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SHORT COMMUNICATION

Effects of conidial densities and spray volume of *Metarhizium anisopliae* and *Beauveria bassiana* fungal suspensions on conidial viability, droplet size and deposition coverage in bioassay using a novel bioassay spray system

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A newly developed spray tower was used to characterise droplet distribution and coverage of conidial suspensions of *Metarhizium anisopliae* ATCC 62176 and *Beauveria bassiana* NI8 with different spray volumes. ATCC 62176 and NI8 had different spray models which could be caused by the surface physicochemical characteristics of the strains and conidia.

Keywords: bioassay; conidial viability; droplet distribution; *Beauveria bassiana*; *Metarhizium anisopliae*

A novel bioassay system was recently developed to investigate the effectiveness of two biocontrol agents, *Metarhizium brunneum* (formerly *M. anisopliae* F52, Bischoff, Rehner, & Humber, 2009) and *M. anisopliae* ATCC 62176, for the control of the red imported fire ant (Jin, Streett, Huang, & Ugine, 2012). The apparatus includes a top mounted high-efficiency particulate air filter, a vertical cylindrical chamber and a turning table on the bottom which is driven by an AC motor. Compressed air is pumped into a 1/4J air atomising nozzle that has both liquid and air feeds. A precise amount of a biopesticide formulation can be injected into the nozzle by a micropipette and sprayed straight down as a fine mist onto the target. The target insects are on either a vertical or flat surface in a spray arena on the top of a turning table. This bioassay system is highly sensitive and reliable using two *Metarhizium* species against the imported fire ant (Jin et al., 2012).

Although this system possesses all the required features for a bioassay system, further studies are needed to investigate if this bioassay system has adverse effects on the conidial viability during the spray employing a variety of entomopathogenic fungal suspensions with different conidial densities. In addition, the factors that affect the distribution of droplet size and deposit coverage of a sprayable formulation of a biocontrol agent are also important in predicting future bioassay experiments.

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Water sensitive paper (WSP) cards are frequently used for visual representation and laboratory analysis of droplet size and distribution for pesticide tests (Nansen, Hinson, Davidson, Vaughn, & Gharalari, 2010; Thomson & Lyn, 2011). WSP has a coating which stains dark blue by aqueous droplets. However, droplet stain diameters must be adjusted through the use of a spread factor to determine the actual droplet size associated with the stain (Thomson & Lyn, 2011). This study was conducted to investigate (1) conidial viability changes during the bioassay spray employing a variety of suspensions of two entomopathogenic fungi, *M. anisopliae* and *Beauveria bassiana* and (2) the effects of conidial density and spray volume of these fungal suspensions on the droplet size and deposit coverage using WSP cards.

Metarhizium anisopliae ATCC 62176 (ESC 1) (Dubois, Lund, Bauer, & Hajek, 2008) and *B. bassiana* NI8 were used in this study. NI8 was provided by the Southern Insect Management Research Unit, USDA-ARS, Mid South Area, Stoneville, Mississippi, and ATCC 62176 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The fungi were first grown on potato dextrose agar (PDA) plates at 26°C for one week until densely sporulated. Aerial conidia were harvested by flushing with 10 ml of 0.01% Ethal TDA (polyoxyethylene tridecyl ether, Ethox Chemical, LLC, Greenville, SC) with different hydrophilic–lipophilic balance (HLB) numbers. TDA surfactant with a HLB number 10 was used for ATCC 62176 (Jin, Grigas, Johnson, Perry, & Miller, 1999) and the HLB number 8 was used for NI8 (Jin, Streett, Dunlap, & Lyn, 2008). Subsequent dilutions were made in sterile deionised water to achieve conidial densities of 1×10^7, 1×10^8 and 1×10^9 conidia/ml of each fungus.

A spray system developed by Jin et al. (2012) was used to conduct this study. The spray tower air pressure gauge was set at 69 kPa, and air flow to the nozzle was adjusted to 10 L/minute. These same settings were previously used by Jin et al. (2012) with success. Three conidial concentrations, 1×10^7, 1×10^8 and 1×10^9 conidia/ml of each fungus, were sprayed in combination with four different volumes, 25, 50, 75 and 100 μl beginning with the least concentrated and the lowest volume (10^7 conidia/ml $\times 25$ μl) moving up to the most concentrated suspension $\times$ highest volume (10^9 conidia/ml $\times 100$μl). There were 24 spray combinations and each was repeated five times.

Experiments were conducted to determine if there was any adverse effect during the spray on the conidial viability. Germination of ATCC 62176 and NI8 was tested before the spray using the methods described by Jin et al. (2008). During each spray, a Petri dish containing PDA medium (100 mm $\times$ 15 mm) was placed in the centre of the turning table which was set still to collect conidia and to reduce the spread factor. Following the spraying, each plate was sealed with a strip of parafilm 'M' and then kept at room temperature for 12 hours. Germination rate was determined using a light microscope. Successful germination was recorded when the germ tube was present and equal to or longer than the conidial length by counting 200 conidia each plate.

To evaluate the distribution of droplet size and deposit coverage, a WSP card (76×52 mm^2, Spraying Systems Co., Wheaton, IL) was placed in the centre of a 100×15 mm Petri dish which was then placed in the centre of the stationary turning table on the bottom of the spray tower. Following each spray, the WSP card was removed using forceps as soon as the card dried and kept in a plastic bag for later evaluation. All treated WSP cards were scanned using a Portable Scanning System

for Spray Deposit Qualification (Zhu, Salyani, & Fox, 2011). The distribution of droplet size was expressed as volume median diameter (vmd), representing 50% of the spray volume or mass contained in droplets smaller than this value. Vmd is the most important parameter that characterises the performance of a spray system. Percent coverage on the WSP card is a relative ratio of sprayed deposit area to the card area. This is obtained by scanning a card to locate the area of each droplet within the scanned portion, summing those areas and dividing the total by the card area scanned. This parameter is also an important indicator of the combination of experiment setting and treatment.

All data except viability were natural log transformed and analysed using the general linear model from SAS 9.2 (Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2006). Independent variables were strains, conidia density and spray volume. The dependent variables were vmd and ln vmd, per cent coverage and ln per cent coverage as well as viability (germination percentages). Means for all dependent variables were natural log transformed. ln vmd and ln per cent coverage were separated by Duncan's Multiple Range Test ($\alpha = 0.01$).

The germination of both fungi were 99% or higher before and after spraying regardless of conidial density and spray volume. Bioassays employing a spray tower with a high gear atomizer may cause viability loss of conidia due to heat generated by atomising or high pressure from the compressed air. Our results showed that this newly developed bioassay system had no adverse effects on viability of the two tested fungi regardless of conidial densities and spray volumes.

Modelling and data collection are critical for obtaining accurate and validated models to predict the behaviour of pesticides through aerial application in forests and fields (Teske & Thistle, 2004). The same concept can also be applied to bioassay of a biopesticide in the laboratory. Employing the WSP cards in bioassays enabled us to collect data for modelling studies. For ATCC 62176, natural log transformation of vmd and per cent coverage produced better distribution models of droplet size ($R^2 = 0.73$) and coverage ($R^2 = 0.84$) than without transformation ($R^2 = 0.55$ and 0.74, respectively) (Table 1). Conidial densities of tested suspensions of ATCC 62176 had no effects on vmd, ln vmd, per cent coverage and ln per cent coverage. The effects of spray volumes of ATCC 62176 conidial suspensions on the distribution of droplet size and coverage were highly significant regardless of the transformation ($p < 0.0001$). However, NI8 performed differently. Although natural log transformation of vmd produced a better distribution model of droplet size ($R^2 = 0.81$) than the model produced without transformation ($R^2 = 0.68$) (Table 1), there was no difference between per cent coverage and ln per cent coverage, $R^2 = 0.80$ and 0.81, respectively. Conidial densities of tested suspensions of NI8 had a significant effect on ln vmd, per cent coverage and ln per cent coverage ($p < 0.01$). Spray volume had highly significant effects on vmd, ln vmd, coverage and ln coverage ($p < 0.0001$) regardless of the conidial densities. There was a significant difference between *M. anisopliae* and *B. bassiana* ($p < 0.0001$) in their effects of conidial densities on droplet size distribution and deposition coverage in bioassay using our bioassay spray system. Water-based formulations of any biopesticide using conidia or any other spores as active ingredient are suspensions, not solutions. Conidia of both *M. anisopliae* and *B. bassiana* are highly hydrophobic and have different hydrophobicity (Jin et al., 2008, 2009). Conidia of *B. bassiana* have higher hydrophobicity than conidia of *M. anisopliae*. Conidia of *M. anisopliae* can be wetted much faster than

Table 1. Statistical analysis: effects of conidia density and spray volume of suspensions of *Metarhizium anisopliae* ATCC 62176 and *Beauveria bassiana* NI8 on volume median diameter (vmd), ln vmd, per cent coverage and ln per cent coverage employing a novel bioassay system.

	vmd ($R^2 = 0.55$)		ln vmd ($R^2 = 0.73$)		Per cent coverage ($R^2 = 0.74$)		ln per cent coverage ($R^2 = 0.84$)	
ATCC 62176	F value	$p > F$	F value	$p > F$	F value	$p > F$	F value	$p > F$
Conidia density (conidia/ml)	1.39	0.26	1.81	0.18	1.67	0.20	1.57	0.22
Spray volume (μl)	13.07	0.0001	31.04	0.0001	36.50	0.0001	67.24	0.0001
	vmd ($R^2 = 0.68$)		ln vmd ($R^2 = 0.81$)		Per cent coverage ($R^2 = 0.80$)		ln per cent coverage ($R^2 = 0.81$)	
NI8	F value	$p > F$	F value	$p > F$	F value	$p > F$	F value	$p > F$
Conidia density (conidia/ml)	3.20	0.0503	5.32	0.0085	6.41	0.0036	7.31	0.0018
Spray volume (μl)	25.61	0.0001	52.98	0.0001	49.18	0.0001	53.81	0.0001

Note: The spray tower air pressure gauge was set at 69 kPa, and air flow to the nozzle was adjusted to 10 L/minute.

conidia of *B. bassiana*. This conidial surface hydrophobicity may play an important role in explaining why conidial density has a significant influence on droplet distribution and coverage. Fungal propagules such as conidia, chlamydospores or even hyphal fragments all have completely different surface characters. Consideration must be given to the physicochemical characteristics of strains and spore types during bioassay studies of biopesticides.

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