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SHORT COMMUNICATION

Development of a novel bioassay system to assess the effectiveness of entomopathogenic fungi against imported fire ants

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A novel spray tower was developed for bioassay of biopesticide formulations. The virulence of *Metarhizium brunneum* and *Metarhizium anisopliae* ATCC 62176 was evaluated against imported fire ants. Both isolates were virulent but *M. brunneum* was more effective against imported fire ants. Results proved this apparatus was reliable, sensitive and accurate.

Keywords: spray tower; fire ant; *Metarhizium brunneum*; *M. anisopliae*; biopesticide formulation

Introduction

Imported fire ants, *Solenopsis invicta* Buren (red imported fire ant), *S. richteri* Forel (black imported fire ant) and their hybrid, are serious pests affecting humans, wildlife, crops and livestock, causing billions of dollars in losses (Vinson 1997; Wojcik et al. 2001; Sánchez-Peña, Patrock, and Gilbert 2005; Gutrich, VanGelder, and Loope 2007). The development of new technologies utilising multiple strategies as a combination of non-chemical and chemical methods to reduce fire ant populations are desirable for the management of fire ants (Williams, Collins, and Oi 2001; Drees et al. 2006). Biological control using entomopathogenic fungi may prove to be an effective management option in the Integrated Pest Management (IPM) programmes.

In the efforts to assess the efficacy of entomopathogenic fungi, Burgerjon's spray tower (Burgerjon 1956) has been used as the standardised delivery equipment by many researchers since the 1950s (Ferron and Robert 1975; Vandenberg, Ramos, and Altre 1998; Desneux, Denoyelle, and Kaiser 2006; Ugine, Wraight, and Sanderson 2007; Castrillo et al. 2008; Quesada-Moraga, Martin-Carballo, Garrido-Jurada, and Santiago-Alvarez 2008; Wraight, Ramos, Avery, Jaronski, and Vandenberg 2010). Burgerjon's spray tower was designed to uniformly spray either dust or aqueous formulations of biopesticides onto target insects which were on either vertical or flat

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surfaces on a turning plate driven by a motor under sterile conditions to conduct laboratory bioassays. This apparatus has two chambers, one is an inclined chamber connected to a vertical chamber. Compressed air is pumped into the inclined chamber by passing through water and a filter to remove contaminants. The apparatus can be lifted by a balance weight for cleaning and changing samples. The fine mist or dust of a biopesticide formulation is sprayed into the inclined chamber at a 120° angle and settles on to the targets in a vertical chamber by gravity after a distance of travel. The bottom of the vertical chamber is sealed by water to prevent contamination. The air with extra mist or dust of the biological formulation is then removed from the spray chamber and passes through an opening with flame to kill microbes before being released into outside air. It can be difficult to regulate the air pressure and adjust the spray angle that can only drive the sprayed particles from the inclined chamber right into the centre of the vertical chamber and then let them drop by gravity. Considerable amounts of sprayed formulation droplets may adhere to the wall of these two chambers, and only a portion of them can be landed on the target insects, and thus could affect the experimental efficacy and accuracy. It is difficult or impossible to use Burgerjon's spray tower to conduct bioassays employing ultra low volume (ULV) formulations. The development of filtration technology allows us to simplify Burgerjon's spray tower. A filter which is a pre-sterilised dispersible unit or an autoclavable unit with a pore size of 0.2 μm will effectively remove all fungal and bacterial contaminants. Moreover, bioassays can also be conducted in a specially designed Clean Room to avoid any contaminants. A typical clean room is a sealed environment with negative air pressure created by fans on the top to draw air into the room through a group of high efficiency particulate air (HEPA) filters around the bottom of the clean room. These HEPA filters can remove more than 99.9% of particles that are larger than 0.2 μm. There are also a group of HEPA filters on the top of the clean room that prevent microbes from being released into the outside air. This report describes a novel spray tower system and the use of this system to assess the efficacy of an entomopathogenic fungus, *Metarhizium anisopliae* against the red imported fire ant in the laboratory.

Design of the novel spray tower

The newly designed spray tower is simple, straightforward and efficient, which sprays aqueous droplets of a biopesticide straight down to land uniformly on the target insects (Figure 1). The apparatus and operation are under sterile conditions enclosed in a HEPA unit. The target insects are on either a vertical or flat surface in a spray arena on the top of a turning table driven by an AC motor (Central Scientific Co., Chicago, IL, USA). This apparatus has only one vertical cylindrical chamber, and the compressed air is pumped by an air pump (Grainger 6B313, GAST Manufacturing Inc., Benton Harbor, MI, USA) into a ¼ J air atomizing nozzle (Low-Capacity Model, Spraying Systems Co., Chicago, IL, USA) by passing through an air regulator with a condensation filter to send the contaminants and wastes to the waste pan. The ¼ J air atomising nozzle has both liquid and air feeds so that a precise amount of a biopesticide formulation can be injected into the nozzle by a micropipette and then sprayed straight down as a fine mist onto the target insects

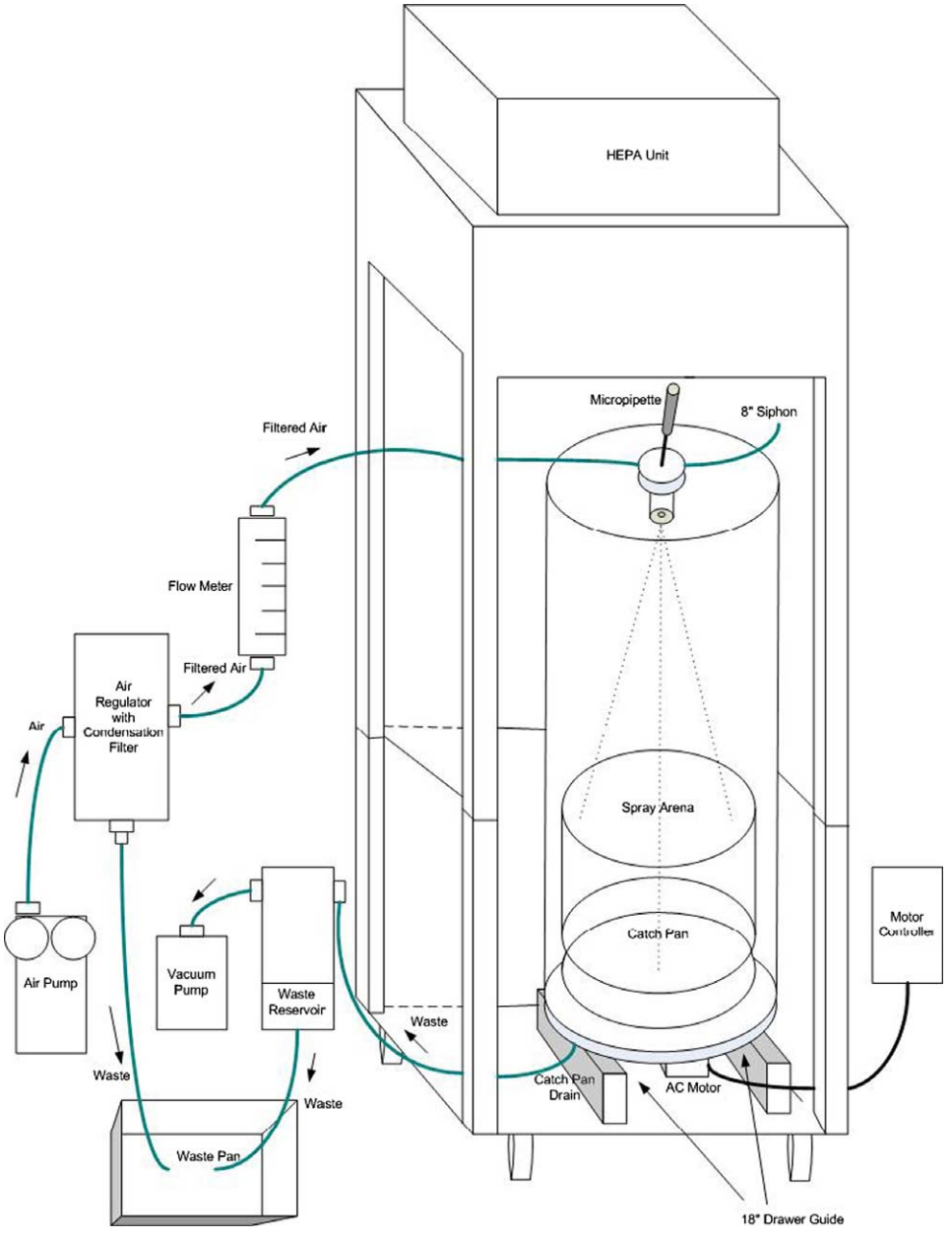

Figure 1. Schematic diagram of the novel spray tower.

in the spray arena. On the bottom of the turning plate, a catch pan drain allows contaminants and wastes to be vacuumed out through a waste reservoir to the waste pan. The tower and chamber can be cleaned and samples can be changed directly from the front end of the HEPA unit.

Fire ant collection and acclimation

Fire ants were field collected and maintained in the laboratory as described by Banks et al. (1981). Smaller workers (3–5 mm) were used in the bioassay, and groups of 35–40 workers were allowed to acclimate in 150 mm × 25 mm vented Petri dishes for 2–3 days prior to application. The Petri dish wall was coated with Fluon® (Polytetrafluoroethylene, AGO Chemicals America, Inc., Moorestown, NJ, USA) to prevent the escape of fire ants (Chen and Wei 2007). There was a darkened nest condo and a 2 ml Kimble shell vial tube containing a 10% sucrose solution with a sterile cotton stopper in the acclimation Petri dish. Fresh air entered the dish through a vent (4 cm in diameter) screen mounted in the centre of each Petri dish cover. The darkened condo was a small FALCON® Petri dish (35 × 10 mm, Becton Dickinson Labware, Becton Dickinson Company, Franklin Lakes, NJ, USA), and the cover was spray painted black. Castone White Dental Stone (Number 99043, Dentsply Trubye, York, PA, USA) was used to construct the condo station. Two hundred millilitres of Castone powder was thoroughly mixed with 80 ml of sterile water in a 400 ml beaker, and the mixture was immediately poured into the FALCON® dishes. A brood pit was set by using a spoon or scoop in the centre. Two or three tunnels were made on the side of the FALCON® dish for the fire ants to walk in and out. The sample size in each dish was reduced to 30 individuals before treatment application.

Preparation of fungal suspensions

Two commercialised *Metarhizium* strains, *M. brunneum* [previously known as *M. anisopliae* F52 and revised by Bischoff et al. (2009)], and *M anisopliae sensu lato* ATCC 62176 (Dubois, Lund, Bauer, and Hajek 2008) were used in these studies. *M. brunneum* was provided by the Southern Insect Management Research Unit, USDA-ARS, Mid South Area, Stonville, Mississippi, and ATCC 62176 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Jin, Streett, Dunlap, and Lyn (2008) and Jin, Ugine, Chen, and Streett (2009) first introduced Griffin's (1949, 1954) hydrophilic–lipophilic balance HLB number method into the formulation development of mycoinsecticides employing hydrophobic aerial conidia of entomopathogenic fungi, *Beauveria bassiana* and *M. brunneum*, from solid fermentation. The optimised surfactant reduced wetting time, increased conidia counts in suspension, and synchronised conidial germination (Jin et al. 2008). Jin et al. (2009) also found that Ethal TDA (polyoxyethylene tridecyl ether, Ethox Chemical, LLC, Greenville, SC, USA). HLB 8 solution was the superior wetting agent for *M. brunneum*. Therefore, these two strains were first grown on Sabouraud Dextrose Agar plates at 26°C for two weeks, and then conidia were washed by 0.01% Ethal TDA HLB 8 solution to supply a fresh conidial preparation for each study.

In order to measure the germination of harvested conidia, a sterile cotton swab was used to touch the conidia and placed in a test tube containing 3 ml of 0.01% Ethal TDA HLB 8 solution. The test tube and the cotton swab were vortexed for 10 seconds. The swab was then used to spread the conidia onto one quadrant of a Potato Dextrose Agar (PDA) plate. A new sterile swab was used to spread the conidia from one part of the PDA plate to the next until the third part where the germination rate could be determined. The plates were incubated at 26°C for 18

hours, and successful germination was recorded when the germ tube was present and equal to or longer than the conidial length. Three plates were counted by observing 150 conidia from several areas on each plate at 200 × with a light microscope. The germination percentages of the conidia used for preparing different suspension densities was greater than 95%. Conidial densities were counted under a microscope with a hemacytometer at 400 × magnification, and a series dilution technique was employed to make seven suspensions with conidial densities of 4×10^2, 4×10^3, 4×10^4, 4×10^5, 4×10^6, 4×10^7 and 4×10^8 conidia/ml. A 0.01% solution of Ethal TDA HLB 8 was used as control. All fungal treatments were applied with our novel spray tower (Figure 1) using a ¼ J air atomizing nozzle.

Experimental design and statistical analysis

Two exposure experiments were conducted to compare different treatment methods. These treatment methods included either a direct spray application to the ants (D) or indirect exposure by allowing ants to remain on an air-dried treated surface (I) for 24 hours. The air output from the spray tower was constant at 10 psi and treatments were applied with a micropipette to the nozzle. Petri dish bottoms (150 mm × 25 mm) were used to confine the fire ants in the spray arenas and were positioned in the centre on the rotating turntable (104 rpm). The Petri dish wall was coated with Fluon®. Sixty workers were transferred to the spray arena as one replicate. Spray deposition at the surface was 0.026 μl/mm^2. After either the direct (D) or the indirect (I) treatment, the ants were transferred to clean 150 mm × 25 mm vented Petri dishes with a darkened nest condo and provisioned with a 10% sucrose solution through a saturated foam. Treated ants were maintained at 28°C and 80% RH, and dead individuals were surface sterilised with 1.0% bleach (containing 5.25% of NaClO) for 30 seconds and then rinsed with sterile deionised water. Each surface sterilised fire ant cadaver was transferred to a transparent 30 ml medicine cup (Southern Container Corp., Mooresville, NC, USA) containing a piece of Aquafoam (Syndicate Sales, Inc., Kokomo, IN, USA) on the bottom that was saturated with sterile water. Each cup was capped with a hard paper lid (LPC Corp., Fairfield, NJ, USA) and the cups were incubated at 28°C and 80% R.H. for 10 days to examine *M. anisopliae* sporulation. Numbers of sporulated fire ant cadavers were used to assess per cent mortality. All treatments in a replicate were evaluated at the same time and were repeated three times. The bioassay was a randomised complete block with a factorial treatment structure of two *Metarhizium* strains × two treatment exposures × eight conidial densities. A linear trend was used to explain the differences in conidial density by fitting mortality as a linear function of the log conidial density. A general linear mixed model with SAS software was used to perform the analysis and the least square post hoc tests to identify significant differences at 0.05 levels (Littell, Milliken, Stroup, Wolfinger, and Schabenberger 2006). The model included fixed effects for strains, exposure treatments, strains × exposure treatments and conidial densities (as log linear trend). Random effects were replicates and lack of fit (LOF). Fixed effects that allowed the conidial density trend to be different for each strain and exposure treatment were removed from the model because they were not significant ($p \geq 0.05$). Random effect for LOF consisted of different Density means that was not explained by the log linear trend for each strain × exposure treatment

combination. Although the analyses were conducted on the counts of dead ants, the graphs represented per cent mortalities.

There are criteria for the establishment of a new spray tower to be used in bioassay research projects. An excellent spray tower must possess (1) reliability, (2) sensitivity, (3) validity, (4) sterility and (5) mobility (Burgerjon 1956; FAO 2001a, b). Biological control of pests is focused on the development of microbial-based pesticides that may result in reduced usage of hazardous chemicals, and therefore bioassay results must be validated for further consideration in either discovery phase or product development phase. While reliability tells us the consistency of bioassay results generated by our new spray tower under the same experimental conditions, validity indicates the accuracy of our results.

Different methods of application were evaluated in a laboratory bioassay employing a strain of *B. bassiana* for the control of the imported fire ants by Siebeneicher, Vinson, and Kenerley (1992). Their results showed that spray application with an artist air brush was not effective and practical for conducting bioassay. Results (Figure 2a) from this study indicated that our spray tower was highly effective and reliable because the experimental results were repeatable and no significant differences ($p > 0.05$) existed among the three replications conducted at different times under the same experimental conditions. The reliability of our spray tower was further demonstrated by the fact that there were no significant differences ($p > 0.05$) between the two exposure experiments which showed the spray tower system delivered an accurate amount of conidia of *M. brunneum* or *M. anisopliae* ATCC 62176 in each application through either direct or indirect applications, and these conidia retained their viability and were highly efficacious. In this study, we would identify if there was a significant difference between the two *Metarhizium* strains against the imported fire ants.

Data analysis of our results showed that there was a significant difference ($df_{\text{numerator}} = 1$, $df_{\text{denominator}} = 27$, $F = 6.00$, $p < 0.05$) between these two biological control strains against the red imported fire ants. *M. brunneum* was more virulent than *M. anisopliae* ATCC 62176 and caused a higher mortality. Although this study included a series of conidial density with only 10-fold increase in each suspension from 4×10^2 to 4×10^8 conidia/ml, mortalities as a log linear function of conidial density was highly significant ($df_{\text{numerator}} = 1$, $df_{\text{denominator}} = 27$, $F = 748.39$, $p < 0.05$). Preliminary ANOVA allowed the slope of Mortality $= f$(log conidial density) to be different for each *Metarhizium* strain $\times$ treatment explore combinations. Based on f-test for homogeneity slopes, neither *Metarhizium* strains nor treatment explores had a significant effect on the slopes, and therefore final ANOVA used a common slope and different intercepts for each *Metarizium* strain $\times$ treatment explore combination (Figure 2b). This further proved that our novel spray tower was reliable, sensitive and accurate in conducting bioassay employing entomopathogenic fungi against imported fire ants under the same experimental conditions. The size of this spray tower is quite small (Figure 1) and sterilisation can be completed easily. Moreover, it can be moved from a laboratory to another to carry out a variety of bioassays employing different biological control agents for insect pests and plant disease management. With careful selection of air atomizer nozzles, this apparatus can be used to conduct accurate bioassays of ULV formulations of biopesticides.

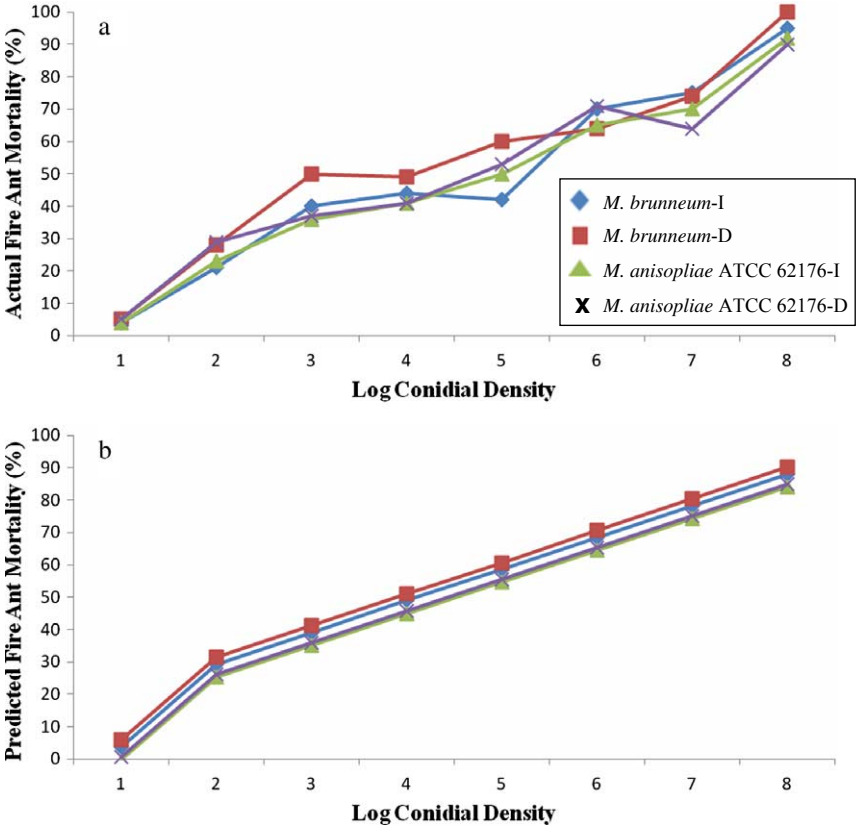

Figure 2. (a) Percent mortalities of the imported fire ant caused by a variety of conidial densities of *M. brunneum* and *M. anisopliae* ATCC 62176 with two different treatment methods, direct or indirect spray. (b) Predicted per cent mortalities of the imported fire ant should be caused by the conidial densities of *M. brunneum* and *M. anisopliae* ATCC 62176 with two different treatment methods, direct or indirect spray based on *f*-test for homogeneity slopes.

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