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ORIGINAL ARTICLE

Sublethal concentrations of spinosad synergize the pathogenicity of fungi to larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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Abstract. We evaluated the efficacy of four entomopathogenic fungi (EPF) and their compatibility with the bioinsecticide spinosad for control of *Ephestia kuehniella* (Zeller) under laboratory conditions. Three EPF, including *Beauveria bassiana* (Balsamo-Criveili) Vuillemin isolates Z1 and Iran 1395C, *Lecanicillium* (= *Verticillium*) *lecanii* (Zimmerman) Zare & Gams, isolate Iran 229, and *Purpureocillium* (*Paecilomyces*) *lilacinum* (Thom) Luangs-ard, Hywel-Jones & Samson, isolate Iran 1026 were tested against third and fifth larval instars of *Ephestia kuehniella* using a filter paper bioassay. Mortality caused by the EPF ranged from 63.3–72.5% for third instars and 50–65.5% for fifth instars, with LT_{50} ranging from 8.4–10.5 d and 10.1–12.9 d, respectively. The effect of spinosad at LC_{10} (= 26.2 ppm) on EPF spore germination was evaluated and found to be negligible, ranging from 0% for *B. bassiana* Z1 to 5.7% for *P. lilacinum*. The LC_{50} values for spinosad against third and fifth instar *E. kuehniella* larvae were 452.5 and 1446 ppm, respectively. Subsequently, spinosad at LC_{10} was applied to third instar *E. kuehniella* larvae 24 h before application of the EPFs at LC_{50}. The addition of spinosad to applications of *L. lecanii* and *B. bassiana* Z1 and Iran1395C isolates synergized their pathogenicity to *E. kuehniella* larvae, whereas the effect was merely additive for *P. lilacinum*. Our results suggest that these EPF isolates can be used effectively in combination with spinosad for management of *E. kuehniella* in stored products.

INTRODUCTION

The Mediterranean flour moth, *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), is a cosmopolitan pest of many stored products, especially grains and flours (Ben-Lalli et al., 2011). Feeding and web-spinning by moth larvae fouls stored products and causes significant economic losses (Lynn & Ferkovich, 2004). Control of stored-product pests in commodites currently relies heavily on the use of chemical pesticides, especially fumigants, but this can result in toxic residues and contamination of food products. Given the high costs of these pesticides, and the inevitable evolution of resistance in target pests, there is an urgent need for alternative, more environmentally friendly, management tactics, such as biological control (Talukder, 2009; Pimentel et al., 2010).

Entomopathogenic fungi (EPF) are potential biological control agents that have been used with some success against stored product pests (Draganova & Markova, 2006; Buda & Peciulyte 2008; Barra et al., 2013; Batta & Kavallieratos, 2018). These microorganisms infect arthropods when their spores attach to a host and germinate, producing appressoria that then penetrate the host cuticle, enabling subsequent growth of mycelia thoughout the arthropod body (Gabarty et al., 2014; Mora et al., 2017). The efficacy of various EPF *against E. kuehniella* larvae has been frequently demonstrated in laboratory trials. For example, Faraji et al. (2013) tested 10 isolates of *Beauveria bassiana* (Balsamo-Criveili) Vuillemin, and *Metarhizium anisopliae* (Metchnikoff) Sorokin for pathogenicity against *E. kuehniella* larvae. All isolates of *B. bassiana* and *M. anisopliae* were pathogenic to *E. kuehniella*, but with varying efficacy. The C-IIA7 isolate of *B. bassiana* and B-VM1 isolate of *M. anisopliae* exhibited the lowest LT_{50} values of 107 and 93 h, respectively. The results of Draganova & Markova (2006) demonstrated the virulence of four *B. bassiana* isolates, two *M. anisopliae* isolates, and one isolate of *Lecanicillium* (= *Verticillium*) *lecanii* (Zimm.) Zare & Gams against *E. kuehniella* larvae. The isolate 383Bb of *B. bassiana* was the most virulent of those tested.

Another promising alternative to synthetic chemical pesticides for protection of stored products are biopesticides of natural origin (Shishir et al., 2015). One such biopesticides is spinosad, a natural insecticide produced by the soil actinomycete bacterium *Saccharopolyspora spinosa* (Mertz

& Yao, 1990). Spinosad kills insects by hyperexcitation of the nervous system (Snyder et al., 2007) and its efficacy against *E. kuehniella* and other stored product pests has been demonstrated in previous studies (Mutambuki et al., 2003; Hertlein et al., 2011; Pozidi-Metaxa & Athanassiou, 2013). Interestingly, larvae of *E. kuehniella* show a preference for remaining on surfaces treated with spinosad, a response which might help improve its uptake and efficacy (Athanassiou et al., 2018), and spinosad would appear to be compatible with the parasitoid *Habrobracon hebetor*, which can also be used for control of *E. kuehniella* (Mahdavi et al., 2015).

Spinosad has a successful history of application against stored product pests (Subramanyam et al., 2014; Nayak & Daglish, 2017) and is often applied in combination with low doses of diatomaceous earth to improve its efficacy (Machekano et al., 2017, 2019; Gad et al., 2021) The combined use of this naturally-derived insecticide and an EPF could potentially increase the efficiency of pest control while minimizing adverse chemical impacts (Paula et al., 2011; Sain et al., 2019). However, the possibility exists that certain insecticides could inhibit the germination or fungal growth of EPF, rendering them incompatible for joint application (da Silva et al., 2013). Therefore, the present study was conducted to evaluate the virulence of different species of EPF against *E. kuehniella* larvae, and their compatibility with spinosad, to determine the potential utility of combination applications of these agents for management of *E. kuehniella* in stored products. We hypothesized that the concentration of spinosad required to produce a given level of mortality would be higher for later stage larvae than for earlier stages, so we assayed toxicity for both third and fifth instars.

MATERIALS AND METHODS

Insect rearing

Eggs of *E. kuehniella* were obtained from the Prominent Insectarium in Ahvaz, Khuzestan Province, Iran. and placed in plastic containers (10 × 6 × 3 cm) containing wheat flour and bran (10 : 1) and held at 26 ± 1°C, 65 ± 5% RH, in continuous darkness until the desired larval stages (third and fifth instars) were harvested for use in the bioassays.

Fungal cultures

The EPF used in bioassays were *B. bassiana* isolates 'Z1' and 'Iran 1395C', the *L. lecanii* isolate 'Iran 229', and the *Purpureocillium lilacinum* (Thom) Luangs-ard, Houbraken, Hywel-Jones & Samson isolate 'Iran 1026'. The isolate *B. bassiana* 'Iran 1395C' was obtained from the Institute of Iranian Plant Protection, Tehran, Iran. Isolates of *B. bassiana* Z1, *P. lilacinum* Iran 1026, and *L. lecanii* Iran 229, were initially isolated from larvae of *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae) in Nazlu, Urmia, Iran by Dr. Youbert Ghosta, at the University of Urmia. All fungal isolates were cultured in the laboratory on potato-dextrose agar (PDA), at 26 ± 1°C and 16L : 8D photoperiod for two weeks. Afterward, the conidia were scraped from the surface of the fungal cultures and placed in a glass bottle containing 0.02% Tween 80 (Merck, Germany). Subsequently, each suspension was vortexed for 2 min and filtered through a single layer of jaconet to separate the mycelia. The concentrations of conidia in these homogenous suspensions was determined using a Neubauer haemocytometer (Precicolor, HBG; Germany) to be 5.56×10^9 conidia ml^{-1} for isolate Z1, 3.26×10^9 conidia ml^{-1} for Iran 1395C, 4.67×10^9 conidia ml^{-1} for Iran 229, and 1.13×10^9 conidia ml^{-1} for Iran 1026.

Assays of EPF virulence against *E. kuehniella* larvae

The *B. bassiana* isolates Z1 and Iran 1395C, the *L. lecanii* Iran 229 isolate, and the *P. lilacinum* Iran 1026 isolate, were tested against third and fifth instar *E. kuehniella* larvae at the full concentration of the isolated suspension (as above). Larvae were exposed to 0.7 ml of conidial suspension absorbed onto filter paper discs (60 mm diam) in plastic Petri dishes (n = 10 larvae per dish, three replicates per concentration) following the procedure of Draganova et al. (2007). Control larvae were exposed to paper discs treated with sterile distilled water containing 0.02% Tween 80. After 24 h of exposure, wheat flour was added to Petri dishes as a food source. The dishes were then sealed with Parafilm® and incubated at 26 ± 2°C in the dark. The numbers of live and dead larvae (discolored and/or with mycelial growth evident on the surface) were counted every other day for 14 d.

Assays of spinosad toxicity to *E. kuehniella* larvae

Spinosad (SP), brand name "Tracer® 24% SC", was obtained from Dow AgroSciences, UK and bioassays were performed on third and fifth instar *E. kuehniella* larvae. Five concentrations were selected for testing, based on the results of preliminary trials. Serial dilutions of the formulated compound were prepared on the day of the bioassay using distilled water containing 0.02% Tween 80, plus a water control with Tween 80 only. Each concentration was assayed in three replicates, with 10 larvae per replicate and mortality was recorded 96 h after exposure. This bioassay was conducted twice with the same concentrations and the methodology and conditions used were the same as those described above to assess pathogenicity.

Germination of EPF exposed to spinosad

In this experiment, we assayed the germination of EPF when exposed to spinosad at the LC_{10} (26.2 ppm) for third instar *E. kuehniella* larvae. This concentration was selected on the assumption that additive mortality contributed by the fungi would permit use of a fractional dose of spinosad compared to what would be required to exert effective control alone. The insecticide was dissolved in sterile distilled water containing Tween 80 (0.02%) at the desired concentration and conidia of each EPF were suspended in the aqueous solution of insecticide. Then, 100 µl of each fungal suspension, containing about 1×10^7 conidia ml^{-1}, was spread onto a thin layer of 0.9% water-agar medium in a sterile plastic Petri dish (6 cm diam); conidia suspended in distilled water served as the control. The Petri dishes were incubated at 26 ± 1°C in the dark for 24 h, at which time one hundred conidia were selected at random on each Petri dish and the percentage germinated conidia was quantified according to the methods of Marcuzzo & Eli (2016). The experiment was repeated twice with 7 replicates in each case. The percentage of conidial germination inhibition was calculated in comparison to the control using the formula of Hokkanen & Kotiluoto (1992):

$$I(\%) = \frac{C - P}{C} \times 100$$

where I, C, and P are the percentage of conidial germination inhibition, conidial germination of fungus in the control, and conidial germination of fungus in pesticidal medium, respectively.

Combined application of EPF and spinosad against *E. kuehniella*

This experiment tested the efficacy of fungal isolates for the control of *E. kuehniella* when combined with a low concentration of spinosad (LC_{10} = 26.20 ppm). The bioassay was conducted with third instar *E. kuehniella* larvae which are significantly more sensitive than fifth instar larvae to the EPF isolates we tested. Larvae were first exposed to spinosad following the same methodology described above. After 24 h, the larvae were exposed to *B. bassiana* isolates Z1 and Iran 1395C, *L. lecanii* Iran 229, and *P. lilacinum* Iran 1026 at concentrations of 3.6×10^9, 2.63×10^9, 3.81×10^9, and 0.83×10^9 conidia ml^{-1}, respectively using the bioassay method described above. Additional treatments exposed larvae to the fungus alone, the spinosad alone, or a water control. Mortality of larvae was recorded daily for 14 days following the fungal treatment; each treatment was replicated six times with 10 larvae per replicate and larval mortality was corrected for control mortality using Abbott's formula (Abbott, 1925). The corrected mortality data were subjected to one-way ANOVA and then analyzed as a randomized complete block design using the GLM procedure of SAS (SAS Institute, 2003), with means separated by Fisher's LSD test ($\alpha = 0.05$).

Statistical analyses

Cumulative mortality of *E. kuehniella* larvae in the EPF virulence assay was first corrected for control mortality (Abbott, 1925) and then analyzed by 2-way ANOVA with 'treatment' and 'larval stage' as independent factors after passing tests for homogeneity of variance (Levine's test) and homoscedasticity (Bartlett's test). The time necessary to produce 50% mortality (LT_{50}) was estimated by probit analysis (SAS Institute, 2003). The data on toxicity of spinosad to *E. kuehniella* larvae were subjected to probit analysis using SAS software (SAS Institute, 2003) to estimate the median lethal concentration (LC_{50}) and its corresponding 95% confidence intervals (95% CI).

RESULTS

Assays of EPF virulence against *E. kuehniella* larvae

All EPF isolates were pathogenic to third and fifth instar *E. kuehniella* larvae, with mortality rates ranging from 50 to 72.5% (Table 1). Cummulative mortality was not affected by either EPF treatment ($F = 1.06$; df = 1,71; $p = 0.374$) or larval stage ($F = 2.45$; df = 1,71; $p = 0.122$), and interaction between these factors is not significant ($F = 0.14$; df = 3,71; $p = 0.934$). The shortest estimated LT_{50} value was for third instar larvae exposed to *B. bassiana* Iran 1395C, but this was not significantly different from LT_{50} values obtained for the three other EPFs.

Assays of spinosad toxicity to *E. kuehniella* larvae

A significantly lower LC_{50} value was obtained for third instar *E. kuehniella* larvae than for fifth instars (Table 2). This confirmed our hypothesis that later stage larvae would require exposure to a higher concentration than earlier stage larvae to obtain a similar level of mortality.

Table 1. Cumulative mortality and LT_{50} values for third and fifth instar *Ephestia kuehniella* larvae exposed to entomopathogenic fungi at the undiluted concentrations isolated from cultures (5.56×10^9, 3.26×10^9, 4.67×10^9 and 1.13×10^9 for *Beauveria bassiana* Z1, *B. bassiana* 1395C, *Lecanicillium lecanii* Iran 229, and *Paecilomyces lilacinum* Iran 1026, respectively).

Larval stage	% mortality[a]	LT_{50}[b] (95% CI)	Slope ± SE
B. bassiana Z1			
Third	67.5	10.5 (9.4–12.2)	2.7 ± 0.4
Fifth	63.5	10.1 (8.1–13.6)	4.4 ± 1.0
B. bassiana Iran 1395C			
Third	72.5	8.4 (7.5–9.5)	2.5 ± 0.3
Fifth	65.5	10.5 (8.4–14.7)	1.3 ± 0.2
L. lecanii Iran 229			
Third	63.3	10.1 (9.1–11.2)	3.3 ± 0.4
Fifth	50	12.9 (10.6–36.5)	4.8 ± 1.5
P. lilacinum Iran 1026			
Third	67.8	9.1 (7.9–10.8)	2.1 ± 0.3
Fifth	59.9	11.3 (9.03–17.6)	2.8 ± 0.6

[a]cumulative mortality at 14 d post exposure. [b]Median lethal time and 95% confidence intervals (CI) were estimated by logistic regression.

Germination of EPF exposed to spinosad

Germination inhibition of fungal conidia following exposure to LC_{10} of spinosad ranged from 0.0% for *B. bassiana* Z1, to 3.9 ± 1.6% for *B. bassiana* Iran 1395C, to 3.03 ± 0.66% for *L. lecanii* isolate Iran 229, to 5.7 ± 1.7% for *P. lilacinum* Iran 1026. None of these values represented significant reductions when compared to controls ($F = 2.79$; df = 3,24; $p = 0.063$).

Combined application of EPF and spinosad against *E. kuehniella*

The mortality of third instar *E. kuehniella* larvae was significantly different among treatments ($F = 17.34$; df = 8,45; $p < 0.001$). Spinosad alone at LC_{10} caused less than 20% mortality of larvae, compared to 60–70% mortality for the various fungal isolates (Fig. 1). However, when combined with spinosad at LC_{10}, *B. bassiana* Z1, *B. bassiana* Iran 1395C, and *L. lecanii* all produced mortalities approaching 100%, with the combination of spinsoad plus *P. lilacinum* producing ca. 80% mortality. Thus, the application of SP in combination with either of the *B. bassiana* isolates or the *L. lecanii* isolate had a synergistic effect on *E. kuehniella* mortality (i.e., more than additive), whereas in combination with *P. lilacinum* it had merely an additive effect on *mortality.*

Table 2. Toxicity of spinosad to third and fifth instar *Ephestia kuehniella* larvae.

Instar	Slope ± SE	LC_{10} (95% CI)*	LC_{50} (95% CI)**	LC_{90} (95% CI)	χ^2 (df)
Third	1.03 ± 0.21	26.2 (3.5–65.1)	452.5 (287.0–672.3)	7816.0 (3424–47361)	1.13 (3)
Fifth	1.20 ± 0.34	122.9 (6.0–300)	1446.0 (1029–2148)	17019.0 (6685–409081)	1.07 (3)

*LC_{10} = lethal concentration that killed 10% of the tested population, with 95% confidence intervals (CI). ** LC_{50} = lethal concentration that killed 50% of the tested population, with 95% confidence intervals (CI).

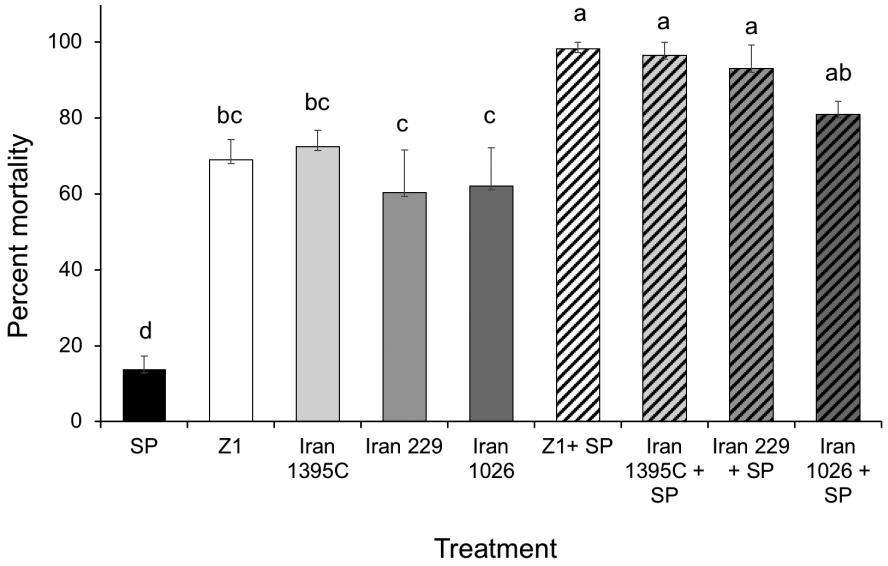

Fig. 1. Mortality of third instar *E. kuehniella* larvae when exposed to spinosad alone (SP), *B. bassiana* Z1 (Z1), *B. bassiana* Iran 1395C (1395C), *P. lilacinum* Iran 1026 (Iran 1026), *L. lecanii* Iran 229 (Iran 229) or various pairwise combinations of these. Columns bearing different letters were significantly different (Fisher's LSD test, $p < 0.05$).

DISCUSSION

All fungal isolates tested were equally effective against *E. kuehniella larvae* with no significant differences among them when applied alone. The efficacy of EPF for control of *E. kuehniella* and other stored product moths has been reported previously (Bahmani et al., 2012, 2020; Sabour et al., 2012; Sohrabi et al., 2019). Draganova & Markova (2006) tested one isolate of *L. lecanii* (*V. lecanii*), four isolates of *B. bassiana*, and two isolates of *M. anisopliae* against *E. kuehniella* and observed more lethal effects from the *B. bassiana* isolates, with only one (*B. bassiana* 383) producing mortality over 70% with an average LT_{50} value of five days. Faraji et al. (2013) reported more than 80% mortality of third instar *E. kuehniella* larvae when treated with five *B. bassiana* isolates at 1×10^8 conidia ml^{-1}, with LT_{50} values ranging from 107 to 154 h. These different results are likely attributable to multiple causes, including the genetic diversity of the isolates, the origin of collections, differences in methodology used, and possibly differential susceptibility of *E. kuehniella* source populations. Based on our findings, younger (third instar) larva were more susceptible to spinosad than were older (fifth instar) larvae. This result suggests that the timing of any application relative to the age demography of the target pest population will affect control efficacy, and that applications should be made as earlier as possible in an infestation when most larvae are still young and susceptible. Although larval stage did not affect EPF susceptibility significantly in this study, a generally greater susceptibility of early instar larvae to EPF has been reported by other researchers (Navon & Ascher, 2000; Erler & Ates, 2015).

The toxicity of spinosad was significantly greater against third instar *E. kuehniella* larvae when compared to fifth instars. Mollaie et al. (2011) reported that spinosad at 0.1–1 mg/kg completely prevented larval survival and adult emergence of *E. kuehniella*. In another study, Pozidi-Metaxa & Athanassiou (2013) reported 89–94% mortality of *E. kuehniella* larvae after 25 days of exposure to a 1 ppm concentration of spinosad at three temperatures.

Conidial germination in the presence of an insecticide is an important criteria of their potential compatibility for joint application (Oliviera et al., 2003). In the present study, spinosad at LC_{10} (26.2 ppm) did not inhibit germination of the EPF tested, and was thus judged to be safe for the various EPF. Similarly, Asi et al. (2010) found that spinosad was compatible with *M. anisopliae* and *Isaria* (*Paecilomysces*) *fumosoroseus* and was less inhibitory to conidial germination and mycelia growth of these fungi compared to various other insecticides that spanned a wide range of modes of action. Ericsson et al. (2007) detected a nonsignificant increase in the growth rate of *M. anisopliae* at low concentrations of spinosad, but reduced growth rates of the fungus at concentrations of 192 ppm or higher.

Previous studies have reported additive mortality of insect pests with combinations of EPF and various bioinsecticides. Shakarami et al. (2015) reported that a mixture of *B. bassiana* (6.3×10^4 conidia ml^{-1}) and essential oil of *Citrus vulgaris* (111 µl l^{-1}) had a synergistic effect in controlling third instar larvae of *E. kuehniella*. Similarly, Bahmani et al. (2020) found that a mixture of *B. bassiana* and the microbial insecticide *Bacillus thuringiensis* kurstaki (BtK), each applied at their LC_{50} concentration, resulted in superior control of *E. kuehniella* larvae compared to separate applications. In another study, combined applications of sublethal concentrations of spinosad (1.5–6.0 ppm g^{-1} sand) and *M. anisopliae* (10^4 conidia g^{-1} sand) caused high mortality and reduced feeding in two wireworm species, *Agriotes lineatus* (L.), and *Agriotes obscurus* (L.) (Ericsson et al., 2007). Spinosad has also been applied in combination with *B. bassiana* against *Tribolium confusum*, although the results suggested efficacy similar to the same products applied alone (Athanassiou et al., 2016). In the

present study, applications of sublethal concentrations of spinosad significantly increased the susceptibility of *E. kuehniella* larvae to infection by the two *B. bassiana* isolates and the *L. lecanii* isolate, although the infectivity of *P. lilacinum* appeared unaffected. It has been proposed that additive or synergistic interactions with EPF maybe arise because the insecticide inhibits detoxifying mechanisms an infected insect might otherwise use to clear fungal toxins, thus accelerating its death (Ericsson et al., 2007).

Based on the results of this study, we concluded that these isolates of *B. bassiana*, *L. lecanii*, and *P. lilacinum* have the potential to be integrated with spinosad for management of *E. kuehniella* in stored products, and that these EPF should be applied while larvae are still in early stages of development. Low concentration spinosad (LC_{10}) synergized the pathogenicity of *B. bassiana* isolates Z1 and Iran 1395C, and the *L. lecanii* isolate Iran 229, as they caused greater than additive mortality to *E. kuehniella* larvae when considering the effects of these treatments separately. Such combined treatments could reduce the amount of spinosad needed to control *E. kuehniella* and diminish selection pressure on the pest to evolve resistance to the insecticide. Further studies are warranted to determine optimal combinations of EPF and spinosad for controlling *E. kuehniella* infestations on specific products under actual storage conditions.

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