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# Co-formulation of *Beauveria bassiana* with natural substances to control pollen beetles – Synergy between fungal spores and colza oil

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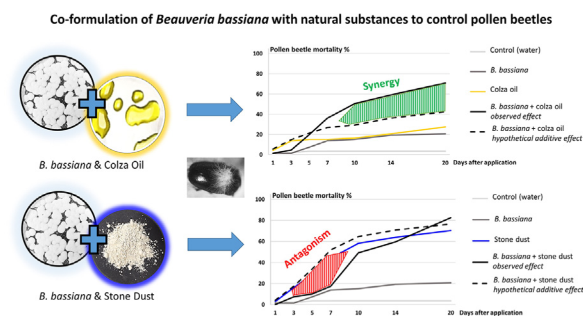
## GRAPHICAL ABSTRACT




## ABSTRACT

Pollen beetles (*Brassicogethes* spp.) are a major pest in colza (*Brassica napus* L., also known as oilseed rape) and show increasing resistance to commonly used insecticides. Entomopathogenic fungi (EPF) such as *Beauveria bassiana* have potential as a sustainable alternative means of control. However, field applications of fungal spores targeting the immigrated beetles on the crop have not been efficient to date. To enhance the effectiveness of fungal spores in insect pest control, this study focused on co-formulations of *B. bassiana* blastospores with vegetable oil and stone dust, which have already shown effects against pollen beetles as single treatments in previous studies. To estimate synergistic effects of these combinations, we developed a novel statistical approach based on a generalized linear mixed effect model and a parametric bootstrapping method. The combination of *B. bassiana* and colza oil increased pollen beetle mortality much more than expected from the additive effect of single applications, indicating a strong synergistic effect. In contrast, the combination of *B. bassiana* and stone dust showed an antagonistic effect, i.e. mortality was lower than expected from the additive effect of single substances.

Our study confirms the suitability of a combination of entomopathogenic fungi with vegetable oil and demonstrates that combinations may exhibit a higher level of pest control than expected from single substances.

## 1. Introduction

Colza (*Brassica napus* L., also known as oilseed rape) is an important agricultural crop valued for its high oil content. The oil is mainly used for technical purposes such as biofuel, but is also important for the food industry in convenience products or as frying oil. Due to the beneficial composition of fatty acids, it is increasingly sold as edible oil. Accompanying the growing demand for organically produced food products in Europe, organically produced colza oil has grown in popularity. Its cultivation, however, is limited by challenging cultivation requirements, such as high nitrogen supply and especially by limited pest control possibilities. Pollen beetles (*Brassicogethes* spp., formerly

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named *Meligethes* spp., Coleoptera: Nitidulidae) represent an important pest problem, causing substantial yield loss when present in large numbers (Ekbom and Borg, 1996). The two species that cause damage in Europe are *B. aeneus* (Fabricus) and *B. viridescens* (Fabricus). Adults emerge in spring and infest the colza during its bud stage. They feed on pollen and lay their eggs in the developing flower buds. This damages the reproductive parts of the flowers, which leads to a reduction in pod formation and ultimately in yield loss (Hansen, 2004). The growing insect resistance to pyrethroids coupled with a general demand for less pesticide use in agriculture by the public and political bodies make the quest for alternative control strategies to reduce pollen beetle infestation increasingly urgent (Slater et al., 2011).

Alternative direct control approaches include entomopathogenic fungi (EPF) such as *Metarhizium anisopliae* or *Beauveria bassiana*, as well as natural substances like vegetable oil or stone dust (Hokkanen, 1989; Dorn et al., 2013). Previous work has indicated that *B. bassiana* is the most common natural EPF found in pollen beetles collected in the field in Switzerland (Pilz, 2005). Direct exposure of pollen beetles to *Beauveria bassiana* is highly effective in laboratory trials, but has so far shown only limited suppression of pollen beetle abundance in field trials (Kuske et al., 2013). This low efficacy might be caused by adverse environmental conditions for EPF in the field, such as inactivation of the fungus spores by desiccation or UV radiation (Ignoffo, 1992; Moore et al., 1993). Previous studies have shown that it is possible to increase the efficacy of EPF in open-field applications by formulation with efficacy-enhancing agents that improve fungus spore survival and infection potential (Fernandes et al., 2015; Kaiser et al., 2019).

The aim of the present study was to evaluate co-formulations of *B. bassiana* blastospores with two natural substances that have already shown effects against pollen beetles as single treatments in previous studies: vegetable oil and stone dust. An emulsified colza oil has been shown to cause pollen beetle mortality of 75% in the laboratory one day after treatment (Dorn et al., 2013). However, the effect of this treatment in field applications against pollen beetles was inconsistent. A dry application of stone dust resulted in 100% mortality in the laboratory, and also led to a significant reduction of pollen beetle abundance in field trials shortly after application (Daniel et al., 2013). Here, we study the potential of these two substances in a combined application with *B. bassiana* blastospores to develop a sustainable alternative to control pollen beetles. Moreover, we were specifically interested in quantifying potential synergistic interactions. To evaluate such synergistic or antagonistic effects between single substances with mortality as outcome, we developed a novel statistical approach based on a generalized linear mixed effect model and a parametric bootstrapping method. Finally, we examined treated pollen beetles with a scanning electron microscope, to detect any visual differences after single and combined treatments.

## 2. Material & methods

### 2.1. Pollen beetles

Newly emerged pollen beetles (*Brassicogethes* spp.) naturally occurring in a winter colza crop at our study site (Zurich Affoltern, Switzerland, 47° 25.8′ N, 8° 31.2′ E) were collected in June. Beetles were placed in plastic boxes containing turf as a shelter, and flower pollen (Reformhaus Dropa, Zurich, Switzerland) and water for nutrition. Until the start of the experiments, they were incubated in a climate chamber at 6 °C, 85% RH and a L14:D10 light cycle, and the boxes moistened regularly. One day before the start of the experiments, beetles were transferred to room temperature to acclimatize. For treatments, beetles were placed into acrylic glass boxes with mesh fitted into the lids (bottom area 10 × 8 cm, height 13 cm, top area 10 × 12 cm). The boxes were equipped with a cotton wool plug (40 mm × 14 mm, IVF Hartmann AG, Neuhausen, Switzerland) with one end reaching into the beetle box and the other end soaked in a tap water reservoir for permanent water supply. An experimental unit consisted of one such box containing 15 active pollen beetles.

### 2.2. Fungal strain and spore production

The *Beauveria bassiana* strain ART2587 was collected from a mycosed pollen beetle (*Brassicogethes* sp.) in Zurich, Switzerland (Pilz, 2005). The single-spore isolate is stored at −70 °C in 10% skim milk (Difco, Becton Dickinson, New Jersey, USA) in the strain collection of Agroscope, Zurich. Before the start of the experiments, the strain was passed through a pollen beetle and re-isolated from single conidia colonies. For conidia production, the strain was grown on complete medium containing 10 g glucose, 0.36 g $KH_2PO_4$, 1.78 g $Na_2HPO_4$, 1 g KCl, 0.6 g $MgSO_4{\cdot}7H_20$, 0.7 g $NH_4NO_3$, 5 g yeast extract, and 20 g agar in 1000 ml of distilled water (Riba and Ravelojoana, 1984). To produce blastospores for the experiments, conidia were collected from 7-day-old plates using an inoculation loop and transferred to a liquid medium containing 20 g corn steep liquor powder, 2.26 g $KH_2PO_4$, 3.8 g $Na_2HPO_4$, and 30 g sucrose in 1000 ml of distilled water (modified after (Butt and Goettel, 2000). Liquid cultures were incubated at 25 °C in a rotary shaker at 150 rpm for 3 days. To separate the blastospores from the mycelium, the liquid cultures were filtered through four layers of sterile cotton gauze (Paul Hartmann AG, Heidenheim, Germany). The blastospores were concentrated using a centrifuge (7744*g* for 8 min) and enumerated for the assays using a cell counting chamber (Thoma, 0.1 mm, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

### 2.3. Formulations and application in the spray chamber

Pollen beetles were treated with single substances or co-formulations in a spray chamber (Spray Lab, Schachtner Geraetetechnik, Ludwigsburg, Germany). Spray applications were performed with one nozzle (TeeJet 8002EVS) at a rate of 650 l/ha at 4 bar. The beetles were treated directly in the boxes by placing these into the spray chamber. Single treatments consisted of 1) 2% emulsified colza oil (Telmion®, surfactant containing 85% colza oil; Omya AG, Oftringen, Switzerland) in water; 2) 7.5% (equivalent to 45 kg/ha) stone dust suspension in water (Klinospray, containing silica (70%), potassium oxide (2.8%), calcium (2.5%); Unipoint AG, Ossingen, Switzerland); and 3) *B. bassiana* spores at $1.55 \times 10^8$ spores/ml, which corresponds to $10^{14}$ spores/ha field application rate with the settings used in the spray chamber. For co-formulations, *B. bassiana* spores were combined with either 2% colza oil or 7.5% stone dust to yield a final spore concentration of $1.55 \times 10^8$ spores/ml. In addition, each trial included a water-only treatment and an insecticide treatment (0.2 l/ha Audienz®, containing 480 g/l Spinosad, Omya AG, Oftringen, Switzerland). All treatments were replicated three times per trial, and the whole experiment was performed in two independent trials.

After application, flower pollen and tissue strips were added to the pollen beetle boxes for nutrition and shelter. Boxes were incubated in a climate chamber at 22 °C, 70% RH, and a L14:D10 light cycle. Beetle mortality was recorded on days 1, 3, 5, 7, 14 and 20 after application. Dead beetles were removed from boxes, incubated on a moist filter paper and observed for *B. bassiana* mycosis for 14 days.

### 2.4. Statistical analyses

Mortality and mycosis of pollen beetles under the different treatments were analyzed separately with generalized linear models fitted by the Laplace approximation using the package “lme4” (version 1.1–17) in the statistical software R (version 3.2.3). (Bates et al., 2014; R Development Core Team, 2016) The status of the beetles in the experimental boxes (numbers alive or killed; mycosed or non-mycosed) was the dependent variable and assumed to be binomially distributed. The two trials of the experiment were included as a random factor.

To test for synergistic or antagonistic effects with mortality as

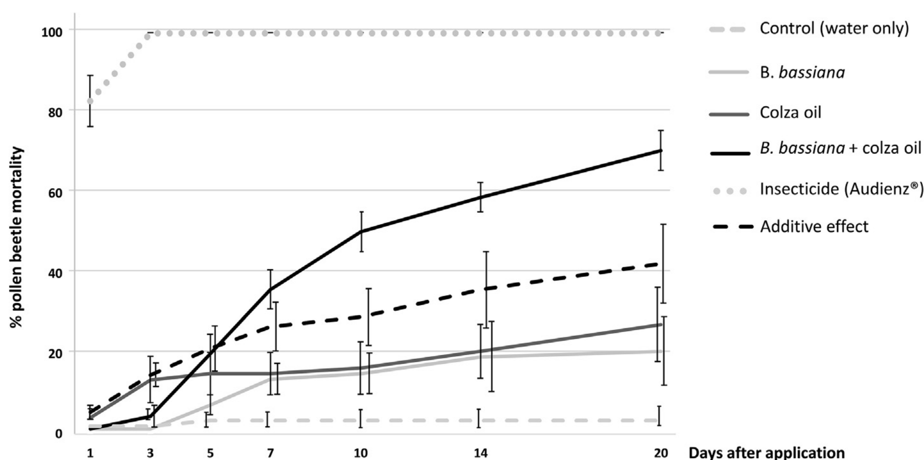


**Fig. 1.** Cumulative pollen beetle mortality after treatment with single substances or a co-formulation of *B. bassiana* blastospores ($1 \times 10^{14}$/ha) with an emulsified colza oil (2% Telmion®, surfactant containing 85% colza oil). Calculated additive mortality is based on observed mortalities caused by single substances. Mortalities and standard errors are based on values from two experiments with three replicates of each treatment.

outcome, we developed a novel statistical approach that adequately accounts for the non-independence of data (beetles in the same experimental boxes). In general, to detect potential synergistic or antagonistic interactions between two treatments, we must compare the mortality rate of the combined treatment to the mortality rate that would be expected in the absence of interaction, what we call the "additive" mortality. The expected additive mortality $M_{\text{additive}}$can be computed as $M_{\text{additive}} = M_{\text{fungus}} + M_{\text{oil}} \times (1 - M_{\text{fungus}})$,where $M_{\text{fungus}}$is the mortality rate due to the fungus alone and $M_{\text{oil}}$ the rate due to the oil alone (Finney, 1964).

This expected additive mortality is subtracted from the observed mortality when both treatments are combined ($M_{\text{combined}}$). One then needs to assess the statistical significance of the difference ($\Delta M = M_{\text{combined}} - M_{\text{additive}}$).The null hypothesis of no synergistic or antagonistic interaction would be $\Delta M = 0$. A positive difference implies a synergistic effect of the two treatments, while a negative difference indicates an antagonistic effect.

Traditionally, the statistical significance of $\Delta M$ is assessed using a $\chi^2$ statistic with one degree of freedom (Ansari et al., 2006; Pelizza et al., 2015). This approach suffers from two shortcomings. First, it assumes independence of the individual organisms tested. This is rarely the case in experimental set-ups where many individuals are subjected to the same treatment simultaneously or several trials are conducted. A $\chi^2$ test cannot fully account for the structure of the replicates and multiple trials. Second, in a $\chi^2$ test, the observed frequency (here the observed combined mortality) has to be compared to a fixed theoretical value. In our case, this value is the additive mortality$M_{\text{additive}}$, which is not a precise theoretical value, but needs to be computed from observations and, thus, suffers from statistical uncertainty. To cope with these two limitations, we developed a new, two-step procedure to fit the data and assess the statistical difference in mortality $\Delta M$.

In a first step, we fitted the observed count of dead and alive beetles using a generalized linear mixed effect model with binomial distribution, which can fully integrate the non-independence of replicates and, as the experiment was carried out twice, the trial as a random factor. Based on the fitted parameters, we then computed the different observed mortalities ($M_{\text{fungus}}$, $M_{\text{oil}}$, $M_{\text{combined}}$), from which we calculated the expected additive mortality$M_{\text{additive}}$ and the difference with respect to the observed combined mortality $\Delta M$. In a second step, using a parametric bootstrap, we computed the 95% confidence interval of the difference in mortality $\Delta M$. If zero fell outside the 95% confidence interval, we considered that there was a statistically significant interaction between the two treatments. A detailed derivation of the novel test including the R code and test data are given in the Supplementary Materials (Supplementary Material 1).

### 2.5. Scanning electron microscopy imaging of treated pollen beetles

Distribution and adhesion of fungal spores to elytra and body surfaces of pollen beetles were examined after treatments with single substances and co-formulations by cryo-scanning electron microscopy (cryo-SEM). Four beetles per treatment were positioned on a cryo-holder (Bal-Tec/Leica, Vienna) with a small brush and fixed with colloidal silver liquid (Plano GmbH, Germany). After drying, this block was cooled in liquid nitrogen. In order to avoid shearing forces due to bubbling, the beetles were immersed in the liquid only after the block was cold. The holder was then transferred into a precooled (−120 °C) freeze-fracturing system (BAF 060, Bal-Tec/Leica, Vienna) at $1 \times 10^{-7}$ mbar. Samples were freeze-etched for 15 min at −95 °C and $3 \times 10^{-7}$ mbar and then coated with 2 nm tungsten at an elevation angle of 45°, followed by 4 nm under continuous elevation angle changes (45° to 90°). Transfer to the precooled cryo-SEM was carried out under high vacuum ($< 5 \times 10^{-7}$ mbar) with an air-lock shuttle. Cryo-SEM was performed in a field emission SEM Leo Gemini 1530 (Carl Zeiss, Germany) equipped with a cold stage to maintain the specimen temperature at −120 °C (VCT Cryostage, Bal-Tec/Leica). SE2 signals at an acceleration voltage of 2 kV and a working distance of 6–7 mm were used for image formation.

## 3. Results

### 3.1. Susceptibility of pollen beetles to single substances and combined application of B. bassiana blastospores with an emulsified colza oil

The control mortality (treated with water alone) remained low, with 3.5 ± 2.1% at day 20, whereas the treatment with the insecticide caused 100% mortality at day 3 (Fig. 1). The two single treatment applications with water-suspended *B. bassiana* blastospores or the emulsified colza oil caused a comparable mortality of 20.8 ± 8.5% (mean ± SE) and 27.4 ± 9.2% at day 20, respectively.

The co-formulation of *B. bassiana* blastospores combined with the emulsified colza oil caused a pollen beetle mortality of 70.8 ± 5.0% at day 20. This mortality was significantly higher than the mortalities caused by the two single treatments alone (*B. bassiana* blastospores: $p = 0.0025$; emulsified colza oil: $p = 0.0043$). Furthermore, from day 10 onwards, the observed mortality was also significantly higher than the theoretical additive mortality, providing strong evidence of a synergistic effect of the combination treatment on pollen beetle mortality (Fig. 3). The theoretical additive mortality of the two single substances would be 42.5% at day 20. At this date, the synergistic effect accounted for a significant increase in mortality of 28.3% ($\Delta M = M_{\text{combined}} - M_{\text{additive}} = 0.28$; 95% confidence interval CI: 0.13–0.43).

Mycosis (visible growth of *B. bassiana* on beetle cadavers) showed a

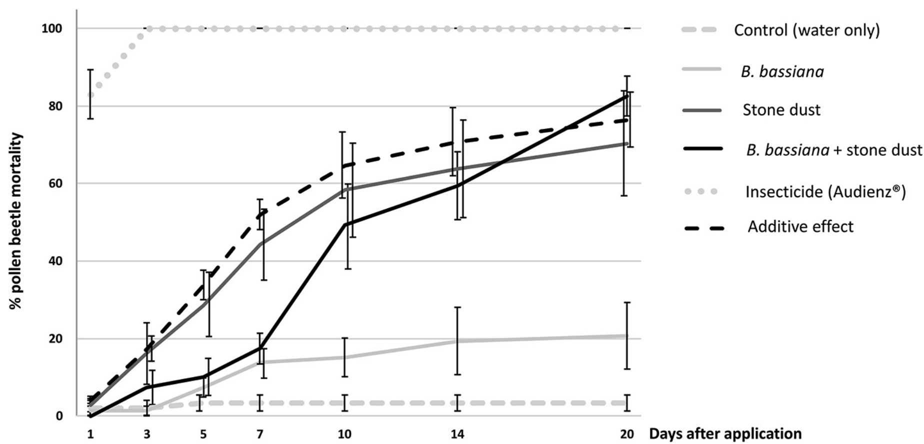


**Fig. 2.** Cumulative pollen beetle mortality after treatment with single substances or a co-formulation of *B. bassiana* blastospores ($1 \times 10^{14}$/ha) with stone dust (45 kg/ha KlinoSpray). Calculated additive mortality is based on observed mortalities caused by single substances. Mortalities and standard errors are based on values from two experiments with three replicates of each treatment.

similar pattern. The proportion of beetle cadavers showing mycosis was significantly higher in the combination treatment (79.9 ± 8.9%, mean ± SE) than with *B. bassiana* applied alone (62.5 ± 18.0%) ($p$ = 0.0464).

### 3.2. Susceptibility of pollen beetles to single substances and combined application of B. bassiana blastospores with stone dust

The treatment of pollen beetles with a stone dust suspension as a single treatment caused a mortality of 70.4 ± 13.4% (mean ± SE) at day 20 after application (Fig. 2). The co-formulation of *B. bassiana* blastospores with stone dust resulted in no increase in mortality compared to the single treatment with stone dust, and in fact, the mortality with the co-formulation was lower over the first 14 days. There was a significant antagonistic effect from days 3 to 7 ($\Delta M_{day7} = M_{combined} - M_{additive} = -0.34$; 95% CI: −0.5 to −0.17) (Fig. 3). At day 20, the combination treatment resulted in mortality of 82.7 ± 5.2%. The theoretical additive effect of the two single treatments at day 20 would be 76.5%, which is at a similar level as the actual mortality observed in the combination treatment.

The proportion of pollen beetle cadavers showing mycosis did not differ significantly between the combination treatment (67.81 ± 15.5%) and the treatment with *B. bassiana* blastospores only (62.5 ± 18.0%) ($p$ = 0.2852).

### 3.3. Scanning electron microscopy images

The co-formulation of *B. bassiana* spores with oil was easily recognizable on scanning electron microscopy images (Fig. 4C). The oil film covered the beetle's cuticula, with flattened hairs sticking to the surface. There was, however, no evident difference in the amount of spores deposited on the elytra in the treatment with spores suspended in oil or in water (compare Fig. 4A and C). In contrast, the fissure between elytra appears to be free of spores after treatment with *B. bassiana* suspended in water (Fig. 4B), while many spores are visible in the fissure of the beetle treated with the oil suspension (Fig. 4D). The low number of beetles scanned (four per treatment) only allows for a qualitative comparison of the images.

## 4. Discussion

Our results show that an emulsified colza oil interacts synergistically with *B. bassiana* blastospores and results in increased pollen beetle mycosis and mortality under laboratory conditions. The success of a combined application of EPF and oil for biocontrol has already been demonstrated in several studies. (Prior et al., 1988; Bateman et al., 1993; Ibrahim et al., 1999) For example, application of spores in oil leads to enhanced deposition of hyphal bodies of *Isaria farinosa* (formerly *Paecilomyces farinosus)* on alfalfa leaves and increased mortality of the larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae). (Agudelo and Falcon, 1983) In addition, the LUBILOSA project established the efficacy of an ultra-low volume application against desert locusts with oil containing *Metarhizium acridum* conidia. (Van der Valk, 2007) However, since an oil treatment alone has never been included in these studies, it remained unclear what role the oil played, and also whether the efficacy of EPF combined with oil resulted from a simple additive effect of the two treatments or benefitted from a synergistic interaction. In one notable exception, an emulsified colza oil and *Metarhizium anisopliae* were applied as single and combination treatments for the control of cattle ticks (*Boophilus microplus*, Arachnida: Ixodidae) (Leemon and Jonsson, 2008). Consistent with our results, their combination treatment suggests a synergistic effect, but this was not

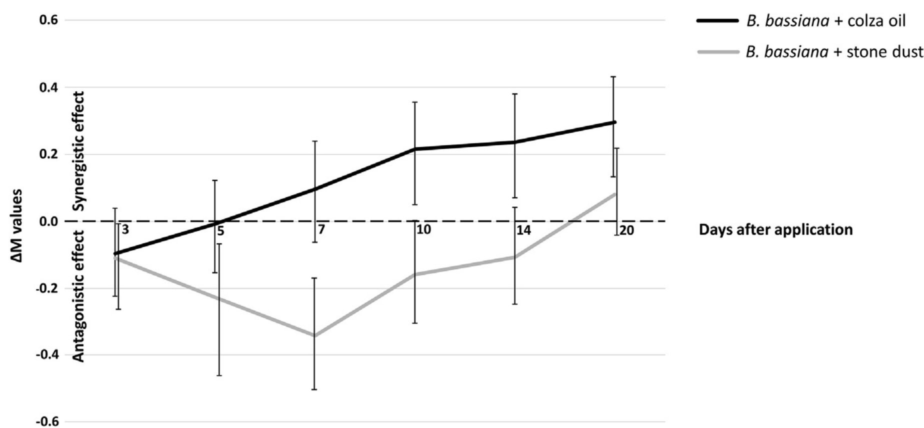


**Fig. 3.** ΔM values ($\Delta M = M_{combined} - M_{additive}$) and the corresponding 95% confidence intervals of the tested co-formulations with *B. bassiana* blastospores combined with either colza oil or stone dust. Values were obtained by the newly developed statistical approach to analyse co-formulations for synergistic or antagonistic interactions based on a generalized linear mixed effect model and a parametric bootstrap. $\Delta M > 0$ indicates a synergistic effect; $\Delta M < 0$ indicates an antagonistic effect.

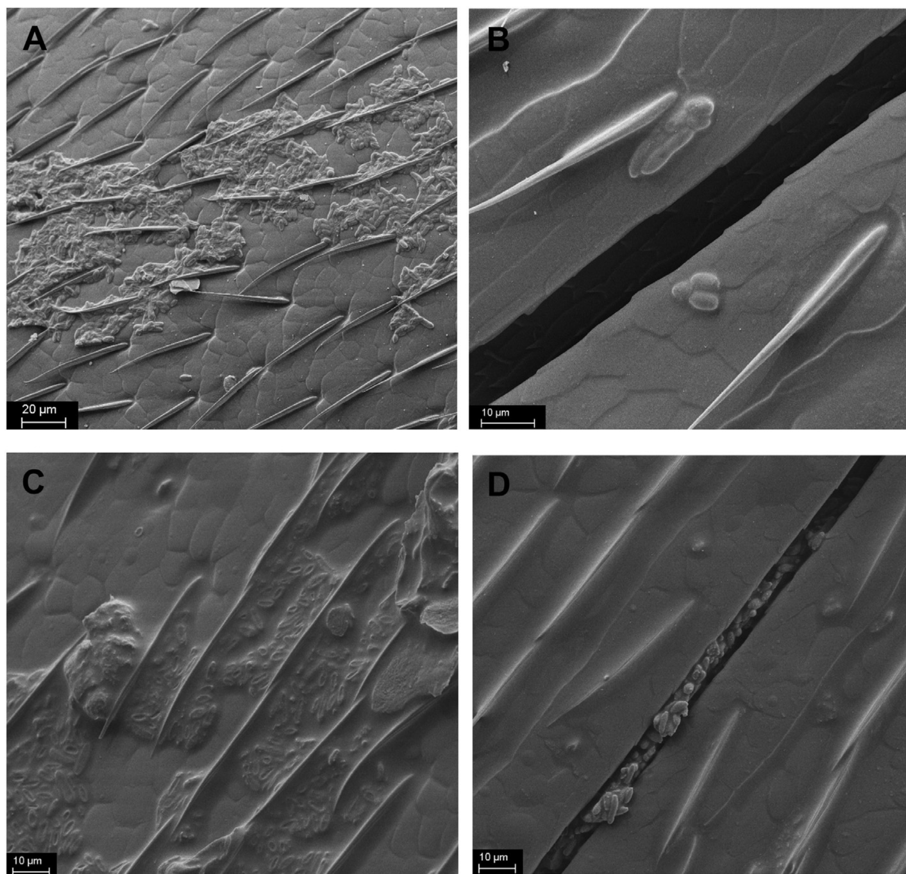


**Fig. 4.** Low temperature scanning electron micrographs of treated pollen beetles in dorsal view. (A) Surface of the elytra (1000×) and (B) fissure between elytra (4000×) after treatment with *B. bassiana* blastospores suspended in water; (C) surface of the elytra and (D) fissure between elytra after combined application of *B. bassiana* blastospores with emulsified colza oil (2000×). Note the absence of spores within the fissure in (B), whereas in (D) the fissure is filled with spores.

statistically analyzed.

Several hypotheses have been proposed to explain how the addition of oil enhances the effect of EPF on target pest mortality. A thin oil layer might prevent desiccation of spores by slowing evaporation, thus giving conidia more time to germinate and infect (Leemon and Jonsson, 2008). However, several studies have shown that germination of EPF and infection of insects are possible in a wide range of relative humidity, so that retention of humidity is unlikely to be the main factor leading to increased mortality of the pest insect (Moore, 1973; Ferron, 1977; Ramoska, 1984).

Oils are known to cause disruption of the protective layer of epicuticular waxes on the insect cuticle or the extraction of cuticle compounds. This could weaken the cuticle and facilitate infection by fungal spores (Ibrahim et al., 1999). Another hypothesis is that oil-formulated spores may better adhere to the lipophilic insect cuticle, which should result in increased deposition of spores (Prior et al., 1988). However, our scanning electron microscopy images of pollen beetles treated with *B. bassiana* spores in water and in oil formulation did not show notably different amounts of spores adhering to the elytra. To the best of our knowledge, increased deposition and persistence of spores has only been reported from treated plant surfaces and not from insect cuticles to date (Butt et al., 2000; Agudelo and Falcon, 1983). Spores may also disseminate more easily to infect susceptible parts of the insect body, such as intersegmental membranes, when formulated in oil (Prior et al., 1988; Ibrahim et al., 1999; Agudelo and Falcon, 1983; Burges and Jones, 1998). Evidence for this effect has been seen in scanning electron microscopy images of *Phaedon cochleariae* beetles (Coleoptera: Chrysomelidae) treated with *M. anisopliae* conidia in aqueous or oil formulations (Ibrahim et al., 1999). Our scanning electron microscopy images suggest a similar mechanism. While the fissure between the elytra appears to be free of spores after treatment with *B. bassiana* suspended in water, it is packed with spores after treatment with spores in combination with oil. This may indicate that more spores reached vulnerable parts of the body when formulated in oil. However, the synergism between the oil and the EPF spores is likely based on a combination of various factors, and deserves further investigation.

The application of stone dust alone had a relatively high impact on pollen beetle mortality already after one week. Stone dust is assumed to act on the epicuticular lipid and wax layer by abrasion and absorption, thereby causing water loss and desiccation (Ebeling and Wagner, 1959; Mewis and Ulrichs, 1999; Akbar et al., 2004; Storm et al., 2016). Several studies have reported greater effects of stone dust on insect mortality when applied as dry powder and under dry conditions, further indicating that insects die due to desiccation (Ebeling and Wagner, 1959; Mucha-Pelzer et al., 2008). Similarly, field studies comparing stone dust as dry powder and wet spray formulation showed greater effects of the dry powder formulation and only significant pollen beetle reduction under dry rainless conditions (Daniel et al., 2013).

The combined treatment of stone dust and *B. bassiana* spores in our case showed lower mortality compared to stone dust alone until day 14 after treatment. This combination thus seemed to initially interact antagonistically. Likewise, a negative effect on the mortality of melon aphids (*Aphis gossypii*) has been reported when clay was added to a wettable powder formulation of *B. bassiana* (Wraight et al., 2016). It was suggested that the clay particles adhere to the fungus spores and thereby impair their effects on the insect. A similar effect may underlie the antagonism observed in our study.

In conclusion, our study demonstrates that a combination of EPF spores with an oil formulation can lead to a higher level of pest control than that expected from the performance of the substances alone. Previous studies already indicated that synergistic interactions of EPF with other biological control agents or natural substances may improve the efficiency of biological pest control (Ansari et al., 2006; Wraight et al., 2016; Tomilova et al., 2016). To the best of our knowledge, we

have delivered a statistic prove for the well-known synergistic effect between fungus and oil for the first time. As with many other data sets, our data are not independent (in our case several beetles in the same experimental unit) and therefore do not fit to the assumptions of a traditional approach based on $\chi^2$ statistics. Our novel approach relies on a parametric bootstrap and a generalized linear mixed effect model and, therefore, can be applied on non-independent data. Moreover, this method takes into account subsequent repetition of trials. A protocol of our statistical analysis is given in the Supplementary material and may easily be adapted for the analysis of data from similar experimental set-ups, where non-independence of data is not achievable in practice.

With regard to the control of pollen beetles, EPF show promise as an alternative or complementary strategy to insecticides. Their efficacy is, however, dependent on adequate formulation to foster spore survival and pest infection when sprayed in open field conditions. Testing of *B. bassiana* against pollen beetles in colza fields, alone and in combination with oil, will be the next step to examine the synergistic effect between EPF and oil under more realistic conditions.

## CRediT authorship contribution statement

**Deborah Kaiser:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft. **Stephan Handschin:** Methodology. **Rudolf P. Rohr:** Methodology, Writing - review & editing. **Sven Bacher:** Methodology, Writing - review & editing, Supervision. **Giselher Grabenweger:** Supervision, Funding acquisition, Methodology, Writing - review & editing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2019.104106.

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**Co-formulation of *Beauveria bassiana* with natural substances to control pollen beetles - synergy between fungal spores and colza oil**

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SUPPLEMENTARY MATERIAL

**Statistical analysis of synergistic or antagonistic effects**

The analysis was made in two steps. In a first step, we fitted the data using a generalised linear mixed effect model (glme) with a binomial distribution. From the model output, we then estimated the probability of mortality due to the fungus treatment, the oil treatment, and the combined treatment. In a second step, we estimated the “additive” mortality, i.e., the expected mortality when both treatments are applied, but in absence of synergistic or antagonistic interactions between the two treatments. The additive mortality is our null hypothesis in this test. Then, the “additive” mortality was compared to the observed combined mortality (the alternative hypothesis). A positive difference between the observed combined mortality and the “additive” mortality indicates a synergistic interaction between the treatments, while a negative difference indicates an antagonistic interaction. To assess the statistical significance of the difference, we used a parametric bootstrapping method based on the glme fit of the first step, to estimate by numerical simulations the 95% confidence interval. The details and the R-code are given in the following.

Step 1: We fitted the count data of dead and alive beetles with a generalized linear mixed effect model with binomial distribution. The explanatory variables are the two treatments and their interaction. Note that the explanatory variables are coded as 1 when the treatment was applied and 0 when the treatment was not applied. Since the experiment was carried out twice, the trial was included as random factor. We used the command “glmer” of the library “lme4”. The R-command reads as:

```
out <- glmer(mortality ~ fungus * oil + (1|trial), family = binomial)
```

For example, on day 20, the output of the model is:

```
summary(out)
Generalised linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: binomial ( logit )
Formula: mortality ~ fungus * oil + (1 | trial)
AIC      BIC      logLik   deviance      df.resid
100.7    106.4    -45.4    90.7          18
Scaled residuals:
Min      1Q       Median   3Q       Max
-1.5419  -0.9704  -0.4674  0.3606   3.5890
Random effects:
Groups Name       Variance     Std.Dev.
trial(Intercept)  0.05464      0.2338
Number of obs: 23, groups: trial, 2
Fixed effects:
                  Estimate     Std. Error    z value  Pr(>|z|)
(Intercept)       -4.0607      1.0219        -3.974   7.08e-05 ***
fungus            2.6833       1.0491        2.558    0.0105 *
oil               3.0524       1.0420        2.929    0.0034 **
fungus:oil        -0.7748      1.1141        -0.695   0.4868
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Correlation of Fixed Effects:
                  (Intr)   fungus   oil
fungus            -0.948
oil               -0.955   0.930
fungus:oil        0.893    -0.942   -0.935
```

The estimated parameters are related to the logit of the probability of mortality due to the fungus alone ($m_{fungus}$), the oil alone ($m_{oil}$), and the combined treatment ($m_{combined}$) as follows:

$$m_{fungus} = \text{(Intercept)} + \text{fungus}$$

$$m_{oil} = \text{(Intercept)} + \text{oil}$$

$$m_{combined} = \text{(Intercept)} + \text{fungus} + \text{oil} + \text{fungus:oil}$$

These logits of mortality probabilities are then transformed into mortality probabilities ($M_{fungus}$, $M_{oil}$, and $M_{combined}$) by the formulas

$$M_{fungus} = 1 / (1 + \exp(-m_{fungus}))$$

$$M_{oil} = 1 / (1 + \exp(-m_{oil}))$$

$$M_{combined} = 1 / (1 + \exp(-m_{fcombined}))$$

Step 2. Based on the inferred mortality probabilities of step 1, we estimated the "additive" mortality probability of the fungus and the oil treatment. This "additive" mortality probability is given by the formula

$$M_{additive} = M_{fungus} + M_{oil} \times (1 – M_{fungus})$$

Then, the "additive" mortality probability was subtracted from the observed combined mortality probability as

$$\Delta M = M_{combined} - M_{additive} = M_{combined} - M_{fungus} - M_{oil} \times (1 – M_{fungus})$$

At this point, it is worth noticing that the difference $\Delta M$ is not equal to the interaction parameter of step1 model, i.e., $\Delta M \neq$ fungus:oil, and neither to the inverse of the logit of the interaction parameter $\Delta M \neq 1 / (1 + \exp(\text{-fungus:oil}))$ (as the parameters are on a logit scale we may also think that $\Delta M$ might be equal to the inverse of the logit of the interaction term). For instance in the above example we computed $\Delta M = 0.296$, while the interaction term fungus:oil = -0.775 and $1 / (1 + \exp(\text{-fungus:oil})) = 0.315$. In particular, this implies that

the statistical significance of the difference cannot directly be deduced from the glme model output. For this reason, we have to numerically simulate the distribution of the difference $\Delta$M, in order to estimate its 95% confidence interval. The simulation was done as follows.

The four parameters of the glme model ((Intercept), fungus, oil, fungus:oil) follow a multivariate Normal (or Gaussian) distribution with mean values given by their estimates (fixed-effect estimates) and variance-covariance matrix computed from their standard error (fixed-effect std. error) and the correlation (correlation of fixed-effect).[1] Both can be extracted directly from the summary of the model output by the following R-commands:

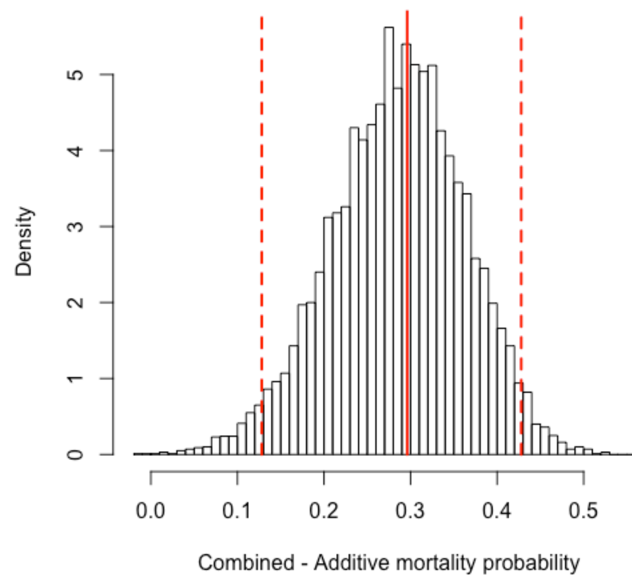
| | |
|---|---|
| mean values: | summary(out)$coefficient[,1] |
| variance-covariance matrix: | summary(out)$vco |

Therefore, we can simulate the distribution of the fitted parameters, which can then be cascaded into the computation of the difference in mortality $\Delta$M. This procedure generates the distribution of the difference $\Delta$M, from which we extracted the 95% confidence interval. We used the library "mvtnorm" to simulate the parameters from a Normal multivariate distribution.[2, 3] The R-code for the simulation reads as follows:

```
library(mvtnorm) #library for multivariate Normal distribution
N_sim <- 10000 #number of simulations
deltaM <- rep(NA,N_sim) #vector to store the output of the simulation
#loop for the simulation
for (i in 1:N_sim){
    #drawing the model parameters
    t <- rmvnorm(1,mean = summary(out)$coefficient[,1],sigma = as.matrix(summary(out)$vco))
    #computation of mortality probability logit
    m_fungus <- t[1] + t[2]
    m_oil <- t[1] + t[3]
    m_combined <- t[1] + t[2] + t[3] + t[4]
    #computation of mortality probability
    M_fungus <- 1 / (1+exp(-m_fungus))
    M_oil <- 1 / (1+exp(-m_oil))
    M_combined <- 1 / (1+exp(-m_combined))
```

```
        #compute the difference between the combined and the "additive" mortality probability
        deltaM[i] <- M_combined - M_fungus - M_oil * (1-M_fungus)
}
```

Then, we can draw the histogram of the simulated distribution of the difference $\Delta M$. For example, with the data on day 20, we obtained the following distribution. The red dashed vertical lines correspond to the 95% C.I., and the red vertical line gives the value of $\Delta M$.




The 95% confidence interval can be extracted as follows:

```
sort(deltaM)[0.025*N_sim] #lower bound of the 95% C.I.
sort(deltaM)[0.975*N_sim] #upper bound of the 95% C.I.
```

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