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Carnauba wax enhances the insecticidal activity of entomopathogenic fungi against the blowfly *Lucilia sericata* (Diptera: Calliphoridae)

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ABSTRACT

Blowfly, *Lucilia sericata* (Diptera: Calliphoridae), is a problematic synanthropic insect pest, a vector of microbial pathogens, and the causal agent of secondary myiasis. Fungal biopesticides are considered eco-friendly tools, alternative to synthetic pesticides, for the control of arthropod pests; however, to date, little is known about their bioactivity against blowflies. In this study, we assessed the insecticidal activity of three well-known entomopathogenic fungi, *Beauveria bassiana*, *Beauveria pseudobassiana* and *Akanthomyces muscarius* against *L. sericata*. In addition, we tested powdered carnauba wax as an electrically charged dust carrier in an attempt to enhance the virulence of fungal spores. Pathogenicity tests on adult flies, by adult immersion in conidial suspension (10^8 conidia mL^{-1}), showed that the median lethal time (LT_{50}) was 5.3, 5.9, and 6.2 days for *B. bassiana*, *A. muscarius* and *B. pseudobassiana*, respectively. In topical tests, when 10^8 dry conidia were mixed with or without carnauba wax, the LT_{50} was 7.7, 10.2, and 14 days without this carrier and 6.9, 8.6, and 13.8 days with it for *B. bassiana*, *B. pseudobassiana* and *A. muscarius*, respectively. Overall, our findings showed that, among the tested fungi, *B. bassiana* was the most virulent when formulated as a dry powder with carnauba wax, which greatly improved fungal efficacy against the blowfly. We discuss the utility of carnauba wax for electrostatic formulation powder of fungal spores in the integrated management of blowflies as an environmentally sustainable tool to reduce the over-reliance on chemical insecticides and their risk of resistance.

1. Introduction

Myiasis is a severe medical and veterinary problem (Monteiro, 2017) caused by the presence of dipterous larvae in living vertebrates' tissues. This parasitism, which can be obligatory or facultative (Hope, 1840), causes pains and suffering to the hosts, serious damage to the tissues and, in more severe cases, permanent mutilation and septicemia (Hall et al., 2016). Human myiasis are mainly caused by fly species belonging to the Calliphoridae. In humans, myiasis are considered to be neglected diseases, since they are most often reported in patients living under low socioeconomic conditions or as a complication of neglected wounds (Hall et al., 2016; Singh and Singh, 2015). *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae), the sheep blowfly, is a problematic synanthropic pest that causes myasis. *Lucilia* larvae grow in necrotic tissues of neglected wounds and occasionally invade healthy tissues (Visciarelli et al., 2007). *L. sericata* represents the most important agent of sheep myiasis in northern Europe (Taylor et al., 2010). In sheep, *L. sericata* eggs are laid preferentially in the moist wool containing remains of urine and feces. After hatching, larvae develop into the wool causing fetid odor wounds that may attract other species of blowflies. In addition to its medical and veterinary importance, *L. sericata* is considered a study model for Calliphoridae flies, as it can be easily maintained under laboratory conditions (Silva et al., 2008).

Currently, the prevention of myiasis relies almost exclusively on synthetic chemical insecticides (Sandeman et al., 2014). However, the inadequate use of these insecticides has increased blowfly resistance (Naqqash et al., 2016), environmental pollution and contamination of animal products (Desneux et al., 2007). The need for new biorational

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insecticides and the market-driven demand for organic products free of chemical residues reflect in the growing use of biocontrol agents. Entomopathogenic fungi may offer an environmentally sustainable alternative strategy for biological control of blowflies. Owing to the fact that few studies have addressed the control of calliphorid flies tied to the lack of available commercial mycoinsecticides to tackle this target around the world (Leathwick et al., 2019), there is an urgent need to seek microbial insecticides that may aid in the sustainable management of these noxious insects.

One of the main challenges for the use of entomopathogenic fungi to control insect pests and vectors resides in the formulation and application of fungal propagules to enhance both their virulence and persistence in the environment (Mascarin et al., 2019). Dry formulations, which consist of a combination of dry propagules and dust carriers may enhance treatment efficacy, facilitate the application, protect the fungal propagules from environmental adverse factors and also stabilize formulation during storage (Faria and Wraight, 2007; Batta, 2016). Recent studies have shown that electrostatic powders can be effective in formulating bioinsecticides, for optimizing the application of chemical pesticides and delivering pheromone for auto-confusion in mating disruption techniques (Barton et al., 2006; Meikle et al., 2008; Huang et al., 2010), since insect cuticle accumulates electrical charges depending on the surface contact and flight time (McGonigle and Jackson, 2002). For instance, Athanassiou et al. (2016) demonstrated that a formulation of the chemical insecticide pirimiphos-methyl with electrostatic powder improved its efficacy (> 90% mortality of storage pests with the lowest concentration of pirimiphos-methyl tested, 0.2 ppm) compared with the commercial formulation.

Carnauba wax - a vegetable wax obtained from the leaves of *Copernicia prunifera* (Miller) H.E.Moore (Arecales: Arecaceae), a palm tree native to Brazil - is the most studied and commonly used electret in the industry due to its dielectric characteristic, which means that it carries permanently both positive and negative charges (Gemant, 1949), and it is also capable of generating an external electrostatic field upon induction. Carnauba wax has been shown as a potentially viable carrier to develop powder-based entomopathogenic fungi delivery systems (Barton et al., 2006; Meikle et al., 2008; Athanassiou et al., 2017): for example, *B. bassiana* mixed with carnauba wax was able to control the bee mite, *Varroa* sp. (Mesostigmata: Varroidae), without affecting bee colony health (Meikle et al., 2008). A dry formulation composed of carnauba wax powder, kaolin and dry conidia of *B. bassiana* was also effective in controlling storage pests during two months in a semi-field study (Athanassiou et al. 2017). In addition, carnauba wax powder had the advantage of being transferred from treated males to non-treated females of *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) during mating behavior (Armsworth et al., 2008; Rogers et al., 2014), which may improve horizontal transmission and dispersal of fungal spores.

In order to select potential biocontrol candidates and to test the efficacy of a conidial delivery system for enhancing the insecticidal activity of entomopathogenic fungi against blowfly adults, the present study aimed to evaluate the insecticidal activity of three hypocrealean (Hypocreales: Cordycipitaceae) fungal pathogens – *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, 1912, *Beauveria pseudobassiana* S.A. Rehner & R.A. Humber, 2011 and *Akanthomyces muscarius* (Petch) Spatafora, Kepler & B. Shrestha, 2017 – against the myiasis-inducing sheep blowfly *L. sericata*. In addition, we tested powdered carnauba wax as an electrically charged dust carrier in an attempt to enhance the virulence of fungal spores as a novel formulation strategy to tackle this pest.

2. Materials and methods

2.1. Insect rearing

L. sericata pupae were purchased from Koppert Italia S.R.L. (Verona, Italy). The pupae were held in cages until the emergence of the adults. The adults were provided with sugar and water *ad libitum* and kept under laboratory conditions (23 °C, 65 ± 5% RH, natural photoperiod) during the tests.

2.2. Culture and molecular identification of fungal isolates

Three isolates belonging to well-known entomopathogenic species were tested: *Beauveria bassiana* (ARSEF 9588) from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA), *Beauveria pseudobassiana* (DSM 6651) from the German Culture Collection (DSMZ, Braunschweig, Germany) and *Akanthomyces muscarius* (I515), these two last deposited in the Fungal Collection of the Plant Pathology & Mycology Lab of the Department of Agriculture, Food and Environment (DAFE) of the University of Pisa, Italy. All isolates were maintained on Potato Dextrose Agar (PDA) under mineral oil at 4 °C and cultivated on PDA supplemented with 1 g L^{-1} yeast extract (PDAY) using Petri plates (90 × 15 mm) in the dark for 15 days at 25 ± 1 °C and RH ≥ 90% for further use in bioassays.

Molecular identification of *B. bassiana* sensu stricto (s.str.) (ARSEF9588) and *A. muscarius* (I515) was performed by the internal transcribed spacers (ITS) sequencing. Genomic DNA from seven-day-old colonies grown on PDA at 25 ± 1 °C was extracted according to the Chelex 100 protocol, as described by Baroncelli et al. (2014). Complete internal transcribed spacers (ITS) 1 and 2 sequences, including the 5.8S gene of the nuclear ribosomal DNA were amplified and sequenced with primers ITS5 and ITS4 (White et al., 1990). Amplification and sequencing were performed according to Sarrocco et al. (2015). Sequences were deposited in GenBank with accession numbers MN960454 and MN960455 for *B. bassiana* s.str. (ARSEF 9588) and *A. muscarius* (I515), respectively. *B. pseudobassiana* DSM 6651 was already identified by ITS sequencing in a previous study (Bedini et al., 2018).

2.3. Pathogenicity test

Freshly produced conidia of each fungal isolate described in section 2.2 were harvested from fully sporulated colonies grown on PDAY, suspended in sterile 0.01% (v/v) Tween 80® aqueous solution and further filtered through a cheesecloth layer; 10 mL of the conidial suspension was adjusted to 10^5, 10^6, 10^7 or 10^8 conidia mL^{-1}. Twenty newly emerged unsexed adults of *L. sericata*, previously cold anesthetized (−4 °C for 3 min), were immersed for 10 s in 10 mL of each conidial suspension. Flies from control group were immersed in 10 mL of 0.01% (v/v) Tween 80® without conidia. Mortality was monitored and recorded daily for 10 days. The experiment was repeated four times on different days using new fungal cultures to ensure reproducibility.

2.4. Formulation of conidia with carnauba wax

Fresh conidia of *B. bassiana*, *B. pseudobassiana* and *A. muscarius* were harvested from PDAY as described above and suspended in 0.01% (v/v) Tween 80®. Conidial suspensions of *B. bassiana* and *A. muscarius* were filtered through a cheesecloth layer and adjusted to the concentration of 10^8 or 10^9 conidia mL^{-1}. Since *B. pseudobassiana* produced fewer conidia than the other isolates, bioassays with *B. pseudobassiana* were performed by using only 10^8 conidia mL^{-1} suspensions. One mL of each conidial suspension was poured into Petri dishes (30 × 10 mm) and put in a desiccator with activated silica gel for five days at 5 ± 0.5 °C. Carnauba wax powder (PVP Sociedade Anônima, Parnaíba, PI, Brazil) was micronized and freeze-dried at −40 °C for 24 h at 0.1 mbar. After drying, 10^8 and 10^9 conidia of each isolate were tested alone, or manually mixed to 0.1 g of the freeze-dried carnauba wax using a metallic spatula; this mixture yielded a final concentration of 10^9 and 10^{10} conidia g^{-1}, respectively. Water activity (a_w) of formulations was measured, and those with $a_w < 0.2$ were considered dried and able to maintain carnauba wax electrostatic properties (Chamber et al., 2018).

Twenty newly emerged unsexed adults of *L. sericata*, previously cold anesthetized (−4 °C for 3 min), were carefully transferred into a glass

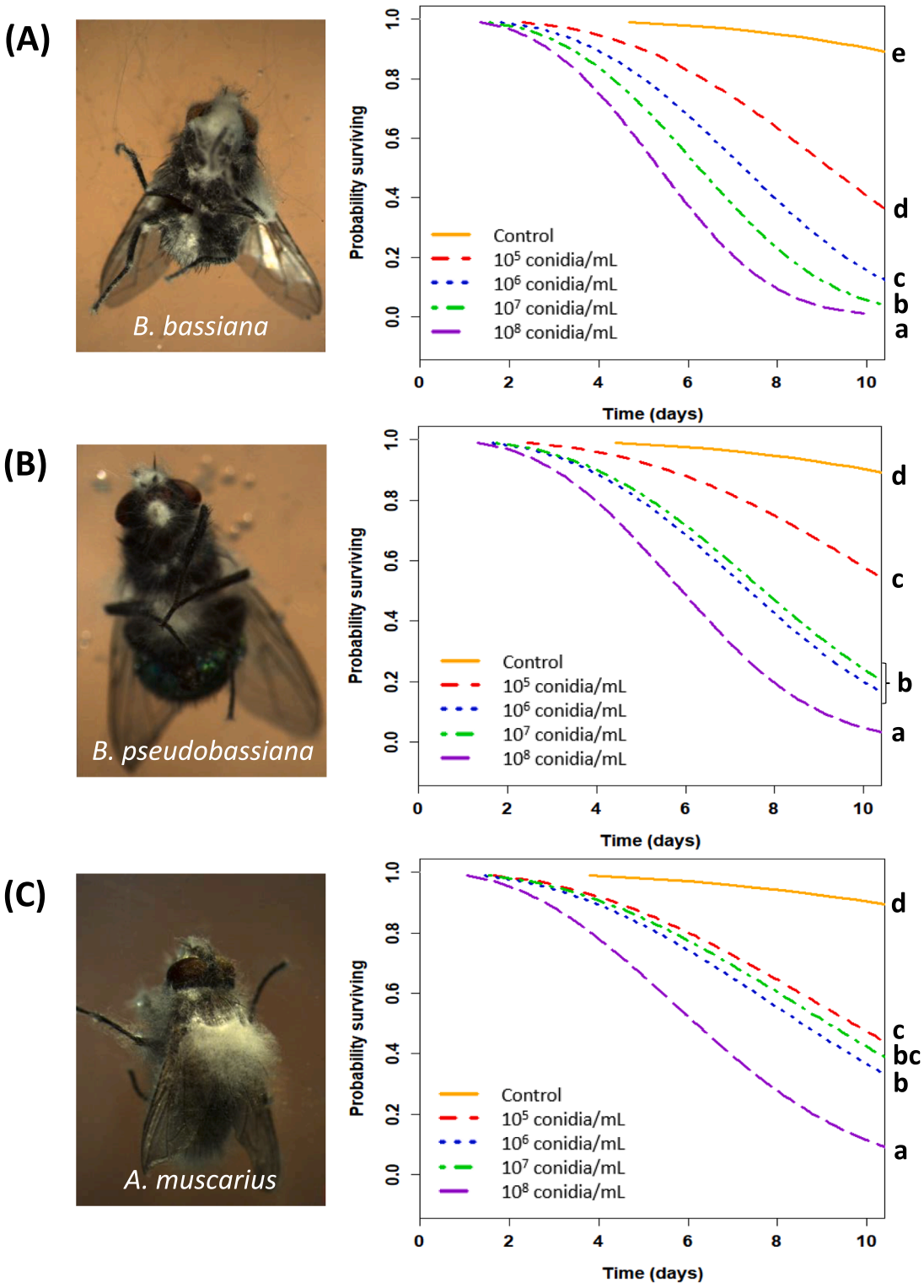

Fig. 1. Survival curves and mycosed flies due to infection by three entomopathogenic fungal isolates. (**A**) *Beauveria bassiana* ARSEF 9588; (**B**) *Beauveria pseudobassiana* DSM 6651; (**C**) *Akanthomyces muscarius* I515. Conidial suspensions were prepared in 0.01% (v/v) Tween 80®. Survival curves were pairwise compared using log-rank test with *P*-values adjusted with Bonferroni-Holm method and considered significant at $P < 0.05$.

Petri dish containing 0.1 g of the carnauba wax formulated with conidia or unformulated dry conidia (both tested at 10^8 and10^9 conidia). The flies were allowed to walk over the dry conidia, or over the carnauba wax formulated with conidia, for 5 min. Two control groups were performed, one treated with 0.1 g of carnauba wax without conidia and another control group that did not receive any treatment. The flies were then removed from the plates and placed inside small plastic cages (20 cm in diameter and 140 cm in height), which were closed on the top with a net cloth. Sugar and water were provided *ad libitum* to the flies during the tests by a water-soaked cotton roll and a container filled with 5 g of granulated sugar. The flies were maintained at 24 ± 1 °C, with RH ≥ 90% under natural photoperiod. Fly mortality was assessed daily

Table 1
Lethal times (LT_{50} and LT_{90}) and their respective confidence intervals (CI 95%) of *Lucilia sericata* adults treated by immersion in conidial suspensions of *Beauveria bassiana*, *Beauveria pseudobassiana* or *Akanthomyces muscarius* at different concentrations (10^5, 10^6, 10^7 or 10^8 conidia mL^{-1}) and incubated under laboratory conditions (24 ± 2 °C, 90 ± 5% RH, and natural photoperiod).

Fungus	Concentration (conidia mL^{-1})	LT_{50} (days)†	CI95% (days)	LT_{90} (days)†	CI95% (days)
B. bassiana	0 (control)	ND*	–	ND*	–
	10^5	9.2a	8.4–10.1	13.6a	12.3–15.0
	10^6	7.2b	6.7–7.8	10.7b	9.9–11.6
	10^7	6.2b	5.8–6.7	9.3b	8.6–10.0
	10^8	5.3c	5.0–5.7	7.9c	7.4–8.5
B. pseudobassiana	0 (control)	ND*	–	ND*	–
	10^5	10.9a	9.7–12.2	16.6a	14.7–18.9
	10^6	7.4b	6.9–8.1	11.3b	10.4–12.4
	10^7	7.8b	7.1–8.4	11.8b	10.9–12.9
	10^8	5.9c	5.5–6.4	9.0c	8.4–9.7
A. muscarius	0 (control)	ND*	–	ND*	–
	10^5	9.7a	8.5–11.0	16.0a	14.0–18.4
	10^6	8.5a	7.6–9.6	14.2a	12.5–16.1
	10^7	9.1a	8.1–10.3	15.1a	13.2–17.3
	10^8	6.2b	5.6–6.8	10.2b	9.2–11.3

(*) ND (not determined) indicates that values could not be calculated because mortality was lower than 50% or 90% at the end of the experiment.
(†) Different letters within each fungal species indicate significant contrasts between spore concentrations, based on the overlap of 95% confidence intervals ($P < 0.05$).

for 15 days. The experiments were repeated three times on different days using new fungal cultures to ensure reproducibility.

2.5. Scanning electron microscopy (SEM)

To compare the potential sites of infection and the spatial distribution of dry or wax-formulated conidia on the cuticle surface of blowfly, *L. sericata* adults were treated with 10^8 dry conidia formulated with carnauba wax or only dry conidia, as described above. Flies were maintained at 24 ± 1 °C, with RH ≥ 90% under natural photoperiod, for 48 h. After this incubation period, each fly was fixed by immersion in 2 mL of fixative [2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, 3% (w/v) sucrose in 0.1 M sodium cacodylate buffer solution, pH 7.2] at 4 °C for 10 days. Thereafter, samples were washed in phosphate buffer, dehydrated in graded series of ethanol solutions and subjected to critical point drying (Autosamdri®-815). Flies were metallized with gold in a sputter-applicator (Denton Vacuum Desk V) and analyzed by a scanning electron microscope (Jeol JSM 6610), located in the multiuser laboratory of high-resolution microscopy (LabMic/UFG, Goiânia-GO, Brazil), at the accelerating voltage of 20 kV.

2.6. Statistical analysis

Goodness-of-fit for model selection was assessed using half-normal plots with simulation envelopes (Moral et al., 2017) along with examination of the smallest Akaike's information criterion (AIC). All analyses were conducted in the free statistical environment R (R Core Team, 2018). Probability of surviving was estimated using the censored survival data of *Lucilia* flies exposed to different fungal species prepared at different concentrations as either aqueous conidial suspensions or as dry conidia. The dry conidia of these fungi were tested alone or combined with carnauba wax forming a dust formulation at two different concentrations. The survival curves obtained from time-mortality data and estimates of median and 90% lethal times (LT_{50} and LT_{90}) with their respective 95% confidence intervals were obtained from Weibull parametric models using the "survival" package (Therneau, 2015). Survival curves were pairwise compared using log-rank test with *P*-values adjusted with Bonferroni-Holm method and considered significant at $P < 0.05$.

Multiple concentration-mortality data recorded after 10 days of exposure were fitted to a generalized linear model with logit link function and a quasi-binomial distribution for errors to take into account the overdispersion parameter [i.e., variance greater than the mean in proportion data (Demétrio et al., 2014)]. In this model, the fixed effects were attributed to fungal species, concentration and their interaction term. Median and 90% lethal concentrations (LC_{50} and LC_{90}) values and their respective 95% confidence intervals for fungal species were estimated using the function 'dose.p' from the "MASS" package (Venables and Ripley, 2002).

3. Results

3.1. Molecular identification of fungal isolates

Amplification of the ITS regions resulted in around 550 bp long sequences for both *B. bassiana* ARSEF 9588 and *A. muscarius* I515 isolates. For isolate ARSEF 9588 the comparison of our ITS sequence (accession number MN960454) with those deposited in GenBank resulted in high similarity percentages (100%) with ITS sequences from other isolates of *B. bassiana* (such as MK862360.1, MF802501.1, MF802497.1, MG515530.1). When ITS sequence of isolate I515 (accession number MN960455) was compared with those available in GenBank, a 100% similarity percentage was achieved with ITS sequence of *A. muscarius* (accession number MH858370.1).

3.2. Pathogenicity to Lucilia sericata adults

Survival curves of flies inoculated with fungal spores differed significantly from the control group (Fig. 1): A) *B. bassiana* ($\chi^2 = 289.36$, $df = 4$, $P < 0.001$), B) *B. pseudobassiana* ($\chi^2 = 214.8$, $df = 4$, $P < 0.001$) and C) *A. muscarius* ($\chi^2 = 131.12$, $df = 4$, $P = 0.002$). The sporulation rate of fly cadavers was higher than 90% in all fungal treatments with no significant differences between fungal isolates ($P > 0.05$).

Lethal times of flies decreased with the increase in conidial concentration for all fungal isolates tested (Table 1). At 10^7 conidia mL^{-1}, the LT_{50} of flies treated with *B. bassiana* was 3 days lower than that of the flies treated with *A. muscarius* (LT_{50} = 6.2 and 9.1 days, respectively). Consistently, smallest value of LT_{90} was observed for the flies treated with *B. bassiana* (LT_{90} = 7.9 days at 10^8 conidia mL^{-1}), whereas, at the same conidial concentration, the LT_{90} values of flies treated with *B. pseudobassiana* and *A. muscarius* were 9.0 and 10.2 days, respectively. In addition, *B. bassiana* applied at 10^7 conidia mL^{-1} also killed faster blowflies as it rendered lower LT_{90} (9.3 days) than *B. pseudobassiana* (11.8 days) and *A. muscarius* (15.1 days). Overall, *B. bassiana* was able to kill faster blowflies than the other two fungal species at 10^7 and 10^8 conidia mL^{-1}.

According to the logistic binomial model, fly mortality was significantly correlated with fungal conidial concentration with slopes significantly different from each other ($\chi^2 = 50.02$, $df = 3$, $P < 0.001$) (Fig. 2). In detail, *B. bassiana* had a significantly higher slope than *A. muscarius*, but not in relation to *B. pseudobassiana* (Table 2). *L. sericata* was indeed more susceptible to *B. bassiana* than to the other two fungi tested ($\chi^2 = 9.51$, $df = 3$, $P = 0.023$), which also resulted in the lowest LC_{50} and LC_{90} values (Table 2). As a result, *B. bassiana* required many fewer conidia to kill 50% and 90% of flies than *B. pseudobassiana* and *A. muscarius*. On the contrary, it was not possible to determine the LC_{90} for *A. muscarius*, because fly mortality did not reach 90%, thereby confirming that this fungus was not as virulent as the two *Beauveria* isolates.

3.3. Virulence of conidia formulated with carnauba wax carrier

Treatments with carnauba wax mixed with dry conidia and conidia alone significantly reduced survival of *L. sericata* adults compared to

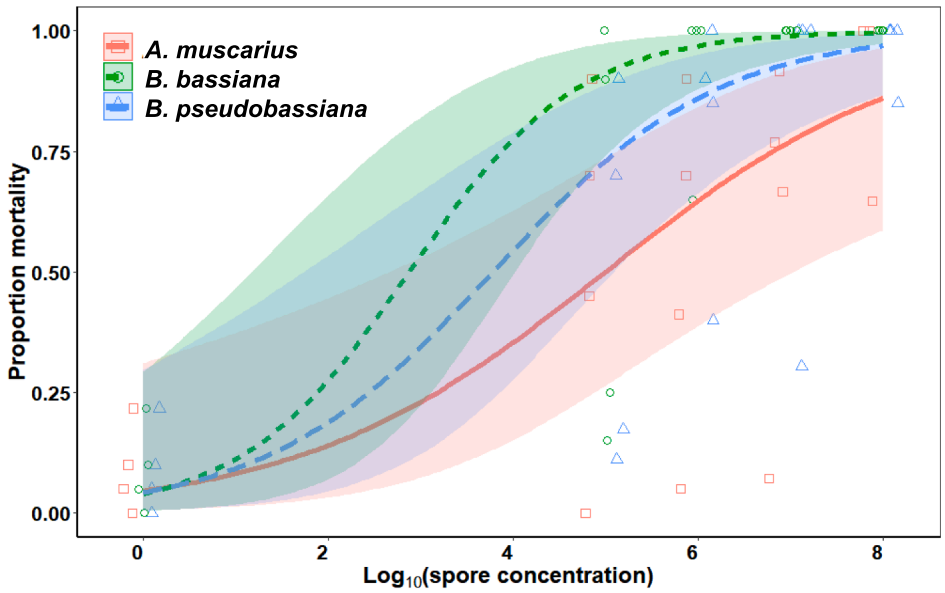

Fig. 2. Mortality of *Lucilia sericata* adults 10 days after exposure to entomopathogenic fungi (*Beauveria bassiana*, *Beauveria pseudobassiana* or *Akanthomyces muscarius*). Concentration-mortality data were fitted to a generalized linear model with quasibinomial distribution for errors to accommodate overdispersion, and the fixed effects were attributed to fungal species, concentration and their interaction term. Median and 90% lethal concentrations (LC_{50} and LC_{90}) values and their respective 95% confidence intervals for fungal species were estimated using the function 'dose.p' from the MASS package in R. Empty symbols represent observation points, and different lines denote predicted mortality curves for each fungal isolate obtained with the model.

wax control free of fungus ($\chi^2 = 295.29$, $df = 4$, $P < 0.001$) (Fig. 3). Actually, carnauba wax alone caused very low mortality to the flies, 6.5 ± 6.5% (Fig. 3); the control group (without treatment) reached the mortality of 15 ± 10%. In addition, regarding comparison between survival curves, the survival rate of the insects treated with either *B. bassiana* or *A. muscarius* conidia mixed with carnauba wax decreased significantly faster than the insects treated with fungal conidia alone when applied at 10^8 conidia ($P = 0.043$) and 10^9 conidia ($P < 0.0001$), respectively (Fig. 3A, C). This result was not observed with flies treated with *B. pseudobassiana* conidia and carnauba wax formulation. In this last case, survival rates of *L. sericata* adults were similar in both treatments, with or without carnauba wax ($P = 0.091$) (Fig. 3B).

Estimated lethal times (LT_{50} and LT_{90}) of flies treated with dry conidia alone were similar to those of flies treated with conidia-based carnauba wax formulation, with the only exception for *A. muscarius* formulated with carnauba wax that enhanced conidia activity by killing faster the flies when exposed to 10^9 conidia compared with dry conidia only (Table 3).

3.4. Scanning electron microscopy (SEM)

Conidia of the entomopathogenic fungi were found clustered and attached mainly to the thorax (Fig. 4A) and wings (Fig. 4B) of the treated flies. Conidia were completely absent from the eyes, head and legs of *L. sericata*. There was no evidence of conidial germination of *B. pseudobassiana* (Fig. 4C) and *A. muscarius* (Fig. 4D) after 48 h of inoculation. Conversely, *B. bassiana* conidia (unformulated) were clearly observed presenting bipolar (bidirectional) germination and appressorial formation on the cuticle of flies (Fig. 4E and 4F).

Carnauba wax particles were irregular in shape and size; wax microaggregates were found on the wings (Fig. 5A) or attached to the thorax bristles (Fig. 5B) with or without conidia. Germinated conidia of *B. bassiana* (Fig. 5C and D) and *A. muscarius* (Fig. 5G and H) were observed when formulated with carnauba wax at 48 h post-application, but not for *B. pseudobassiana* (Fig. 5E and F). This indicates that *B. pseudobassiana* may take longer to resume germination on the insect cuticle than the other fungal species tested here.

4. Discussion

This is the first study that showed *B. bassiana*, *B. pseudobassiana* and *A. muscarius* conidial pathogenicity to *L. sericata* adults, but with different degrees of virulence. Although blowfly larvae correspond to the parasitic stage, sheep fleece is a complex and dynamic environment, including its own microbial flora and a range of skin-secreted compounds that may affect fungal viability (Jackson et al., 2010). Adults have been considered to be the most suitable target stage for the fungal biocontrol of sheep blowfly (Sandeman et al., 2014). In our experiments, fly mortality rates reached 100% after 10 days of incubation after immersion in suspension of *B. bassiana* at 10^7 conidia mL^{-1}. Similar control levels were obtained after treating *L. sericata* adults by immersion with *Metarhizium anisopliae* s.L. (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) at 10^7 conidia mL^{-1} (Wright et al., 2004). Although satisfactory results were obtained here, an immersion treatment of adult flies is not feasible for practical field uses; therefore, in our second study we proposed a dry formulation of the fungal entomopathogens within the aim to develop a dispersal strategy to control calliphorid flies. Moreover, dry formulations of entomopathogenic fungi can survive longer during storage than in liquid preparations (Moore et al., 1995).

Table 2
Virulence of entomopathogenic fungi (*Beauveria bassiana*, *Beauveria pseudobassiana* or *Akanthomyces muscarius*) to *Lucilia sericata* adults treated by immersion in aqueous conidial suspensions and incubated under laboratory conditions (24 ± 2 °C, 90 ± 5% RH, and natural photoperiod). Virulence was measured in median and 90% lethal concentration (LC_{50} and LC_{90}) followed by confidence interval (CI 95%).

Fungus	Slope (± SE)[a]	LC_{50} (conidia mL^{-1})	CI 95%	LC_{90} (conidia mL^{-1})	CI 95%
B. bassiana	1.1 ± 0.2a	7.84×10^2	1.42×10^1–4.15×10^4	7.32×10^4	7.22×10^3–1.76×10^5
B. pseudobassiana	0.82 ± 0.20ab	6.01×10^3	6.02×10^1–6.06×10^5	2.97×10^6	1.74×10^5–9.09×10^6
A. muscarius	0.61 ± 0.19b	9.66×10^4	4.21×10^2–2.26×10^7	ND[b]	5.76×10^6–3.74×10^{13}

[a]Slopes (± standard error) were obtained from the logistic-normal model and different letters indicate significant differences among treatments ($\chi^2 = 50.02$, df = 3, $P < 0.0001$). [b] ND = not determined, as mortality did not reach 90% and thus it was not able to be estimated for LC_{90} of *A. muscarius*.

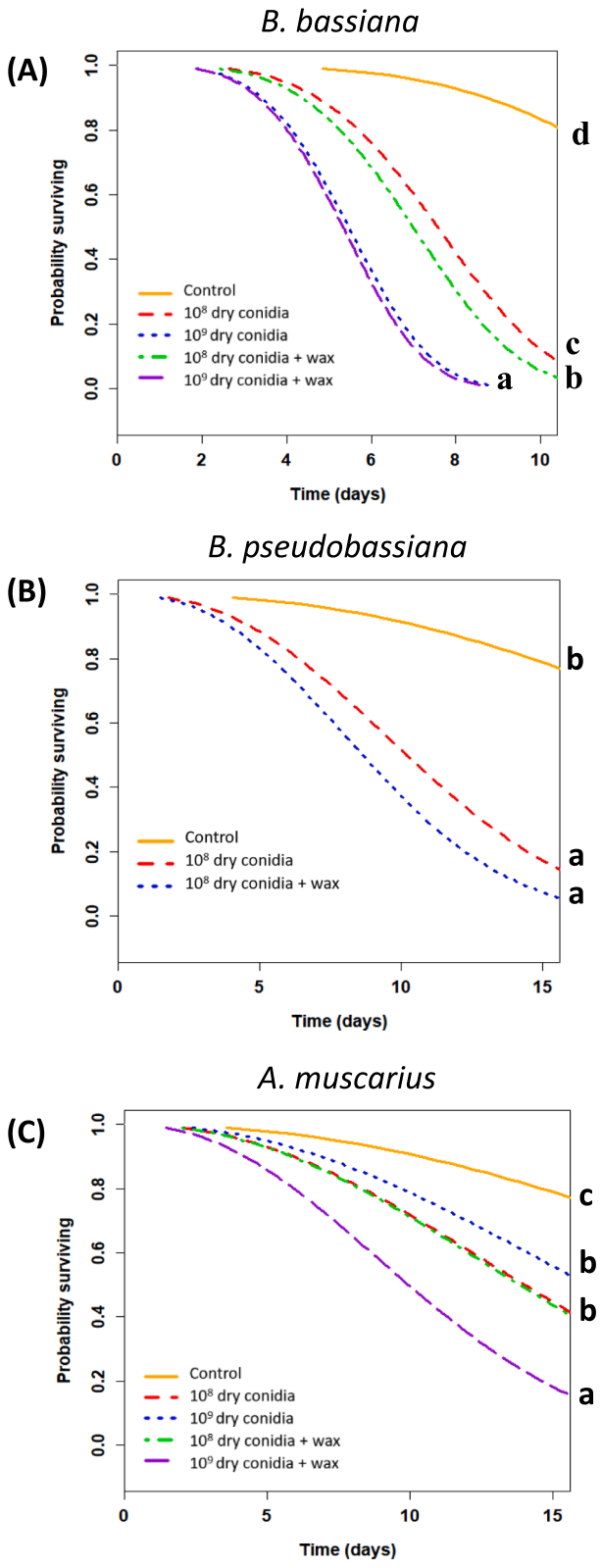

Fig. 3. Survival curves of *Lucilia sericata* adults treated with carnauba wax (control), dry conidia or carnauba wax mixed with dry conidia of different entomopathogenic fungi: (**A**) *Beauveria bassiana*, (**B**) *Beauveria pseudobassiana* and (**C**) *Akanthomyces muscarius*. Survival curves were pairwise compared using log-rank test with *P*-values adjusted with Bonferroni-Holm method and considered significant at $P < 0.05$.

Besides demonstrating the insecticidal activity of these entomopathogenic fungi, in this work we tested carnauba wax as an additive to enhance their effectiveness against blowfly. Our findings suggest that dry conidia of *B. bassiana* and *A. muscarius* exhibited an increased insecticidal performance against blowfly *L. sericata* when mixed with carnauba wax powder, but this virulence was dependent on the conidial concentration. The most pronounced improvement in the virulence using carnauba wax for conidial formulation was achieved with *A. muscarius*, which had been previously shown to be the least virulent fungus in the first bioassays using immersion technique for contamination. These results indicate that carnauba wax powder is able to boost the insect infection rate by the fungal conidia, but conidial concentration and physicochemical properties may affect in different manners the interactions between carnauba wax and fungal virulence. Therefore, additional studies are necessary to unravel these complex interactions between fungal spores and carnauba wax on a target host cuticle.

Improvement in performance of the entomopathogenic fungi may be due to the hydrophobic properties of carnauba wax that could have increased the adhesion of conidia onto the hydrophobic cuticle of insect hosts (David-Henriet et al., 1998; Barreto et al., 2016). In line with our results, Athanassiou et al. (2017) also showed a high efficacy of formulation of *B. bassiana* conidia plus Entostat™ (Exosect Ltd, Winchester, UK) and kaolin against stored-product beetles; this dry powder formulation induced mortality rates comparable to those of the chemical insecticide deltamethrin in field conditions. As suggested by Athanassiou et al. (2017), dry conidia formulated with carnauba wax may favor the protection of conidia against detrimental abiotic factors such as ultraviolet radiation and high temperatures, but we have not tested whether such ecological protection is afforded by carnauba wax on the fungal species studied here.

Carnauba wax may also act as an electrostatic carrier for delivery of infective fungal propagules, such as aerial conidia (Barton et al., 2006). In fact, insects can accumulate electrical charges on the surface of their body during flight by contacting a dielectric surface, transferring ions via dermal glands, and attaching charged particles (McGonigle and Jackson, 2002). Because of that, carnauba wax carrying hydrophobic aerial conidia of entomopathogenic fungi may facilitate adhesion and thus providing better dispersion of these propagules on the host body surface to initiate infection. The adhesion of conidia on the host cuticle is strongly related to the hydrophobicity of the conidia cell wall and the epicuticle of arthropods (Holder and Keyhani, 2005), and electrostatic activity on conidial surface seems to have little influence on the adhesion process (Boucias et al., 1988). In the current study, carnauba wax was micronized and freeze-dried (aw < 0.2), mixed with dry conidia as described by Chamber et al. (2018), and wax electrostatic interaction with conidia occurred by friction using a metallic spatula. In addition, when flies walked on a dielectric surface possibly retained electrostatic charge on their body (McGonigle et al., 2002), and enhanced electrostatic attraction of the formulation with the insect. Although, electrostatic charge of conidia-based carnauba wax formulation was not measured, our results indicated that carnauba wax may act as a good carrier for effectively delivering conidia to the target, resulting in the attachment to the insect body by both electrostatic and hydrophobic properties. However, based on our SEM results, we observed that carnauba wax did not influence the deposition rate of conidia onto the insect surface, showing similar numbers of conidia found per insect body when either formulated or not with this carrier.

Another key feature of entomopathogenic fungi resides in their ability of spreading among the insect population to healthy individuals that have not directly been in contact with the insecticide formulation, thus enhancing the effectiveness of the biological treatment in auto-confusion, or auto-dissemination, systems (Bedini et al., 2018). In this regard, the dispersion of the fungal conidia through a powder formulation may facilitate such individual-individual pathogen transmission, also known as horizontal transmission, which is an important attribute to fungal epizootics under field conditions. Armsworth et al.

Table 3
Lethal times (LT_{50} and LT_{90}) and their respective confidence intervals (CI 95%) of *Lucilia sericata* adults treated with dry conidia mixed with or without carnauba wax and incubated under laboratory conditions (24 ± 2 °C, 90 ± 5% RH under natural photoperiod).

Fungus	Concentration (number of conidia)	LT_{50} (days)	CI 95% (days)	LT_{90} (days)	CI 95% (days)
B. bassiana	0 (only wax)	ND	–	ND	–
	10^8	7.7	7.1–8.1	10.2	9.6–10.9
	10^8 + wax	6.9	6.5–7.4	9.4	8.9–10.0
	10^9	5.5	5.1–5.8	7.4	7.0–7.8
	10^9 + wax	5.3	5.0–5.7	7.2	6.8–7.6
B. pseudobassiana	0 (only wax)	ND	–	ND	–
	10^8	10.2	9.0–11.5	16.8	14.8–19.1
	10^8 + wax	8.6	7.7–9.7	14.2	12.7–16.0
A. muscarius	0 (only wax)	ND	–	ND	–
	10^8	14.0	12.0–16.4	24.2	20.3–28.9
	10^8 + wax	13.8	11.9–16.1	23.9	20.2–28.5
	10^9	16.2	13.8–19.1	28.1	23.3–34.0
	10^9 + wax	9.9*	8.6–11.5	17.2*	14.7–20.0

[a]ND (not determined) indicates that values could not be calculated because mortality was lower than 50% or 90% at the end of the experiment. Asterisk (*) indicates significant difference between treatments (dry conidia only, and dry conidia + carnauba wax) based on the overlap of 95% confidence intervals ($P < 0.05$).

(2008) demonstrated that *C. capitata*, which visited baits with Entostat™ (a carnauba-based product), took powder up on their bodies and also those flies that had not come into direct contact with electrostatic powder were contaminated through social interactions with contaminated flies or contaminated surfaces. Actually, within the first 24 h after treatment, we observed an increase of self-cleaning behavior (auto-grooming) in the flies treated with conidia plus carnauba wax formulation; the absence of conidia, confirmed by SEM investigation, indicates that the flies had routinely cleared their eyes, head and legs during wax-induced grooming behaviors.

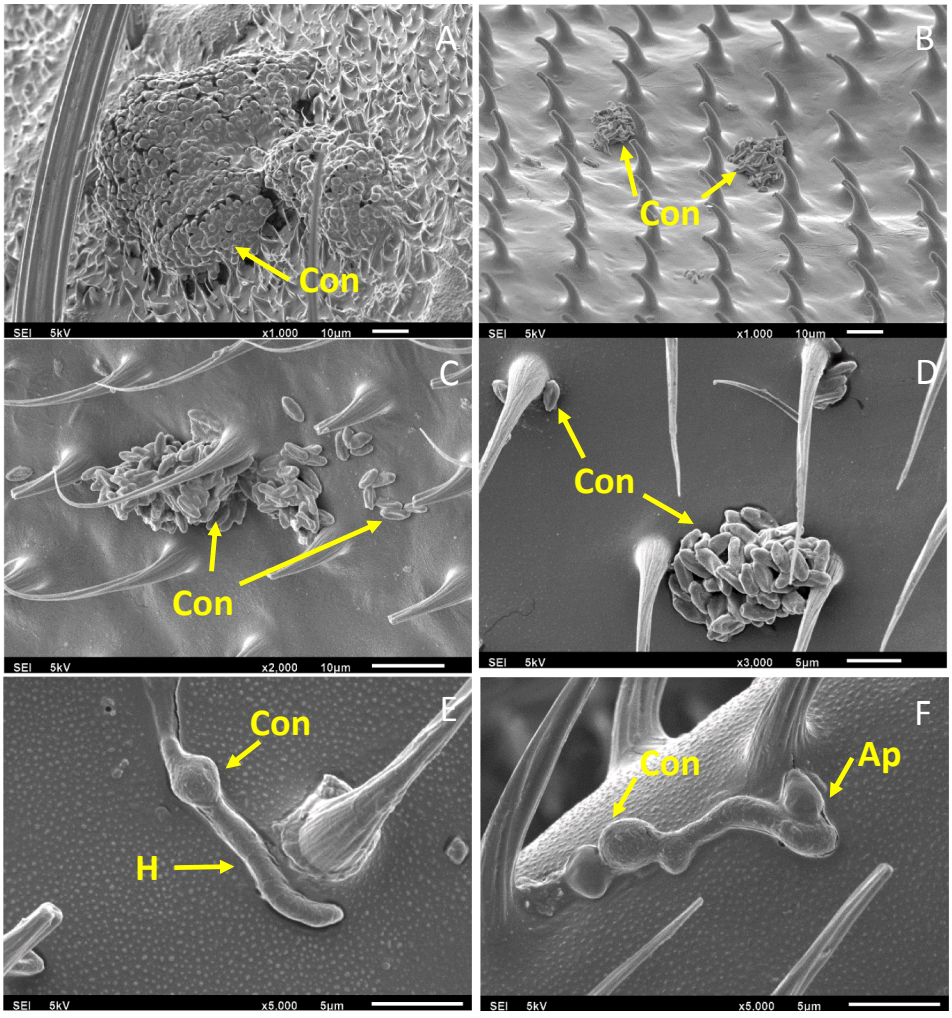

Fig. 4. Scanning electron micrographs of *Lucilia sericata* adults treated with dry conidia of different species of entomopathogenic fungi and incubated at 24 ± 2 °C, with RH = 90% and natural photophase for 48 h. (**A, E** and **F**) *L. sericata* treated with *Beauveria bassiana*; (**C**) *L. sericata* treated with *Beauveria pseudobassiana*; (**B** and **D**) *L. sericata* treated with *Akanthomyces muscarius*. Regions of fly: (**A**) thorax, and (**B** - **F**) wings. Con = conidia (single and clumps), Ap = appressorium, and H = fungal hypha.

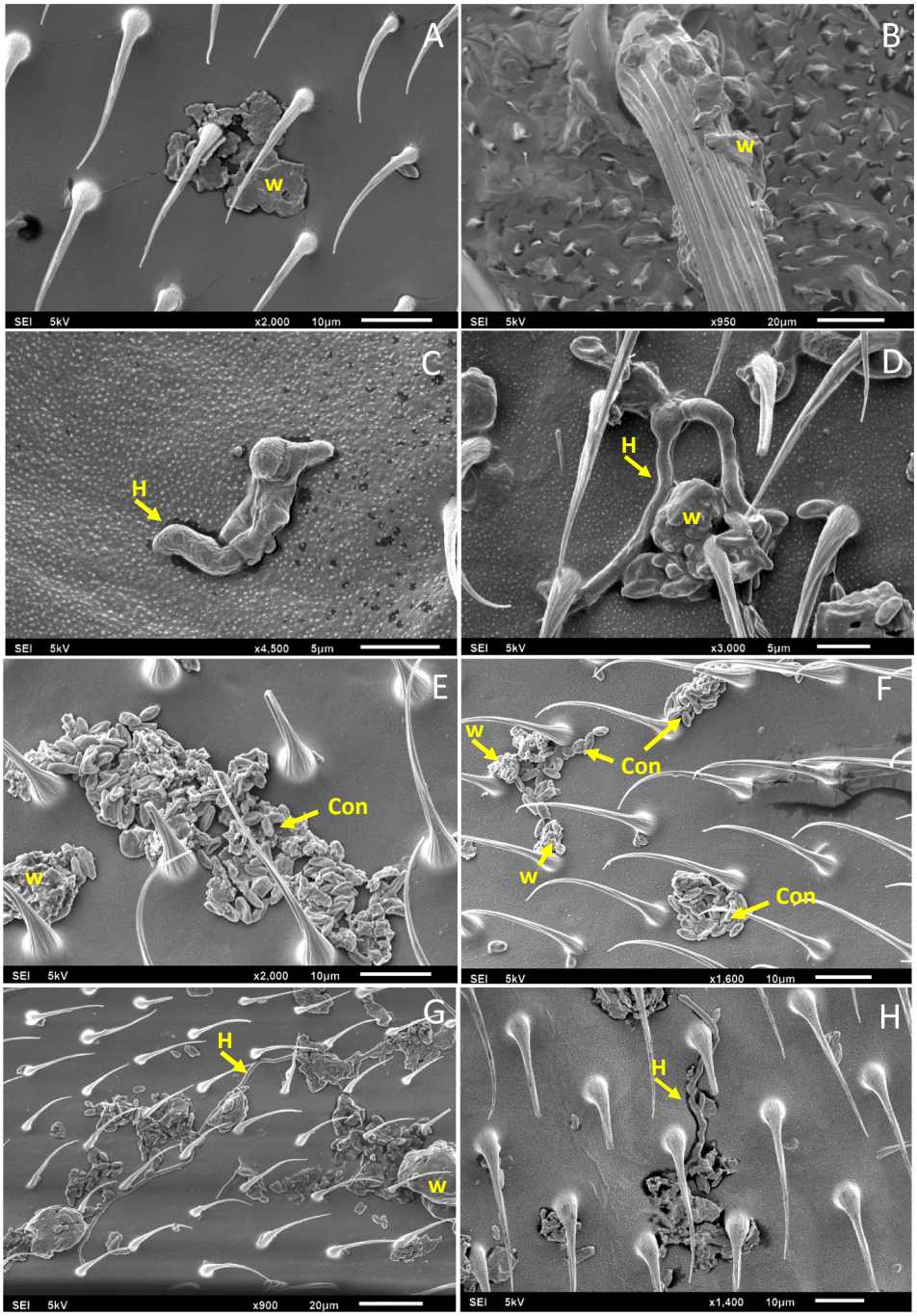

Fig. 5. Scanning electron micrographs of *Lucilia sericata* adults treated with formulation of dry conidia with carnauba wax of different species of entomopathogenic fungi and incubated at 24 ± 2 °C, with RH = 90% under natural photophase for 48 h. (**A**) *L. sericata* wing with carnauba wax; (**B**) *L. sericata* thorax with carnauba wax; (**C** and **D**) *L. sericata* cuticle with *Beauveria bassiana* formulation; (**E** and **F**) *L. sericat* cuticle with *Beauveria pseudobassiana* formulation; (**G** and **H**) *L. sericata* cuticle with *Akanthomyces muscarius* formulation.W = carnauba wax granule; H = fungal hypha.

It is conventionally well-known that the main route of infection of entomopathogenic fungi is through the insect cuticle where conidia - after adhesion to the cuticle by nonspecific hydrophobic and electrostatic interaction - germinate and penetrate the host (Mannino et al., 2019). In our study, SEM observations showed that conidia of *B. bassiana*, unlike the other two fungal species tested, germinated from opposite sides on the insect cuticle. Such bidirectional germination by *B. bassiana* conidia has not been well documented in the literature. For instance, Talaei-Hassanloui et al. (2007) correlated this type of conidial germination with the low virulence of *B. bassiana* isolates because no evidences of fungal penetration were detected. In our study, however, *B. bassiana* had bipolar-germinated conidia during cuticle penetration and exhibited higher efficacy against the blowfly than the other two fungal species tested here; indeed, this result needs further investigation. Lastly, it is also important to note that *A. muscarius* formulated with carnauba wax exhibited an improved virulence in terms of shortening survival time to kill 50% and 90% of fly population with a higher concentration of conidia, but carnauba wax was not able to significantly reduce neither the LT_{50} nor LT_{90} for *B. bassiana* and *B. pseudobassiana*. However, *B. bassiana* conidia formulated with carnauba wax decreased the survival rate of blowflies faster than conidia alone when tested at 10^8 conidia, but not at higher concentration. Besides

that, we noticed that *B. bassiana* attained lower values of LT_{50} and LT_{90} in comparison to the other fungal species (see Table 3), either formulated with or without carnauba wax, indicating that the former is the most virulent candidate for blowfly control. Such incongruence among these three fungi interacting with carnauba wax should be studied in detail to elucidate how this powder formulation might further improve fungal efficacy against this and other fly pests of agricultural and veterinary importance using reduced fungal loads for field application.

In summary, our findings highlight the virulence of *B. bassiana* against adults of *L. sericata* and confirm the ability of dust carnauba wax as an electrostatic carrier that may enhance the insecticidal activity of conidia produced by the entomopathogenic fungi. These results may serve as a baseline to design field strategies for controlling *L. sericata* by facilitating dispersal of entomopathogenic fungal conidia formulated with carnauba wax powder.

Declaration of Competing Interest

None.

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