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Efficacy of Entomopathogenic Fungi Against *Bruchus rufimanus* (Coleoptera: Chrysomelidae) in Laboratory and Field Trials Using Dropleg Spraying Technique

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Abstract: Entomopathogenic fungi (EPF)-based biopesticides have attracted growing interest in pest management as alternatives to neurotoxic insecticides. Their potential was evaluated against various pests, including the broad bean weevil (*Bruchus rufimanus Boheman 1833*), a significant threat to faba bean (*Vicia faba* L.) crops. This study examined the entomotoxic effects and sublethal impacts (on oviposition) of three fungal strains under laboratory conditions: *Beauveria bassiana* (GHA), *Metarhizium brunneum* (USDA 4556), and *M. brunneum* (V275) on *B. rufimanus* adults. Subsequently, a large-scale field trial assessed the efficacy of *B. bassiana* (GHA) against *B. rufimanus* infestations using conventional anti-drift and dropleg spraying methods. The laboratory LT_{50} values ranged from four days for *B. bassiana* to eight days for *M. brunneum* (V275). The mortality rates recorded after ten days ranged from 86.6% for *M. brunneum* (V275) to 96.6% for *B. bassiana* (GHA). The inhibition of oviposition rates ranged from 12% for *M. brunneum* (USDA 4556) to 36% for *B. bassiana* (GHA). Field trials showed that the dropleg nozzles targeted faba bean pods, the oviposition sites of *B. rufimanus*, more effectively than the anti-drift nozzles. However, both fungal and chemical treatments applied via dropleg nozzles offered limited protection, reducing the infestation rates by 7% and 14%, respectively, with only a 3% improvement over anti-drift nozzles. This suggests that the large-scale spraying of chemical or fungal agents, including *B. bassiana* GHA, is not an optimal IPM strategy for managing *B. rufimanus* in faba beans. These laboratory and field results highlight the potential of EPF for managing *B. rufimanus*. However, the limitations of spray-applied plant protection methods underscore the need to redirect research toward more targeted strategies, such as attract-and-infect or endophytic EPF approaches.

Keywords: *Vicia faba*; faba bean; biopesticide; microbial control; *Beauveria bassiana*; *Metarhizium brunneum*

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1. Introduction

Entomopathogenic fungi (EPF) are naturally occurring microorganisms with significant entomotoxic potential against a wide range of insect pests, making them a promising, friendly, and sustainable alternative to chemical insecticides [1,2]. The use of EPF to manage insect pests is attracting much interest due to their direct entomotoxicity and minimized environmental, health, and ecological risks [3,4]. In addition, their ability to colonize plants as endophytes provides antagonistic effects on the insect pest population, triggering the production of toxic metabolites by plants or modifying plant VOC profiles that regulate tripartite interactions between plants, herbivores, and natural enemies [2].

Approximately 700 to 800 species of EPF are concentrated within Ascomycota (Hypocreales) and Zoopagomycota (*Entomophthorales*) phyla [5–7]. Different fungal species within the Hypocreales (Ascomycota) have been formulated and commercialized for large-scale applications against insect pests [8]. Although EPF have demonstrated significant potential as biopesticides in different crops, their application has not been sufficiently explored in the faba bean crop, a legume of increasing interest in Europe [9–11]. Despite numerous environmental benefits, this crop remains highly sensitive to biotic and abiotic stressors, leading to unstable yields from year to year [12–14]. The bean weevil *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae) is among the leading economic biotic threats to faba bean seed quality.

The damage caused by *B. rufimanus* begins with larvae developing within the green forming seeds, later emerging as adults from mature seeds at harvest [12,15]. After emerging in late summer, the adults enter a reproductive diapause through the fall and winter, preparing for a new generation aligned with the spring's next flowering season of faba bean crops [14]. The lifecycle of these weevils then synchronizes with the flowering period (allowing mating after consuming faba bean nectar and pollen) and the fruiting period, during which oviposition occurs on developing green pods [14,16]. This pest, primarily found in temperate zones where faba beans grow, has a univoltine lifecycle, producing one generation per year [12,17]. The endophytic lifecycle of the bean weevil results in seed coat perforation when the adults emerge, degrading the seed quality rather than quantity [18]. These perforations cause notable impacts on the seeds' aesthetic, organoleptic, and nutritional values as well as a decrease in their storage capacity. This damage has high economic repercussions as seed batches are rejected from food and feeding markets, which impose respective quality standards of 3% and 10% of infested seeds [19–22]. No further impacts are caused in storage facilities by this insect as (i) emerging insects are in reproductive diapause and (ii) females of *B. rufimanus* are unable to oviposit on dry seeds [21,23].

The management of *B. rufimanus* poses serious problems for European growers [13]. The only technique used for this is pyrethroid application on crops when the pest is detected concurrently with the first pod's emergence and after at least two consecutive days with a temperature of 20 °C [20]. This control is subject to many limitations, including a decrease in efficiency observed over the last decade, combined with legislative restrictions in Europe, limiting growers' choice to a few approved active ingredients, and limited crop applications [14,23,24]. In addition to the effectiveness and cost limitations, chemical control is affected by negative impacts on non-target beneficial entomofauna by exposing flowers to insecticides [24,25].

To cope with these limitations, a fungus-based biopesticide was suggested as an effective management tool for *B. rufimanus* in faba bean crops destined for the food market due to its observed efficacy on several insect crop pests and minimal risk to beneficial entomofauna [26]. To date, only Sabbour et al. [27] have tested the efficacy of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Verticillium lecanii* against *B. rufimanus.* Their successful results highlighted the effectiveness of fungal treatments in protecting bean seeds against *B. rufimanus* infestation in field trials. They suggested that EPF (particularly *B. bassiana* and *M. anisopliae*) could constitute effective MBCAs formulated into new biopesticides. Unfortunately, practical information, such as the amount of conidia administrated per surface unit, or the mode of application (canopy spraying or seed coating), or the economic cost for an inundative strategy were lacking. Consequently, the implementation of EPF to control *B. rufimanus* in faba bean crops still remains out of reach for growers' cropping itineraries.

Integrating EPF-based biopesticide into agronomic practices should ideally use mechanical tools that farmers already have. In this context, conventional sprayers employed for applying phytosanitary plant products (PPPs) could serve as an effective method for large-scale EPF application in crops. This potential is enhanced by the emergence of innovative spraying technologies, such as dropleg sprayers, which improve the treatment efficacy while minimizing impacts on non-target organisms [28]. In the case of *B. rufimanus* management, it can be hypothesized that such sprayers would maximize plant protection, as the active ingredient would be directly applied under the canopy on the basal pods visited by *B. rufimanus* for egg-laying while protecting the active ingredient from UV radiation or desiccation and minimizing exposure to beneficial entomofauna that visit the flowers in the upper parts of plants.

Within this context, this study explored the efficacy of *B. bassiana* (strain GHA) and *Metarhizium brunneum* (strains V275 and USDA4556) against *B. rufimanus*. Specifically, the lethal and sublethal effects (inhibition of oviposition) on adults of *B. rufimanus* were assessed under laboratory conditions, and the efficiency of the EPF-based biopesticide was tested under field conditions using two spraying techniques, the dropleg and anti-drift nozzles (i.e., the conventional technique). This study's findings are then discussed to provide valuable insights into the potential of the tested EPF species for managing faba bean-associated pests.

2. Materials and Methods

2.1. Evaluation of the Efficacy of Entomopathogenic Fungi in the Laboratory

2.1.1. Insect Rearing

The sexual maturity of *B. rufimanus* poses a considerable challenge, with rearing under controlled conditions being fastidious [29]. Two collection techniques were used to ensure there were enough adults to be tested for lethal and sublethal assessments of EPF strains. The adults used for lethal assessments were collected in emergence traps containing bean seed samples that were harvested from experimental plots of faba bean crops near Gembloux (Belgium) (Location: 50.50° N; 4.74° W, altitude = 178 m). These adults were stored in reproductive diapause under overwintering conditions, under which they can survive for up to six months [30]. The adults used for the sublethal assessment (i.e., the fecundity) were captured in blooming crops at least three weeks after colonization in parcels where winter and spring faba bean cultivars were simultaneously cropped in adjacent parcels. This system, combining spring and winter varieties with staggered phenology, maintained a flowering period long enough to retain the insects within the crops for at least three weeks until reaching sexual maturity [29]. All adults were sexed by observation of the morphological dimorphism of the middle legs of the male's spurs and the presence of the indentation of the male's pygidium [14]. Given that the reproductive status of the two groups of adults used for the analyses of lethal effects and oviposition inhibition differed—an inevitable consequence of the insect's biology preventing laboratory mass rearing— two independent experiments were conducted, assuming similar sensitivity to treatments of both populations. This approach does not affect the ability to evaluate fungal strains for pest management purposes (i.e., either killing adults in reproductive diapause at the beginning of their field infestation, or preventing oviposition of females at sexual maturity).

2.1.2. Fungal Cultures

Given the efficacy of genera *Beauveria* and *Metarhizium* highlighted in the literature, three EPF strains were selected for lethal and sublethal assessment under laboratory conditions including *B. bassiana* GHA, formulated as the active ingredient of the commercial mycoinsecticide BotaniGard ® 22WP (Certis Europe, Brussels, Belgium), which was generated from a culture stock preserved in 10% glycerol solution and stored at −80 °C in cryogenic tubes at the Functional and Evolutionary Entomology Laboratory (Gembloux Agro-Bio Tech, Gembloux, Belgium), and *M. brunneum* strains V275 and USDA 4556, which were kindly provided by the Department of Biosciences, Faculty of Science and Engineering, Swansea University, United Kingdom.

Conidia were collected from the different fungal cultures using a spatula sterilized in Tween 80 (0.05% v/v) (Merck KGaA, Darmstadt, Germany) to prepare fungal inoculums. The fungal suspension was then filtered to remove hyphal debris. The conidial concentration was adjusted to 1×10^8 conidia mL^{-1} from 100-fold dilutions in Tween 80 (0.05% v/v) using a Neubauer® haemocytometer (Assistant, Sondheim vor der Rhön, Germany). The conidial viability of each strain was assessed by plating 100 µL of conidial suspension at 10^5 conidia mL^{-1} onto SDA media incubated at 23 °C for 24 h [31]. Conidial suspensions with a germination rate > 90% were used in the experiments.

2.1.3. Lethal and Sublethal Effects

Two bioassays were carried out under laboratory conditions on 600 adults of *B. rufimanus* to evaluate the lethal (cf. adults in reproductive diapause collected from harvested seeds) and sublethal effects on the inhibition of oviposition (cf. insects manually captured during oviposition period in the field). For both experiments, five treatments were triplicated on batches of 20 sexed adults (10 males and 10 females). These treatments included a negative control corresponding to a solution of 0.05% (v/v) of Tween 80, a positive control corresponding to a solution of 400 µL/L of karate® zeon insecticide (Syngenta, Basel, Switzerland) containing 100 g/L of λ-cyhalotrin (i.e., a 40 mg/L solution of lambacyhalotrin), and the conidial suspension containing 10^8 conidia ml^{-1} of each fungal strains (*B. bassiana* strain GHA—BbGHA; *M. brunneum* strain V275—MbV275; and *M. brunneum* strain USDA 4556—MbUSDA4556).

In all treatments and repetitions, batches of 20 insects were manually sprayed with one milliliter of solution under a truncated cone. The insects, anesthetized with CO_2, were placed on filter paper in 10 mm Petri dishes for spraying [31,32] (Figure 1). After treatment, the insects were transferred to 10 cm × 10 cm plastic boxes containing a 10% sucrose solution and maintained under controlled conditions of 23 °C, a 12 h photoperiod, and 75% relative humidity. The lethal effect of treatments was evaluated by recording mortality at 24 h intervals over 10 days. To test the treatments' effectiveness in inhibiting egg-laying, three green ethanol-disinfected faba bean pods were placed in each replication, and ovipositions and female mortalities were recorded daily (when pods were replaced) in order to determine ponderated ovipositions (i.e., the number of eggs divided by the number of living females) over 14 days, following a published protocol [15].

Dead insects were removed from boxes and disinfected by immersion in 70% ethanol, followed by three rinses in sterile distilled water. Cadavers were placed on moist filter paper in incubators set at 25 °C to confirm fungal infection and observed until mycelium emerged from intersegmental regions.

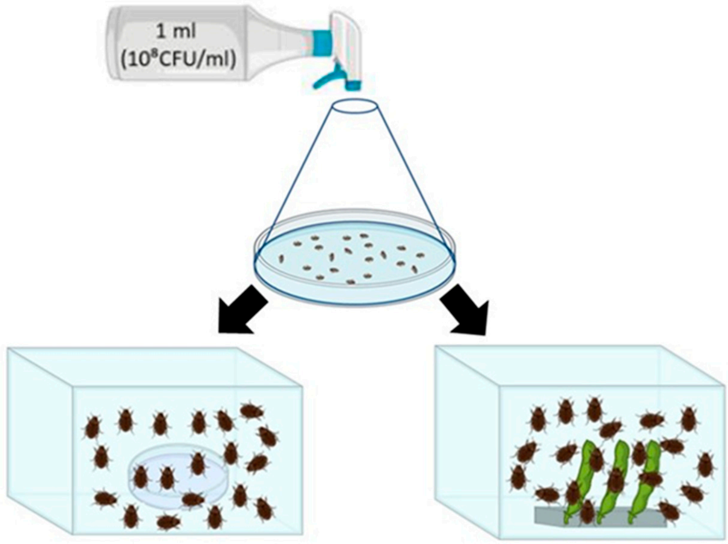

Figure 1. Manual spraying of anesthetized adults of *Bruchus rufimanus* to the different treatments under a truncated cone and arrangement of treated adults in boxes for observation of lethal (i.e., mortalities) and sublethal (oviposition on green pods) effects.

2.2. Field Experiments of EPF in Faba Bean Crops Using Two Spraying Techniques

Following laboratory bioassays, the commercialized form of *B. bassiana* GHA (i.e., a wettable powder containing 4.4 × 10^{13} CFU/kg) was used for a large-scale efficacy experiment against *B. rufimanus* infestations in a faba bean field. This field trial was carried out during the cropping season of 2023 in a spring field bean crop of the experimental farm of Gembloux Agro-Bio Tech (University of Liege, Gembloux, Belgium). The study site covered an area of 7.8 hectares and was located at geographic coordinates 50.50146°; 4.74°, at an altitude of 178 m. This area was known to have high populations of *B. rufimanus* according to previous studies [13,33]. Faba bean cultivar "Fanfare" was sown on the 3rd of March 2023 at a density of 50 seeds/m^2. No chemical insecticides or fungicides (outside of trials) were applied to the study plots, which were chemically weeded after sowing.

Two types of experimental parcels (Figure S1) were delimited in this field trial with two respective objectives. Firstly, two parcels of 60 m × 27 m plots (Figure S2) were used to compare the distribution of plant protection products (PPPs) on the different targeted parts of plants according to their spraying methods, namely, drift-reducing nozzles, which are commonly employed by users, and dropleg UL nozzles. The dropleg UL spray system is made up of the devices used on conventional spray booms, similar to inverted canes, at the end of which are inserted two nozzles enabling spraying to be carried out under the plant canopy in row-sown crops and to reach plant parts that are difficult to access. In addition, the anti-drift or conventional nozzle spraying system uses nozzles connected to a horizontal boom that allows spraying from the top of the crop downwards in a vertical direction [28]. The dropleg nozzle spraying method is considered a promising and effective alternative to conventional spraying suitable for the phytosanitary treatment of crops developing a dense vegetative cover (case of faba bean) thanks to its ability to project sprays under the plant cover and to minimize the impacts on pollinators and auxiliaries visiting the flowers located on the aerial parts [28,34].

In this field study, each type of nozzle was mounted on a 27 m wide boom and spaced every 50 cm (i.e., 54 nozzles) connected to a sprayer with a tank of 200 L (Figure 2). The sprayer applied a rate of 300 L/ha of water, operating at a spraying speed of 7 Km h^{-1}. Plant water distribution was evaluated using two pairs of water-sensitive papers (WSPs) (size: 26 × 76 mm, Teejet technologies, Spraying Systems Co. (R), Wheaton, IL 60187, USA) placed at two heights on fixed stakes (Figure 2a). These heights corresponded to the flower level (upper level) and emerging pods at the base of the plant (lower level). WSPs were angled at 30° from the vertical to reflect pod orientation. Twenty stakes (each bearing two pairs of WSPs) were spaced every 10 m × 5 m within the 60 × 27 m plots, ensuring they were distributed on either side of the sprayer's path (Figure S3). The coverage rates of each water-sensitive paper were measured, providing 40 independent observations per plant level to identify the most effective spraying method, defined as targeting pods that must be protected from *B. rufimanus* oviposition and avoiding PPP application on flowers that are visited by pollinators. ImageJ software (version 1.53e, java 1.8.0_172 (64 bits)) was used to analyze the percentage droplet coverage rates of each WSP. Scanned water-sensitive papers are available in the form of an image database (available on request).

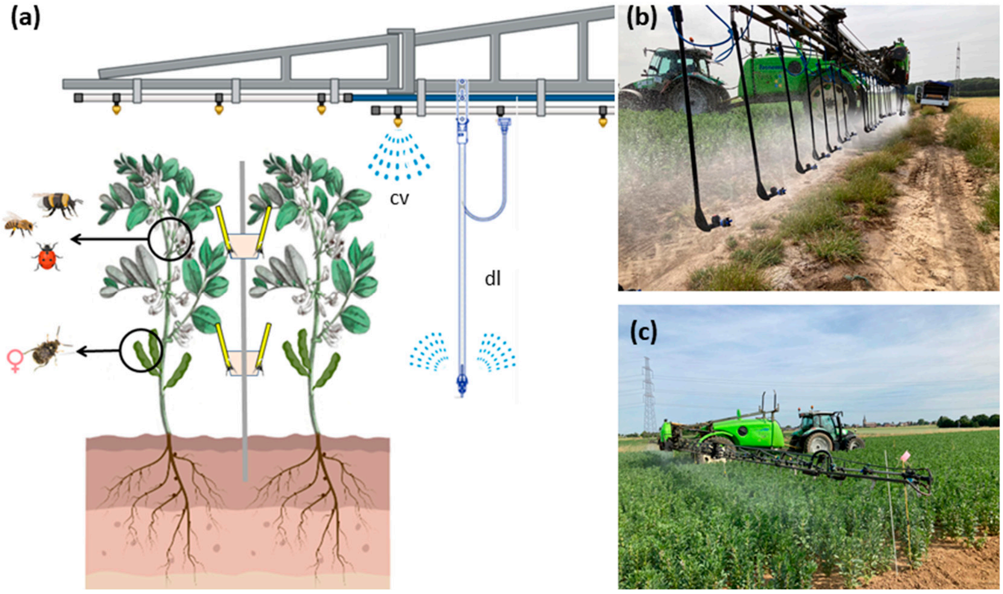

Figure 2. Crop arrangement of WSPs at upper and lower levels of faba bean plants according to auxiliary/*B. rufimanus* behavior and according to the two types of nozzles compared in the trials—anti-drift (cv) and dropleg (dl) nozzles (**a**). Illustration of dropleg nozzles mounted on a 27 m boom (**b**); illustration of anti-drift nozzles mounted on a 27 m boom (**c**). © A. Segers.

Secondly, the EPF protective effect and effect of chemical insecticide against *B. rufimanus* infestations were compared on parcels of 540 m^2 (20 m × 27 m), separated by a buffer crop of 10 m in length. Four treatments were applied on a completely randomized block dispositive with three replicates (Figure S3), including (i) a negative control (i.e., no spraying), (ii) a pyrethroid insecticide (6.25 g/ha of λ-cyhalotrin) sprayed with a drift-reducing nozzle, (iii) a pyrethroid insecticide (6.25 g/ha of λ-cyhalotrin) sprayed with a dropleg nozzle, and (iv) a commercialized form of *B. bassiana* (GHA) (61.2 g/ha of wettable powder containing 4.4×10^{13} CFU/kg) supplied with surfactant (8.1 g/ha of isodecyl alcool ethoxylate, FMC) applied with a dropleg nozzle. Each treatment was simultaneously applied at the sensitive crop stage, i.e., when first pods appeared on faba bean plants [25], and was then replicated at approximately ten-day intervals when climatic conditions afforded insecticidal spraying (i.e., wind speed inferior to 20 Km h^{-1} and no dew on the plants). Three applications of Botanigard 22WP were repeated to cover the egg-laying period of *B. rufimanus* while KARATE ZEON was applied two times. The quantities of active substances and the number of applications used during these field trials were determined by the legislation currently in force in Belgium (https://fytoweb.be/fr lastly accessed on 21 November 2024).

The protective effect of these treatments was evaluated by determining the *B. rufimanus* infestation rates on twelve randomly selected seed samples, each containing 50 faba bean seeds (i.e., a total of 600 seeds per treatment). These seeds were examined macroscopically in the laboratory and counted as 'infested' when the seed's tegument presented larvae necrosis traces or adult emergence holes, and 'non-infested' when the seed's tegument was intact. The proportion of seeds infested by *B. rufimanus* was determined by the total number of seeds determined following published procedures [35,36]. The effect of different treatments was also assessed on crop yields by weighing nine samples of 1000 randomly selected seeds harvested in each plot (i.e., thousand-seed weight—TSW).

2.3. Statistical Analysis

Data collected throughout our study were encoded with Excel 365 version 2010 software. Mortality analyses and comparative statistics of the different treatments were performed with R studio software version 1.3.9.5.9. The median lethal times (LT_{50}) of EPF strains were estimated using a non-parametric method (Kaplan–Meier) from time-to-event (death) observations, which assigns a survival probability based on the observed survival time [37]. Non-parametric univariate analyses were preferred over semi-parametric approaches (e.g., Cox proportional hazards model) or parametric approaches (e.g., Weibull, or probit model) for the following reasons: (1) the dataset is censored (i.e., incomplete observation of survival times), (ii) there were no covariates to account for in relation to survival times; (2) Kaplan–Meier analyses allow for a clear and straightforward visualization of survival curves; and (3) this approach was used in other studies from the literature, which facilitate comparisons of our findings with those obtained for other insect species or fungal strains. Survival curves of different treatments were compared by the log-rank test, a non-parametric test approximately distributed like a chi-square test. These analyses were made using the packages ¨survival¨, ¨survminer¨. The comparative effects of different treatments on the inhibition of oviposition was performed with a non-parametric Kruskal–Wallis test from total ponderated ovipositions, using R multcomp package ($p < 0.05$) [38].

The comparison of the PPP repartitions on different plant levels according to spraying technique (dropleg or anti-drift nozzles) was assessed with a linear mixed model (LMM) associated with Student's *t*-test. This model used logarithmic transformed coverage rates and was performed with the package lme4 [39]. The comparative effects of different field treatments on TSWs and on the infestation rates was assessed with the Kruskal–Wallis test, using the same procedure as for the inhibition of ovipositions.

3. Results

3.1. Lethal and Sublethal Effects Under Laboratory Conditions

The lethal and sublethal effects of *B. bassiana* strain GHA (BbGHA), *M. brunneum* strain V275 (MbV275), and *M. brunneum* strain 175 USDA 4556 (MbUSDA4556) were assessed on a total of 600 adult weevils under laboratory conditions across two bioassays.

The first bioassay involved 300 adult weevils to evaluate the median lethal time (LT_{50}) and mortality rates over a 10-day period. The second bioassay also included 300 sexually mature adults (150 males and 150 females) to assess oviposition inhibition over 14 days.

The comparison of survival curves for treated adult weevils, performed using the log-rank test, revealed a highly significant difference between treatments ($\chi^2 = 105$, Df = 4, $p < 0.001$). Among the tested strains, *B. bassiana* (GHA) was the most virulent, with an LT_{50} of four days ($\chi^2_{(0.95,1)} = 101$, $p < 0.001$) and an adult mortality rate of 96.6% after 10 days.

For the other strains, *M. brunneum* V275 and USDA 4556 exhibited LT_{50} values of eight days ($\chi^2_{(0.95,1)} = 53.5$, $p < 0.001$) and six days ($\chi^2_{(0.95,1)} = 71.4$, $p < 0.001$), respectively, with corresponding adult mortality rates of 86.6% and 88.3% (Figure 3 and Table 1). Fungal emergence from the intersegmental regions of *B. rufimanus* bodies is illustrated in Figure S4.

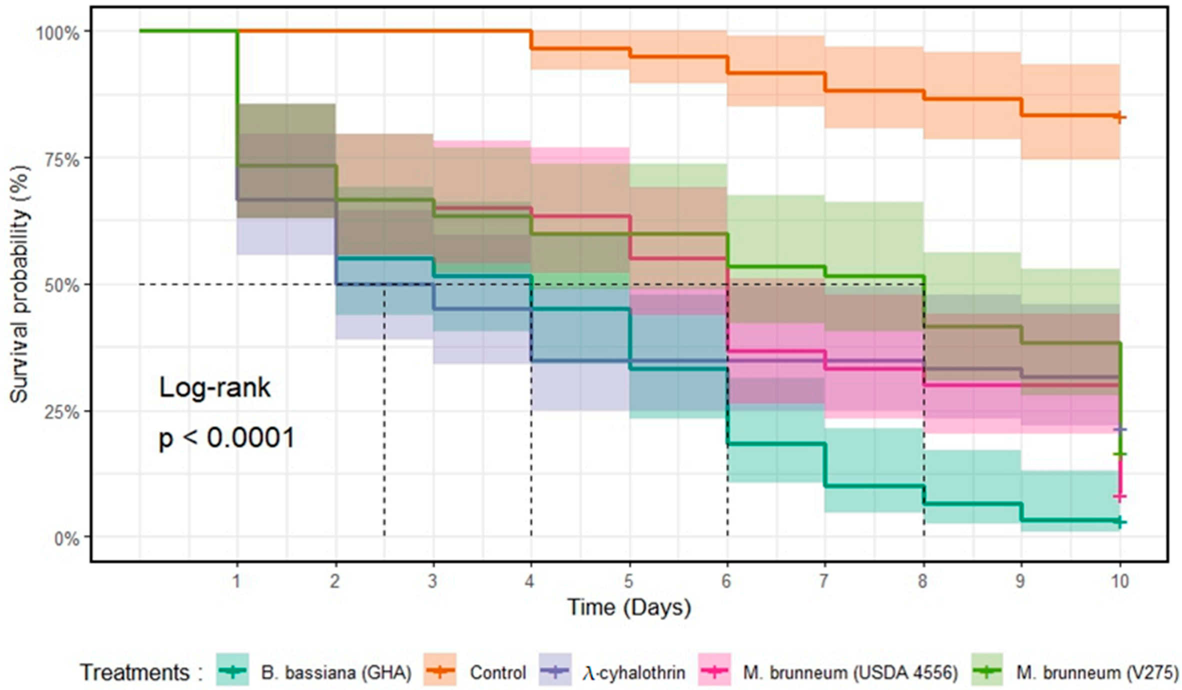

Figure 3. Survival curves after treatments performed on *Bruchus rufimanus* during laboratory experiments. The dotted lines report LT_{50} estimations. Each survival curve is presented with the 95% confidence intervals.

A highly significant difference was observed among the treatments regarding the cumulative ponderated oviposition of *B. rufimanus* (χ^2 = 48.3, Df = 4, $p < 0.001$). All of the tested strains reduced *B. rufimanus* oviposition during the observation period (Figure 4). *Beauveria bassiana* (GHA) induced complete oviposition inhibition after five days, while *M/brunneum* USDA 4556 and V275 achieved total inhibition after seven and eight days, respectively. In comparison, the chemical treatment with λ-cyhalothrin inhibited oviposition within one day. The total cumulative oviposition recorded for each treatment is presented in Table 1, with fecundity inhibition rates ranging from 12% to 36%.

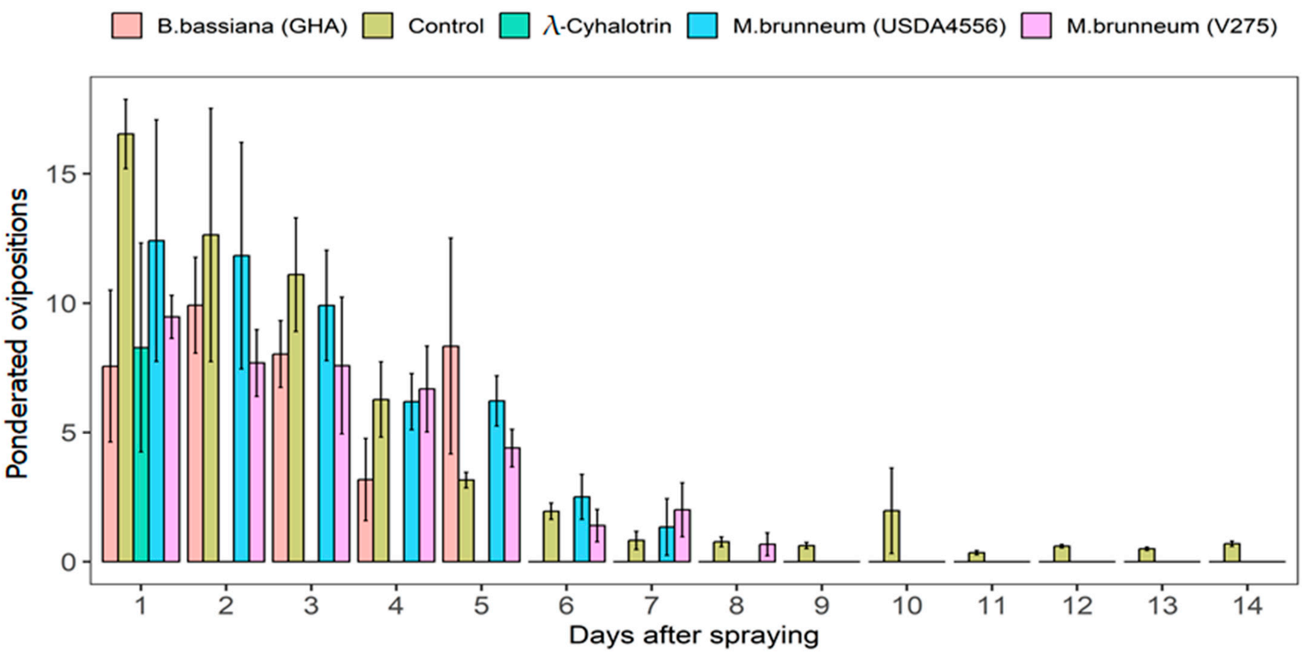

Figure 4. Evolution of ponderated ovipositions (average ± standard deviation).

Table 1. Synthesis of EPF lethality and sublethal (egg-laying inhibition) bioassay results (n = total number of treated adults, Dead_obs = number of events (deaths) observation, Chisq = the chi-square statistic of the log-rank test of equality; Df = degrees of freedom related to the log-rank test of equality; *p* = *p*-value associated with the log-rank test of equality) recorded during laboratory experiments.

	Kaplan–Meier Survival Estimates			Log-Rank Test vs. Control			Total Ovipositions	Oviposition Inhibition Rate
Treatments	**n**	**Dead_obs**	**Median (LT_{50})**	**Chisq**	**Df**	***p*-Value**		
B. bassiana (GHA)	60	58	4	101	1	<0.001	111 eggs	36%
λ-cyhalothrin (40 mg/L)	60	47	2.5	54	1	<0.001	25 eggs	86%
M. brunneum (USDA 4556)	60	55	6	71.4	1	<0.001	151 eggs	12%
M. brunneum (V275)	60	50	8	53.5	1	<0.001	120 eggs	31%
Tween 80 (0.05%)	60	10	>10	/	/	/	174 eggs	-

3.2. Field Trials

3.2.1. Repartition of Spray Density on Faba Bean Crops with Dropleg and Conventional Sprayers

The performance of dropleg nozzles in targeting the pods of faba bean plants was compared to that of conventionally used anti-drift nozzles using WSPs to evaluate coverage rates and assess the distribution of phytosanitary treatments on the upper (flowers) and lower (basal nodes) parts of the plants.

The two spraying techniques (dropleg vs. anti-drift nozzles) showed highly significant differences in spray distribution between the upper and lower levels of the faba bean plants (Df = 156, F = 50.72, $p < 0.001$) (Figure 5). On the lower parts of the plants, the dropleg nozzles achieved a significantly higher mean coverage rate of 17.03 ± 2.72% compared to 3.00 ± 0.51% for the anti-drift nozzles. Conversely, on the upper parts of the plants, the anti-drift nozzles provided a higher mean coverage rate of 5.98 ± 0.54%, compared to 3.96 ± 0.77% with the dropleg nozzles.

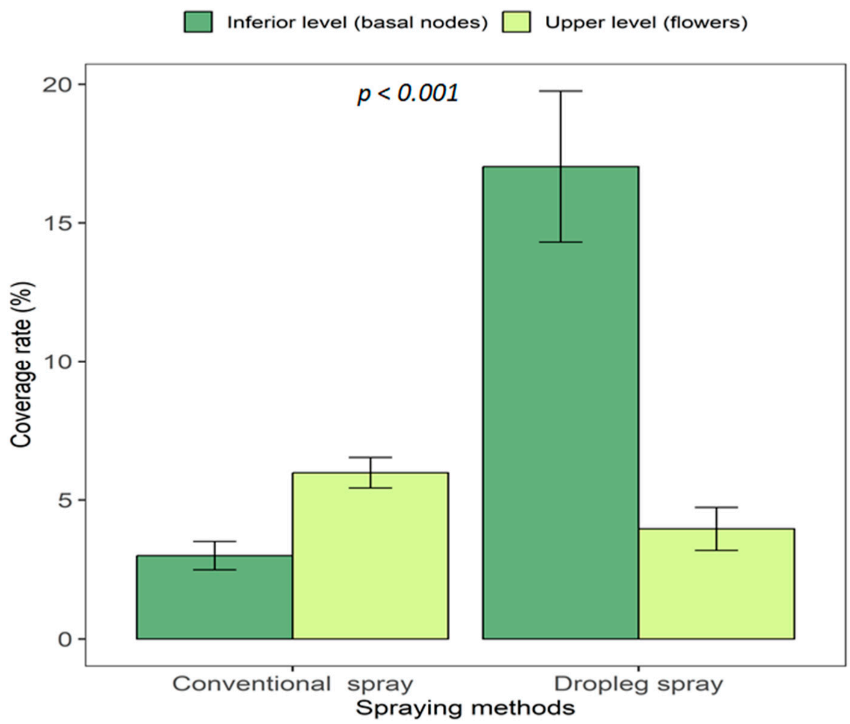

Figure 5. WSP coverage rate (average ± standard deviation).

3.2.2. Comparative Effects of Treatments on Bruchid Infestations and Faba Bean TSW

No significant differences were observed between the treatments in terms of the infestation rates ($p = 0.140$) or thousand-seed weights (TSWs) ($p = 0.180$) (Figure 6a,b). The average infestation rates were as follows: 37.0 ± 20.9% in plots treated with λ-cyhalothrin using dropleg nozzles, 40.0 ± 11.8% in plots treated with λ-cyhalothrin using anti-drift nozzles, 44.0 ± 9.9% in plots treated with *B. bassiana* using dropleg nozzles, and 51.0 ± 15.0% in untreated control plots.

Compared to the untreated control, the most effective treatment for improving the aesthetic quality of the seeds was λ-cyhalothrin applied with dropleg nozzles, which reduced the infestation rates by 14%. This was followed by λ-cyhalothrin applied with anti-drift nozzles, which reduced the infestation rates by 11%, and *B. bassiana* applied with dropleg nozzles, which reduced the infestation rates by 7%. Surprisingly, the use of dropleg nozzles with pyrethroid application resulted in only a 3% reduction in the infestation rates compared to conventional anti-drift nozzles.

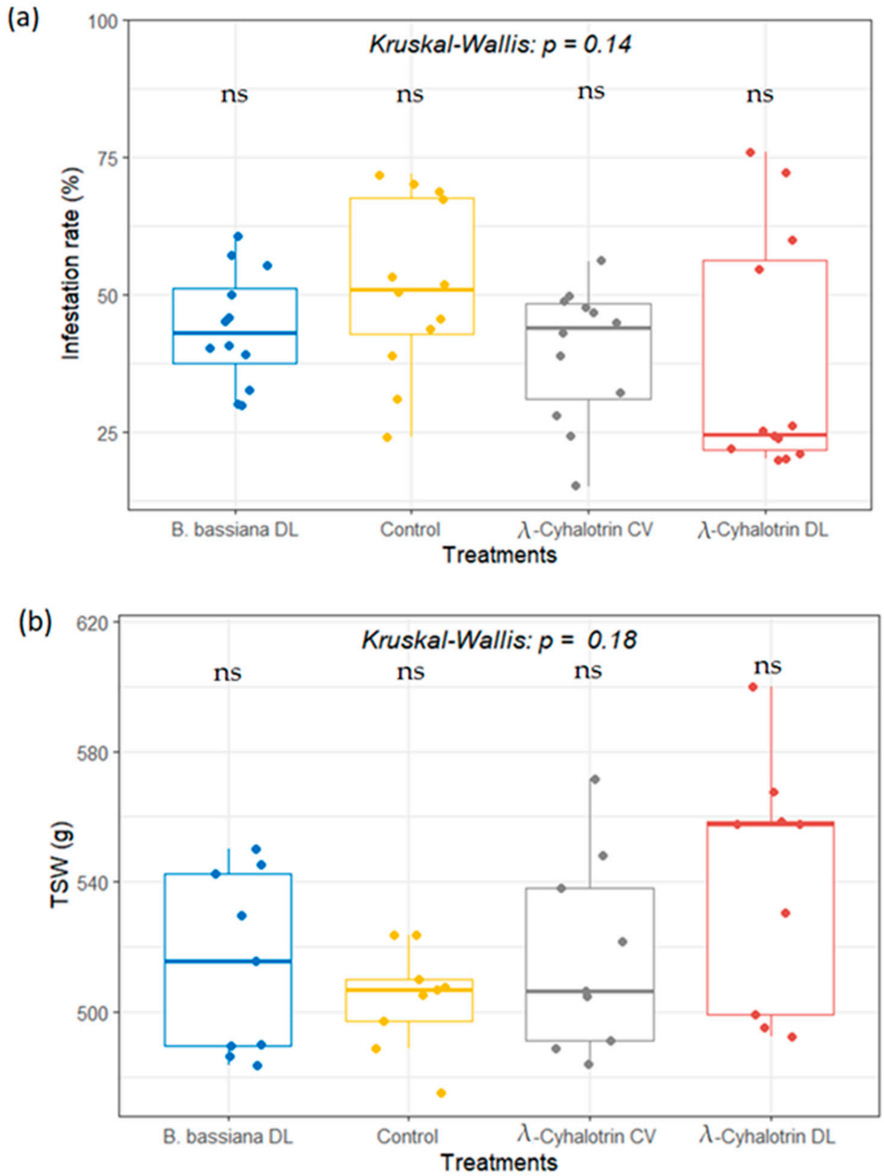

Figure 6. Boxplots of infestation rates (**a**) and TSWs (**b**) observed with different field treatments (cv = anti-drift nozzles, dl = dropleg nozzles, ns = not significant).

In terms of the faba bean productivity, the TSW values were 539.9 ± 37.7 g in the plots treated with λ-cyhalothrin (dropleg nozzles), 517.1 ± 30.0 g in the plots treated with λ-cyhalothrin (anti-drift nozzles), 514.5 ± 27.8 g in the plots treated with *B. bassiana* (dropleg nozzles), and 504.1 ± 15.5 g in the untreated control plots.

4. Discussion

4.1. Lethal and Sublethal Effects of Entomopathogenic Fungi on B. rufimanus Under Laboratory Conditions

Two laboratory experiments were conducted on different insect populations to evaluate the lethal effects of treatments and their sublethal effects on female fecundity. The lethal effects were assessed on insect populations of the same age, maintained after emergence under overwintering conditions in reproductive diapause, to determine which treatment would be most effective against adults colonizing crops. Subsequently, the same treatments were applied to a population of adults collected from the field (of varying ages) after sexual maturation during the oviposition period to evaluate the potential impact of the treatments on reducing fecundity.

These two independent experiments are the consequence of the biological complexity of *B. rufimanus* imposing unavoidable constraints on experiments. Its univoltine lifecycle includes an obligatory diapause that requires specific biotic and abiotic conditions to be terminated, such as the consumption of pollen and nectar from the host plant, a minimum diapause duration of six months, and a 16 h light photoperiod [30,40] Controlling these factors in laboratory conditions is highly challenging, making it difficult to produce a sufficient number of adults with uniform age and reproductive status, as noted by Segers et al. [29]. The only option is to collect sexually mature insects from the field, even though their age is different. This makes it impossible to separate the effects of age from reproductive status (sexual maturity) on survival and fecundity. As a result, the treatment effects on fecundity are subject to greater variability, likely due to differences in the ages of the individuals tested.

The lethal effects and the inhibition of oviposition induced by infection with entomopathogenic fungi were nonetheless successfully demonstrated. The three EPF strains tested in the laboratory showed interesting lethal and sublethal effects on adults of *B. rufimanus*. The best result in terms of lethality (i.e., the shortest LT_{50} value and the high mortality rate) and inhibition of oviposition (i.e., the higher oviposition inhibition rate) was obtained with *B. bassiana* (GHA), with an LT_{50} of four days, an adult mortality rate of 96.6%, and an oviposition inhibition rate corresponding to 36%, compared to an LT_{50} of eight and six days, mortality rates of 86.6% and 88.3%, and oviposition inhibition rates of 31% and 12% obtained with the *M. brunneum* V275 and USDA 4556 strains, respectively.

The high virulence of *B. bassiana* (GHA) observed on *B. rufimanus* in the laboratory experiments could be explained by its high pathogenicity towards a wide range of insect crop pests, including legume seed pests [41,42]. For example, a similar LT_{50} (4.61 days) was obtained by Ozdemir et al. [41] on adults of *Callosobruchus maculatus* treated with *B. bassiana* (TR 217) versus an LT_{50} of 5.34 days obtained with *M. anisopliae* (TR 106). In laboratory experiments, Cherry et al. [42] also observed LT_{50} values ranging from 3.11 to 6.13 days on adults of the same pest species treated with *B. bassiana* strain (0362) and a 100% adult mortality rate six days after treatment. On the other hand, LT_{50} values corresponding to 6 and 5 days were obtained by Dessauvages et al. [31] with the *B. bassiana* strain (GHA) and the new *B. bassiana* isolate GxABT-1, respectively, on the beet aphid *Myzus persicae* under laboratory conditions. Mortality rates of over 90% were also recorded by the author with both microbial agents 8 days after treatment. The oviposition inhibition results show that all of the fungal strains generally caused a reduction in the repeated oviposition of *B. rufimanus* during the 14-day monitoring period. Similar observations were made by Jarrold et al. [43] and Zimmermann [44], who reported that the infection of insect pests with entomopathogenic fungi affects their physiological functions and, specifically, their reproductive capacity. Given the above, EPF are potential candidates for developing microbial biological control agents against *B. rufimanus*.

4.2. Application of EPF in Large-Scale Faba Bean Crops

The development of spray technologies over the past few decades has focused on improving the efficiency of plant protection product (PPP) applications and enhancing crop protection [45]. These advancements aim to minimize spray drift, reduce PPP overapplication during treatments, and mitigate impacts on non-target organisms and the environment, thereby promoting agricultural sustainability [46,47]. Within this context, technologies such as dropleg sprayers have been introduced to optimize crop protection treatments while reducing harm to non-target organisms, including pollinators and beneficial insects [28].

A comparison of dropleg and conventional spraying revealed a highly significant difference ($p < 0.001$) in spray distribution between the lower and upper levels of the plants. The dropleg method achieved a much higher mean coverage rate (17.03 ± 2.72%) on the basal parts of plants, where pods develop and are targeted by *B. rufimanus* for oviposition and where pest damage occurs. In contrast, conventional spraying achieved only 3 ± 0.51% coverage in these basal areas. Conversely, conventional spraying provided better coverage (5.98 ± 0.54%) on the upper parts of the plants, which host flowers visited by pollinators and beneficial insects, compared to dropleg spraying (3.96 ± 0.77%). Similar results have been reported by Roten et al. [48] and Hoffmann et al. [49] in potato and soybean crops. These findings suggest that for managing pests like *B. rufimanus*, which lay eggs on the basal pods of faba bean plants, dropleg nozzles may offer better protection against seed infestations while reducing potential harm to non-target beneficial insects, such as ladybeetles, bees, and hoverflies [23]

However, despite the improved targeting of *B. rufimanus* oviposition sites, no significant reduction in infestation rates was observed, with only a 3% decrease when pyrethroids were applied. Additionally, no significant improvement in seed quality was noted for either the chemical or fungal treatments. A comparison of the *B. bassiana* fungal treatment (GHA) with the chemical insecticide and untreated control showed no significant differences in the infestation rates or thousand-seed weight (TSW) ($p = 0.14$ and $p = 0.18$, respectively). Moreover, three applications EPF did not improve the quality or quantity of the harvested seeds compared to two applications of the chemical insecticide.

These results contrast with those of the laboratory trials, which demonstrated the significant effectiveness of the treatments against adult *B. rufimanus*, both in terms of lethality and fecundity reduction. Two main factors may explain the lack of effectiveness observed in the field trials compared to the laboratory experiments. First, *B. rufimanus* has been exposed to numerous pyrethroid applications in Belgium, the only active substance approved by the European Commission. Conventional spraying has proven insufficient to target critical plant areas effectively, as demonstrated with water-sensitive papers (WSPs). Repeated applications of the same active ingredient, combined with application inefficiencies, likely promoted the selection of pyrethroid-resistant *B. rufimanus* populations, as observed in other faba bean pests, such as *Sitona lineatus* (Coleoptera: Curculionidae) [14,16]. This hypothesis is supported by laboratory results where the λ-cyhalothrin treatments resulted in a mortality rate of only 78%, compared to mortality rates of at least 83.3% for the EPF. Further evidence comes from field observations in neighboring countries, such as the discontinuation of the BruchidCast program by PRGO and Syngenta in the UK in 2021 [50] (https://www.syngenta.co.uk/bruchidcast accessed on 25 January 2024).

Additional factors contributing to the disparity between laboratory and field results include environmental conditions and application parameters. Studies by Jiang et al. [51], Neto et al. [52], and Knežević et al. [53] highlight that the droplet size, application parameters, and environmental factors can significantly affect the effectiveness of dropleg spraying. Larger droplet sizes, for instance, are prone to evaporation and drift at higher working speeds, reducing the spray coverage [53]. Application parameters such as the leaf area volume density and power gradient can also influence droplet deposition [51]. Proper adjustments to spraying parameters—such as the spray rate, nozzle height, angle, and direction—are essential for improving droplet deposition on targeted plant parts and ensuring contact between the active ingredient and pests [52]. Furthermore, environmental factors such as high temperatures, UV radiation, humidity, and rainfall can reduce the persistence and efficacy of both EPF and chemical treatments [54,55]. During field trials, hot and dry conditions (as observed in situ) likely decreased the effectiveness of the treatments.

Considering the increasing prevalence of extreme weather events, such as higher temperatures and altered precipitation patterns driven by climate change [56–58], these findings suggest that inundative delivery methods for EPF, such as conventional or dropleg spraying, to target adults before oviposition may not be a suitable strategy for effective EPF integration into crop management systems.

4.3. Alternative Strategies to Enhance EPF Within Effective IPM Cropping Itineraries

Considering the biology of *B. rufimanus* and the current knowledge of its chemical ecology, control methods based on attract-and-infect strategies or the use of endophytic fungi represent promising approaches for implementing microbial biocontrol agents (MBCAs) in integrated pest management (IPM) strategies.

The attract-and-infect strategy against phytophagous insects involves co-formulating insect pathogens with attractive volatile organic compounds (VOCs), such as sexual or aggregation pheromones and host plant kairomones [59]. Host plant kairomones that attract *B. rufimanus* have been identified in recent studies, and commercial attractants mimicking the scents of *V. faba* flowers and pods are already available (e.g., International Pheromone System Ltd.—Wallasey, UK; and AgriOdor—Orsay, France). This strategy has been successfully applied to other coleopteran pests [60], where insects are lured into devices containing dry fungal conidia. Such an approach offers several advantages, including increased spore deposition on adult insects, potential sublethal effects such as oviposition inhibition, and the possibility of the autodissemination of conidia to sexual partners, thereby extending the method's action range [61,62].

Endophytic fungi, in contrast, are mutualistic microorganisms that inhabit healthy plant tissues and provide protection against herbivory and diseases [26,63]. Using endophytic fungi to control *B. rufimanus* would involve colonizing *V. faba* tissues with fungi capable of deterring feeding, exerting antibiosis effects, or inducing host plant defenses. This approach could effectively target the seminivorous larvae of *B. rufimanus*, the pest's most vulnerable stage [64]. Moreover, endophytic fungi could simultaneously protect plants against diseases, promote *V. faba* growth, and minimize impacts on beneficial organisms [26].

The infestation levels in faba bean crops are also influenced by the choice of variety used [13,64]. Specifically, the phenological cycle of faba bean varieties significantly affects the extent of crop infestation by *B. rufimanus* populations [64]. For example, the oviposition preferences of *B. rufimanus* vary depending on the faba bean genotype [65]. Early-maturing faba bean varieties have been shown to reduce inter-annual *B. rufimanus* populations by reaching seed maturity before pest populations emerge from overwintering sites in spring [23]. Winter faba bean varieties, sown early in autumn and harvested in spring, are less susceptible to infestation than spring varieties because their sensitive phenological stages do not overlap with the pest's emergence and colonization periods.

Previous studies at our experimental site have confirmed high *B. rufimanus* populations [13,23]. These conditions, combined with nearby overwintering sites, favor repeated infestations by pest populations migrating into the experimental plots, as demonstrated previously [66]. Such dynamics are likely to result in high seed infestation levels at harvest [67].

Furthermore, our findings align with the study by Jensen et al. [68], which assessed the impact of inoculating *V. faba* seedlings with the *B. bassiana* (GHA) strain (used in our study) on the reproduction of *Aphis fabae* (Hemiptera: Aphididae). The study reported significantly higher nymph production in the second aphid generation on treated plants compared to untreated ones. Similarly, Barribeau et al. [69] demonstrated that insect pests often respond to the threat of pathogen-induced mortality by increasing reproductive investment. This phenomenon could explain the larval infestations of *B. rufimanus* observed on the seeds harvested from the plots treated with fungal applications.

5. Conclusions

This study demonstrated that entomopathogenic fungi (EPF) are promising biocontrol agents for incorporation into ecofriendly biopesticides. Laboratory tests evaluated three fungal species: *Beauveria bassiana* GHA, *Metarhizium brunneum* (V275), and *M. brunneum* (USDA4556) fungal strains, among which *B. bassiana* GHA exhibited the highest virulence against *B. rufimanus*.

Field trials provided, for the first time, evidence that dropleg spraying in faba bean crops achieves a better spray distribution to the basal nodes of the plants—where pods are located during the crop's sensitive stage for *B. rufimanus* oviposition—compared to conventional anti-drift nozzles. However, both the fungal and chemical treatments offered limited protection, reducing the infestation rates by only 7% and 14%, respectively. These results suggest that the large-scale spraying of chemical or fungal active ingredients, such as the inundative application of *B. bassiana* GHA, is not an optimal strategy to control *B. rufimanus* in faba bean crops.

To improve the sustainability of faba bean cultivation in Western Europe, alternative application methods for EPF should be explored. These approaches could leverage the endophytic properties of EPF or utilize the chemical ecology of *B. rufimanus*, such as attract-and-infect strategies. Combining these methods with the selection of suitable faba bean cultivars would further support the development of effective and sustainable pest control solutions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15040376/s1, Figure S1: Experimental design of field trials. Red delimitations represent both 60 × 27 plots used for comparison of dropleg nozzles and anti-drift nozzles on water-sensitive papers. Dotted delimitations represent 20 × 27 m plots used for the comparison of phytosanitary treatments on *B. rufimanus* infestations; Figure S2: Water-sensitive paper arrangement within the plots to compare the spray distribution on the upper and lower parts of the plants using drift-reducing nozzles and dropleg nozzles; Figure S3: Experimental design for the comparison of different treatments on *B. rufimanus* infestations; Figure S4: Evolution of the intersegmental emergences of sporulating EPF from *B. rufimanus* that were fatally infected by genus *Beauveria* (**a**) and by genus *Metarhizium* (**b**) over a period of 10 days.

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