavicipitaceous fungus with dry airborne conidia) from dead larvae was 2.7 km h^{-1}. These findings suggest that the wind speeds registered during the present field studies (6.5 km h^{-1}, 3-9 on average) were sufficient to disperse conidia of *M. anisopliae* and *B. bassiana* in the soybean foliage. Our observations during the first three days after application that CFU densities were greater on leaflets from the north and west sectors compared to the other sectors are possibly attributable to prevalence of winds from the east, south, and southeast sides.

Based on the weather station records, the reported dispersal and survival of the entomopathogens in the soybean field plots took place during weather that was generally warm (17-34°C) and sunny. Cloudiness occurred mainly in association with the rainshowers. Specific effects of microclimatic variables (e.g. temperature, irradiance, water potentials) on populations of the entomopathogens on soybean foliage, however, are not well understood. Conidia remaining on the boundary layer, defined as the transition zone above the leaf surface, are exposed to localized microclimate conditions, which are difficult to evaluate in relation to spore population dynamics (Southwick & Ferro, 1984; Jaronski, 2010).

The beneficial effects of mineral and vegetable oils on entomopathogenic fungi include a protective effect against ultraviolet light (Moore et al., 1993) and a greater temperature tolerance than that observed for aqueous suspensions (Hedgecock et al., 1995). In addition, oil suspensions improved medium-term storage of *M. anisopliae* conidia (Alves et al., 2002) and enhanced infectivity of conidia (Prior et al., 1988; Bateman et al., 1993). The oil formulation significantly affected phylloplane CFU counts in all *Beauveria* field experiments conducted. In addition, no visible phytotoxic effects were observed in the sprayed plants. Oil-based formulations of conidia seem to have several advantages over dust formulations for field applications. Formulations of the biocontrol agents with carriers that avoid clumping and favor minimal droplet size may increase wind dispersion of conidia in the crop and consequently, increase the possibility of insect infection or endophytic establishment. However, more extensive studies are needed regarding the physiological interactions of oils at the phylloplane on conidia or hyphal material of entomopathogenic fungi in order to improve their field persistence for pest or disease control.

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