

Laboratory evaluation of entomopathogenic fungi as biological control agents against the bark beetle *Pityogenes scitus* Blandford (Coleoptera: Curculionidae) in Kashmir

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ABSTRACT

The bark beetles (Coleoptera: Curculionidae) are widely recognised as one of the most damaging group of forest pests. Entomopathogenic fungi have shown great potential for the management of some bark beetle species. The efficacy of three entomopathogenic fungi, namely, *Beauveria bassiana* (Balsamo) Vuillemin, *Metarrhizium anisopliae* sensu lato (Metchnikoff) Sorokin and *Lecanicillium lecanii* (Zimmerman) Zare and Gams was tested against the bark beetle *Pityogenes scitus* Blandford under the laboratory conditions. An insecticide – cyclone 505 EC, was also used as positive control in the experiment. Each fungal suspension contained 1.0×10^9 spores of fungi in 1 ml. In treated branches, *B. bassiana* and *M. anisopliae* caused higher percentage of mortalities, that is, 58.33% and 48%, respectively, after 10 days of treatment and 85% and 71%, respectively, after 20 days of treatment. In petri plate assay, *B. bassiana*, *M. anisopliae* and *L. lecanii* caused 100%, 100% and 73.33% of mortality respectively. The percentage of mortality caused by treated insecticide was 79.16%. The results obtained in the present study are promising; however, no recommendations concerning the potential use of these fungal pathogens in forest protection can be given, and further research studies are needed in this respect, especially under field conditions.

KEY WORDS

Pityogenes scitus, biological control, mortality, laboratory conditions

INTRODUCTION

The bark beetles (Coleoptera: Curculionidae) are amongst the most destructive wood boring insects that have induced considerable damage to the forest

ecosystems globally. Majority of the species require weakened or recently felled trees for reproduction, but some species such as *Pityogenes scitus* (Coleoptera: Curculionidae: Scolytinae) could infest healthy trees as well, especially when climate conditions promote

outbreak in pest population density (Yu et al. 2015). After attacking suitable host tree species, the development phase of bark beetles occurs within the bark, which includes mating, gallery construction, oviposition and brood development. Most of the vascular cambium is destroyed, and the tree is irreversibly stressed (Lieutier et al. 2009). However, the sapwood still remains functional; the canopy is alive with green foliage, and it takes weeks or months until the canopy fades and the whole tree dies (Paine et al. 1997). In addition to causing widespread tree mortality, beetle pests substantially change forest structure, composition and function. Colonised trees become hosts to dozens of arthropod, nematode and vertebrate species (Raffa et al. 2008). As biological control is becoming more acceptable as a practical science and the danger of long-term use of chemical pesticides is fully appreciated, there has been a resurgence of interest in using fungal pathogens to combat insect pests. In contrast to all other pathogen groups associated with bark beetles, entomopathogenic fungi have been well studied and evaluated as bark beetle control agents (Popa et al. 2012). There are minimal effects of entomopathogenic fungi on non-targets, and they offer a safer alternative for use in integrated pest management (IPM) than chemical insecticides (Wegensteiner 1996; Inglis et al. 2001; Batta 2007; Buhroo et al. 2016).

Entomopathogenic fungi similar to most fungal pathogens infect their host through the external cuticle. This mode of infection is the unique characteristic of the fungi. The infective units are the conidia, borne on specialised stalks called conidiophores; sporulation and germination require high humidity and adequate temperatures. Fungi gain access to the insect directly through the insect's integument. After germination of the conidium on the insect's cuticle, the fungus penetrates the integument and proliferates throughout the host, ultimately resulting in death of the infected host. Host specificity of entomopathogenic fungi varies considerably; some species have a broad host range and others are more restricted (Vincent et al. 2007; Vega et al. 2012; Mudronekova et al. 2013). In the present study, a standardised bioassay was developed and the efficacy of three entomopathogenic fungi, namely, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin (Ascomycota: Hypocre-

ales: Clavicipitaceae) and *Lecanicillium lecanii* (Zimmerman) Zare and Gams (Ascomycota: Hypocreales: Cordycipitaceae), against *P. scitus* was investigated under laboratory conditions.

MATERIAL AND METHODS

Fungal treatment of *Pinus wallichiana* branches infested with *P. scitus*

Naturally infested branches of *Pinus wallichiana* A.B. Jacks (Pinaceae) were collected in June 2017 from a severely infested pine stand located near forest check point, Tangmarg ($34^{\circ}03.797' N$, $74^{\circ}24.948' E$, Elevation 7552 ft) in Baramulla District, Jammu and Kashmir, India. The purpose of selection of severely infested branches with *Pityogenes scitus* was to ensure that the treatments would penetrate spontaneously after the application. A total of 90 branches (27.71 ± 1.58 (standard deviation) cm in length and 3.09 ± 0.52 (SD) in diameter) infested with *P. scitus*, categorised into five groups (G1–G5), were used in the experiment. Each replicate represented three infested branches, and six replicates per experimental treatment were used (Tab. 1).

Table 1. Treatments used against naturally infested branches of *Pinus wallichiana* with the bark beetle, *Pityogenes scitus* under laboratory conditions ($28 \pm 3.12^{\circ}C$ and $77.64 \pm 7.45\%$ RH). Standard deviation (SD) is added in parentheses

Sample No.	Groups*	No. of branches in each replicate/total	No. of debarked branches		Treatments	Quantity (ml/log)
			after 10 days	after 20 days		
1	G1	3/18	9	9	<i>Beauveria bassiana</i>	500
2	G2	3/18	9	9	<i>Metarhizium anisopliae</i>	500
3	G3	3/18	9	9	<i>Lecanicillium lecanii</i>	500
4	G4	3/18	9	9	Insecticide	500
5	G5	3/18	9	9	Distilled water	500

* Each group represents 18 infested branches with 6 replicates per experimental treatment. Each replica contains three infested branches treated with 5 treatments

The commercial biopreparation of entomopathogenic fungi was obtained from Green Life Biotech Laboratory, Somanur, Coimbatore, India. They included *B. bassiana*, *M. anisopliae* and *L. lecanii*. The insecticide used was Cyclone 505 EC (positive control) (active ingredients: Chlorpyriphos 50% + Cypermethrin 5%), which was applied following the standard directions for use, and distilled water sprayed on infested branches served as control during the course of experimentation.

The fungal preparation was diluted in water: 1 ml biopreparation/1000 ml water with four drops of a common detergent as a wetting agent. Each fungal suspension contained 1.0×10^9 spores of fungi in 1 ml. The fungal suspensions were applied using a hand sprayer at 500 ml per log (Tab. 1). High volumes of fungal suspensions were used for effective treatment so that suspensions would penetrate spontaneously after application. After 10 days, 9 branches from 3 treated replicates in each group were carefully debarked, and the percentage of mortality of *P. scitus* was calculated and compared (Tab. 1). The same procedure was applied for calculating percentage of mortality of *P. scitus* after 20 days of treatment.

Fungal treatment of *P. scitus* adults (Petri plate assay)

In this method, a total of 15 petri dishes containing filter papers were used; three replicates were maintained for each treatment. The treatments were performed by applying two rapid jetting sprays standardised at 1.0 ml per replicate using a small calibrated hand sprayer (1 L capacity) equipped with a nozzle suited to low-volume spray application (Batta 2007). In each petri dish, 40 adults of *P. scitus* were introduced before spraying. The same spray volumes (1 ml per replicate) were applied in the other treatments (Tab. 2). The mortality percentage from each treated group was evaluated after 2, 4 and 6 days of treatment. This mortality was shown either by the lack of movement of treated adults within 5-min. period of continuous observation or by the appearance of mycelial growth on the bodies of dead adults. The beetles were then incubated in petri dishes under humid conditions at $28 \pm 3.12^\circ\text{C}$ and $77.64 \pm 7.45\%$ relative humidity (RH) for 1 week to promote mycelial growth with the conidia and the conidiophores on their bodies.

Table 2. Treatments used against the bark beetle *Pityogenes scitus* using petri plate assay under laboratory conditions ($28 \pm 3.12^\circ\text{C}$ and $77.64 \pm 7.45\%$ RH). Standard deviation (SD) is added in parentheses

Sample No.	Groups*	No. of Petri dishes in each group/ beetles	Treatments	Quantity (ml/petri dish)
1	G1	3/120	<i>Beauveria bassiana</i>	1.0
2	G2	3/120	<i>Metarhizium anisopliae</i>	1.0
3	G3	3/120	<i>Lecanicillium lecanii</i>	1.0
4	G4	3/120	Insecticide	1.0
5	G5	3/120	Distilled water	1.0

* Each group represents 3 petri dishes with 40 adult beetles of *P. scitus* treated with 5 treatments

STATISTICAL ANALYSES

Statistical analyses were performed using OriginPro (Version 15) software. Data obtained on means of percentage of mortality of *P. scitus* adults in the different experimental treatments were analysed using analysis of variance (ANOVA), and means of the treatment effects were separated using Fisher's Least Significant Difference (LSD) pair-wise multiple comparison test.

RESULTS

The results on mortality caused by entomopathogenic fungi were recorded in treated branches and petri plate assay. In treated branches, after 10 days of treatment (Tab. 3), at spore concentration of 1.0×10^9 , percentage of mortality of *P. scitus* observed was 58.33% (± 4.27 Standard error) with *B. bassiana*, 48% (± 4.03 SE) with *M. anisopliae*, 30.76% (± 3.61 SE) with *L. lecanii* and 13% (± 1.72 SE) with insecticide (positive control). Treatments with *B. bassiana* and *M. anisopliae* resulted in significantly higher mortality ($p < 0.05$) than with other treatments. There were no significant differences between the mortalities caused by *B. bassiana* and *M. anisopliae*. After 20 days of treatment (Tab. 3), percentage of mortality reached a maximum of 85% (± 5.59 SE) with *B. bassiana*, 71% (± 6.03 SE) with *M. anisopliae*, 64.61% (± 6.61 SE) with *L. lecanii* and 12.22% (± 2.71 SE) with

Table 3. Mean percentage of mortality of *Pityogenes scitus* individuals in experiment by comparing the efficiency of different treatments applied on infested branches of *Pinus wallichiana* under laboratory conditions ($28\pm3.12^\circ\text{C}$ and $77.64\pm7.45\%$ RH). Means in the column followed by different letters are significantly different at $p < 0.05$ as indicated by Fisher's Least Significant Difference (LSD) pair-wise multiple comparison test

Groups*	Treatment	Mean mortality \pm Standard error (%)				Death of <i>P. scitus</i> individuals/total	
		after 10 days	difference	after 20 days	difference	after 10 days	after 20 days
G1	<i>Beauveria bassiana</i>	58.33 \pm 4.27	a	85 \pm 5.59	a	70/120	68/80
G2	<i>Metarhizium anisopliae</i>	48 \pm 4.03	ac	71 \pm 6.03	a	48/100	78/110
G3	<i>Lecanicillium lecanii</i>	30.76 \pm 3.61	bc	64.61 \pm 6.61	a	40/130	84/130
G4	Insecticide	13 \pm 1.72	d	12.22 \pm 2.71	b	13/100	11/90
G5	Distilled water	6.66 \pm 2	d	17.5 \pm 2.88	b	6/90	21/120

* Each group represents 18 infested branches with 6 replicates per experimental treatment. Each replica contains three infested branches treated with 5 treatments

insecticide. No significant differences were observed within fungal treatments; however, all treated fungal species caused significantly higher mortality than other treatments, that is, insecticide and distilled water. The present results indicated high ability of the fungal mycelium to grow saprophytically on the treated branches, on the bodies of infected beetles, and in the galleries of *P. scitus* adults. The results also showed higher efficacy of treatments of *B. bassiana* against *P. scitus* adults infesting *P. wallichiana*.

In petri plate assay, after 2 days of treatment (Tab. 4), at spore concentration of 1.0×10^9 , percentage of mortality of *P. scitus* observed was 47.5% (±1.0 SE) with *B. bassiana*, 50.83% (±2.08 SE) with *M. anisopliae*, 30.83% (±2.0 SE) with *L. lecanii* and

66.66% (±3.78 SE) with insecticide. Treatment with insecticide resulted in significantly higher mortality ($p < 0.05$) than with other treatments. However, no significant differences ($p < 0.05$) were observed between the percentages of mortality caused by *B. bassiana* and *M. anisopliae*. After 4 days of treatment (Tab. 4), percentage of mortality of *P. scitus* observed was 83.33% (±1.52 SE) with *B. bassiana*, 89.16% (±1.52 SE) with *M. anisopliae*, 44.16% (±1.52 SE) with *L. lecanii* and 72.5% (±4.35 SE) with insecticide. Treatments with *M. anisopliae*, *B. bassiana* and insecticide caused significantly higher mortality ($p < 0.05$) than with other treatments. No significant differences ($p < 0.05$) were observed between the mortalities caused by *B. bassiana* and *M. anisopliae* but both the species caused signifi-

Table 4. Mean percentage of mortality of *Pityogenes scitus* adults using petri plate assay under laboratory conditions ($28\pm3.12^\circ\text{C}$ and $77.64\pm7.45\%$ RH). Means in the column followed by different letters are significantly different at $p < 0.05$ as indicated by Fisher's Least Significant Difference (LSD) pair-wise multiple comparison test

Groups*	Treatment	Mean mortality \pm Standard error (%)					
		after 2 days	difference	after 4 days	difference	after 6 days	difference
G1	<i>Beauveria bassiana</i>	47.50 \pm 1.0	a	83.33 \pm 1.52	a	100 \pm 0.00	a
G2	<i>Metarhizium anisopliae</i>	50.83 \pm 2.08	a	89.16 \pm 1.52	a	100 \pm 0.00	a
G3	<i>Lecanicillium lecanii</i>	30.83 \pm 2.08	b	44.16 \pm 1.52	b	73.33 \pm 1.52	b
G4	Insecticide	66.66 \pm 3.78	c	72.50 \pm 4.35	c	79.16 \pm 3.78	b
G5	Distilled water	3.33 \pm 0.57	d	9.16 \pm 0.57	d	11.66 \pm 1.15	c

* Each group represents 3 petri dishes with 40 adult beetles of *P. scitus* treated with 5 treatments

cantly higher mortality ($p < 0.05$) than other treatments. After 6 days of treatment (Tab. 4), percentage of mortality of *P. scitus* reached a maximum of 100% (± 0.00 SE) with *B. bassiana*, 100% (± 0.00 SE) with *M. anisopliae*, 73.33% (± 1.52 SE) with *L. lecanii* and 79.16% (± 4.35 SE) with insecticide. Treatment with *B. bassiana* and *M. anisopliae* resulted in significantly higher mortality ($p < 0.05$) than other treatments. There were no significant differences between the mortalities caused by *B. bassiana* and *M. anisopliae*. Incubation of treated *P. scitus* adults with three fungal species, namely, *B. bassiana*, *M. anisopliae* and *L. lecanii* in petri dishes under humid conditions at $28 \pm 3.12^\circ\text{C}$ and $77.64 \pm 7.45\%$ RH for 1 week has revealed the appearance of white mycelium on the bodies of infected beetles with conidiophores and conidia typical to the fungi as compared with the uninfected ones.

DISCUSSION

In the present study, all fungal treatments used showed high efficacy in treated branches infested with *P. scitus*. However, *B. bassiana* and *M. anisopliae* caused higher percentages of mortality, that is, 58.33% and 48%, respectively, after 10 days of treatment and 85% and 71%, respectively, after 20 days of treatment. Such efficacy could be explained by the ability of the fungus mycelium to grow saprophytically in the galleries of *P. scitus* and the ability of the conidia borne by this mycelium to infect the beetles inside the galleries. Kirschner (2001) reported many species of pathogenic fungi in the galleries of bark beetles from different tree species. Several species of pathogenic fungi have been reported to infect bark beetles and are able to kill all stages of beetle pests (Wegensteiner 2007). Numerous commercial isolates of entomopathogenic fungi have been evaluated widely as confirmed by the earlier studies of Gusteleva in 1980 and 1984, who evaluated the commercial product boverin against adult *Ips subelongatus* (Motschulsky) and achieved 89% mortality. As in the studies of Castrillo et al. (2011) that were performed with commercial isolates; *B. bassiana* Naturalis and *M. brunneum* F52 were found to be more virulent than *B. bassiana* GHA, against the bark beetle, *Xylosandrus germanus* (Blandford). Similar studies were performed by Doberski (1981), who found that isolates of *B. bassiana* were more virulent against

larvae and adults of *Scolytus scolytus* (Fabricius) than *Isaria farinosa* (Holmsk.) Fr. and *M. anisopliae*. Earlier studies on the virulence of *B. bassiana*, *M. anisopliae* against scolytine bark beetles are in line with the results of the present study as Markova (2000) compared the virulence of *B. bassiana*, *M. anisopliae*, *I. farinosa* and *Cordyceps confragosa* (Mains) against *Ips typographus* (Linnaeus) and found that the most aggressive species was *M. anisopliae* (100% mortality in 4 days) and the least aggressive was *C. confragosa* (still causing 90% mortality after 5 days). In another study with *I. typographus*, it was highly susceptible to both *B. bassiana* and *M. anisopliae*; 90–99% mortality was achieved in inoculated individuals (Mudroneckova et al. 2013). In bioassays against *Dendroctonus micans* (Erichson), one isolate of *B. bassiana* was more virulent than *M. anisopliae* isolates, *I. fumosorosea*, *Evlachovaea* sp., and other isolates of *B. bassiana*, particularly against larvae (Sevim et al. 2010). Spraying *B. bassiana* conidial suspensions onto the bark of *I. typographus* infested logs caused 29% mortality (Jakus and Blazenc 2011). It was also observed that amongst the three species of entomopathogenic fungi tested, *L. lecanii* was found to be less effective than the other two fungi against the *P. scitus* under laboratory conditions.

All three fungal treatments using petri plate assay showed high efficacy against the adults of the bark beetle, *P. scitus*. All treated fungal species, that is, *B. bassiana*, *M. anisopliae* and *L. lecanii*, caused higher percentages of mortality, after 6 days of treatment, that is, 100%, 100% and 73.33%, respectively, than control treated with distilled water (11.66%). Numerous bioassays have been performed with different fungal species (and isolates) against different species of bark beetles, all demonstrating the potential to achieve high mortalities in bark beetle populations in the laboratory. The most widely studied species is *B. bassiana*, which has been bioassayed against a number of different bark beetle species. Doane (1959) reported *B. bassiana* induced mortality in larvae (92%), pupae (87%) and adults (61%) of *Scolytus multistriatus* (Marsham). *B. bassiana* also caused 100% mortality in *Trypodendron lineatum* (Olivier) larvae within 6–8 days (Novak and Samsinsakova 1962) and was highly pathogenic to *I. typographus* and *S. ratzeburgi* (Janson) (Bałazy 1966). Higher mortality was induced by *B. bassiana* in *S. scolytus*; the LD₅₀ was reported as 1×10^6 conidia/ml after 5 days (at 23°C

and 100% RH) (Barson 1976). *B. bassiana* caused high mortality rates in bioassays against *Dendroctonus micans* ($\geq 90\%$) (Tanyeli 2010). Promising activity of *B. bassiana* against bark beetles was supported by earlier studies as isolate of *B. bassiana* from *I. duplicatus* (Sahlberg) sprayed on to log sections infested with *I. typographus* caused 60% mortality (Andrei et al. 2013) and caused 100% mortality in the same species within four days in the laboratory (Dinu et al. 2012). The activity of *B. bassiana* extended interspecifically as evaluation of *B. bassiana* against *D. valens* (LeConte) cause up to 100% mortality within 4.60 days (Zhang et al. 2011) and inoculation of *I. sexdentatus* (Boerner) with dry conidial powders or conidial suspensions of *B. bassiana* caused more than 90% mortality within a few days in young, immature adults and also old adults (Steinwender et al. 2010). In only study, with an isolate from the *L. lecanii* complex was found to be infective to *S. scolytus* larvae, achieving 100% mortality within 5 days at a conidial concentration of 4.5×10^6 conidia/ml (Bałazy 1963; Barson 1976). It was also observed that treated insecticide (cyclone) in petri plate assay also caused higher mortality (79.16%) after 6 days of treatment as compared with control treatment under laboratory conditions. Grosman and Upton (2006) evaluated the effectiveness of dinotefuran, emamectin benzoate (Denim), fipronil and imidacloprid (IMA-jet) for preventing *Ips* spp. attacks and brood development on standing, stressed trees and bolt sections of loblolly pine (*Pinus taeda* L.) in East Texas. Both emamectin benzoate and fipronil significantly and successfully reduced *Ips* spp. colonisation and levels of mortality in stressed trees. However, on commercial forest land, insecticides are costly and difficult to apply on a large scale. They can also disrupt the effect of natural enemies, and their effectiveness in controlling beetle outbreaks has been variable.

CONCLUSIONS

For many years, the focus of application of entomopathogenic fungi for their role in reducing the insect pest problems has been increasing. The results of our experiments carried out on the bark beetle *P. scitus* are promising; however, no recommendations concerning the potential use of these fungal pathogens in forest pro-

tection can be given, and further field experiments are warranted to investigate their efficacy under more practical conditions. Further research on entomopathogenic fungi could provide better understanding of pathogen–host interactions, especially pathogen transmission and spreading in field conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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