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Article

Efficacy of entomopathogenic fungi against the fruit fly *Drosophila suzukii* and their side effects on predator and pollinator insects

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With 3 figures

Abstract: Abstract: Entomopathogenic fungi (EPF) are insecticide alternatives for pest control. Their ability to easily adhere and quickly penetrate the insect cuticle is a key factor for their selection, which has received too little consideration so far. Here, we evaluated the impact of five EPF on the survival of *Drosophila suzukii*, a worldwide invasive pest of soft-skinned fruits. The most efficient EPF was then selected, and a second efficacy assay was performed by exposing *D. suzukii* adults to the EPF for different durations: 10 seconds, 1 minute, 10 minutes, 1 hour and 3 hours. Finally, EPF safety was assessed on two non-target beneficial insects frequently encountered in the same crops affected by *D. suzukii*, namely *Orius laevigatus* and *Bombus terrestris*. We found *Beauvaria bassiana* to be the most efficient EPF, killing over 95% of the flies within 10 days. The exposure time impacted the mortality rates: 50% of the flies died within 4 days after a 3-hours exposure to *B. bassiana*, whereas 6 days were needed to reach the same result with 10 seconds of exposure. Whatever the exposure time, this EPF always needed ten days to be lethal for more than 95% of individuals. *Beauvaria bassiana* was not lethal for the non-target species. Thus, *B. bassiana* is an option to control *D. suzukii* without harming beneficial insects. Further studies are now needed under real cultivation conditions to assess whether *B. bassiana* can be included in biocontrol strategies against *D. suzukii*.

Keywords: Spotted-Wing Drosophila; microbial control; attract-and-kill; biological control; invasive species; *Orius laevigatus*; *Bombus terrestris*

1 Introduction

Globalization and monoculture promote the introduction and establishment of invasive insect species (Meyerson & Mooney 2007; Pyšek & Richardson 2010). Some of them can become agricultural pests, unthreatened by natural enemies and benefiting from unlimited resources. This is particularly true for *Drosophila suzukii* (Diptera Drosophilae), a fly species native to Asia, which has expanded its range since 2008, establishing itself in the Americas, Europe and the North of Africa (Cini et al. 2012; Boughdad et al. 2021). Models predict that its area will continue to grow to include Oceania and the rest of the African continent in the future (Boughdad et al. 2021). Although *D. suzukii* uses wild host plants (e.g. strawberry tree, bittersweet, woolly nightshade) as refuges during winter, their main hosts include important fruit crops such as blueberries, strawberries and raspberries (Bellamy et al. 2013). Females lay eggs in ripe or ripening soft-skinned fruits making them unmarketable (Walsh et al. 2011). This species has a high fecundity (Asplen et al. 2015), a short reproduction cycle (Cini et al. 2012) with adults living up to 9 weeks. Thus, populations grow exponentially and can reach up to 15 generations in a single year (Asplen et al. 2015; Cini et al. 2012).

Nowadays, *D. suzukii* is mainly controlled by insecticides, Spinosad being the most widely used (Cahenzli et al. 2018). The emergence of resistant populations (Kenis et al. 2016) and side-effects on non-target organisms (Williams et al. 2003) are some of the reasons that justify the development of alternative control measures. This can include a combination of physical barriers (e.g. netting) (Leach et al. 2016), cultural practices (e.g. tolerant varieties, destruction

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of infested fruits) (Walsh et al. 2011), mass trapping using odorant and/or visual lures (Galland 2022) and natural enemies (Lee et al. 2019; Wang et al. 2020).

Entomopathogenic fungi (EPF) have been in use for more than 150 years and their effectiveness has now been reported for against a variety of pests. The most efficient strains typically belong to the genera Metarhizium and Beauveria, that are applied as an inundative approach. EPF invade their hosts by direct penetration of the host cuticle requiring the adhesion of conidia to the host surface and production of several cuticle hydrolytic enzymes. This step is then followed by the proliferation of hyphae throughout the body cavity and ends with the death of the insect (Sharma et al. 2020). The EPF then emerges from the host, producing conidia able to infect a new insect (Sharma & Sharma 2021). EPF have been reported to be effective against several insect pests, belonging to various feeding guilds including moths (Wang et al. 2021a), thrips, aphids (Yeo et al. 2003), weevils (Yasin et al. 2019), and many others. As regard to *Drosophila suzukii*, very contrasting results have been reported in the literature, ranging from 0 to 100% for the same fungal species (Cuthbertson et al. 2014; Woltz et al. 2015; Cossentine et al. 2016; Cahenzli et al. 2018; Rhodes et al. 2018). These differences can be attributed to the method of application of the EPFs (e.g. spraying conidia on insects or leaves, dipping fruits …) (Jaber 2018). Another key factor to be considered for selection of efficient EPF is the ability of these fungi to easily adhere and quickly penetrate the insect cuticle (Litwin et al. 2020). Unfortunately, this factor has been little considered to date. In fruit flies, adults have an active and mobile lifestyle. Two weaknesses are regularly observed among previous studies carried out on fruit flies: (1) the concentration of conidia is often too high to be representative of an action carried out in the field and (2) the duration of exposure of the insect target to conidia, which regularly exceeds the exposure time. However, EPN conidia must be able to adhere quickly to the targeted insect, typically within seconds. Therefore, the aim of the present study was to evaluate the efficacy of five EPF species as potential control agents against *D. suzukii* under laboratory conditions. The most efficient EPF was then selected and exposure time (as low as 10 seconds exposure) leading to insect death explored. Finally, its safety was assessed on two non-target beneficial insects frequently encountered in the same crops affected by *D. suzukii*, namely *Orius laevigatus* and *Bombus terrestris*.

2 Materials and methods

2.1 Insects rearing

Drosophila suzukii was maintained in groups of 20–30 adults in plastic vials (9.5 cm height, 3 cm diameter) partially filled with artificial diet (1 L distilled water, 60 g sucrose, 100 g cornmeal, 7 g agar, 15 g torula yeast, 0.75 g nipagin diluted in 5 mL ethanol, 4.8 mL propionic acid) that was changed every week. Vials were kept in a quarantine laboratory at 23 ± 1 °C, 80 ± 5 % relative humidity and a photoperiod of 16:8 (day/night). The Anthocorid predator *Orius laevigatus* and the bumblebee *Bombus terrestris* were purchased from Biobest (Westerlo, Belgium) and used for the experiments directly after their reception.

2.2 Entomopathogenic fungi

The EPFs *Beauveria bassiana* MUCL 1555, *Metarhizium anisopliae* MUCL 6859, *Metarhizium brunneum* MUCL 9645, *Lecanicillium lecanii* MUCL 8115 and *Isaria fumosoroseus* MUCL 15122, were purchased from the BCCM/MUCL collection (https://bccm.belspo.be/about-us/bccm-mucl). The strains were kept in sterilized (121°C for 15 minutes) agar V8 medium (V8, vegetable juice, Continental foods, Belgium) in Petri plates (90 mm diameter, Sardstedt, Germany), in the dark at 24°C until use.

2.3 EPFs bioassay

The screening experiment was conducted in multiwell plates with well dimensions chosen according to the size of the tested insect: an area of 0.32 cm^2 was selected for *D. suzukii* and *O. laevigatus*, while an area of 9.6 cm^2 was selected for *B. terrestris* (96-wells-F or 6-wells-F, sterile, VWR, Radnor, PA, USA). All multiwell plates were filled with agar V8 medium (100 µL of vegetable juice, 1.5 g of $CaCO^3$ and 7.5 g agar) before sterilization at 121°C for 15 min. They were then inoculated with 2 µl of peptone water containing 10^6 conidia/µL of each EPF species and incubated in the dark at 24°C for fungal growth. Two controls were included in the design: One consisting in multiwell plates filled with 2 µL of peptone water, named control^{pept}. Another consisting in multiwall plates filled with 10 µL Spinosad (Edialux®, at 480 g/L) the day before the bioassay (Pavlova et al. 2017), knowing to be lethal against *D. suzukii* (Cahenzli et al. 2018), *B. terrestris* (Besard et al. 2011) and *Orius sp.* (Dağlı & Bahşi 2009), and named control^{Spin}.

Two successive experiments were conducted at the same time. Experiment 1 aimed at comparing the lethality of the five EPF species on *D. suzukii*. A single fly was introduced in each well (n = 40 per treatment with a sex ratio 1:1) for 3 hours. The flies were then transferred individually to rearing tubes and their mortality assessed over 10 days. After 10 more days (day 20), for the vials that contained females, the number of new adults was evaluated to assess the fertility of flies exposed to the EPFs. Experiment 2 aimed at evaluating the impact of exposure time on fungal lethality. This experiment was conducted with the most efficient EPF from experiment 1 (i.e. *B. bassiana*). The same protocol as above was used, but the flies were exposed to the EPF for different durations: 10 seconds, 1 minute, 10 minutes or 1 hour (n = 40 per duration, with a sex ratio 1:1). Their mortality was then evaluated each day for 10 days.

2.4 Susceptibility of beneficial insects to B. bassiana

Bumblebees *B. terrestris* and predatory bugs *O. laevigatus* were exposed to *B. bassiana* for 3 hours in multiwall plates following the above-mentioned protocol. Bumblebees were then placed in a plastic nest box in microcolonies of 6 individuals originating from the same hive. These microcolonies were maintained at ± 25°C in the dark (Mommaerts et al. 2009) and were fed twice a week with cotton soaked in honey and water (60:40) and with pollen balls (pollen with honey coated with beeswax) (Mommaerts et al. 2009; Ramanaidu & Cutler 2013). Forty-eight bumblebees, originating from three hives, were distributed into 8 microcolonies per treatment (*B. bassiana*, control^{pept}, control^{Spin}). Their mortality was assessed every day for 20 days. Each dead bumblebee was removed from the microcolony to avoid a potential new source of infection. Similarly, *O. laevigatus* were placed individually in tubes and were fed with green beans and *Ephestia juehnilla* eggs. Their mortality was assessed every day during 10 days (Portilla et al. 2017). Thirty individuals were tested per treatment (*B. bassiana*, control^{pept}, control^{Spin}).

2.5 Statistical analysis

All analyses were performed using R and the following packages: survival, ggplot2, survminer, car, lsmeans, and lm4. All models were selected by sequential comparison of the nested sub-models and stepwise elimination of non-significant variables. When a global effect was detected, a post-hoc contrast analysis was performed. To facilitate comparisons, the effect size indices (Hazard Ratio and Cliff's delta δ) and the corresponding 95% confidence intervals were also reported. The lethal effects of EPFs were analyzed with Cox regressions, assuming a binomial distribution. Firstly, the effects of different treatments (Fungus, control^{pept}, control^{Spin}) with an exposure of 3 hours, on the survival of *D. suzukii* were analyzed. In a second step, the exposure time (3 hours, 1 hour, 10 minutes, 1 minute and 10 seconds) for the effective EPF on the survival of *D. suzukii* was analyzed following similar regression Cox, as reference 3 hours of contact. For the susceptibility of *B. terrestris* and *O. laevigatus* experiment, the effect on bumblebee and bug mortality of *B. bassiana* was investigated with Cox regression. For bumblebees, microcolony and original hive are included in the models as a random factor. To evaluate the effect of EPF on fertility, a General Linear Model Mix (GLMM) was performed, assuming a Poisson distribution, to compare the number of offspring according to the treatment. The number of days of survival was integrated as a random factor.

3 Results

3.1 Effects of EPFs on *D. suzukii* survival rate

The survival rate of *D. suzukii* differed between treatments (Fig. 1, Cox model, $\chi^2_6 = 151.510$, $p < 0.001$). Beside the control^{Spin}, *B. bassiana* was the only EPF inducing significant mortality in *D. suzukii* as compared to the control^{pept}. With this EPF, all the flies died within 8 days after exposure (Fig. 1). The other EPFs had no significant effect on mortality, compared to the control^{pept} (Fig. 1). The duration of exposure to *B. bassiana* significantly impacted the survival rate of *D. suzukii*: a significantly higher mortality was noticed with an increasing time of exposure (Fig. 2, Cox model, $\chi^2_4 = 0.575$, $p = 0.966$). Half of the *D. suzukii* population died within 4 days after a 3-hour, 1 hour and 10 minutes exposure to *B. bassiana,* whereas 6 days were needed to reach the same result with 1 minute and 10 seconds of exposure (Fig. 2). However, ten days were always needed to be lethal to > 95% of the individuals, independently of exposure time (Fig. 2).

3.2 Effects of EPFs on *D. suzukii* fecundity

The number of offspring significantly differed between the treatments (GLMM, $\chi^2_6 = 509.841$, $p < 0.001$). The control^{Spin} reduced significantly the number of offspring as compared to the other treatments (mean = 1.169; se = 0.449 ±; $p < 0.001$; Cliff's $\delta = 0.601$). Similarly, *B. bassiana* significantly reduced the number of offspring as compared to the control^{Pept} (mean = 13.783084; se = 1.439 ±; $p < 0.001$; Cliff's $\delta = 0.182$), after an exposure time of 3 hours, while the other EPFs had no significant effect on fecundity as compared to the control^{Pept}: *M. anisopliae* (mean = 16.347; se = 1.575 ±; $p = 0.155$; Cliff's $\delta = 0.145$), *M. brunneum* (mean = 23.566; se = 2.019 ±; $p = 0.155$; Cliff's $\delta = -0.032$), *I. fumosoroseus* (mean = 19.099; se = 2.058 ±; $p = 0.872$; Cliff's $\delta = 0.009$) and *L. lecanii* (mean = 16.007; se = 1.623 ±; $p = 0.053$; Cliff's $\delta = 0.143$).

3.3 Effects of *B. bassiana* on *O. laevigatus* and *B. terrestris*

The treatment significantly affected the survival rate of both insects (Fig. 3, *O. laevigatus*: Cox model, $\chi^2_2 = 87.087$, $p < 0.001$ / *B. terrestris*: Cox model, $\chi^2_2 = 156.38$, $p < 0.001$), but this significant effect was due to the Control^{Spin} that killed both insects within a period of two days (Fig. 3), while *B. bassiana* had no impact on survival probability of bumblebees and predatory bug (Fig. 3). The microcolonies and the original hive don't influence the mortality (microcolonies: Cox model, $\chi^2_1 = 0.1662$, $p = 0.687$ / original hive: Cox model, $\chi^2_2 = 5.751$, $p = 0.056$).

4 Discussion

Beauveria bassiana was the only fungus species able to lead to *D. suzukii* mortality. Conversely, the other EPFs had no effects. This contradicts other studies showing that *I. fumosoreus* (Cossentine et al. 2016; Naranjo-Lázaro et al. 2014), *L. lecanii* (Cossentine et al. 2016), *M. anisopliae*) and *M. brunneum* (Cossentine et al. 2016; Woltz et al.

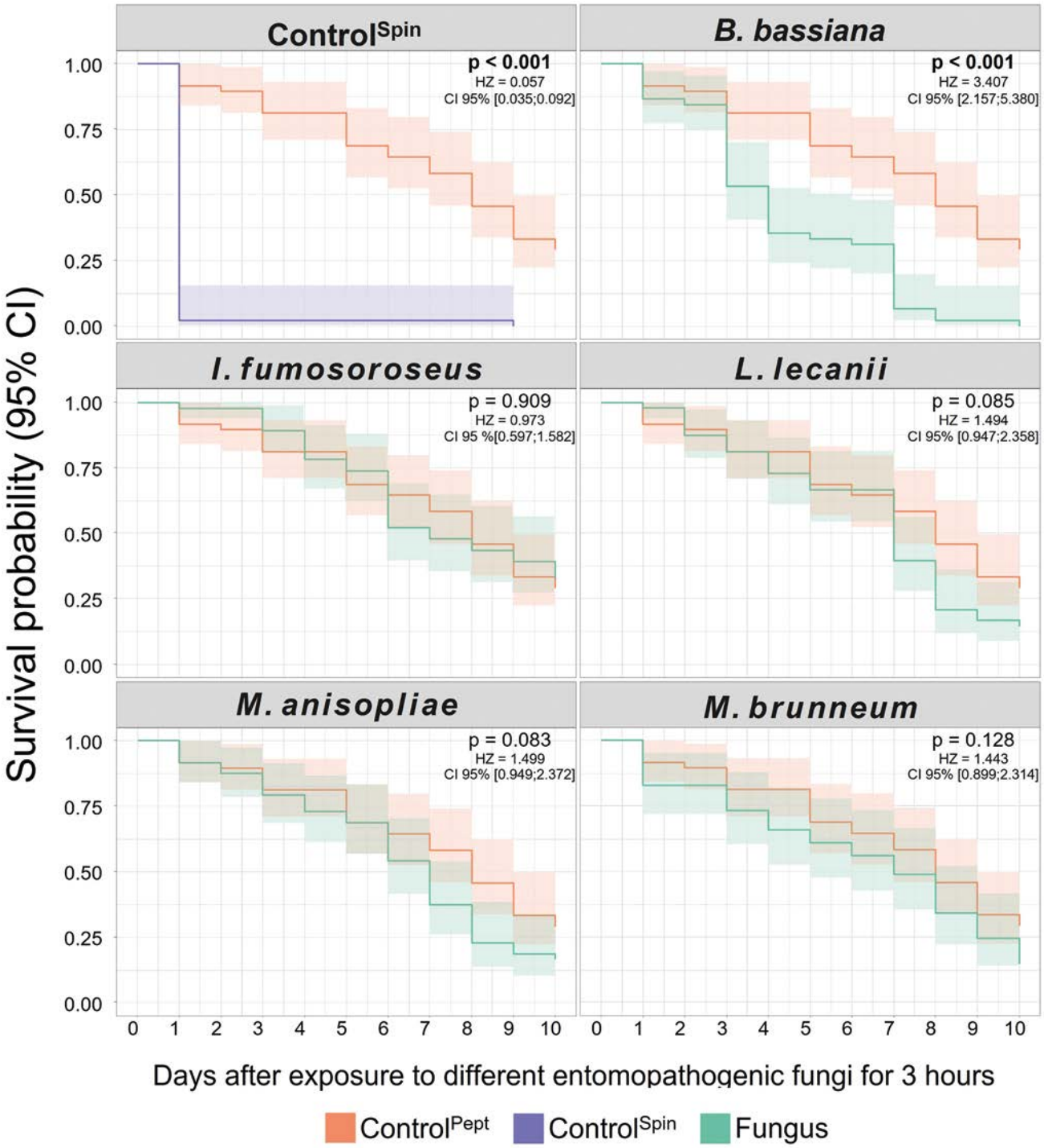

Fig. 1. Probability of *D. suzukii* survival (95% CI) following contact during 3 h with the different EPFs (green), or Spinosad (control^{Spin} (blue)) or water peptone (control^{Pept} (orange)) as a function of days after exposure. P-value (p), hazard ratio (HZ) and Confidence Interval at 95% (CI 95%) are reported between EPF or control^{Spin} treatment and control^{Pept}. Significant differences at 5% (Cox model, $\chi^2_6 = 151.510$, $p < 0.001$) are marked in bold.

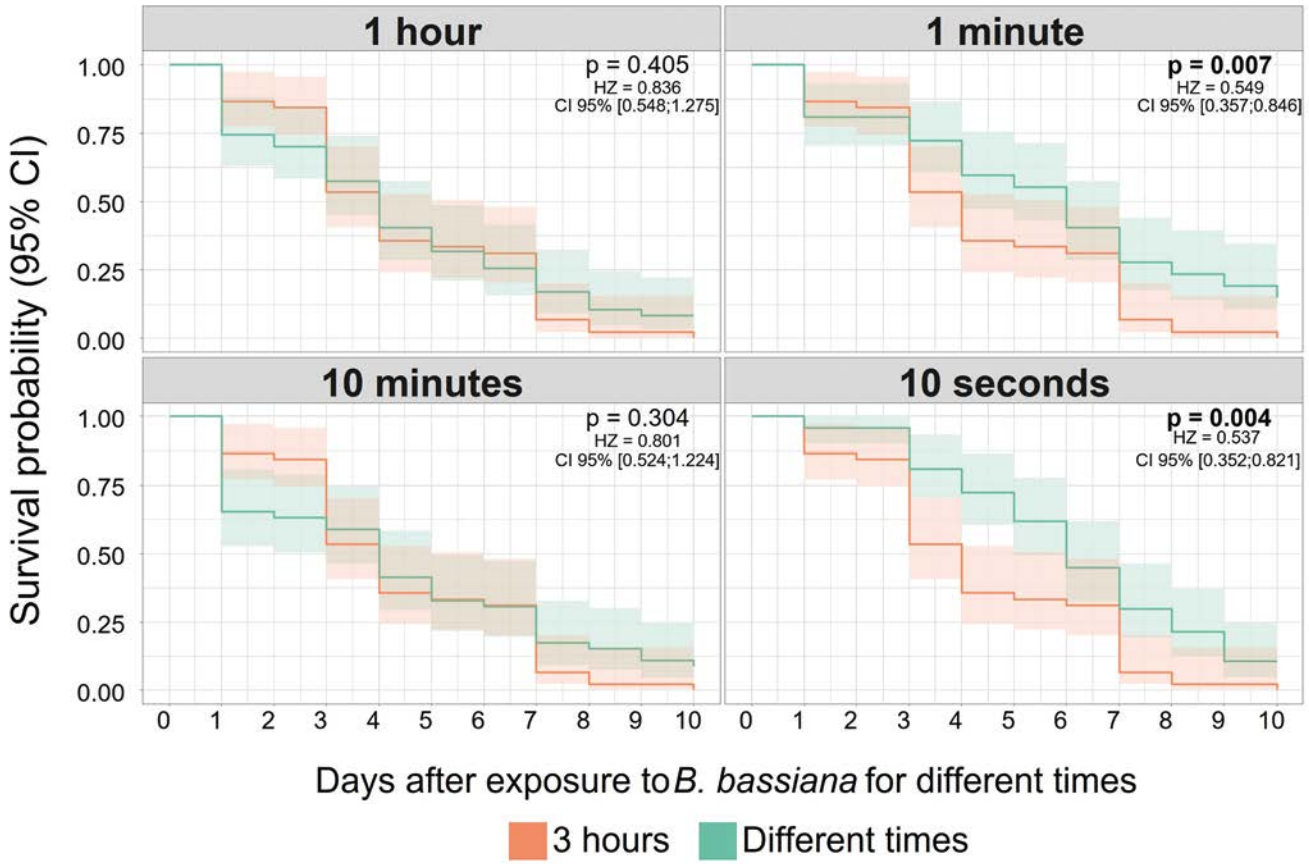

Fig. 2. Probability of *D. suzukii* survival (95% CI) following different times of contact with B. bassiana as a function of days after exposure. The provided P-value (p), hazard ratio (HZ) and Confidence Interval at 95% (CI 95%) refer to the comparison with the survival probability observed after 3 hours of exposure. Significant differences at 5% (Cox model, $\chi^2_4 = 0.575$, p = 0.966) are marked in bold.

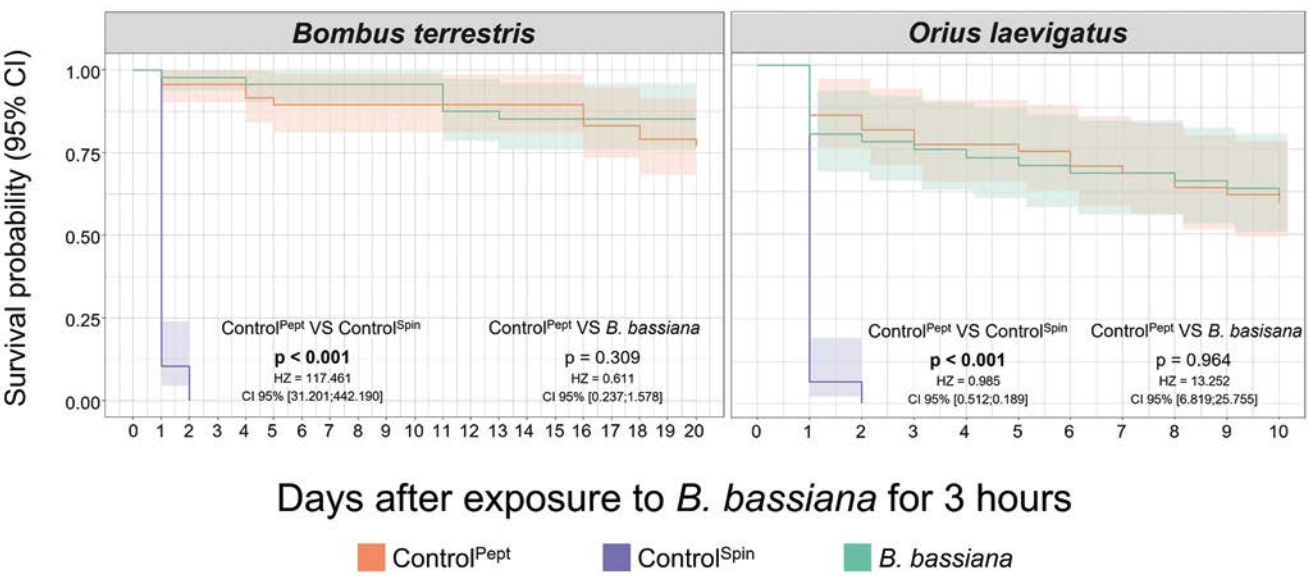

Fig. 3. Probability of *Bombus terrestris* and *Orius laevigatus* survival (95% CI) following contact during 3 h with *B. bassiana* (green), or Spinosad (control^{Spin} (blue)) or water peptone (control^{Pept} (orange)) as a function of days after exposure. P-value (p), hazard ratio (HZ) and Confidence Interval at 95% (CI 95%) are reported between *B. bassiana* or controlSpin treatment and controlPept. Significant differences at 5%, (*O. laevigatus*: Cox's model, $\chi^2_2 = 87.087$, p < 0.001 / *B. terrestris*: Cox model, $\chi^2_2 = 156.38$, p < 0.001) are marked in bold.

2015; Yousef et al. 2018) increase significantly the mortality. These differences can probably be explained by at least three factors. The first one is the method of application of the EPF, which are very different among the studies and mainly explain the differences in fungi efficacy as demonstrated by Cahenzli et al. (2018): applying EFP directly on the fly body or enclosing for 24 h a fruit fly in a vial previously sprayed with the EPF lead to a much higher mortality than letting the fly walk on a treated fruit. In most studies, the EPFs are sprayed directly on the adult flies (Cuthbertson et al. 2014; Naranjo-Lázaro et al. 2014; Woltz et al. 2015; Cahenzli et al. 2018), the formers are also much less realistic in field conditions. Thus, we have selected an EPF based on a method whose application in the field seems more practical to us. The second reason is the lower concentrations of conidia (10^6 conidia/mL) and shorter duration of exposure (3 hours maximum) used in the present study. In most studies, conidia concentrations were up to 10^9/mL and exposure time 24 to 48 h (Cahenzli et al. 2018; Cossentine et al. 2016). Finally, it is not excluded that the fungal strain is important as suggested by Furuie et al. (2022). Indeed these authors showed significant differences in mortality (from 5 to 40%) between 15 strains belonging to the same *B. bassiana* species. Thus, fungal strain rather than species can be an important factor in choosing an effective EPF.

In accordance with numerous studies (Cahenzli et al. 2018; Cossentine et al. 2016; Cuthbertson et al. 2014; Naranjo-Lázaro et al. 2014; Rhodes et al. 2018; Woltz et al. 2015), we found that *B. bassiana* is a good candidate to control *D. suzukii* because death occurs within 4 to 7 days after infection, although a second generation could still appear (Lee et al. 2019; Sharma & Sharma 2021). The enzymatic arsenal (including chitinases, lipases and proteases) of *B. bassiana*, allowing the degradation of the insect cuticle, is among the main factors explaining the success of this EPF in controlling insects (Gebremariam et al. 2022). These enzymes enable the rapid hydrolysis of the molecules constituting the cuticle and accelerate the EPF lethality. The particularly high pathogenicity of *B. bassiana* could also be explained by the toxins released by this EPF in the haemolymph. Even if the mechanism of pathogenicity cannot be generalized because the same toxin may have a different mechanism of action depending on the associated host (Wang et al. 2012), the virulence of *B. bassiana* is associated to 3 main toxins: beauvericin, beauveralid and oospore in (Wang et al. 2021b). These secondary metabolites inhibit the immune system of the insect and are therefore key elements in the virulence of EPFs (Pedrini 2022; Rafaluk et al. 2017).

While several studies have shown a link between Canadian concentration and fly mortality (Cossentine et al. 2016; Naranjo-Lázaro et al. 2014), our study is the first to consider duration of exposure. We obtained 100% mortality of *D. suzukii* after 3 h exposure and even 90% after 10 s exposure. We assume that a long exposure results in better conidial adhesion, although microscopic analysis would have been necessary to confirm this hypothesis. For an efficient treatment in the field, it is thus essential to increase the contact time between *D. suzukii* and EPF. One could imagine coupling EPF with attractants such as acetic acid, ethanol, acetoin or ethyl acetate (Galland et al. 2020; Larson et al. 2020, 2021; Lasa et al. 2020; Toledo-Hernández et al. 2021). Such a lure and infection strategy, involving a self-infection device based on yeast (as attractant) and EPF, has shown promising results on *D. suzukii* (Yousef et al. 2018).

The safety of humans and non-target organisms is a major public concern related to the use of EPF for the biological control of pests. The inability of fungal spores to persist in the atmosphere for a long duration is the main reason for EPF not to display adverse effects on human health (Weng et al. 2019). The compatibility of EPF with many other biological control agents, especially predators (Canassa et al. 2019) and parasitoids (Wang et al. 2021a) of the targeted pest has repeatedly been reported. Although EPFs pose minimal risk to non-target species, their virulence must be tested on beneficial insects, on a case-by-case basis. In the present study *B. bassiana* MUCL 1555 showed no lethal effect on these two non-target insects, the stink bug *Orius laevigatus* and the bumblebee *Bombus terrestris*, also present under greenhouses, where *D. suzukii* are often observed to cause major damages. That could be attributed to the fact that these insects have strongly sclerified body segments preventing the adhesion of spores to their cuticle (Sharma & Sharma 2021). Such information is crucial since biocontrol methods can work more effectively when used in conjunction with one another. Thus, to enhance the success of individual biological control strategies, it is essential to employ them in a coordinated fashion alongside traditional or cultural practices. This collaborative approach fosters synergies that benefit both biocontrol agents and leads to a substantial reduction in pest populations.

In conclusion, *B. bassiana* MUCL 1555 was the only tested fungus to lead to *D. suzukii* mortality. We demonstrate a significant lethal effect after a very short period of exposure to the fungus (10 seconds) suggesting a great capacity of adhere and penetrate the insect cuticle. Moreover, this fungus shows no adverse effects on non-target insect species. We conclude that *B. bassiana* MUCL 1555 has great potential as a biocontrol agent and could be involved in IPM programs after optimization of field trials.

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