Laboratory evaluation of a native strain of *Beauveria bassiana* for controlling *Dermanyssus gallinae* (de Geer, 1778) (Acari: Dermanyssidae)

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Abstract

The poultry red mite, *Dermanyssus gallinae* (De Geer, 1778) (Acari: Dermanyssidae) is one of the most economically important ectoparasites of laying hens worldwide. Chemical control of this mite may result in environmental and food contamination, as well as the development of drug resistance. High virulence of *Beauveria bassiana* sensu lato strains isolated from naturally infected hosts or from their environment has been demonstrated towards many arthropod species, including ticks . However, a limited number of studies have assessed the use of *B. bassiana* for the control of *D. gallinae* s.l. and none of them have employed native strains. This study reports the pathogenicity of a native strain of *B. bassiana* (CD1123) against nymphs and adults of *D. gallinae*. Batches of nymph and adult mites (i.e., n = 720 for each stage) for treated groups (TGs) were placed on paper soaked with a 0.1% tween 80 suspension of *B. bassiana* (CIS, 10^5, 10^7 and 10^9 conidia/ml), whilst 240 untreated control mites for each stage (CG) were exposed only to 0.1% tween 80. The mites in TG showed a higher mortality at all stages ($P<0.01$) when compared to CG, depending on the time of exposure and the conidial concentration. A 100% mortality rate was recorded using a CIS of 10^9 conidia/ml 12 days post infection (DPI) in adults and 14 DPI in nymphs. *B. bassiana* suspension containing 10^9 conidia/ml was highly virulent towards nymph and adult stages of *D. gallinae*, therefore representing a possible promising natural products to be used in alternative or in combination to other acaricidal compounds currently used for controlling the red mite.

Keywords: *Beauveria bassiana*, biological control, *Dermanyssus gallinae*, entomopathogenic fungus, *in vitro* studies.

1. Introduction

Dermanyssus gallinae (De Geer, 1778) (Acari: Dermanyssidae), known as the poultry red mite or chicken mite, is considered a significant threat to egg-laying hens in many parts of the world, including Europe, Japan, and China (Chauve, 1998; Wang et al., 2010; Sparagano et al., 2014). This mite species displays relative plasticity in terms of host specificity, being associated primarily with birds, but also with mammals, including humans (Cafiero et al., 2009, 2011; reviewed by George et al., 2015). The lifecycle of the parasite usually takes about 1 or 2 weeks under favourable conditions, and a weekly doubling of populations is possible in egg-laying facilities where optimal environmental conditions (i.e., temperature from 10 to 35°C and humidity >70%) exist (Maurer and Baumgartner, 1992; Nordernfors et al., 1999). Both nymph and adult stages of these mites feed on the host, and then move into nearby cracks and crevices to digest the blood-meal and to moult or lay eggs (Nakamae et al., 1997; Sparagano et al., 2014). However, in the environment mites can live without feeding for up to 9 months (Norderfors et al., 1999). Heavy infestations by this pest may cause severe damage to the poultry industry, varying from decreased growth rates, egg production and feed conversion to high animal mortality (Chauve, 1998; Hoglung et al. 1995; Sparagano et al., 2014). In addition, *D. gallinae* can be a vector of microorganisms such as *Salmonella* spp., avian spirochetes, and other pathogens of livestock (Valiente Moro et al., 2009; Sparagano et al., 2014). In humans, *D. gallinae* can cause pruritic dermatitis, representing an occupational hazard for poultry workers (Cafiero et al., 2011). The control of red mites relies on the use of synthetic acaricides such as organophosphosphates, carbamates and pyrethroids (Chauve, 1998; Sparagano et al., 2014). Nonetheless, resistance phenomena of this mite to these compounds have been reported (Marangi et al., 2009; Sparagano et al., 2014). In addition, misuse/abuse of the chemicals often results in the presence of pesticide residues in the organs and tissues of poultry, which are sold at the end of their production cycle (Marangi et al., 2012). Therefore, the use of synthetic products has been limited in order to minimize the risk of chemical residues in food products and the environment (Tavassoli et al., 2011; Sparagano et al., 2014).

78 The study of alternative compounds for controlling this infestation is been considered timely
79 (Tavassoli et al., 2011; reviewed by Sparagano et al., 2014). Entomopathogenic fungi have been
80 investigated for their potential in the biological control of arthropods, due to their ability to
81 penetrate the integument of mites (Tavassoli et al., 2008, 2011; Kaoud, 2010; Steenberg and
82 Kilpinen, 2014). In particular, *Metarhizium anisopliae* s.l. and *Beauveria bassiana* s.l. can infect
83 mites, but their virulence depends on host, fungal strain and environmental conditions (e.g.,
84 humidity >60%, protection from UV-A and UV-B radiation, temperature between 25-35°C,-Teng,
85 1962; Tavassoli et al., 2008, 2011; Huang and Feng, 2009; Kaoud, 2010; Steenberg and Kilpinen,
86 2014; Braga et al., 2015).It has been shown that the native strains (i.e., isolated from the
87 environment or naturally infected hosts) of *B. bassiana* s.l. and *M. anisopliae* s.l. were more
88 virulent against different species of ticks than non-native strains (Fernandes et al., 2012; Perinotto et
89 al., 2012; Cafarchia et al., 2015). However, none of the studies available in the literature on the use
90 of *B. bassiana* s.l. against *D. gallinae* (Kaoud, 2010; Steenberg and Kilpinen, 2014) were designed
91 to test "native" strains of this fungus, representing a major gap in the control of red mites.
92 Therefore, the aim of this study was to investigate the *in vitro* effects of a locally isolated strain of
93 *B. bassiana* (CD1123) on nymphs and adults of *D. gallinae*.
94
95 **2. Material and methods**
96 *2.1. Mite samples*
97 Mites were collected from the same egg-laying hen farmin Bitritto (41°03′00″N 16°50′00″E, 102 m
98 a.s.l.), in the province of Bari, southern Italy, in three different times. The farm was naturally
99 infested by the parasite, and no standard treatments were conducted two months before the
100 collection. Mites were stored in sealed plastic bags and delivered to the Department of Veterinary
101 Medicine, Unit of Parasitology and Mycology, University of Bari, Italy. After morphological
102 identification as *D. gallinae* (Moss et al., 1968) and its confirmation with the pictorial key provided
103 by Di Palma et al. (2012), mites were divided in two groups adults and nymphs (i.e., protonymphs

and deutonymphs) and finally stored at 20°C to be used for the experiment within 24 hours of collection.

2.2. Beauveria bassiana *origin and conidial infection suspension*

The locally isolated strain of *B. bassiana* (CD1123) - herein after referred as "native" strain - was obtained from naturally infected *Rhipicephalus sanguineus* sensu lato adult ticks collected in a private dog shelter in Putignano, province of Bari, Italy and morphologically and molecularly identified as described previously (Cafarchia et al., 2015). The *B. bassiana* strain was maintained on Potato Dextrose Agar (PDA) and kept at 4° C. The conidial infection suspension (CIS) of *B. bassiana* was obtained by culturing 15 strains on PDA for 3 weeks at 26 °C. Conidia were harvested by washing the plates with sterile distilled water containing 0.1% tween 80 (Prette et al., 2005; Reis et al., 2005; Campos et al., 2010; Perinotto et al., 2012; Cafarchia et al., 2015) and turbidity was adjusted spectrophotometrically (Biosan DEN 1) to a McFarland optical density of 4.5, (1–5 x 10^5 conidia/ml), 6.5 (1–5 x 10^7 conidia/ml) and 10 (1–5 x 10^9 conidia/ml). The amount of conidia was evaluated by quantitative plate counts of colony forming unit (CFU)/ml in PDA.

2.3 Laboratory bioassays and data analysis

A total of 960 adults and 960 nymphs were tested. All bioassays consisted of four groups of mites, one control group – CG and three treated groups – TG (i.e., one for each CIS). Each group was composed of four subgroups of twenty mites. Mites (i.e., adult and first and second nymph stages) were subjected to the same treatment and were put into bioassay rooms (BR) composed of Petri dishes (60 mm diameter) containing filter paper (Whatman N. 1, 10x10 mm Labor, 67 g/m^2, Tecnochimica Moderna, Italy) of the same diameter. The filter paper was soaked with 0.2 ml of each CIS (i.e., 10^5, 10^7 and 10^9 conidia/ml) for the TGs and with 0.2 ml of sterile distilled water plus 0.1% tween 80 for the CGs. The mites were placed on paper soaked with either CIS or control solution. The Petri dishes were covered with a lid, sealed with parafilm and stored at 25± 1°C (RH

80 ± 5%). Mortality was evaluated daily until 100% mortality was recorded in TGs. Mites were considered dead if they exhibited no movement after repeated mechanical stimulation with an entomological pin by three different examiners. One dead mite for each group was cultured on PDA for verifying the presence of viable fungus. The death caused by fungal infection was checked according to Koch's postulate. All experiments were repeated in triplicate. The dead mites were not removed from the bioassay room. The mortality data of CG and TG were compared and were analysed using chi-square tests, with 5% significance ($p<0.05$) (Sampaio, 2002).

3. Results

The *in vitro* effect of *B. bassiana* (CD1123) against nymphs and adults of *D. gallinae* are reported in Figures 1-2. No statistical differences among replicates were recorded ($p<0.01$). A statistically higher mortality of red mites at all stages was recorded in TG than in CG. The mortality rate increased significantly ($P<0.01$) according to the time of exposure and conidial concentration. A mortality rate higher than 50% in adults was recorded at 8, 6 and 4 days post-infection (DPI) using a CIS of 10^5, 10^7 and 10^9conidia/ml, respectively. The mortality rate of nymphs was significantly lower than that of adults at all exposure times when using CIS of 10^5 and 10^7conidia/ml. A 100% mortality was recorded using CIS of 10^9 conidia/ml after 12 DPI in adults and 14 DPI in nymphs (**Figures 1, 2**). The 100% mortality was recorded at 24 and 22 DPI in nymphs and at 22 and 20 DPI in adults using 10^5 and 10^7 CIS, respectively.. White fungal mycelium started to emerge on the surface of nymphs and adults of *D. gallinae* of TGs 3 DPI. Fertile conidiophores appeared from 5 DPI in TGs, but only on the surface of dead mites (**Fig. 3**). At stereomicroscope observation and on culture, no fungal growth was observed on the CG mites.

4. Discussion

The results presented suggest that the "native" strain of *B. bassiana* – obtained from naturally infected *Rhipicephalus sanguineus* sensu lato adult? ticks from the same province- herein tested is

156 highly virulent towards *D. gallinae,* therefore being of potential use for the control of infestations
157 caused by this mite. Indeed, all CISs were highly effective against adult and nymph stages and the
158 effects on vitality were dependent on the exposure time and conidial concentration. Recently, the
159 use of entomopathogenic fungi for biological control of arthropods has been increase in order to
160 overcome the limitations posed by conventional control methods (e.g., development of pesticide
161 resistance and risks related to chemical residues in food products). Meanwhile, *M. anisopliae* s.l.
162 and *B. bassiana* s.l. have been tested as biological control agents because of their wide distribution
163 and low risk to non-target organisms and to the environment (Sun et al., 2013). However, it has
164 been shown that successful use of these fungi depends on fungal strain, formulation and application
165 at an appropriate dosage and time, and also on the presence of a susceptible host stage (Lacey et al.,
166 2001; Fernandes et al., 2012). In the present study the pathogenicity of a "native" strain of *B.*
167 *bassiana* (CD1123) against *D. gallinae* nymphs and adults was investigated and the results obtained
168 are in agreement with those previously determined using selected strains of *M. anisopliae* and
169 higher than those obtained by *B. bassiana* alone or in association with various desiccant dusts
170 (Tavassoli et al., 2008, Steenberg and Kilpinen, 2014).~~.~~ Since the mortality rate for all
171 developmental stages of *D. gallinae* (i.e., 100% for adults and nymphs within 12 and 14 days,
172 respectively) is the highest ever recorded in the international literature (Tavassoli et al., 2008;
173 Steenberg and Kilpinen, 2014), the "native" strain of *B. bassiana* (CD1123) obtained from naturally
174 infected *Rhipicephalus sanguineus* sensu lato adult? ticks from the same area - may be more
175 effective than those previous tested, in controlling mite populations in the field environment. The
176 same strain was also highly efficacious in controlling ticks (Cafarchia et al., 2015). Being 3 days the
177 useful time for starting the infection process by highly virulent fungal strains (Maketon et al., 2008;
178 Steenberg and Kilpinen, 2014), the results herein obtained indicates that *B. bassiana* CD1123 is
179 highly virulent against *D. gallinae*.
180 The mortality rate of *D. gallinae* increased according to the time of exposure, also suggesting that
181 *B. bassiana* could provide long-term control, as this fungal species also reproduces using mites as a

182 medium. Indeed, the presence of viable *B. bassiana* from the dead mites as well as its proliferation
183 within 5 days on the dead mites suggests that the fungus might persist on mites and might generate
184 new infective conidia for healthy mites in the same population (Lekimme et al., 2008; Cafarchia et
185 al., 2015). In addition, the environmental conditions of egg-laying hen farm which are favourable
186 for population growth of red mites (i.e., temperature ranging from 17° to 32°C and RH from 40% to
187 80%) (Nordenfors et al. 1999) also positively affect the growth and the conidial germination of *B.*
188 *bassiana* (Fernandes et al., 2008; Huang and Feng, 2009), thus indicating the potential employment
189 of this fungus against *D. gallinae* in the field environment. However, the main limitation for the
190 employment of *B. bassiana* as an acaricide is directly linked to the time required to be effective.
191 Usually, a concentration of *B. bassiana* conidia equal to or higher than 10^7 conidia/ml is useful to
192 cause 100% mortality within 15 days, depending on the fungal strain and host species (Smith et al.,
193 2000; Alves et al., 2002; Lekimme et al., 2006; Tavassoli et al., 2008, 2011). In the present study, a
194 more rapid mortality was achieved at the highest concentration of conidia (i.e., 10^9 conidia/ml, 12
195 days for 100% mortality for adults). In addition, the same set solution caused higher and faster
196 mortality than those registered with CIS of 10^5-10^7 conidia/ml also in the nymphal stage of *D.*
197 *gallinae,* which seems to be less susceptible to *B. bassiana*. The reduced mortality of nymphs, in
198 comparison with other stages, was previously reported for *R. sanguineus* s.l. treated with *M.*
199 *anisopliae* or *B. bassiana* (Samish et al 2001; Fernandes et al., 2012; Cafarchia et al., 2015) and
200 might be due to a different cuticle composition in nymphs, which influence fungal penetration
201 (FAO protocol, 1984; Fernandes et al., 2012). Indeed, the lipid composition of arthropod cuticles
202 selectively affects the conidial germination and the formation of appressoria, which are important
203 events in interactions between entomopathogenic fungi and their arthropod hosts (Fernandes et al.,
204 2012; Ment et al., 2012; Cafarchia et al., 2015). In addition, the presence of exuvium in the
205 nymphal stage might limit the adhesion of conidia to the nymphs' cuticle, thereby prolonging the
206 infection time (FAO protocol, 1984; Wu et al., 2014).
207

5. Conclusions

The results of the current study demonstrate that a CIS of 10^9 conidia/ml of a "native" strain of *B. bassiana* is highly virulent towards nymphs and adults of *D. gallinae*, thus suggesting that this fungus may be effective in controlling mite populations in the environment. Nonetheless, further laboratory and field studies are required to determine the best dose, route of application and frequency of treatment for the use of this fungus as a bio-control agent in poultry houses. In addition, against *D. gallinae*, the application of *B. bassiana*, in combination with chemicals or even with natural *D. gallinae* compounds (i.e., essential oils, silicates) should also be investigated in order to increase the efficacy of the fungus, thus providing an integrated pest management strategy against mite infestations in poultry houses and working towards reducing the hazards related to the excessive use of chemical products.

Acknowledgements

We kindly thank Dr Bronwyn Campbell (University of Bari) for revising the English text.

Competing interests

The authors declare that they have no competing interests.

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Figure legend

Fig. 1. Mortality rate of adults of *Dermanyssus gallinae* with different concentrations of the "native" strain of *Beauveria bassiana* after different days post infection. The statistically not significant differences are indicated with the same letters.

Fig. 2. Mortality rate of nymphs of *Dermanyssus gallinae* with different concentrations of the native strain of *Beauveria bassiana* after different days post infection. The statistically not significant differences are indicated with the same letters.

Fig. 3. Mycelium and conidiophores of *Beauveria bassiana* on adult of *Dermanyssus gallinae* at 5 days post infection