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Laboratory and semi-field evaluation of *Beauveria bassiana* (Ascomycota: Hypocreales) against the lettuce aphid, *Nasonovia ribisnigri* (Hemiptera: Aphididae)

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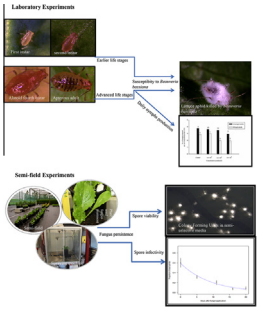
HIGHLIGHTS

- The lettuce aphid is an economically important pest of lettuce worldwide.
- *Beauveria bassiana* can potentially be used for lettuce aphid biocontrol.
- Young aphids were generally less susceptible to *B. bassiana* than older aphids.
- *B. bassiana* significantly decreased the rate of lettuce aphid nymph production.
- Persistence of viable spores was positively correlated with spore infectivity.

GRAPHICAL ABSTRACT



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ABSTRACT

The lettuce aphid, *Nasonovia ribisnigri* (Mosley), is an economically important pest of lettuce worldwide. The entomopathogenic fungus *Beauveria bassiana* strain GHA has recently been reported as a potential biocontrol candidate for use against the lettuce aphid. This study provides information on the mortality inflicted by *B. bassiana* when applied against different life stages of the lettuce aphid under laboratory conditions and how fungus infection affects the aphid fecundity. In addition, temporal changes in persistence of fungus inoculum applied to foliage of young lettuce plants under semi-field conditions was analysed. Immature life stages were generally the least susceptible to fungal infection and the susceptibility of all stages was dose-dependent, with the highest mortality occurring at the highest dose. *B. bassiana* significantly affected the rate of nymph production by the lettuce aphid, with the highest effect seen when the alatoid fourth instar of *N. ribisnigri* was inoculated with *B. bassiana*. The persistence of *B. bassiana* conidia on lettuce foliage was not influenced by leaf position. Within 5 days, the cumulative percentage decline in the conidial population was 38% which declined further to 92% and 99% on day 11 and 20 post-spraying, respectively. In accordance, the infectivity to second instar lettuce aphid nymphs of *B. bassiana* conidia deposited on leaves declined according to an exponential decay model predicting an intercept of 0.59 ± 0.03 (S.E), a reduction in aphid mortality at a rate of 11% with each increasing day after fungal application and a fungus half-life of 6.34 ± 0.69 days.

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1. Introduction

The lettuce aphid, *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae), a lettuce specialist herbivore, native of temperate regions of Europe, has become one of the most serious pests of lettuce worldwide within the last decades (Blackman and Eastop, 2000). It has a cryptic habitat preferring to attack mostly in the lettuce hearts as compared with other aphid species infesting lettuce (Mackenzie and Vernon, 1988; Palumbo, 2000; Liu, 2004). *N. ribisnigri* reduces the lettuce yield directly by causing leaf distortion, reducing seedling vigour and deforming lettuce heads (Stufkens and Teulon, 2003). Furthermore, the presence of *N. ribisnigri* decreases the percentage of harvested heads that can go to market, which can have serious financial implications for the producers (Van de Steene et al., 2003; Kift et al., 2004).

Control strategies for *N. ribisnigri* populations depend to a large extent on the use of insecticides (Rufingier et al., 1997). The need for alternative methods to control *N. ribisnigri* has, however, been stimulated due to increased insecticide resistance (Barber et al., 1999) and environmental and human health concerns regarding residual effects of pesticides (Sances et al., 1993). Potential biological control methods for lettuce aphids include the use of predators such as lacewings (Quentin et al., 1995; Shrestha and Enkegaard, 2013), syrphids (Hopper et al., 2011) and parasitoids (Nebreda et al., 2005; Shrestha et al., 2014). A further potential method for lettuce aphid biocontrol is the use of fungal pathogens (Fournier and Brodeur, 2000; Åsman, 2007; Diaz et al., 2009).

Regarding entomopathogenic fungi, detailed laboratory studies have been carried out with different species of *Lecanicillium* (Ascomycota: Hypocreales) demonstrating the ability of these fungi to inflict mortality in *N. ribisnigri* (Fournier and Brodeur, 2000; Åsman, 2007; Diaz et al., 2009). However, tests in greenhouses or under field conditions have failed to show efficacious levels of control (Fournier and Brodeur, 2000; Åsman, 2007). Another fungus, *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) is pathogenic to many pest species, including aphids (Ugine et al., 2007; Hesketh et al., 2008; Castrillo et al., 2010), and the strain GHA, commercially available as Botanigard®, seems to be a potential biocontrol candidate for use against the lettuce aphid (DEFRA, 2007; Yearbook, 2009). Thus, a study carried out under field conditions in Spain documented that *N. ribisnigri* numbers were reduced by 53–68%, depending on dosage as compared with a control treatment (Yearbook, 2009), while laboratory and field tests in UK indicated that this fungal strain may provide better control of *N. ribisnigri* compared to other commercial fungal species and other strains of *B. bassiana* (DEFRA, 2007). To further evaluate the potential of *B. bassiana* against *N. ribisnigri* studies on aspects influencing the ability of this entomopathogenic fungus to control target pest populations are required.

One such aspect is the susceptibility of different aphid developmental stages to fungal infection (Schmitz et al., 1993; Kim and Roberts, 2012; Jandricic et al., 2014). Entomopathogenic fungi infect their host via cuticular penetration and since aphids change developmental stage at relatively short intervals (down to 1 or 2 d) (Diaz and Fereres, 2005), they may therefore escape fungal infection by shedding the cuticle in the moulting process (Liu et al., 2003; Kim and Roberts, 2012). The differential susceptibility of different aphid stages may also be related to differences in physiology or cuticle biochemical composition as shown for other arthropods (Tang et al., 1999; Kirkland et al., 2004). Another important aspect is the influence of fungal infection on the reproductive performance (Wang and Knudsen, 1993; Xu and Feng, 2002; Baverstock et al., 2006; Kim, 2007; Ganassi et al., 2010). Aphids infected with fungus at an immature developmental stage may develop into nymph producing adults before they die from fungus infection (Fournier and Brodeur, 2000; Kim, 2007) and infected adults will likewise produce nymphs before dying (Wang and Knudsen, 1993; Baverstock et al., 2006; Ganassi et al., 2010). Consequently, the fecundity of fungus-infected aphids may be increased, decreased or not influenced by the fungus infection and such information is valuable regarding the prediction of the ability of a fungal pathogen to suppress the growth of aphid populations. In addition, the persistence (propagule viability and infectivity over time) of fungal inoculum in the host habitat is of major importance for the efficacy of fungi as biocontrol agents (Jaques, 1983; Inglis et al., 1993; Gatarayiha et al., 2010). Variation in persistence on leaf surfaces of different plant species have been documented and is reported to be influenced by a number of factors such as plant morphology (Inglis et al., 1993; Kouassi et al., 2003; Gatarayiha et al., 2010), plant surface chemistry (Meekes et al., 2000) and abiotic conditions (Daoust and Pereira, 1986; Ignoffo, 1992; Inglis et al., 1995).

None of the above mentioned aspects have been investigated for *B. bassiana* in relation to the lettuce aphid. This study therefore aimed to investigate (1) the effect of *B. bassiana* treatment on different developmental stages of *N. ribisnigri*, (2) the influence on lettuce aphid fecundity and (3) the persistence over time of *B. bassiana* inoculum on lettuce leaves under semi-field conditions as estimated by in vitro quantification of viable conidia (Colony Forming Units (CFUs)) and aphid infectivity assays.

2. Material and methods

2.1. Plants

Iceberg lettuce, *Lactuca sativa* L. cv. 'Mirette', was used as a source of plant material for the experiments. Seeds were sown on Jiffy-strip trays and maintained in a glasshouse at 15–18 °C, 55–70% RH and natural light conditions. After two weeks, plants were transplanted in large sized pots (2 L) filled with peat soil (admixed with perlite and vermiculite). These plants were either used within 6–10 days for production of aphid cohorts and for use in bioassays (detached leaflets; lab and semi-field experiments) or maintained for additional 3 days in a glasshouse and subsequently transported to the semi-field experimental site.

2.2. Insects

A stock culture of *N. ribisnigri*, originally supplied by Dr. Gemma Hough (Warwick Crop Centre, University of Warwick, UK), was reared on Iceberg lettuce plants in nylon net cages ($68 \times 75 \times 82$ cm) in a controlled environment glasshouse compartment at 16:8 L:D, 20 ± 1 °C and 70 ± 5% RH.

To obtain cohorts of the same stages of lettuce aphids for laboratory (first instar, second instar, alatoid fourth instar and newly moulted unwinged adults) or semi-field experiments (second instar nymphs), adult aphids were inoculated onto uninfested Iceberg lettuce plants with a fine camel hair brush and maintained in similar cages and at similar conditions as above. After 24 h, the resulting first instar nymphs were either used directly for the experiments or transferred onto new clean plants (50–60 aphids per plant) and maintained as above for another 3 days or 8 days for production of second instar or alatoid fourth instar, respectively. Eight days after birth apterous fourth instars (fourth instar nymphs without wing buds) will moult into unwinged adults (Diaz and Fereres, 2005) and these newly moulted unwinged adults were used for experimentation as well. The two groups of insects in the test thus had a similar numerically age but differed in developmental stage.

2.3. Fungus material

B. bassiana strain GHA (BotaniGard®), formulated as paraffinic oil-based emulsifiable suspension was provided by Borregaard BioPlant, Aarhus, Denmark. The conidial concentration in the product was determined by counting conidia suspended in 0.01% Tween 80 in a compound microscope using a hemocytometer (0.0625 mm^2; Fuchs-Rosenthal Merck Eurolab) to calibrate a suspension of 1×10^8 conidia/ml for the laboratory bioassay experiment. Lower concentrations were prepared by serial dilution with 0.01% Tween 80. For the semi-field experiment, the recommended dose for greenhouse or field applications that corresponds to 1.44×10^7 conidia/ml formulated product was used. The viability of conidia was checked by spreading 2 ml of 1×10^6 conidia/ml spore suspension on a plate with 2% Sabouraud dextrose agar (SDA) and estimating percentage germination in a light microscope after 18 h incubation at room temperature. The viability check was carried out immediately prior to the initiation of each experiment. Conidia germination always exceeded 95%.

2.4. Laboratory experiments: mortality and fecundity

The bioassay experiment was performed to quantify the susceptibility of different developmental stages of *N. ribisnigri* to three spore concentrations of *B. bassiana* (high: 1×10^8, medium: 1×10^6 and low: 1×10^4 conidia/ml) and a control of 0.01% Tween 80. Furthermore, sublethal effects (Torrada-Leon et al., 2006) of fungal infection on the reproductive capacity of two life stages of the lettuce aphid (alatoid fourth instar and newly moulted apterous adults) were studied by measuring daily nymph production.

Aphids of same stage were placed in groups of 7 aphid individuals in a 30 ml plastic cup capped with a mesh screen lid. Each group of aphids was swirled for 5 s in 5 ml spore suspension and the liquid drained off in a Büchner funnel and discarded (Hall, 1976). The aphids were subsequently transferred with a fine camel hair brush to a detached lettuce leaf. The base of the leaf was wrapped with moist cotton, inserted into an 1.5 ml Eppendorf tube with demineralised water and placed at the bottom of a Petri dish (diameter: 15 cm) lined with filter paper. The Petri dish was then sealed with parafilm to prevent escape of the aphids. In this system, described previously by Yokomi and Gottwald (1988), the leaf remained in satisfactory condition for at least one week. However, if necessary, the filter paper was replaced and a new fresh lettuce leaf in an Eppendorf tube was placed close to the old one to allow the aphids to translocate by themselves. Dishes were incubated in a climate cabinet at 22 °C and 16:8 L:D. The bioassay experiment was performed twice. The numbers of replicates (one replicate equals one cup) per treatment were 3 and 9, respectively, in the first and second experimental run (providing a total of 12 replicates per treatment), while data on fecundity were only recorded in the second experimental run.

Starting two days after the treatment, aphid mortality and the numbers of new-born nymphs in each aphid group (alatoid fourth instar and newly moulted apterous adults) were checked daily for 9 days and nymphs produced were counted and removed. Dead aphids were removed and placed on moist filter paper in a Petri dish to check for sporulation.

2.5. Semi-field experiments: colony forming units (CFUs) and infectivity

Two methods were employed to quantify the persistence of fungus spores on lettuce foliage: CFU enumeration and infectivity assays using second instar lettuce aphids.

2.5.1. Experimental plots

The semi-field experimental plots were established at Research Centre Flakkebjerg, Department of Agroecology, Aarhus University, Denmark. The site was plastic roofed with concrete floor and bounded by a windbreak to reduce air velocity. There were two experimental plots (treated and untreated), approx. 9 m^2 per plot. In each experimental plot, 2 rows of potted lettuce plants were maintained with spacing of 60×30 cm. The untreated plot was approx. 10–12 m from the treated plot. The numbers of plants were 50 for the treated plot and 25 for the untreated plot. Plants were drip irrigated daily for half an hour mornings and evenings during the whole experimental period. Minimum, maximum and mean hourly temperature, relative humidity levels and solar radiation were measured automatically (Grand weather Station, Metric A/S, Denmark) (Fig. 1).

2.5.2. Application of conidia

For the application of *B. bassiana* conidia, plants established in the semi-field experimental site 6 days previously were transported to a spraying room. At the time of fungus application the plants were 23 days old after seeding and had 3–4 unfolded leaves. Leaves developed from the central portion of plants were denoted as inner leaves and the leaves developed from peripheral layers as outer leaves. The numbers of leaves on each treated plant were recorded at the time of spraying to ensure that outer and at least 1 inner leaf was present on all plants. A knapsack sprayer (pressure of 40 PSI) equipped with a hollow cone fine nozzle (1553-10) was used to apply the conidial suspension of 1.44×10^7 conidia/ml of *B. bassiana* to the plants. Each plant was sprayed for 4 s to run off with 35 ml spore suspension by placing it on an automatic spinning disc in the centre of a spraying cabin. The complete spraying procedure was accomplished between 0800 am and 1400 pm. The sprayed plants were left in the room for 3 h and subsequently transported back into their original place in the semi-field experimental site. Control plants were left untreated.

2.5.3. Leaf collection and preparation

From treated and untreated plants, outer and inner leaves of the same plant were randomly collected at each sampling time. The times of sampling were immediately after (denoted day 0), 5, 11, 16 and 20 days post application for the infectivity assay and day 0, 5, 11 and 20 days post application for the CFU test. The plants were selected randomly for sampling and care was taken to choose leaves that were present at the time of treatment with *B. bassiana*. Each leaf sample (inner leaf or outer leaf) was kept separately in a plastic bag and immediately transported to the laboratory for further analysis. A cork borer was used to make a leaf disc (diameter = 2 cm) randomly positioned on each sampled leaf. The cork borer was sterilised with ethanol (96%) between making each leaf disc.

2.5.4. Experimental procedure for quantification of CFUs

The number of *B. bassiana* conidia in outer or inner leaves of fungus treated lettuce plants (5 plants sampled each sampling time) were quantified on plates with semi selective growth medium containing streptomycin, tetracycline, cycloheximide and dodine (Meyling and Eilenberg, 2006) following washing of leaf discs as described by Inglis et al. (1993). Each leaf disc was washed separately in 30 ml of 0.01 M phosphate buffer solution (pH 7.0) containing 0.01% Tween 80 in a 200 ml sterile tissue culture flask at ambient room temperature for 2 h on a rotary shaker at 300 rpm. After shaking, the wash solution was serially diluted threefold to yield a dilution series of 1:10, 1:100 and 1:1000. 200 µl aliquots from each dilution were spread onto plates (3 plates per dilution) with semi selective medium. Plates were sealed with parafilm and incubated at 22 °C and 16:8 L:D in a controlled

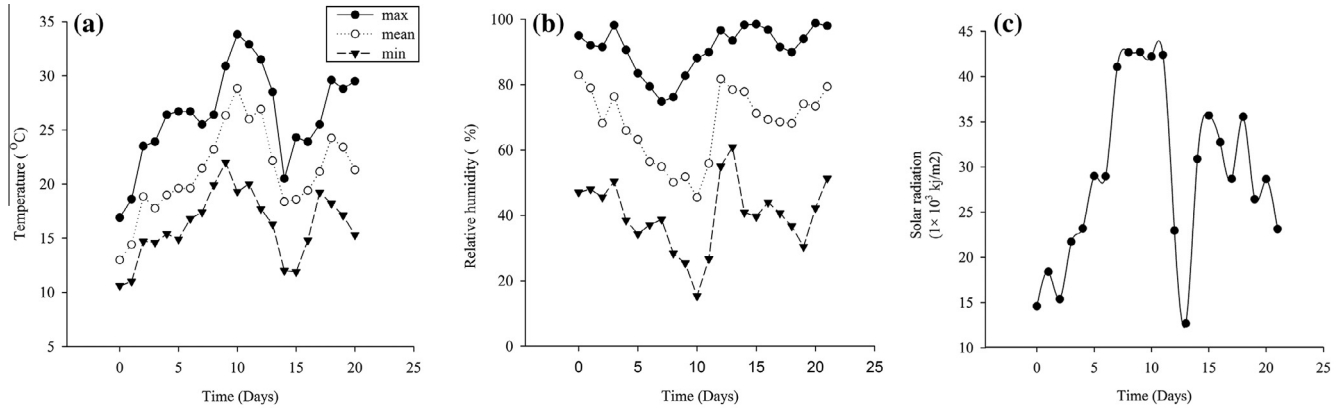

Fig. 1. Weather conditions during the experiment in semi-field unit: (a) daily maximum, minimum and mean temperature; (b) daily maximum, minimum and mean relative humidity; (c) daily cumulative hourly incoming solar radiation.

climate cabinet for 7 days. The number of colony forming units of *B. bassiana* was determined at an appropriate dilution, usually the 1:10 dilution, and this value was used in calculation of CFUs per leaf disc. The recovery rate on the selective medium was found to be 79%. For quantification of CFUs on untreated plants, 20 leaf discs (10 from outer leaves and 10 from inner leaves) were pooled, washed together and processed as described above.

2.5.5. Experimental procedure for infectivity test with lettuce aphids

The infectivity of *B. bassiana* against *N. ribisnigri* nymphs was tested on outer or inner leaves of fungus treated lettuce plants (4 plants sampled at each sampling time). A cohort of 10 second instar nymphs was transferred to each leaf disc (diameter = 2 cm) and placed at the bottom of a Petri dish (diameter = 9 cm) lined with moist filter paper. The Petri dish was then sealed with parafilm and kept in a climate cabinet at 22 °C and 16:8 L:D. After 24 h, the leaf disc with aphids was transferred onto a young detached lettuce leaf and the subsequent handling of the aphids were as described above for the laboratory bioassay experiment (see Section 2.4). The same procedure was followed for untreated plants. Aphid mortality was recorded at 1–2 days intervals for 6 days. Dead aphids were placed on moist filter paper in Petri dishes to check for sporulation.

2.6. Statistical analysis

The data were analysed in R 2.15.1 (R Development Core Team, 2011). For all data (except persistence data from the infectivity assay), a test with a normal quantile-quantile plot was performed to confirm normality of data and equality of variance and where appropriate, Tukey contrast pairwise multiple comparisons were used to test for significant differences in means (Hothorn et al., 2008).

2.6.1. Mortality and fecundity

Data on cumulative mortality were corrected for the mortality in the controls (Abbott, 1925) and the number of nymphs produced per surviving adult per day was calculated (total numbers of nymphs produced/total number of exposed aphids or surviving adults for each day). Mortality data were angular transformed. Two way analysis of variance (ANOVA) was performed in order to determine the overall influence of lettuce aphid developmental stage and spore concentration on aphid mortality. One way analysis of variance (ANOVA) was carried out to determine (1) the effect on mortality across aphid developmental stages, (2) the effect on mortality across spore concentrations, (3) the effect of fungal infection on total nymph production per surviving adult per day across spore concentrations and (4) the effect of fungal infection on daily nymph production per surviving adult per day across the spore concentrations on each day.

2.6.2. CFUs and infectivity

There were very few or no *B. bassiana* conidia recovered from leaf samples collected from untreated plants. Therefore, this part of the data set on CFUs was excluded from analysis. The percentage reduction of conidia was calculated relative to the initial conidia population (assessed immediately after spraying) as follows:

Conidial density reduction (%)

$$= \frac{\mathrm{CFU}_s t_0 - \mathrm{CFU}_s t_1}{\mathrm{CFU}_s t_0} \times 100;$$

where $\mathrm{CFU}_s t_0$ represents the colony forming units of conidia recovered immediately after fungus spray and $\mathrm{CFU}_s t_1$ is the colony forming units of conidia recovered at each sampling time (Gatarayiha et al., 2010). The data was angular transformed prior to statistical analysis.

The data was fitted to a linear mixed model with sampling time interval, leaf position and CFUs per replicate as fixed effects (categorical variables converted to factors), the variation in CFUs (1|Unit) as random effect and the mean CFUs per plant as response variable using the function "lmer". The mean CFU per plant was calculated using the "Summaryby" work package (doBy). The model was then reduced if possible, with stepwise removal of factors having no effect and the Kenward–Roger test was used using the function "KRmodcomp" to compare the models (Halekoh and Højsgaard, 2012). The values of angular transformed data were back transformed at the end of analysis.

Data for infectivity to aphids was fitted to a logistic regression model with the function "glm" and family "binomial". Two categorical variables of data, time and leaf position were converted into factors. The model was then simplified with stepwise removal of factors having no effect and the lack of fitness test was performed to compare the models. As the model was formulated in terms of transformed probabilities (logit transformation to the log-odds scale), the result was back transformed to an original probability scale after analysis. Subsequently, the final model was fitted to an exponential decay model with lower limit at 0 (2 parameters) with the package "drc" (Ritz and Streibig, 2012):

$$f(x) = d * \exp(-x/e)$$

where d (intercept) is the proportion of dead aphids at day 0 and e is the measure of decay rate which was used to calculate the relative decay rate and the half-life of the fungus. A similar procedure was

followed for analysis of aphid mortality data from untreated lettuce plants.

3. Results

3.1. Laboratory experiment

3.1.1. Susceptibility of N. ribisnigri developmental stages to B. bassiana

Overall this bioassay study demonstrated significant main effects on lettuce aphid mortality for both spore concentration level ($F = 107.22$; $df = 2$; $P < 0.0001$) and host stage ($F = 16.25$; $df = 3$; $P < 0.001$) without a significant interaction effect ($F = 1.71$; $df = 6$; $P > 0.05$).

Across the developmental stages of *N. ribisnigri*, significant differences in aphid mortality occurred when aphids were treated with either spore concentration (conidia/ml): 1.0×10^4 ($F = 3.19$; $df = 3$; $P < 0.05$), 1.0×10^6 ($F = 10.86$; $df = 3$; $P < 0.001$) and 1.0×10^8 ($F = 4.16$; $df = 3$; $P < 0.05$). Generally mortality increased with the aphid stage with adults being the most susceptible to fungal infection (Table 1). However, mortality differences between alatoid fourth instar and newly moulted apterous adults and also between second and first instar nymphs were not significant across all concentration levels (Table 1).

Within each aphid developmental stage treated with high, medium or low spore concentrations of *B. bassiana*, there was a significant difference in mortality inflicted by *B. bassiana*: newly moulted apterous adults ($F = 26.50$; $df = 2$; $P < 0.001$), alatoid fourth instar ($F = 35.60$; $df = 2$; $P < 0.001$), second instar nymphs ($F = 17.49$; $df = 2$; $P < 0.001$) and first instar nymphs ($F = 35.34$; $df = 2$; $P < 0.001$). The mortality caused by the fungus was dose-dependent with the highest mortality recorded with highest concentration, except that no significant difference was observed in the mortality of apterous adults treated with high or medium spore concentration (Table 1). Mean levels of mortality in fungus treated groups ranged from 11% to 77% for first instar nymphs, 15% to 73% for second instar nymphs, 20% to 95% for alatoid fourth instar and 34% to 94% for newly moulted apterous adults (low dose to high dose, respectively; Table 1). The average control mortality (±SE) for first instar, second instar, alatoid fourth instar nymphs and newly moulted apterous adults of *N. ribisnigri* were: 4.32 ± 2.40, 7.74 ± 2.94, 9.05 ± 2.25 and 10.91 ± 1.90% respectively, 9 days post-inoculation. The mortality data were supported by data on sporulation (Table 1); no fungus infected specimens were found in the control treatments.

3.1.2. Effect of B. bassiana infection on aphid fecundity

3.1.2.1. Unwinged adults. Newly moulted apterous adults of *N. ribisnigri* inoculated with different *B. bassiana* spore concentrations were significantly affected in terms of average nymph production capacity per surviving adult per day over the 9 days post inoculation period ($F = 4.13$; $df = 3$; $P < 0.01$). Adults exposed to the high spore concentration produced significantly fewer nymphs (mean ± SE: 2.93 ± 0.27) than those treated with the control dose (mean ± SE: 3.76 ± 0.12), although no significant differences was observed between control, low (mean ± SE: 3.64 ± 0.12) and medium (mean ± SE: 3.48 ± 0.18) spore concentrations or between high and medium spore concentrations (Fig. 2). However, no significant difference was detected on daily mean nymph production per unwinged adult across spore concentrations (Table 2).

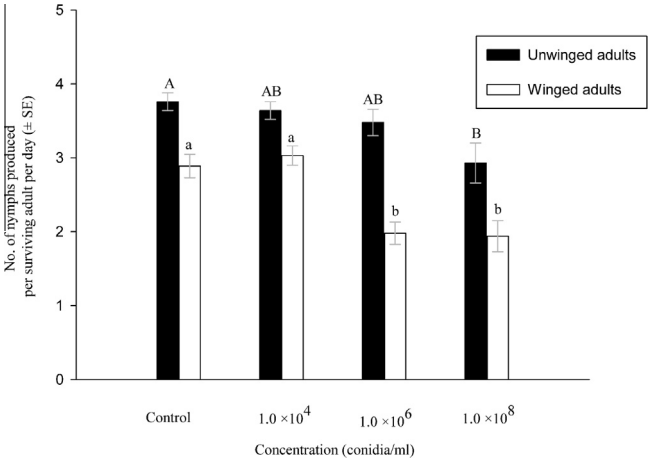

Fig. 2. Mean daily nymph production per surviving winged or unwinged adult (±SE) of *Nasonovia ribisnigri* inoculated with different spore concentrations of *Beauveria bassiana*, over the 9 days post inoculation period. Unwinged adults were newly moulted and the winged adults at alatoid fourth instar stage at the time of fungus treatment. Bars bearing the same letters are not significantly different (Tukey test, $P > 0.05$).

3.1.2.2. Alatoid fourth instars. Alatoid fourth instar of *N. ribisnigri* (expected to develop into winged adults within the following day) exposed to different spore concentrations of *B. bassiana* were significantly affected in terms of average nymph production per winged adult per day ($F = 12.99$; $df = 3$; $P < 0.001$). The number of nymphs produced by a winged adult in the control (2.89 ± 0.16) was significantly higher than those produced by aphids exposed to high (mean ± SE: 1.94 ± 0.21) and medium (mean ± SE: 1.98 ± 0.15) spore concentrations of *B. bassiana*, although no significant difference was found between control and low (mean ± SE: 3.03 ± 0.13) spore concentrations (Fig. 2). When the daily average nymph production per adult was analysed, the number of nymphs produced by aphids in the control were significantly higher than those produced by aphids exposed to the highest spore concentration on day 3, 5, 6 and 8 (Table 3).

3.2. Semi-field experiments

3.2.1. Quantification of colony forming units of B. bassiana on lettuce plants

The mean numbers (±SE) of viable spores of *B. bassiana* on inner and outer lettuce leaves, as quantified immediately after the fungal spray, were $1.54 \times 10^5 \pm 3.30 \times 10^2$ and $1.02 \times 10^5 \pm 3.54 \times 10^2$ per leaf disc, respectively. There was a tendency to a more slow

Table 1
Susceptibility of different developmental stages of *Nasonovia ribisnigri* to *Beauveria bassiana*. Mean percentage of corrected mortality (±SE) 9 days post inoculation.

Concentration (conidia/ml)	Developmental stages			
	Apterous adults	Alatoid fourth instar	Second instar	First instar
1.0×10^8 (High)	94.00 ± 4.75*Aa* (81/84)	94.85 ± 2.80*Aab* (79/83)	72.85 ± 7.43*Ab* (53/84)	76.57 ± 6.74*Aab* (56/78)
1.0×10^6 (Medium)	81.51 ± 6.40*Aa* (66/81)	73.86 ± 8.10*Bab* (61/84)	46.41 ± 7.19*Bbc* (37/82)	29.51 ± 4.70*Bc* (26/79)
1.0×10^4 (Low)	34.16 ± 6.69*Ba* (7/77)	19.90 ± 4.97*Cab* (6/81)	14.98 ± 3.88*Cab* (1/84)	10.68 ± 2.85*Cb* (3/74)

Mean values within columns bearing the same upper case letter and within rows bearing the same lower case letters are not significantly different (Tukey test, $P > 0.05$). The values in parentheses denote the total number of mycosed aphids out of total number of tested aphids. Data pooled over the two experimental runs. The total numbers of replicates were 12 per treatment.

Table 2
Mean daily nymph production per surviving unwinged adult (±SE) of *Nasonovia ribisnigri* inoculated with different spore concentrations of *Beauveria bassiana*.

Days after inoculation	Mean nymph production per unwinged adult			
	1×10^8	1×10^6	1×10^4	Control
2	1.16 ± 0.23*b* (62)	2.63 ± 0.15*a* (60)	2.68 ± 0.40*a* (56)	1.95 ± 0.13*ab* (62)
3	2.68 ± 0.46*a* (52)	3.09 ± 0.38*a* (60)	3.76 ± 0.25*a* (56)	3.78 ± 0.28*a* (62)
4	4.15 ± 0.55*a* (23)	3.59 ± 0.36*a* (45)	3.75 ± 0.31*a* (52)	4.00 ± 0.17*a* (61)
5	4.08 ± 0.55*a* (15)	4.19 ± 0.50*a* (23)	3.82 ± 0.31*a* (52)	3.88 ± 0.21*a* (61)
6	2.56 ± 0.22*a* (8)	3.66 ± 0.58*a* (17)	3.71 ± 0.21*a* (51)	4.22 ± 0.20*a* (60)
7	3.83 ± 0.83*a* (4)	3.94 ± 0.67*a* (15)	3.12 ± 0.32*a* (50)	3.76 ± 0.18*a* (59)
8	4.33 ± NA*a* (3)	4.01 ± 0.94*a* (14)	4.53 ± 0.35*a* (44)	4.55 ± 0.49*a* (58)
9	4.00 ± NA*a* (3)	3.01 ± 0.76*a* (12)	3.76 ± 0.26*a* (41)	3.98 ± 0.29*a* (58)
Total nymph production	26.80	28.14	29.14	30.12

Mean values within a row bearing the same letters were not significantly different (Tukey test, $P > 0.05$). The values in parentheses denote the total number of live aphids per day. The total numbers of replicates were 9 per treatment.

decline in *B. bassiana* spore viability on inner leaves as compared to outer leaves (Fig. 3), but the leaf position effect was not significant ($F = 1.91$; $df = 1$; $P > 0.05$). No significant interaction ($F = 3.68$; $df = 2$; $P > 0.05$) between the leaf position and time of leaf sampling was found and the data for inner and outer leaves were consequently combined.

Time was the only factor that significantly affected the persistence of the applied *B. bassiana* spores on the lettuce leaves ($F = 33.29$; $df = 2$; $P < 0.001$). Within 5 days, the cumulative percentage decline in the conidial population for inner and outer leaves combined was 38%. After 11 days post-spraying the conidial population had sharply declined by 92%, a value not significantly different from the decline of 99% observed after 20 days (Table 4).

3.2.2. Quantification of *B. bassiana* infectivity against *N. ribisnigri* nymphs on lettuce leaf discs

Time played a significant role in the persistence measured as spore infectivity of *B. bassiana* against *N. ribisnigri* ($\chi^2 = 76.96$; $P < 0.001$), whereas leaf position was without effect on aphid mortality ($\chi^2 = 2.75$; $P > 0.05$). Similarly, there was also no significant interaction between time and leaf position ($\chi^2 = 0.49$; $P > 0.05$).

The data combined for inner and outer leaves and fitted with the exponential decay model ($R^2 = 0.96$) provided an estimated intercept of 0.59 ± 0.03 (SE) and a decay rate (e) of 9.15 ± 0.99 (SE), corresponding to a fungus half-life of 6.34 ± 0.69 days (Fig. 4) and aphid mortality declining at a rate of 11% with each increasing day after fungal application. No significant difference was found in aphid mortality in the untreated plot across the sampling interval ($P > 0.05$), the mean mortality being less than 8%. The mean percentage sporulation for aphids dying after being incubated on fungus treated leaf discs sampled on day 0, 5, 11, 16 and 20 days post application were 88%, 48%, 20%, 17% and 29% respectively.

4. Discussion

4.1. Susceptibility of *N. ribisnigri* developmental stages to *B. bassiana*

This study showed that first and second instars of *N. ribisnigri* were generally less susceptible to infection with *B. bassiana* than alatoid fourth instar and apterous adult. Furthermore, the mortality in all stages was dose-dependent, with the highest mortality occurring at the highest dose. Similar dose-mortality responses for different developmental stages have been reported in many other pest species (Feng et al., 1985; Chiuo and Hou, 1993; Wekesa et al., 2006).

These results are in line with previous findings reporting a lower mortality caused by *B. bassiana* in young instars of the green peach potato aphid, *Myzus persicae* (Sulzer) (Liu et al., 2003), foxglove aphid *Aulacorthum solani* (Kaltenbach) and cotton aphid *Aphis gossypii* (Glover) (Jandricic et al., 2014) as compared with other life stages. Furthermore, for other fungal species belonging to the same fungal group, Kim and Roberts (2012) reported similar results with less susceptibility to *Lecanicillium attenuatum* [formerly *Verticillium lecanii* (Zimm.) Viegas] of first instar nymphs of *A. gossypii* than of adults.

Difference in mortality between host stages of insects after fungal infection has been reported to be influenced by moulting time, with fast moulting developmental stages being less susceptible (Ekesi and Maniania, 2000; Wekesa et al., 2006; Kim and Roberts, 2012), changes in cuticle biochemical composition during development, where presence of e.g. toxic compounds may inhibit conidia germination (Kirkland et al., 2004), or insect body size with conidial load increasing with body surface (Tang et al., 1999; Kim and Roberts, 2012). We suggest that the low mortality rate of first and second instar lettuce aphids could be due to multiple moulting in young instars (within 2–3 days after treatment) causing fewer spores to adhere to the new cuticle, perhaps in combination with a low germination rate on the cuticle of young instars of lettuce aphid. Decreased spore adhesion was observed for the cotton aphid by Kim and Roberts (2012), who reported that the number of spores adhering to early instars declined with increasing time after treatment due to moulting. Furthermore, the smaller body size of the young instars might have played a role in the lower infectivity

Table 3
Mean daily nymph production per surviving winged adult (±SE) of *Nasonovia ribisnigri* treated as alatoid 4th instar with different spore concentrations of *Beauveria bassiana*.

Days after inoculation	Mean nymph production per winged adult			
	1×10^8	1×10^6	1×10^4	Control
2	0.00 ± 0.00*b* (61)	0.29 ± 0.08*b* (62)	0.83 ± 0.09*a* (58)	0.20 ± 0.09*b* (57)
3	1.07 ± 0.17*b* (58)	1.58 ± 0.16*ab* (62)	2.28 ± 0.16*a* (58)	2.33 ± 0.30*a* (56)
4	3.39 ± 0.19*a* (37)	2.85 ± 0.28*a* (49)	3.64 ± 0.23*a* (57)	3.71 ± 0.19*a* (55)
5	2.68 ± 0.40*bc* (31)	2.25 ± 0.37*c* (43)	3.73 ± 0.34*ab*(56)	4.05 ± 0.19*a* (55)
6	1.86 ± 0.51*b* (14)	2.71 ± 0.41*ab* (26)	3.72 ± 0.30*a* (55)	3.24 ± 0.17*a* (55)
7	3.08 ± 0.58*a* (9)	2.58 ± 0.49*a* (21)	3.57 ± 0.22*a* (53)	3.10 ± 0.23*a* (55)
8	1.66 ± 1.20*b* (3)	1.96 ± 0.35*ab* (18)	3.50 ± 0.14*a* (49)	3.60 ± 0.38*a* (54)
9	2 ± 2*ab* (2)	1.40 ± 0.43*b* (10)	3.06 ± 0.15*a* (47)	2.98 ± 0.19*ab* (51)
Total nymph production	15.75	15.63	24.30	23.22

Mean values within a row bearing the same letters were not significantly different (Tukey test, $P > 0.05$). The values in parentheses denote the total number of live aphids per day. The total numbers of replicates were 9 per treatment.

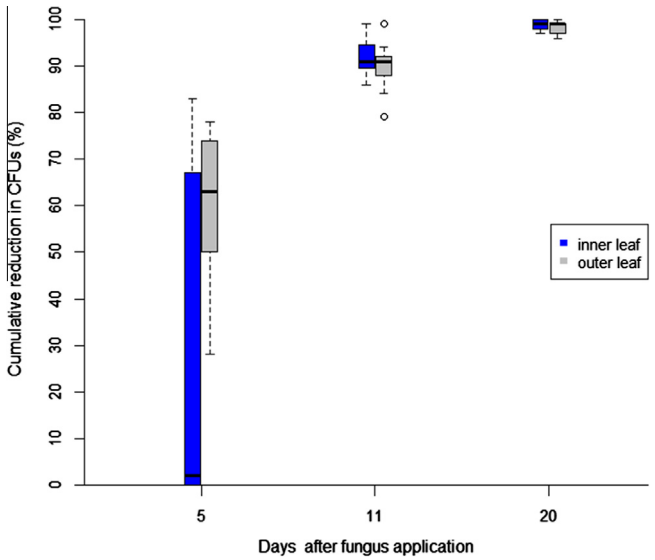

Fig. 3. Box plots summarising the raw data of cumulative percentage reduction in colony forming units of *B. bassiana* on lettuce leaves. Boxes and whiskers represent the minimum, maximum and upper/lower quartiles, while the median and outliers are represented by the horizontal lines and dots respectively.

by lowering the relative conidial load (Tang et al., 1999; Kim and Roberts, 2012).

4.2. Effect of B. bassiana infection on adult fecundity

The daily nymph production in lettuce aphid adults (apterous and alatoid morph type) found in control groups were comparable to results obtained in previous laboratory studies (Diaz and Fereres, 2005). Within treated aphid groups, *B. bassiana* significantly affected the rate of nymph production for apterous or alatoid adults. However, the effect of *B. bassiana* on adult fecundity seemed more pronounced when the alatoid fourth instar of *N. ribisnigri* was inoculated with *B. bassiana* compared to the newly moulted apterous adult of *N. ribisnigri*, despite the former group producing fewer nymphs in total and also starting reproducing later than the latter group.

Several studies have examined the reduction in daily nymph production of aphids treated with entomopathogenic fungi. Fungal treatment of adult aphids have in some cases resulted in reduction in nymph production as observed for instance by Ganassi et al. (2010) for wheat aphids *Schizaphis graminum* (Rondani) treated with *Lecanicillium lecanii*, (Zimmerman) Zare and Gams, and by Baverstock et al. (2006) for pea aphids *Acyrthosiphon pisum* (Harris), treated with *B. bassiana.* Reduction in nymph production may also occur after treatment of younger aphid stages, as demonstrated for third instar *N. ribisnigri* (Fournier and Brodeur, 2000) and potato aphids, *Macrosiphum euphorbiae* (Thomas), treated with Vertalec® (*Lecanicillium longisporum* (Petch) Zara and W.Gams) (Askary et al., 1998).

Table 4
Cumulative percentage reduction of conidial populations of *Beauveria bassiana* on lettuce plants after fungus application.

Time	Cumulative percentage reduction		
	Mean estimates ± S.E	Lower limit	Upper limit
5 days	38.11 ± 4.62a	23.61 ± 4.62	53.79 ± 4.62
11 days	91.83 ± 4.62b	81.25 ± 4.62	98.27 ± 4.62
20 days	98.89 ± 4.62b	93.21 ± 4.62	99.73 ± 4.62

Data for inner and outer leaves combined. Mean values within columns bearing the letter are not significantly different (Tukey test, $P > 0.05$).

However, sublethal effects of entomopathogenic fungi on aphid reproduction seem to depend on aphid species and/or the fungal species. Thus, Wang and Knudsen (1993) found no impact on reproduction of treatment of apterous adult of the Russian wheat aphid, *Diuraphis noxia* (Kurdyumov) with *B. bassiana* and Kim (2007) found no effect on reproduction after treatment of first and third instar of *A. gossypii* with *L. attenuatum*.

In addition, fungal effects on aphid reproduction depend on the fungal isolate as shown by Yokomi and Gottwald (1988) who demonstrated effects on nymph production of cotton aphids *A. gossypii*, *M. persicae* and spirea aphids *Aphis spiraecola* (Patch) after treatment of apterous adults with only two out of three isolates of *V. lecanii*.

Concerning possible mechanisms responsible for the sublethal effect of entomopathogenic fungi on fecundity, these fungi are known to influence the host insect via a combination of events including mechanical damage by hyphal growth, nutrient depletion and production of toxins (Hajek and St Leger, 1994). Any of these processes at a sublethal level could negatively affect the reproductive system of the host.

4.3. Persistence of B. bassiana on lettuce leaf surface: CFUs and infectivity

Determining the ability of entomopathogenic fungi to persist in the habitat of its host is important for the evaluation of their efficacy as biocontrol agents (Jaques, 1983). Knowledge on this aspect is crucial for development of effective and economic application strategies, including specifically the timing and frequency of spray applications (Castrillo et al., 2010). The persistence of conidia, measured in this study by CFUs counts and nymph mortality, showed that *B. bassiana* propagule numbers as well as their infectivity to lettuce aphid nymphs declined over time on lettuce foliage in a semi-field environment.

4.3.1. Conidia viability

Several studies have been performed to determine the conidia viability of *B. bassiana* on different crop species under field and greenhouse conditions. Under field conditions, populations of

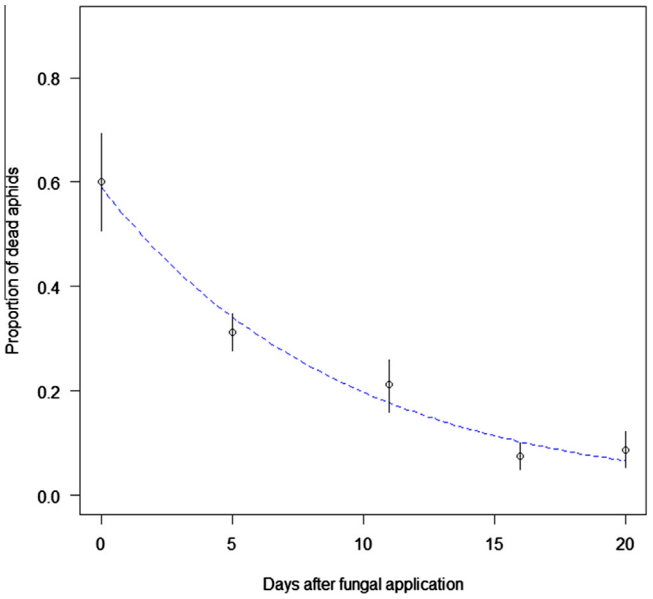

Fig. 4. The decline over time in infectivity of *Beauveria bassiana* towards lettuce aphid nymphs (*N. ribisnigri*) on lettuce leaf discs. The solid points represent the observed responses (±SE) and the dotted line the response predicted by the exponential decay model.

B. bassiana conidia have been reported to decline at various levels over time: 75% reduction in 4 days on crested wheat grass (*Agropyron cristatum* L.) and alfalfa (*Medicago sativa* L.) (Inglis et al., 1993); 50% reduction in 1–2 days and no conidia viable after one week in cowpea (*Vigna ungiculata* L.) (Daoust and Pereira, 1986); 50–60% reduction on ash (*Fraxinus* spp.) (Castrillo et al., 2010) in 5 days; and declines of 86% and 40% on celery (*Apium graveolens* L.) and lettuce (*Lactuca sativa* L. var. "longifolia"), respectively in 5 days (Kouassi et al., 2003). The present study showed that more than 60% of the initial conidial population was viable on lettuce leaves 5 days after fungus application as measured by CFU counts. This relatively long persistence of *B. bassiana* might be explained by properties of the lettuce plant favouring adhesion of fungal conidia. Thus, Kouassi et al. (2003) reported that the surface of lettuce leaves has many cavities, depressions and stomata which may offer binding or clustering sites for propagules and thus promote adhesion.

Regarding persistence beyond the first few days, only 8% and 1% of the initial conidial population in the present study were recovered 11 and 20 days after fungal application, respectively, which is in contrast to recoveries of 47% and 38% after similar time lapses in the study of Kouassi et al. (2003). These differences can be a result of using different fungal strains, different lettuce cultivars or from differences in abiotic factor such as temperature and solar radiation which increased sharply in our study in the period 5–11 days after fungal application (Fig. 1). Most probably, increased solar radiation during this period may have adversely affected the conidial survival as it has been reported to be detrimental to conidia on phylloplanes and is known to be important particularly in the inactivation of *B. bassiana* (Daoust and Pereira, 1986; Inglis et al., 1993). With respect to increased temperature, it presumably had a less deleterious effect on conidial viability since the temperatures recorded in this period were within the range (20–33 °C) of minimal effect (Ignoffo, 1992). In addition, the fungus inoculum applied to the lettuce in our study was probably diluted during leaf expansion (Inyang et al., 1998) as we used young lettuce plants that grow faster than the older plants used in the study of Kouassi et al. (2003).

4.3.2. Conidia infectivity

This study showed that the mean percentage mortality of second instar nymphs of *N. ribisnigri*, a life stage less susceptible to fungus infection compared to older instars as demonstrated in the bioassay, declined from 59% on the day of application to 7–8% on day 16–21 after application, when confined on lettuce leaves previously treated with *B. bassiana*. Our results are comparable to other studies of persistence of *B. bassiana* on different plant species when measured in infectivity assays with various pest species. However, the rates of decline vary considerably (Gardner et al., 1977; Ignoffo et al., 1979; Inglis et al., 1993; Castrillo et al., 2010). For instance, Gardner et al. (1977) reported 83%, 44% and no infectivity of *B. bassiana* applied against *Spodoptera frugiperda* (J.E. Smith) (Noctuidae) in soybean (*Glycine max* L.) at day 0, 5 and 10 after fungus application, and Ignoffo et al. (1979) found 75% and 3% infectivity of *B. bassiana* applied against *Trichoplusia ni* (Hübner) (Noctuidae) in the same plant species 0 and 1 day after fungus application, respectively. The data obtained here for the decline of fungus inoculum on lettuce foliage showed that the *B. bassiana* GHA isolate declines at a rate of 11% with each increasing day after fungal application and that the half-life of fungus is approximately 6 days in conditions similar to those encountered in the semi-field unit. If the experiment had been conducted outdoors, rainfall could potentially have influenced the decline even further. Furthermore, also aphid behaviour depends on abiotic factors (Dill et al., 1990) and thus the pick-up of conidia and the effect of the inoculum in the field may rely not only on effects directly on the conidia on the leaves but also indirectly via the aphids behaviour and susceptibility (Yeo et al., 2003).

We used two methods for monitoring the fungus persistence over time. While the CFU enumeration is a relatively fast procedure compared to the infectivity assays, the latter provide additional information on whether the inoculum levels detected are actually capable of killing aphids. We observed that results correlated when the two methods were used. There was a significant decline in CFUs 5 days after fungal application, and a similar decline was found in infectivity to aphids of conidia deposited on lettuce foliage. Similar relationships have been reported in other studies (Inglis et al., 1993; Meekes et al., 2000; Kouassi et al., 2003) while only a few studies showed no correlation between results obtained with the two methods (James et al., 1995; Meekes et al., 2000).

5. Conclusion

In conclusion, *B. bassiana* infect the first, second, alatoid fourth instar and apterous adult of *N. ribisnigri* and further seem to decrease the fecundity of the aphid. Due to *B. bassiana* being most virulent against older developmental stages of the lettuce aphid, possibilities for combining its use with other biocontrol agents with a potential to control earlier aphid stages should be examined. In addition, the results on the temporal pattern of fungal persistence with an estimated fungus half-life of 6.34 days indicate that application of *B. bassiana* should be repeated within a week after the initial application to increase control levels.

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