Research Article

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Laboratory Test of Three Isolates of *Beauveria bassiana* (Bals.) Vuill. Against the Invasive Sawfly *Aproceros leucopoda* Takeuki, 1939 (Hymenoptera: Argidae)

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Abstract: The invasive sawfly *Aproceros leucopoda* is an important defoliator pest of elm trees in Romania. The susceptibility of *A. leucopoda* to three isolates of the entomopathogenic fungus *Beauveria bassiana* (BbIt, BbAl1/10 and BbS1.07) with different origins regarding the insect host, was evaluated. The bioassay was performed by exposing field collected larvae and pupae to fungal liquid culture consisting of $6x10^9$ propagules/ml, sprayed on leaves. The mortality was recorded every 2-3 days for 11 days. There were no significant differences between mortalities caused by the isolates BbIt and BbAl1/10. The insects were highly susceptible to both isolates BbIt and BbAl1/10, with mortality rates of 97.4% and 75.6%, respectively. The values of the median lethal time (LT_{50}) were 3.9 and 3.7 days, respectively. The sensibility of the larvae of *A. leucopoda* to the fungal isolate BbS1.07 was very low. When pupae were treated with the isolate BbAl1/10, a mortality rate of 63% was recorded at the 7^{th} day, and the LT_{50} value was 5.5 days.

Key words: Entomopathogenic fungus, *Beauveria bassiana*, *Aproceros leucopoda*, virulence

Introduction

The East Asian sawfly *Aproceros leucopoda* Takeuki, 1939 (Hymenoptera: Argidae) is a defoliating insect, which has produced severe defoliations on the elms *Ulmus glabra* Huds (Blank et al. 2010) and *Ulmus minor* Mill. (Balacenoiu et al. 2017) in Romania. The most damaging are the larvae that begin to devour the leaf immediately after hatching. Data about the control methods of this invader are rare. Blank et al. (2010) mentioned that methods of chemical control of this species do not give satisfactory results but the introduction of natural and specialised parasitoids may result in effective control. In Romania, different parasitoids were identified from the eggs of *A. leucopoda*, *Blondelia nigripes* (Fallén), *Asecodes (Teleopterus) erxias* (Walker) (Pricop et al. 2012), and *Mastrus deminuens* (Hartig.) (Cardaş et al. 2011).

The rapid development of life cycle enhanced by parthenogenetic reproduction of the insect represents a challenge even for microbial control. An entomopathogenic fungus with a rapid speed of action, which naturally regulates the insect population may be a useful tool in controlling *A. leucopoda*. The Hyphomycetes fungus *Beauveria bassiana* (Bals.) Vuill., the most commonly reported fungus isolated from dead and moribund insects in nature (Niranjana et al. 2008) can represent a potential biological control agent of *A. leucopoda* in the Romanian forests where the elm is present.

The characterisation of entomopathogenic strains in terms of virulence towards a target pest and the identification of highly virulent strains are the most important criteria for their effective use in biological control. This is why the evaluation of insecticidal activity of entomopathogenic fungi, under laboratory and field conditions, has been the subject of many studies, in order to provide excellent strain resource for pest control.

The aim of our study was to investigate the virulence of three isolates of the entomopathogenic fungus *B. bassiana,* with different origins in respect to the host species, against larvae and pupae of *A. leucopoda*.

Materials and Methods

Collection and preparation of materials for the experiment

The isolates used in the experiment belong to the Fungal Collection of Entomopathogenic Microorganisms maintained at the Research and Development Institute for Plant Protection, Romania, and are also deposited at NCAIM (Corvinus University of Budapest, Hungary). The *B. bassiana* isolate BbAl1/10 (NCAIM P (F) 001392) was initially obtained from a cadaver of *A. leucopoda* on an elm (*U. glabra*) leaf in a deciduous forest at Botosani (Romania); the *B. bassiana* BbIt isolate (NCAIM P (F) 001392) was from *Ips typographus* (L.) (Coleoptera: Scolytidae) on a spruce trunk in a coniferous forest at Suceava (Romania); and the *B. bassiana* BbS1.07 isolate (NCAIM P (F) 001353) was from *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) in a potato crop at Ialomita (Romania). The identification of the isolates was performed according to Barnett (1960) and Humber (1997).

The propagules (blastospores) of the isolates used as infectious units in bioassay were produced by inoculating 250 ml of Goral media ($NaNO_3$ 5g/l, KH_2PO_4 5g/l, $MgSO_4$ 2g/l, and corn syrup 0.8g/l) in 500 ml Erlenmayer flasks with an aqueous conidial suspension to obtain a final concentration of $1x10^6$ conidia/ ml, followed by incubation on a rotary shaker at 150 rpm at 25°C. After five days of incubation, the flasks were stored at 4°C until the use. Before the treatment application, the propagule concentration was adjusted with distilated water to a concentration of $6x10^9$ propagules/ ml.

The test insects were collected in May 2011, from heavily infested elm forests (*U. glabra*) in one region of East Romania, Poienesti (N 46°38'; E 27°32'). The branches with larvae and pupae (loosely spun cocoons attached to the lower surface of the elm leaves) were collected and transported in closed bags provided with wet cotton wool plugs.

Experiment procedures

To evaluate the susceptibility of larvae to the *B. bassiana* isolates, the larvae were gently transferred on fresh elm branches with leaves (three replicates of 15-20 larvae), in pots with water. Before the transfer of larvae, the leaves were inoculated *in situ* with fungal propagules (13 ml) by direct application on leaves attached to the branch, until complete covering of leaves. After treatment, the elm branches were maintained at laboratory conditions (25°C±2°C, 50-60% RH, 16:8 L/D). The control consisted of larvae transfered on elm branches previously treated with distilled water.

A second bioassay to evaluate the susceptibility of *A. leucopoda* pupae to *B. bassiana* was performed. For this, the leaves with one or two pupae attached were treated with BbAl1/10 isolate (with distilled water for control) by spraying until complete covering the leaves. The treated leaves were then placed individually into a sterile rearing box (170x115 mm) and incubated at 25°C.

There were three replicates (boxes) per treatment (one isolate and control) and 20 individuals per box.

The insect mortality was recorded every 2-3 days for 11 days; the development of the insects was observed until complete metamorphosis. The dead insects were placed into humid chambers and incubated to encourage fungal sporulation, which allowed the confirmation of infection by *B. bassiana*.

Data analysis

The larval mortality was corrected with the control mortality, using the Schneider-Orelly formula (Ehabsoft 2012) and expressed as cumulative percent mortality. Then the pooled data were analysed, using one-way ANOVA, and mean values compared, using Tukey HSD. Two-way ANOVA followed by a Tukey-Kramer test was used to analyse the differences between the strains at different times of observations. The mean values were considered significantly different at p-level<0.05. Prior to applying analysis of variance, the conditions of normality and homogeneity distribution were tested.

The virulence of each isolate was estimated by the values of the median lethal time (LT_{50}), calculated by probit analysis (Finney 1971). All the analyses were carried out with Biostat v5 AnalistSoft Inc.

Results and Discussion

In the first experiment, three different *B. bassiana* isolates were tested on larvae of *A. leucopoda*. The efficacy (cumulative mortality) and LT_{50} induced by each isolate at a concentration of $6x10^9$ propagules /ml after 11 days are presented in Table 1. For the isolates BbAl1/10 and BbIt, the fungal treatment showed a significant effect on the larval mortality. The isolate BbIt was responsible for 97.4% ±1.62 cumulative mortality in the treated larvae, with no significant differences from the BbAl1/10 isolate (P=0.053; Tukey HSD), which caused a cumulative mortality of 75.6% ±6.4.

The larvae of *A. leucopoda* were less susceptible to the isolate BbS1.07, the mean mortality due to mycosis was 7.3% ±3.69, the number of dead

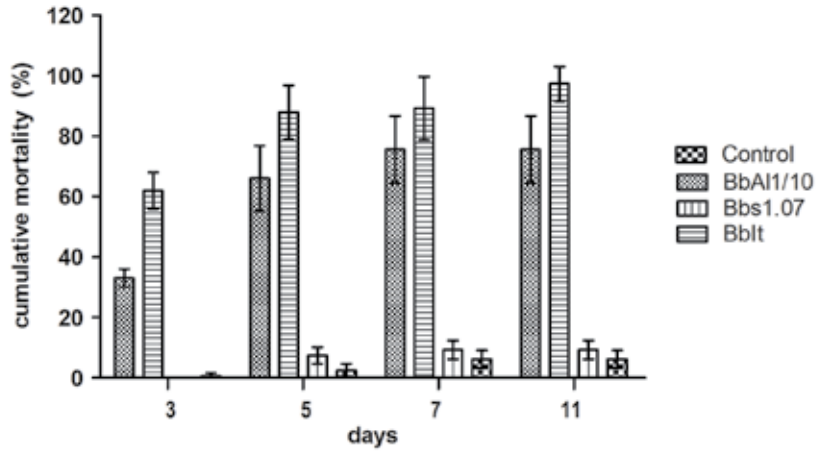

Fig. 1. Efficacy (cumulative mortality % mean ±SD) of the three isolates of *Beauveria bassiana* against the larvae of *Aproceros leucopoda* at $6x10^9$ propagules/ml

Table 1. Virulence of the three isolates of *Beauveria bassiana* in bioassays against the larvae of *Aproceros leucopoda* and the efficacy of treatment with blastospore suspensions of the isolates ($6x10^9$ blastospores/ml). For the control and BbS1.07 isolates no LT_{50} could be calculated because of the low percent mortality

Isolate of *B. bassiana*	**Efficacy (Cumulative mortality, %) (mean± SE)**			**Virulence (Values of median lethal time) (LT_{50}) (d)**	**Confidence intervals of LT_{50} (d)**	**Regression coefficient (β±SE)**
	Total	Larvae	Pupae			
Control	6.2±1.6	0	6.23±1.6	–	–	–
BbAl1/10 (*A. leucopoda*)	75.6±6.4	23.6±4.7	51.9±4.8	3.9	3.3–4.3	4.17±0.7
BbS1.07 *(L. decemlineata*	7.3±3.6	5.4±3.0	1.9±1.9	–	–	
BbIt (*I. typographus*)	97.4±1.6	18.4±8.3	79.0±7.5	3.7	3.1–4.3	6.04±1.3

Table 2. Virulence of the BbAl 1/10 isolate of *Beauveria bassiana* against the pupae of *Aproceros leucopoda* and the efficacy of treatment with blastospore suspension of the isolate ($6x10^9$ blastospores/ml)

Isolate of *B. bassiana*	**Efficacy (Cumulative mortality, %) (mean± SE)**			**Virulence (Values of median lethal time) (LT_{50})**	**Confidence interval of LT_{50}**	**Regression coefficient (β±SE)**
	Total	Pupated larvae	Adults			
Control	0	0	0	–	–	–
BbAl1/10	63±11.2	32.8±9.4	31.2±2.9	5.5	4.5–7.8	2.1±0.6

individuals did not differ from that of the untreated individuals, which showed 6.2% ±1.69 mean mortality (Table 1). No mycosed individuals were found in control treatments.

The period required to cause 50% insect mortality (median lethal time) was established only for the isolates BbIt and BbAl1/10. The BbS1.07 isolate exceeded the test period of 11 days. The comparison between the virulence of the isolates BbIt and BbAl1/10 showed that there were no differences in the virulence of both isolates. The similarity in virulence in these two *B. bassiana* isolates was indicated by the overlapping of the 95% confidence intervals, from 3.3 to 4.3 and from 3.1 to 4.3 days, respectively (Table 1). The reduced median lethal time for the isolates BbIt (3.7 days) and BbAl1/10 (3.9 days) could be due to the rapid fungus germination and invasion of the insect body. Jaronski (2010) mentioned that fungal spores after adhesion on the cuticle, invade the body of the hosts within 24h. On the other hand, the laboratory conditions can represent stress factors which may influence the susceptibility of the tested insect.

The infection of the treated larvae by *B. bassiana* resulted in illness of the larvae, but most of

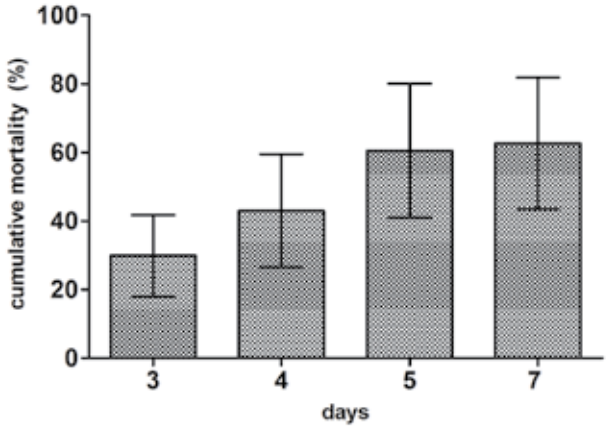

Fig. 2. Efficacy (cumulative mortality % mean ±SD) of the BbAl1/10 isolate of *Beauveria bassiana* in bioassays against the pupae of *Aproceros leucopoda* at $6x10^9$ propagules/ml

them survived to the next stage of development and died as pupae.

In the two-way ANOVA, the cumulative percent of infection differed significantly between the isolates ($F_{2, 28}$=372.6, p<0.0001) and between the four periods of observations ($F_{3, 28}$=27.14, p<0.0001), starting to be strongly visible since the 3rd day when the percent mortality caused by the BbIt isolate (59%) was almost twice as high as that in the BbAl isolate (29.6%) (Fig. 1). In the cadavers resulted from the BbIt infection visible sporulated mycelium was developed, more abundant than in those killed by the other two isolates. On the 7th day after the treatment, no significant difference (p=0.075, Tukey-Kramer) was registered between the cumulative percent of infection caused by the BbIt and BbAl1/07 isolates. On the 11th day of observations, the mycosed individuals were observed only in the pupal stage.

In the second experiment, the BbAl 1/10 isolate of *B. bassiana* was tested against pupae of *A. leucopoda*. The mortality rates of the treated pupae followed the same trend of increasing mortality as in the first bioassay with the same isolate, with the largest difference being observed on the 7th day, when a 13% lower mortality rate was observed in the pupae versus the treated larvae (Fig. 2).

The cumulative mortality and LT_{50} induced by each isolate at a concentration of $6x10^9$ propagules /ml after seven days are presented in Table 2. The isolate BbAl 1/10 was responsible for 63%±11.2 cumulative mortality. The infection by *B. bassiana* on the treated pupae resulted in illness of both larvae and adults, in similar proportions. No mortalities were observed in the control treatment.

Virulence, in terms of LT_{50} and LC_{50}, varies not only from one species to another, but also from one strain to another (Mwamburi et al. 2010), even registering high genetic variability (Lopes et al. 2011) and significant differences of insecticide potential, depending on the physiological and enzymatic properties (Leland et al. 2005). Virulence tests on insects belonging to different orders have proved that the virulence must be considered in selecting biological control agents.

The recent results published by Khosravi et al. (2015) have demonstrated different LC_{50} and LT_{50} values of four *B. bassiana* isolates tested on larvae of the rose sawfly, *Arge rosae* L. (Hymenoptera: Argidae). The most virulent isolate induces mean larval mortality of 70% at a conidial suspension concentration of 10^7 conidia/ml. Significant differences in virulence were reported when *B. bassiana* strains were tested on lepidopteran pests. The larval mortality of the diamondback moth (*Plutella xylostella* L.) experimentally infested with conidia of ten *B. bassiana* strains has values ranging from 14.5 to 53.3% (Talaei-Hassanloui et al. 2006). Based on virulence criteria, Lihong et al. (2009) selected only five isolates from the 33 isolates tested on the diamondback moth larvae; the most virulent strain has an LC_{50} (7 days) value of 0.47x10 spores/ml, the LT_{50} value (1.0x10 spores/ml) is 1.57+0.027. The *B. bassiana* strains screened out in field conditions on the other lepidopteran pest, the masson pine moth (*Dendrolimus punctatus* Walker), have also proved differences in virulence. The results interpreted on the grounds of the LT_{50} and LC_{50} values show differences in virulence between the twentieth strains tested; the most virulent strains recorded are those with LT_{50} values of 7.63, 7.62, 7.88 d; and LC_{50} of $0.63x10^6$, $0.96x10^6$ and $0.78x10^6$ conidia/ml, respectively (YiXun et al. 2016). Different virulence of *B. bassiana* isolates against lepidopteran pests was reported also by Wraight et al. (2010).

Jones et al. (1996) conducted studies on the strain virulence against isopteran pests and demonstrated the different virulence of seven isolates of *B. bassiana* and *M. anisopliae* against subterranean termites (*Coptotermes formosanus* Shiraki), with the LT_{50} values ranging from 0.13 to 4.5 days.

With regard to the coleopteran pests, the different virulence of *B. bassiana* strains has been demonstrated. Johny et al. (2012) conducted a virulence screening of 23 isolates of *Beauveria* spp. against the invasive wood boring beetle (*Agrillus planipennis* Firmaire), and reported virulence differences. Based on the LC_{50} values, as well as on

the mean survival time at $2x10^6$ conidia/ml, a single isolate is significantly more virulent and produced more conidia on *A. planipennis* cadavers than the other isolates. Tests have been carried out to evaluate the virulence against *L. decemlineata,* of nine *B. bassiana* isolates originated from different host insects: *Tenebrio molitor* L.*, Operophtera brumata* L.*, Phytodecta fornicata* Brügg*, Cassida nebulosa* L.*, Sitona lineatus* L., and *Hypera variabilis* Herbat*)* (Andrei 1998). The *B. bassiana* isolates from *L. decemlineata* show greater virulence against the Colorado potato beetle larvae than the isolates originated in the other insects, with the LC_{50} values of 1.5, 5.3 and 6.8 (x 10^6 conidia/ml), respectively.

The results of Quesada-Morava et al. (2016), based on the fourth instar mortality rate of two whitefly species, are significant for the studies on the *B. bassiana* virulence against hemipteran pests insects. Twenty-five native *B. bassiana* isolates and a commercially available mycoinsecticide (based on *B. bassiana*) have been evaluated for virulence against the whitefly, *Bemisia tabaci* Gennadius, and greenhouse whitefly, *Trialeurodes vaporariorum* Westwood. The conidial suspension at a concentration of 1×10^7 conidia/ml induced mortality rates range from 3 to 85%. Other virulence tests on hemipteran pests demonstrated that the twenty-three *B. bassiana* isolates screened on the third instar nymphs of *Triatoma infestans* Klug show significantly different virulence among isolates. The cumulative mortality 15 days after treatment varies from 17.5 to 97.5%, and the estimates of 50% survival time vary from 6 to 11 days (Luz et al. 1998).

Conclusions

The results from this work demonstrate that the larvae and pupae of *A. leucopoda* are susceptible to *B. bassiana* isolates under laboratory conditions, when treated with $6x10^9$ propagules/ml.

This is the first report about a bioassay against *A. leucopoda*, with using three different isolates, one of which was originated in *A. leucopoda*. The results of the study are encouraging to continue by evaluating the use of the isolates BbIt si BbAl1/10 of *B. bassiana* against *A. leucopoda* in field tests.

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