Evaluation of Entomopathogenic Fungi Against the Western Flower Thrips (Thysanoptera: Thripidae) Under Laboratory Conditions[1]

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Abstract Western flower thrips, *Frankliniella occidentalis* (Pergande), is an economically important pest of greenhouse production systems, and entomopathogenic fungi may be used to suppress populations of the western flower thrips. The objectives of our study were (a) to evaluate the efficacy of products containing three different entomopathogenic fungi (*Beauveria bassiana* [BotaniGard®], *Isaria fumosoroseus* [NoFly™], and *Metarhizium anisopliae* [Met52®]) against western flower thrips larvae and adults and (b) to assess effectiveness of combinations of entomopathogenic fungi and the insect growth regulator azadirachtin against western flower thrips larvae. In two laboratory bioassays western flower thrips larvae and adults were exposed to the three entomopathogenic fungi at two label rates (maximum and minimum), and two product shelf-life conditions (unexpired and expired). Bioassay 1 involved a 120-h incubation period; bioassay 2 entailed a 216-h incubation period. A third bioassay evaluated any synergistic effects of combining the entomopathogenic fungi with azadirachtin on western flower thrips larvae. Results indicated that (a) by 120 h of incubation, adult western flower thrips were more susceptible than larvae to maximum rates of unexpired *B. bassiana* and *I. fumosoroseus*; (b) unexpired products of *B. bassiana* and *I. fumosoroseus* had higher percentages of adult western flower thrips mortality than expired products at the maximum label rate; and (c) azadirachtin combined with unexpired *M. anisopliae* at the maximum label rate resulted in greater mortality of western flower thrips larvae than *B. bassiana* and *I. fumosoroseus*. However, there was no evidence of any enhanced efficacy when combining azadirachtin with *B. bassiana* or *I. fumosoroseus*.

Key Words *Beauveria bassiana*, *Isaria fumosoroseus*, *Metarhizium anisopliae*, azadirachtin, laboratory bioassay

Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), is an economically important insect pest of greenhouse-grown horticultural crops (Lewis 1997, Robb and Parrella 1995). Management of western flower thrips is difficult because of high female reproductive capacity, short life cycle, and broad host range (Fery and Schalk 1991, Jensen 2000, Seaton et al. 1997). The primary concern of greenhouse producers regarding western flower thrips is the direct and indirect damage they cause to horticultural crops (Jensen 2000, Kirk 2002, Seaton et al. 1997). Direct damage is a consequence of feeding

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with their piercing-sucking mouthparts, resulting in discoloration and deformation of flowers and foliage (Chisholm and Lewis 1984, van Dijken et al. 1994) and can have negative effects on marketability and consumer acceptance (Jensen 2000). Indirect damage is associated with adult western flower thrips vectoring tospoviruses such as *Tomato spotted wilt* and *Impatiens necrotic spot* virus (Allen and Broadbent 1986, Daughtrey et al. 1997, Stobbs et al. 1992). Due to both direct and indirect damage, greenhouse producers have a very low tolerance for the presence of western flower thrips populations in horticultural crops (Parrella and Jones 1987).

Currently, insecticides are the primary means of suppressing western flower thrips populations. However, the continuous use of insecticides during the growing season places intensive selection pressure on western flower thrips populations, consequently leading to development of insecticide resistance (Cloyd 2009). Indeed, development of resistance to a variety of insecticides with different modes of action has been documented in certain western flower thrips populations, which can impact the ability of greenhouse producers to suppress populations of western flower thrips (Broadbent and Pree 1997, Herron and James 2005, Immaraju et al. 1992, Loughner et al. 2005). Due to insecticide resistance and the limited number of effective insecticides available for western flower thrips suppression (Reitz and Funderburk 2012), there is an increased interest in the use of predatory mites, insects, and entomopathogenic fungi to suppress western flower thrips populations (Blaeser et al. 2004, Cloyd 2009, Vestergaard et al. 1995). The use of entomopathogenic fungi may be a viable option to suppress western flower thrips populations. The incorporation of entomopathogenic fungi into a plant protection strategy against western flower thrips may reduce selection pressure from insecticide applications, thus potentially decreasing the incidence of insecticide resistance (Kivett et al. 2015).

Entomopathogenic fungi, which are typically applied as sprays to the plant foliage, may reduce selection pressure due to the unique mode of action by which they induce insect mortality. Generally, fungal spores germinate and hyphae penetrate the insect cuticle by enzymatic degradation and mechanical pressure (Clarkson and Charnley 1996, Gillespie and Claydon 1989), and then the fungus consumes the internal tissues of the host insect (Hall et al. 1984). In general, mortality depends on host susceptibility, and is dose-dependent, with higher spore concentrations resulting in quicker kill and higher insect mortality (James et al. 1998, Vestergaard et al. 1995).

A potential disadvantage of entomopathogenic fungi is their relatively short shelf life compared to conventional insecticides (Osborne and Oetting 1989). For instance, according to the label for *Isaria fumosoroseus* (Wize) Brown & Smith strain FE 9901 (NoFly™, Novozymes, Salem, VA), the product performs best when used within 3 mo of the manufactured date. The label for *Metarhizium anisopliae* (Metschnikoff) Sorokin strain F52 (Met52® EC, Novozymes) indicates that the product should be used within 1 yr and/or by the expiration date noted on the label. The *Beauveria bassiana* (Bals.) Vuill. strain GHA (BotaniGard 22WP, BioWorks, Inc., Victor, NY) label only provides an expiration date. There is no information available on the efficacy of these products beyond the manufacturers' recommended expiration date. Given their relatively short shelf lives, it is important to understand how products associated with entomopathogenic fungi may perform

when used some time after the expiration date as efficacy (virulence) may decline over time.

Furthermore, response to entomopathogenic fungi exposure differs between larvae and adults of western flower thrips (Vestergaard et al. 1995). Adults would be expected to be more susceptible to entomopathogenic fungi because they spend more time feeding in flowers than larvae and, in general, the high relative humidity creates ideal conditions for fungal infection of adults (Cloyd 2009, Murphy et al. 1998). Moreover, larvae have thicker cuticles and molt, whereas adults do not molt (Maniania et al. 2001, Shipp et al. 2003, Ugine et al. 2005, Vestergaard et al. 1995), which could explain lower larval susceptibility as fungal spores may not have enough time to successfully penetrate the cuticle before ecdysis (Maniania et al. 2001). Azadirachtin, an insect growth regulator (ecdysone antagonist), delays the molting process of larvae, thus presumably allowing more time for hyphal penetration through the cuticle (Akbar et al. 2005, Hernandez et al. 2012, Schmutterer 1990). However, there is limited information available on the efficacy of combining entomopathogenic fungi and azadirachtin against western flower thrips larvae.

The first objective of this study was to evaluate the efficacy of products associated with entomopathogenic fungi against western flower thrips larvae and adults under laboratory conditions, considering different label rates (maximum and minimum), and assessing product effectiveness beyond the designated expiration date. The second objective was to assess the efficacy of combining entomopathogenic fungi with azadirachtin against western flower thrips larvae. Results from the experiments reported here are intended to serve only as a proof-of-concept in order to assess the biological activity of the entomopathogenic fungi. Therefore, additional studies are needed in actual greenhouses before providing relevant recommendations to commercial greenhouse producers.

Materials and Methods

Rearing western flower thrips. Colonies of western flower thrips were maintained in the Department of Entomology at Kansas State University (Manhattan, KS). Both larvae and adults were reared in plastic containers (11.5 × 17 × 9 cm [length × width × height]) ventilated with insect screening 50 × 24 (0.2 × 0.8 mm; Greentek, Edgerton, WI) and placed on a laboratory bench under a 24:0 (light:dark) hour photoperiod at 25 ± 3°C. Green beans (*Phaseolus vulgaris* L.) were used as a food source. Green beans were purchased from a commercial supermarket (Dillons, Manhattan, KS), soaked in soapy water for 10 min, then rinsed with distilled water and allowed to dry. After drying, green beans were placed into a clean, ventilated plastic container where first-instar larvae began emerging from the eggs after 2 d. Western flower thrips adults were transferred to a clean container every 2 to 3 d, and provided with two to five fresh beans as a food source and/or oviposition site for adult females. Two-to-three-day-old beans were either discarded or maintained for egg-hatching. Specimens used in this research are deposited as voucher number 237 in the Kansas State University Museum of Entomological and Prairie Arthropod Research (Manhattan, KS).

Laboratory bioassays. Laboratory bioassays were designed to evaluate the effect of selected entomopathogenic fungi (*Beauveria bassiana* [Bals.] Vuill. strain GHA, *Isaria fumosoroseus* [Wize] Brown & Smith strain FE 9901, and *Metarhizium anisopliae* [Metschnikoff] Sorokin strain F52) on western flower thrips larvae and adults. Bioassays 1 and 2 were conducted using a three-way factorial treatment design consisting of all combinations of the three entomopathogenic fungi (*B. bassiana*, *I. fumosoroseus*, and *M. anisopliae*), two label rates (maximum and minimum), and two shelf-life conditions: (unexpired [prior to the manufacturer's designated expiration date] and expired [sometime beyond the expiration date]). There was also a water control, resulting in a total of 13 treatment groups. For the first bioassay, *B. bassiana* strain GHA (BotaniGard 22WP, BioWorks, Inc.) was 19 mo past the expiration date on the label, and both *I. fumosoroseus* strain FE 9901 (NoFly, Novozymes) and *M. anisopliae* strain F52 (Met52 EC, Novozymes) were 10 mo past the designated label expiration date. For the second bioassay, *B. bassiana* was 21 mo past the expiration date on the label, and both *I. fumosoroseus* and *M. anisopliae* were 12 mo past the label-designated expiration date.

Suspensions of the entomopathogenic fungi *B. bassiana* and *I. fumosoroseus* were prepared by adding the maximum and minimum label rates (0.48 g and 0.24 g, respectively) to 200 ml of water. Suspensions of the entomopathogenic fungus *M. anisopliae* were prepared by adding the maximum and minimum label rates (0.5 ml and 0.12 ml, respectively) to 200 ml of water. Tween™ 20 Enzyme Grade (Fisher Scientific; Pittsburgh, PA) was added to *I. fumosoroseus* suspensions at a rate of 30 μl per 200 ml of water to help disperse the spores in water and counteract formulation issues (Kivett 2015).

Glass Petri dishes (100 × 15 mm) were lined with a 9-cm-diameter piece of P8 filter paper (Fisher Scientific), and treated with 1 ml of the assigned treatment group suspension or a water control dispensed uniformly around the entire filter paper with a sterile 1-ml plastic syringe. Immediately afterward, 15 western flower thrips adults (mixture of sexes and ages) and 15 second-instar larvae (2 to 4 d old) (Bioassay 1) or 15 western flower thrips adults (Bioassay 2) were randomly placed into each glass petri dish pretreated with the appropriate treatment group. A 2.5-cm piece of green bean, cut lengthwise into quarter sections, was provided as a food source and replaced every 2 d. A foam circle (Darice, Inc., Strongsville, OH) was fitted into the lid of each glass Petri dish, and the lid was sealed with Parafilm® (Pechiney Plastic Packaging Company, Chicago, IL) to prevent western flower thrips from escaping and insure a high relative humidity. For the duration of Bioassays 1 and 2, petri dishes were maintained in a controlled environmental growth chamber (Conviron, Winnipeg, Canada) under 0:24-h (light:dark) photoperiod and 24 ± 3°C. In each bioassay, the number of dead western flower thrips larvae and adults were recorded every 24 h during the 120-h (Bioassay 1) or 216-h (Bioassay 2) incubation period (time allotted for entomopathogenic fungi to infect and kill western flower thrips larvae and adults). The extended incubation period for Bioassay 2 was intended to take into account the delayed activity of the entomopathogenic fungus *M. anisopliae* observed in Bioassay 1. In order to determine mortality, individuals were gently prodded with a fine-tipped paintbrush. Any western flower thrips larvae or adults that did not move immediately were considered dead. The bioassays were set up as a randomized complete block design in which a block was defined as a set of 13 glass Petri dishes representing all treatment groups managed

simultaneously. Complete blocks of 13 petri dishes each (one per treatment group) were staggered, to be initiated, at 2-d intervals in order to facilitate logistical execution of the bioassays. There were a total of six blocks for Bioassays 1 and 2.

Bioassay 3 was designed to evaluate whether the combination of azadirachtin with each entomopathogenic fungus product would enhance mortality of western flower thrips larvae. Treatments included each of the entomopathogenic fungi (*B. bassiana*, *I. fumosoroseus*, and *M. anisopliae*) with and without azadirachtin (AzaGuard™, Biosafe Systems, LLC, East Hartford, CT), as well as azadirachtin alone in the absence of any entomopathogenic fungi, and a water control, for a total of eight treatment groups. For Bioassay 3, all entomopathogenic fungi products used had an unexpired shelf life; that is, prior to the manufacturer's designated expiration date and applied at the maximum label rate. Suspensions of *B. bassiana* and *I. fumosoroseus* were prepared by adding the maximum label rate of 0.48 g to 200 ml of water. Suspensions of *M. anisopliae* were prepared by adding the maximum label rate of 0.5 ml to 200 ml of water. Tween 20 Enzyme Grade was added to the *I. fumosoroseus* suspension at a rate of 30 μl per 200 ml of water to aid in dispersing the spores in the mixture as requested by the manufacturer. Treatments that included azadirachtin were processed by adding the highest label rate of 0.25 ml of azadirachtin to 200 ml of the entomopathogenic fungi mixtures. Treatments were randomly assigned to glass petri dishes prepared as described for Bioassays 1 and 2, and dispensed uniformly onto the filter paper. Fifteen newly emerged western flower thrips second-instar larvae were placed into each petri dish. For the duration of Bioassay 3, Petri dishes were maintained at room temperature (22 ± 1°C) under 9:15-h (light:dark) photoperiod. The number of dead western flower thrips larvae in each petri dish was recorded, similar to the previous bioassays, every 24 h for the 144-h incubation period associated with the treatments. Bioassay 3 was set up as a randomized complete block design in which a block was defined as a set of eight Petri dishes representing all treatment groups handled simultaneously. Complete blocks consisted of eight petri dishes with each staggered at 2-d intervals to facilitate logistical execution of the bioassay. There were a total of six blocks in Bioassay 3.

Statistical analysis. For each bioassay, generalized linear mixed models were fitted assuming a binomial response variable defined as the number of dead western flower thrips larvae or adults at a given incubation period among those placed in each Petri dish. In Bioassay 1, separate analyses were conducted for larvae and adults. In each model, a logit link was used to connect the binomial probability of western flower thrips mortality with the linear predictor, which included the fixed effects of treatment (13 treatment groups for Bioassays 1 and 2 versus 8 treatment groups for Bioassay 3), time, and their interactions. The 24-h time point corresponding to western flower thrips adult mortality (Bioassays 1 and 2) and the 24-, 48-, and 72-h time points corresponding to western flower thrips larval mortality (Bioassays 2 and 3) were excluded from analyses due to extreme category problems (0% mortality, all individuals were alive at these time points), which prevented convergence and model fitting. In Bioassay 1, we conducted an additional analysis on data corresponding to the 12-h incubation period to compare mortality of larvae and adults. In this case, the linear predictors included the fixed effects of treatment (13 levels, consisting of combinations of entomopathogenic fungi, rate, and shelf life, as well as the water control), life stage (larvae versus

adults), and their two-way interaction. For all models, random effects in each linear predictor included the blocking factor and subsequent cross-product with treatment, in order to recognize Petri dish as the appropriate experimental unit for treatment and also as the unit of repeated measures over time.

In all cases, overdispersion was evaluated using the maximum-likelihood based fit statistic Pearson χ^2/df. There was no evidence of overdispersion in any of the models. The final statistical models used for inference were fitted using residual pseudolikelihood. A Kenward Roger's procedure was used to estimate degrees of freedom and make the corresponding adjustments to estimate standard errors. Likewise, a Laplace approximation to maximum likelihood was used to fit the final model for Bioassay 2 due to convergence problems associated with quasi-complete separation of data points for some treatments at the later time points of the incubation period. All statistical models were fitted using the GLIMMIX procedure of SAS (SAS Institute 2013) implemented using Newton-Raphson with ridging as the optimization technique. Tailored contrasts were built to assess main effects and interactions of entomopathogenic fungi, azadirachtin treatment, label rate (maximum and minimum), and product shelf-life condition (unexpired and expired), as appropriate in each case. All relevant pairwise comparisons were conducted using Bonferroni adjustments ($P \leq 0.05$) to prevent inflation of Type I error rate due to multiple comparisons.

Results

Bioassay 1. The estimated mean percentage of mortality of adult western flower thrips, after exposure to unexpired and expired entomopathogenic fungal products over the 120-h incubation period, is presented in Table 1. Treatment effects on adult western flower thrips mortality differed during the incubation period, as indicated by a significant two-way interaction between treatment and time ($P < 0.001$). More specifically, for the incubation periods of 48 and 72 h, adult western flower thrips mortality in response to any of the treatments was not significantly different from the water control. However, by 96 h and beyond, treatments with both unexpired *B. bassiana* and *I. fumosoroseus* showed greater adult mortality than the water control (Table 1). Furthermore, by 96 h of incubation, mortality of adult western flower thrips to unexpired *B. bassiana* was more than twice as much as the corresponding expired product ($P = 0.00012$), again, regardless of the label rate used. By 120 h of incubation, unexpired product of either *B. bassiana* or *I. fumosoroseus* resulted in at least a two-fold increase in mortality compared to the expired counterparts ($P < 0.01$), regardless of label rate used. Moreover, at 96 and 120 h of incubation, unexpired product of *B. bassiana* and *I. fumosoroseus* (at either maximum or minimum label rates) outperformed unexpired *M. anisopliae* associated with adult western flower thrips mortality ($P < 0.0001$; Fig. 1). In contrast, for the expired entomopathogenic fungal products, *I. fumosoroseus* provided a significantly higher adult western flower thrips mortality than *B. bassiana* ($P = 0.029$) and *M. anisopliae* ($P = 0.026$) at 120 h of incubation although there was no evidence of any treatment differences at shorter incubations (Fig. 1). Adult western flower thrips mortality associated with *M. anisopliae* was not significantly different from the water control for the 120-h incubation period evaluated in Bioassay 1 (Fig. 1).

Table 1. Estimated mean mortality (% ± SEM) of western flower thrips, *Frankliniella occidentalis*, adults after 48, 72, 96, and 120 h of incubation with unexpired and expired products of entomopathogenic fungi (*Beauveria bassiana*, *Isaria fumosoroseus*, and *Metarhizium anisopliae*) at maximum and minimum label rates (Bioassay 1).

			Hours of Incubation			
Entomopathogenic Fungi	**Rate***	**Shelf-Life Condition**	**48**	**72**	**96**	**120**
Beauveria bassiana	Max	Expired	2.2 ± 1.7	4.4 ± 2.8	10.1 ± 4.9	19.3 ± 7.6
	Max	Unexpired	3.2 ± 2.2	18.0 ± 7.2	36.7 ± 10.7**	66.5 ± 10.3**
	Min	Expired	3.9 ± 2.5	3.9 ± 2.5	3.9 ± 2.5	7.9 ± 4.0
	Min	Unexpired	3.2 ± 2.2	19.3 ± 7.5	47.1 ± 11.3**	71.8 ± 9.4**
Isaria fumosoroseus	Max	Expired	1.0 ± 0.8	2.1 ± 1.4	19.5 ± 7.8	41.6 ± 11.6
	Max	Unexpired	0.9 ± 1.0	4.8 ± 2.8	52.8 ± 11.3**	85.0 ± 6.4**
	Min	Expired	1.3 ± 1.1	1.3 ± 1.1	14.2 ± 6.2	33.8 ± 10.5
	Min	Unexpired	2.5 ± 1.6	4.6 ± 2.6	29.5 ± 9.9**	61.7 ± 11.1**
Metarhizium anisopliae	Max	Expired	3.6 ± 2.3	6.4 ± 3.4	13.4 ± 5.8	20.8 ± 7.9
	Max	Unexpired	4.7 ± 2.8	8.7 ± 4.3	9.7 ± 4.6	16.9 ± 6.9
	Min	Expired	2.1 ± 1.7	3.2 ± 2.3	5.4 ± 3.3	6.6 ± 3.7
	Min	Unexpired	0.9 ± 1.0	1.9 ± 1.5	2.8 ± 2.0	3.8 ± 2.4
Water	–	Control	1.5 ± 1.3	2.3 ± 1.7	5.7 ± 3.1	8.4 ± 4.2

* Maximum and minimum labeled rates (per 200 ml): *Beauveria bassiana* = 0.48 and 0.24 g, *Isaria fumosoroseus* = 0.48 and 0.24 g, and *Metarhizium anisopliae* = 0.5 and 0.125 ml.

** Indicates significant difference from the water control at a given time point (either 96 or 120 h of incubation).

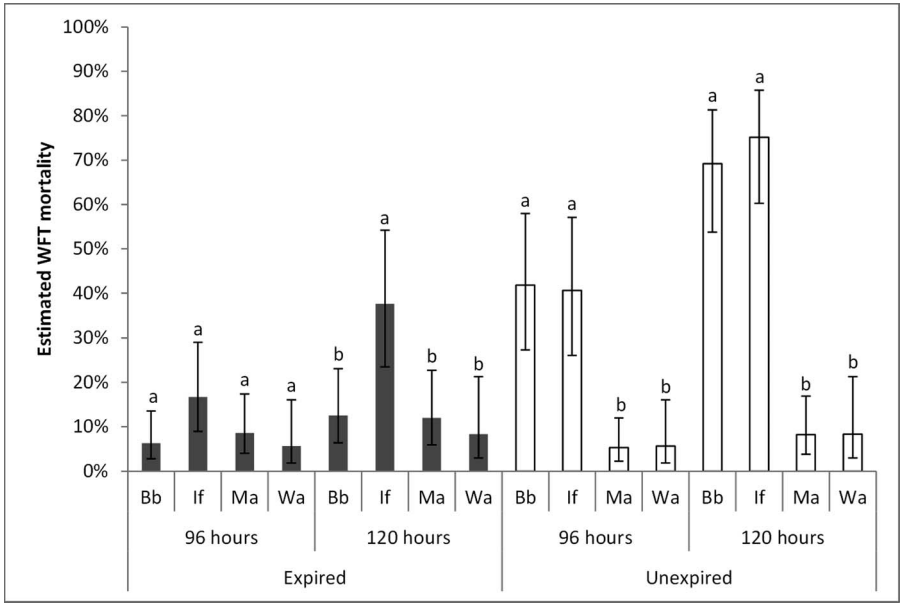

Fig. 1. Estimated mean percentage of mortality (±95% confidence intervals) of adult western flower thrips (WFT), *Frankliniella occidentalis*, at 96 and 120 h of exposure to the entomopathogenic fungi *Beauveria bassiana* (Bb), *Isaria fumosoroseus* (If), and *Metarhizium anisopliae* (Ma) for unexpired and expired products averaged across maximum and minimum label rates, and water (Wa) (Bioassay 1). Estimated means marked with a different lowercase letter (a, b) within shelf-life conditions at a given time point indicate significant differences in western flower thrips mortality ($P \leq 0.05$) between entomopathogenic fungal products. Data correspond to Bioassay 1.

At 24, 48, or 72 h of incubation, we observed 0% mortality of western flower thrips larvae. Larval mortality was apparent starting at 96 h of incubation, although estimated larval mortality remained low (≤24%) for all treatments throughout the 120-h incubation period. The estimated mean percentage of mortality of western flower thrips larvae to unexpired and expired entomopathogenic fungi products evaluated over the 120-h incubation period is shown in Table 2. We observed a significant effect of the entomopathogenic fungi on western flower thrips larval mortality ($P = 0.0008$), which was particularly noticeable at 120 h of incubation. Overall, larval mortality due to *I. fumosoroseus* was greater than that due to *M. anisopliae* ($P = 0.012$), regardless of label rate (maximum or minimum) or product condition (unexpired or expired).

By the end of the incubation period for Bioassay 1 (120 h), we found evidence of differential treatment effects associated with western flower thrips larval and adult mortality (interaction; $P = 0.023$). The estimated mean percentages of mortality of larvae and adults when exposed to the entomopathogenic fungi treatments after

Table 2. Estimated mean mortality (% ± SEM) of western flower thrips, *Frankliniella occidentalis*, larvae after 96 and 120 h of incubation with unexpired or expired products of entomopathogenic fungi (*Beauveria bassiana*, *Isaria fumosoroseus*, and *Metarhizium anisopliae*) at maximum and minimum label rates (Bioassay 1).

Entomopathogenic Fungi	Rate*	Shelf-Life Condition	Hours of Incubation	
			96	120
Beauveria bassiana	Max	Expired	3.2 ± 2.3	4.5 ± 3.0
	Max	Unexpired	13.1 ± 6.3	15.4 ± 7.1
	Min	Expired	2.8 ± 2.1	5.9 ± 3.5
	Min	Unexpired	5.7 ± 3.4	6.7 ± 3.9
Isaria fumosoroseus	Max	Expired	4.4 ± 2.7	11.3 ± 5.7
	Max	Unexpired	21.2 ± 8.8	23.7 ± 9.4
	Min	Expired	13.0 ± 6.3	22.7 ± 9.3
	Min	Unexpired	10.8 ± 5.5	19.5 ± 8.4
Metarhizium anisopliae	Max	Expired	4.1 ± 2.7	5.2 ± 3.2
	Max	Unexpired	1.0 ± 1.0	2.0 ± 1.7
	Min	Expired	3.2 ± 2.2	4.0 ± 2.6
	Min	Unexpired	4.3 ± 2.7	4.3 ± 2.7
Water	—	Control	2.8 ± 2.0	2.8 ± 2.0

* Maximum and minimum labeled rates (per 200 ml): *Beauveria bassiana* = 0.48 and 0.24 g, *Isaria fumosoroseus* = 0.48 and 0.24 g, and *Metarhizium anisopliae* = 0.5 and 0.125 ml.

120 h of incubation are presented in Fig. 2. Fresh *B. bassiana* resulted in significantly greater mortality of adults than larvae at both the maximum and minimum label rates ($P = 0.007$ and $P < 0.0001$, respectively) (Fig. 2). Similarly, unexpired *I. fumosoroseus* at the maximum label rate resulted in significantly greater mortality of adults than larvae ($P = 0.0005$) (Fig. 2). There was no evidence of any differences in mortality associated with the life stages (larvae and adults) when expired *B. bassiana* or *I. fumosoroseus* were used, nor was there evidence for differences with unexpired or expired *M. anisopliae*.

Bioassay 2. When the incubation period was extended to 216 h, we found evidence of differential treatment effects on adult western flower thrips mortality (interaction; $P < 0.0001$; Fig. 3), over the course of the experiment, but no evidence of any treatment effects by 48 h of incubation. However, by 72 h of incubation, and at all later time points, mean percentage of adult western flower thrips mortality, when exposed to unexpired *B. bassiana* or *I. fumosoroseus*, significantly increased relative to their expired counterpart products (Fig. 3). More specifically, unexpired *B. bassiana* resulted in significantly greater percentage of mortality of adult western

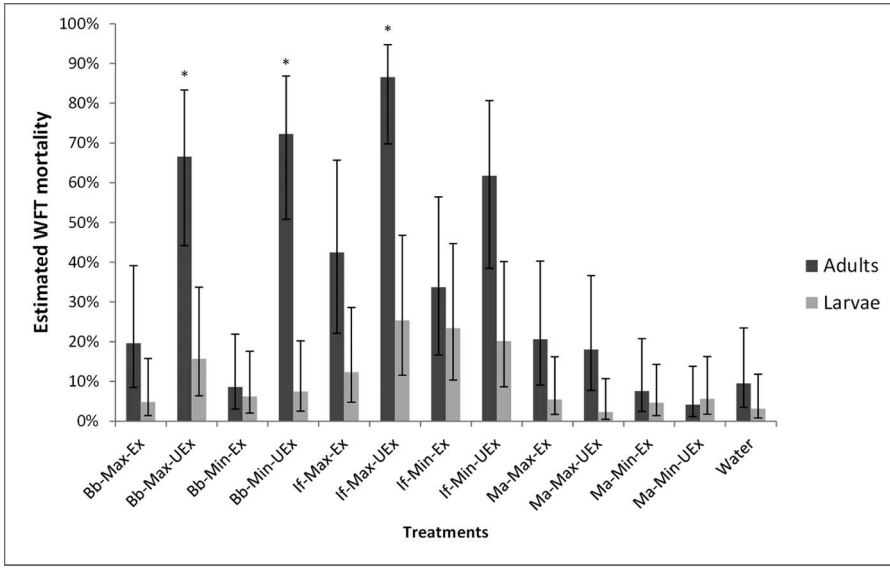

Fig. 2. Estimated mean percentage of mortality (±95% confidence intervals) associated with western flower thrips (WFT), *Frankliniella occidentalis*, adults compared to larvae when exposed to unexpired (UEx) and expired (Ex) products of the entomopathogenic fungi *Beauveria bassiana* (Bb), *Isaria fumosoroseus* (If), and *Metarhizium anisopliae* (Ma) at the maximum (Max) and minimum (Min) label rates by 120 h of incubation, and water (Bioassay 1). * Indicates significant differences ($P \leq 0.05$) in percentage of mortality between larvae and adults of western flower thrips within a given treatment at 120 h of incubation. Maximum and minimum labeled rates (per 200 ml): *Beauveria bassiana* = 0.48 g and 0.24 g, *Isaria fumosoroseus* = 0.48 g and 0.24 g, and *Metarhizium anisopliae* = 0.5 and 0.125 ml. Data correspond to Bioassay 1.

flower thrips than the corresponding expired product at 72 ($P = 0.0003$), 96 ($P < 0.0001$), and 120 ($P < 0.0001$) h of incubation, with the mean percentage of mortality differences greatest at the 120-h incubation period (Fig. 3). Likewise, unexpired *I. fumosoroseus* resulted in significantly higher adult western flower thrips mortality than expired at 72 ($P = 0.007$), 96 ($P = 0.0007$), and 120 ($P = 0.01$) h of incubation although the greatest difference was observed at 96 h (Fig. 3). For both entomopathogenic fungi, maximum adult western flower thrips mortality occurred more rapidly when using unexpired compared to the expired products. Overall, by 144 h of incubation, mean adult western flower thrips mortality to unexpired *I. fumosoroseus* was above 99%, whereas mortality to unexpired *B. bassiana* reached 92% by 168 h of incubation. However, only at 216 h of incubation was the estimated mean adult mortality for expired *I. fumosoroseus* and *B. bassiana* approximately 99% and 75%, respectively (Fig. 3).

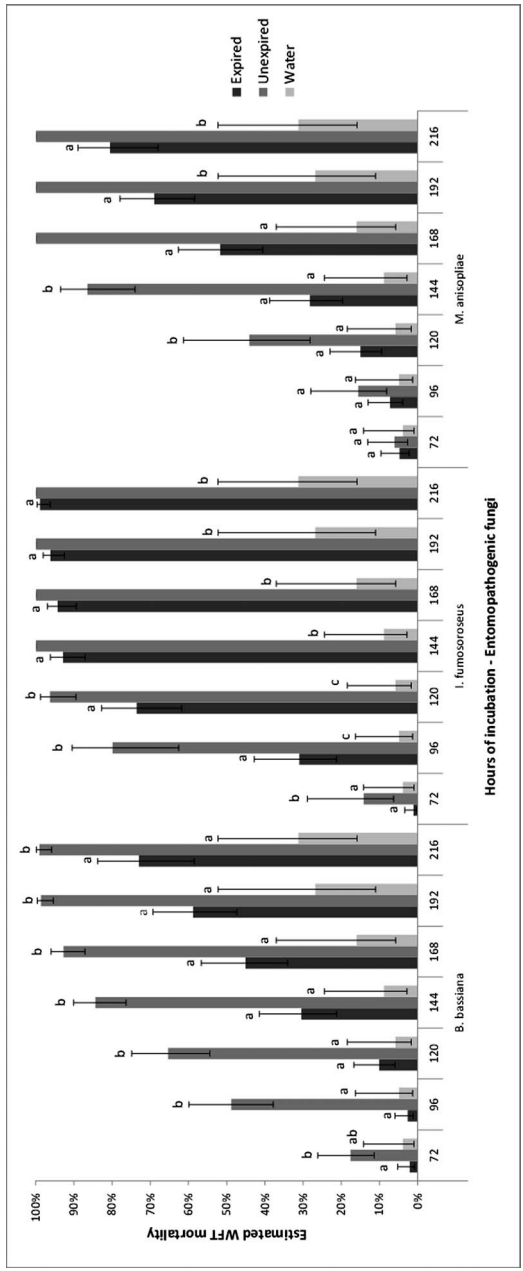

Fig. 3. Estimated mean percentage of mortality (±95% confidence intervals) of adult western flower thrips (WFT), *Frankliniella occidentalis*, after exposure to unexpired compared to expired *Beauveria bassiana*, *Isaria fumosoroseus*, and *Metarhizium anisopliae* at 72, 96, 120, 144, 168, 192, and 216 h of incubation averaged across maximum and minimum label rates, and water (Bioassay 2). Different lowercase letters (a, b, c) within each entomopathogenic fungus (*Beauveria bassiana*, *Isaria fumosoroseus*, and *Metarhizium anisopliae*) indicate significant differences in mortality ($P \leq 0.05$) associated with western flower thrips among water, expired, and unexpired products of a given entomopathogenic fungus at a selected incubation time. Data correspond to Bioassay 2.

Unexpired product of *M. anisopliae* at 144 and 168 h of incubation resulted in an estimated mean percentage of mortality of western flower thrips adults of at least 96% and 100%, respectively. However, for expired *M. anisopliae*, estimated adult western flower thrips mortality was approximately 86% by 216 h of incubation, although only at the maximum label rate. For *M. anisopliae*, the effect of shelf life on mortality of adult western flower thrips was only significant at 120 h of incubation ($P = 0.004$), despite the relatively low magnitude of the mortality estimate (44%; Fig. 3). However, at 192 and 216 h of incubation, both of the *M. anisopliae* treatments (unexpired and expired) provided greater mortality than the water control (Fig. 3).

Relative to the water control, the effect of *B. bassiana* on estimated mean mortality of adult western flower thrips (4.8 ± 3.1% versus 60.2 ± 7.4%, respectively) was apparent starting at 96 h of incubation although only for unexpired products used at the maximum label rate. Also, by 96 h of incubation the effect of unexpired *I. fumosoroseus* on estimated mean adult western flower thrips mortality at either the maximum or minimum label rates (86.9 ± 6.7% or 70.6 ± 10.8%, respectively) was apparent relative to the water control (4.8 ± 3.1%). The effect of unexpired *M. anisopliae* at the maximum label rate on adult mortality (96.1 ± 2.8%) was noticeable relative to the water control (8.8 ± 4.9%) only after 144 h of incubation.

Bioassay 3. The estimated mean percentage of larval mortality of western flower thrips in response to the entomopathogenic fungi combined with azadirachtin during the 144-h incubation period is presented in Fig. 4. The effect of azadirachtin on larval mortality was different across the entomopathogenic fungi (two-way interaction, $P < 0.0001$; Fig. 4). For *M. anisopliae*, azadirachtin enhanced efficacy ($P = 0.0004$) approximately 20-fold by 144 h of incubation, from mean western flower thrips larval mortality estimates of 1.9 ± 1.5% (without azadirachtin) to 34.7 ± 12.2% (with azadirachtin). When *M. anisopliae* was combined with azadirachtin, larval mortality, by 144 h of incubation, was the highest of all the entomopathogenic fungi ($P < 0.05$). However, there was no evidence of any synergistic effects of azadirachtin on the efficacy of either *B. bassiana* ($P = 0.38$) or *I. fumosoroseus* ($P = 0.071$) associated with western flower thrips larval mortality.

Discussion

This study evaluated the efficacy of entomopathogenic fungi against the larval and adult life stages of the western flower thrips using the maximum and minimum label rates of unexpired and expired products. Furthermore, potential synergistic effects of azadirachtin with each entomopathogenic fungus on larval mortality were assessed. While not representative of conditions typically encountered in a commercial greenhouse, this laboratory study reflects a controlled environmental scenario primarily related to temperature and relative humidity (in the petri dishes), as a means of evaluating proof of concept regarding biological activity of entomopathogenic fungi. That is, the environmental conditions in the laboratory were expected to favor entomopathogenic fungal infection, thus presumptively allowing for a best-case scenario of the fungi's efficacy against western flower thrips. Despite their limitations, laboratory studies constitute initial steps in the research process prior to testing under field conditions. As such, follow-up research

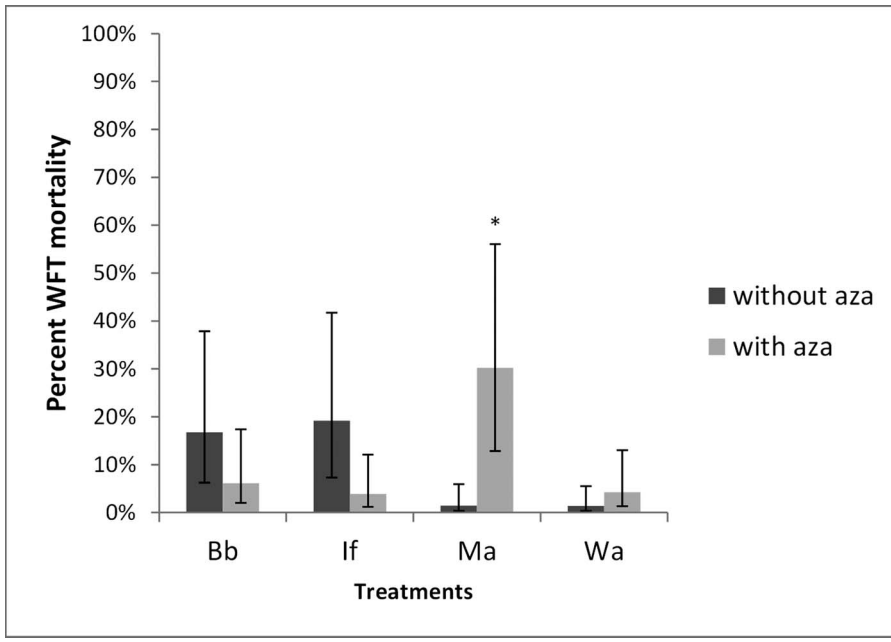

Fig. 4. Estimated mean percentage of mortality (±95% confidence intervals) of western flower thrips (WFT), *Frankliniella occidentalis*, larva when exposed to the entomopathogenic fungi *Beauveria bassiana* (Bb), *Isaria fumosoroseus* (If), and *Metarhizium anisopliae* (Ma) at the maximum label rate, and water (Wa) combined with and without azadirachtin (aza) (Bioassay 3). These mean percentage of mortality estimates associated with combinations of entomopathogenic fungi and azadirachtin were averaged across 96, 120, and 144 h of incubation. * Indicates a significant effect ($P \leq 0.05$) of azadirachtin when combined with an entomopathogenic fungus on western flower thrips larval mortality.

will be needed to determine the efficacy of entomopathogenic fungi against western flower thrips under more realistic conditions, such as those in a greenhouse, where environmental conditions (e.g., temperature and relative humidity) are likely to vary and where western flower thrips can interact directly with horticultural crops. In fact, in a greenhouse experiment, Kivett et al. (2015) found that a rotation program that involved the three entomopathogenic fungi evaluated in our laboratory experiment was comparable (based on efficacy) in suppressing populations of western flower thrips to standard insecticides commonly used in rotation programs; indirectly indicating that the fungal spores were viable.

Our results provide evidence for (a) the importance of shelf life of entomopathogenic fungi, particularly *B. bassiana* and *I. fumosoroseus*, for adult mortality of western flower thrips; (b) a delayed effect of *M. anisopliae* on adult western flower

thrips mortality, relative to *B. bassiana* or *I. fumosoroseus*; (c) enhanced susceptibility of western flower thrips adults relative to larvae to unexpired products of *B. bassiana* and *I. fumosoroseus* at a 120-h incubation period when exposed to the maximum label rate, although there was no evidence of any effect affiliated with *M. anisopliae*; and (d) a synergistic effect of azadirachtin when combined with unexpired *M. anisopliae* by enhancing larval mortality of western flower thrips.

Our study is the first to compare the efficacy of entomopathogenic fungi for control of western flower thrips when used before and after their respective expiration dates. Consistent with our results, Ansari and Butt (2011) found that percentage of conidial germination of *M. anisopliae* strain F52 declined nearly 50% after 4 mo stored at 4°C and nearly 70% after 4 mo stored at 20°C. Expired products used in this study were at least 10 mo past the expiration date as indicated on the label, and were stored at approximately 4°C. In Bioassay 1, unexpired products of *B. bassiana* and *I. fumosoroseus* resulted in significantly higher adult western flower thrips mortality than the expired products, regardless of the rate used (maximum or minimum). In Bioassay 2, unexpired product for all three entomopathogenic fungi resulted in higher western flower thrips adult mortality when compared to expired products at 120 h of incubation. Based on our results, even under ideal conditions for fungal development, entomopathogenic fungal products should be used prior to their expiration date and should be properly stored as indicated on the label. If entomopathogenic fungal products are used sometime beyond the expiration date, decreased efficacy against western flower thrips should be expected.

Interestingly, *M. anisopliae* exhibited a delayed effect on western flower thrips adult mortality compared to the other two species of entomopathogenic fungi. As a result, it may be reasonable to expect more plant damage from western flower thrips adults after applying *M. anisopliae* (Met52) due to the delayed activity relative to the other entomopathogenic fungi. In Bioassay 1, unexpired *B. bassiana* and *I. fumosoroseus* resulted in $>$70% mean estimated adult western flower thrips mortality by 120 h of incubation, whereas unexpired *M. anisopliae* resulted in approximately 17% mean adult western flower thrips mortality at the same incubation period. Thus, we conducted a follow-up experiment (Bioassay 2) with a prolonged incubation period that extended up to 216 h. After accounting for label rates, we observed that unexpired *M. anisopliae* did not reach 100% adult western flower thrips mortality until 168 h of incubation, whereas adult mortality associated with *I. fumosoroseus* was nearly 100% by 120 h of incubation. Likewise, unexpired *B. bassiana* did not reach maximum adult western flower thrips mortality (99%) until 192 h of incubation. Similarly, Vestergaard et al. (1995) observed 94% mortality of western flower thrips adults exposed to *M. anisopliae* after 168 h at 20 $\pm$ 1°C. Consequently, based on the results of our study, *M. anisopliae* appears to have a prolonged lag time resulting in delayed initiation of adult western flower thrips mortality, whereas the effects associated with the other entomopathogenic fungi occurred earlier and steadily increased over time. It is conceivable that any delay in initiation of activity may be due to a formulation difference affiliated with *M. anisopliae* (Met52), which is an emulsifiable concentrate, whereas *B. bassiana* (BotaniGard) and *I. fumosoroseus* (NoFly) are both wettable powders. However, Ugine et al. (2005) found that the technical grade of *B. bassiana* GHA strain, which contained a formulation of pure conidia, provided the same level of mortality as the

formulated product. The effect of formulation on entomopathogenic fungi efficacy against western flower thrips needs to be investigated.

Certain inert ingredients such as surfactants found in formulated products can, by themselves, be toxic to pests, in addition to the active ingredient (Cowles et al. 2000). If this were the case, it is possible that the products associated with *B. bassiana* (BotaniGard) and *I. fumosoroseus* (NoFly) may contain inert ingredients that cause western flower thrips mortality by themselves, in addition to the direct effect of the entomopathogenic fungi. However, additional studies need to be conducted to determine whether the inert ingredients actually have activity on western flower thrips. Moreover, *M. anisopliae* may be a slower-acting entomopathogenic fungus than *B. bassiana* or *I. fumosoroseus* although Vestergaard et al (1995) indicated that certain isolates of *M. anisopliae* caused rapid death of western flower thrips. Further research is warranted to compare speed of activity and mode of infection associated with these entomopathogenic fungi species. In fact, even within a single fungal species, considerable differences in infection rates have been reported for different strains (Erler and Ates 2015).

Our study also provided evidence indicating that western flower thrips adults were more susceptible than larvae to unexpired *B. bassiana* and *I. fumosoroseus*. Western flower thrips adults were approximately 60% more susceptible (based on mortality) than the larvae to unexpired *B. bassiana* and *I. fumosoroseus*. These results are consistent with previous findings where the larvae were found to be less susceptible to infection due to having thicker cuticles and to fungal spores being shed along with the cuticle during ecdysis (Shipp et al. 2003, Ugine et al. 2005, Vestergaard et al. 1995). Taken together, these physiological parameters associated with the larvae may have reduced the efficacy of the entomopathogenic fungi.

Plant protection during the crop production cycle usually requires that entomopathogenic fungi be applied early. However, if early applications are not possible, then one may consider synergizing the effectiveness of entomopathogenic fungi against western flower thrips larvae with azadirachtin, an insect growth regulator that inhibits or delays the molting process (Islam et al. 2010, James 2003, Schmutterer 1990). Reports indicate that by delaying the molting process, the hyphae of the entomopathogenic fungi may be able to penetrate the cuticle and more effectively infect western flower thrips larvae before ecdysis (Akbar et al. 2005, Hernandez et al. 2012). However, in our study, the expected synergistic effect of azadirachtin with the entomopathogenic fungi was limited to only *M. anisopliae*, whereas no evidence for any synergistic or additive effects was apparent for *B. bassiana* or *I. fumosoroseus*. Akbar et al. (2005) obtained similar results in which adding azadirachtin (Neemix® 4.5, Certis USA, Columbia, MD) to *B. bassiana* resulted in decreased larval mortality of the red flour beetle, *Tribolium castaneum* (Herbst). Furthermore, an initial greenhouse study, in which azadirachtin (Ornazin® 3% EC, SePRO, Carmel, IN) was added to two formulations of *B. bassiana* (BotaniGard ES and BotaniGard WP) found no evidence of increased mortality affiliated with western flower thrips larvae with or without azadirachtin (R.A.C., unpubl. data). Even under the controlled environmental conditions favoring the entomopathogenic fungi, the enhancing effect of azadirachtin with *M. anisopliae* was rather low (mortality was estimated at approximately 30%). In this study, we did

not evaluate the effect of azadirachtin on adult western flower thrips mortality as azadirachtin is an insect growth regulator and thus is less effective against mature adults that do not molt (Shipp et al. 2003, Vestergaard et al. 1995).

One reason for the lack of effectiveness associated with azadirachtin in combination with *B. bassiana* and *I. fumosoroseus* in our study may be a consequence of azadirachtin inhibiting the growth of certain fungal spores. For example, James (2003) demonstrated that azadirachtin (Neemix) had an inhibitory effect on the growth and germination of *I. fumosoroseus*. Furthermore, studies have demonstrated that azadirachtin can be effective against certain plant pathogenic fungi (Pasini et al. 1997, Singh et al. 1980, Singh and Prithiviraj 1997). The rate of AzaGuard used in the experiment was the maximum label rate. A lower rate may diminish any inhibitory effect that azadirachtin may have on the entomopathogenic fungi (James 2003). Azadirachtin is also known to have repellent effects (Schmutterer 1990); however, it is unlikely that repellency had any impact on the efficacy of the entomopathogenic fungi against western flower thrips larvae given the bioassay conditions. Therefore, the results of this experiment indicate no evidence of any benefit associated with adding azadirachtin to either *B. bassiana* or *I. fumosoroseus*, with only a limited benefit of adding azadirachtin to *M. anisopliae*. The latter is an interesting finding because *M. anisopliae* by itself has slower efficacy, but was the only entomopathogenic fungus that showed synergistic effects when combined with azadirachtin. However, the overall efficacy of the combination was estimated at $<$50% mortality in the best-case scenario, which in all likelihood would be unacceptable under commercial conditions.

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