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An assessment of biological control of *Polygraphus major* Stebbing, 1903 (Coleoptera: Curculionidae) by entomopathogenic fungi

Abdul Lateef KHANDAY, Abdul Ahad BUHROO*

Section of Entomology, Department of Zoology, Faculty of Biological Sciences, University of Kashmir, Srinagar, India

**Corresponding author: lateefkhanday@gmail.com*

Abstract

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Recently the use of fungal entomopathogens against bark beetles has gained increasing attention throughout the world and researchers continue to seek highly pathogenic fungal isolates for controlling beetle pests. In the present study, the efficacy of three entomopathogenic fungi, namely *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin and *Lecanicillium lecanii* (Zimmerman) Zare & Gams, was tested against *Polygraphus major* Stebbing, 1903 under laboratory conditions. Each fungal suspension contained 1.0×10^9 spores of fungi in 1 ml. An insecticide – Cyclone was also used as positive control in the experiment. The mortality caused by these fungi was recorded in treated branches and petri plate assay. In treated branches, *B. bassiana* and *M. anisopliae* s. l. caused higher mortality, i.e. 57.77 and 46%, respectively, after 10 days of treatment and 98 and 92.77%, respectively, after 20 days of treatment. The results of the petri plate assay revealed that *P. major* adults were highly susceptible to both applied fungal species and insecticide. However, *B. bassiana* and *M. anisopliae* s. l. caused higher percentage mortalities after six days of treatment, i.e. 100 and 91.66%, respectively. The percentage mortality caused by application of the insecticide was 69%. *L. lecanii* was observed to be significantly less virulent (mortality 46.66%) in all fugal treatments. After observing the promising nature of the three entomopathogenic fungi by testing them, we arrive at the conclusion that the tested fungi have a potential for the control of *P. major*, and further field experiments are warranted to investigate their efficacy under more practical conditions.

Keywords: pathogenic fungi; bark beetle; cyclone; petri plate assay; mortality

Bark beetles are economically important pests both in conifer forests and broadleaved tree species in the temperate regions of the northern hemisphere. The bark beetle, *Polygraphus major* Stebbing, 1903 (Coleoptera: Curculionidae) is one of the most serious pests of *Pinus wallichiana* A.B. Jacks (Pinaceae) in Kashmir Himalaya (Stebbing 1914; Maiti, Saha 2009). Bark beetles exhibit incredible diversity and complexity in their behaviour, ecology, and population dynamics. After attacking a 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). Close inspection shows a fine reddish-brown boring dust in bark cervices when the beetles bore into the bark of the tree. In addition to their ecological

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roles mainly in nutrient cycling, biodiversity and successional pathways, these economically important pests cause substantial socioeconomic losses, and at times necessitate management responses (Raffa et al. 2015).

The experience of limitation and difficulties of traditional insecticidal control have now-a-days led to a unifying concept that the sound ecological basis of pest suppression is the most rewarding venture to manipulate their population to keep them below the threshold level of their damage. 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 employing fungal pathogens to combat insect pests. Existing research suggests that there are minimal effects of entomopathogenic fungi on non-targets and they offer a safer alternative for use in integrated pest management than chemical insecticides (Wegensteiner 1996; Inglis et al. 2001; Batta 2007; Buhroo et al. 2016).

Among pathogens, entomopathogenic fungi are considered the most promising biocontrol agents because they are currently being developed for control of many agricultural insect pests such as termites (Rath 2000; Sindhu et al. 2011) or public health insects such as mosquitoes (Roberts 1970). In contrast to other pathogenic groups associated with Scolytine beetles, entomopathogenic fungi have been well studied and evaluated as bark beetle control agents (Popa et al. 2012). There are numerous reports of entomopathogenic fungi infecting bark beetles and in all cases they are able to infect and kill all life stages (Jurc 2004; Wegensteiner, Weiser 2004). The infective units are the conidia, borne on specialized stalks called conidiophores; sporulation and germination require high humidity and adequate temperatures. Fungi are exclusively infecting insects by penetration of the mycelium into their cuticle, then growing in the haemocoel causing finally the death of the attacked individuals (Gabarty et al. 2014). Furthermore, the internal mycelium can grow outward and then sporulate on the outer surfaces of cadavers of insects (Murad et al. 2007; Schrank, Vainstein 2010). 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; Mudrončeková et al. 2013).

The objective of the present study was to test the susceptibility of the bark beetle, *P. major,* to the widely used entomopathogenic fungi – *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin (Ascomycota: Hypocreales: Clavicipitaceae), and *Lecanicillium lecanii* (Zimmerman) Zare & Gams (Ascomycota: Hypocreales: Cordycipitaceae) and to study their comparative effectiveness under laboratory conditions.

MATERIAL AND METHODS

Collection of *P. wallichiana* branches used for bioassays. In August 2017, naturally infested branches of *P. wallichiana* with the bark beetle, *P. major*, were collected from a severely infested pine stand located in Nowpora village (33°61.078'N, 75°18.700'E, elevation 1,804 m a.s.l.) in Anantnag District, Jammu & Kashmir (Fig. 1). The infested branches were selected after observing *P. major* infestations (Fig. 1). The sampled branches were then transported in plastic boxes to the Animal House, Department of Zoology, University of Kashmir for assessment of biological control of *P. major* by the entomopathogenic fungi. Spatial information regarding the sample site was recorded in the form of latitude and longitude with the help of handheld GPS eTrex 10 (Garmin, India).

Entomopathogenic fungi used in the treatment. The commercial biopreparations of entomopathogenic fungi were obtained from Green Life Biotech Laboratory, Somanur, Coimbatore, India. They included *B. bassiana*, *M. anisopliae* s. l. and *L. lecanii*. A total of 90 branches, 27.87 ± 0.73 (SD) cm in length and 4.87 ± 0.61 (SD) cm in diameter, naturally infested by *P. major*, categorized into five groups (G1–G5), were used in the experiment. Each replicate represented three infested branches and six replicates per experimental treatment were used (Table 1). The used insecticide was Cyclone 505 EC (positive control) (active ingredients: Chlorpyriphos 50% + Cypermethrin 5%) applied following the standard directions for use and distilled water sprayed on infested branches served as control during the course of experimentation.

A standard protocol for the biopreparation of entomopathogenic fungi was followed as per the method adopted by Jakuš and Blaženec (2011). The fungal preparation was diluted in water: 1 ml biopreparation per 1,000 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 with a hand sprayer at 500 ml per log (Table 1). High volumes of fungal suspensions were used

Fig. 1. Pine stand infested by bark beetles (a), felled *Pinus wallichiana* A.B. Jacks tree infested by *Polygraphus major* Stebbing, 1903 (b, c), sampled branches infested by *P. major* (d), adult beetle of *P. major* (e)

for effective treatment so that suspensions would penetrate spontaneously after application. After 10 days nine branches from three treated replicates in each group were carefully debarked and the percentage mortality of *P. major* was calculated and compared (Table 1). The same procedure was applied for calculating the percentage mortality of *P. major* after 20 days of treatment.

Fungal treatment of *P. major* 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

Table 1. Treatments used against naturally infested branches of *Pinus wallichiana* A.B. Jacks with the bark beetle *Polygraphus major* Stebbing, 1903 under laboratory conditions (29.53 ± 2.81°C and 78.15 ± 7.06% relative humidity)

Sample No.	Group*	No. of branches in each replicate/total	No. of debarked branches		Treatment	Quantity (ml per log)
			after 10 days	after 20 days		
1	G1	3/18	9	9	*Beauveria bassiana* (Balsamo) Vuillemin	500
2	G2	3/18	9	9	*Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin	500
3	G3	3/18	9	9	*Lecanicillium lecanii* (Zimmerman) Zare & Gams	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 six replicates per experimental treatment. Each replicate contains three infested branches with five treatments

Table 2. Treatments used against the bark beetle *Polygraphus major* Stebbing, 1903 using a petri plate assay under laboratory conditions (29.53 ± 2.81°C and 78.15 ± 7.06% relative humidity)

Sample No.	Group*	No. of Petri dishes in each group/beetles	Treatment	Quantity (ml per petri dish)
1	G1	3/120	*Beauveria bassiana* (Balsamo) Vuillemin	1.0
2	G2	3/120	*Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin	1.0
3	G3	3/120	*Lecanicillium lecanii* (Zimmerman) Zare & Gams	1.0
4	G4	3/120	insecticide	1.0
5	G5	3/120	distilled water	1.0

*Each group represents three petri dishes with 40 adult beetles of *P. major* subjected to five treatments

treatments were performed by applying two rapid jetting sprays standardized 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. major* were introduced before spraying. The same spray volumes (1 ml per replicate) were applied in the other treatments (Table 2). The mortality percentage from each treated group was evaluated after 2, 4 and 6 days after treatment. This mortality was shown either by the lack of movement of treated adults within a five-minute 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 29.53 ± 2.81°C and 78.15 ± 7.06% relative humidity for one week to promote mycelial growth with conidia and conidiophores on their bodies. The identification of fungal species was done by consulting literature on their external morphological characters such as colour of the colony, length, arrangement and shape of conidiophores. We follow the nomenclature used by Humber (1997) for identification of the fungi tested in the experiment. The images were taken using an M205A Leica Stereomicroscope (Leica Microsystems GmbH, Germany) with a DFC295 camera (Leica Microsystems GmbH, Germany) and Leica Application Suit software (Version 4.10, 2017) and focused using the same software.

Statistical analyses. Statistical analyses were performed using OriginPro software (Version 2015). Data obtained on means of percentage mortality of *P. major* adults in the different experimental treatments were analysed by ANOVA and means of the treatment effects were separated using Fisher's LSD pairwise multiple comparison test. The significance of differences between means was determined at $P < 0.05$.

RESULTS

The three entomopathogenic fungi were tested against *P. major* at a concentration of 1.0×10^9 spores per millilitre and compared with controls. The results revealed that branches infested with *P. major* were highly susceptible to the three fungal species tested. After ten days of treatment (Fig. 2a, Table 3), the observed percentage mortality of

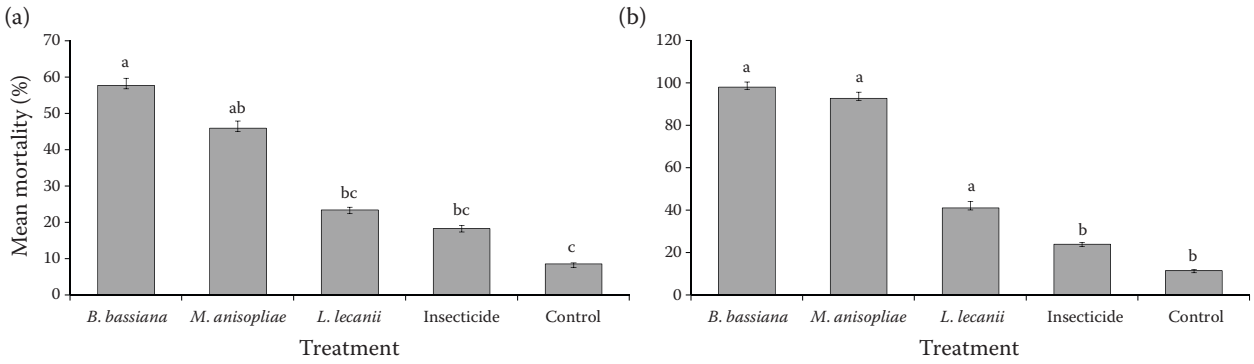

Fig. 2. Mean percentage mortality of *Polygraphus major* Stebbing, 1903 individuals in an experiment comparing the efficiency of different treatments applied to infested branches of *Pinus wallichiana* A.B. Jacks after 10 (a), 20 (b) days of treatment. Standard error is added on bars and the bars that do not share the same superscript are significantly different at $P < 0.05$ as indicated by Fisher's LSD pairwise multiple comparison test

B. bassiana – *Beauveria bassiana* (Balsamo) Vuillemin, *M. anisopliae* – *Metarhizium anisopliae* (Metchnikoff) Sorokin, *L. lecanii* – *Lecanicillium lecanii* (Zimmerman) Zare & Gams

Table 3. Mean percentage mortality of *Polygraphus major* Stebbing, 1903 individuals in an experiment comparing the efficiency of different treatments applied to infested branches of *Pinus wallichiana* A.B. Jacks after 10 and 20 days of treatment under laboratory conditions (29.53 ± 2.81°C and 78.15 ± 7.06% relative humidity)

Sample No.	Group*	Mean mortality ± SE (%)		No. of *P. major* individuals/total		Treatment
		after 10 days	after 20 days	after 10 days	after 20 days	
1	G1	57.77 ± 1.95	98.0 ± 2.41	52/90	49/50	*Beauveria bassiana* (Balsamo) Vuillemin
2	G2	46.0 ± 1.91	92.77 ± 2.92	40/82	77/83	*Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin
3	G3	23.40 ± 0.75	41.77 ± 2.92	11/47	28/68	*Lecanicillium lecanii* (Zimmerman) Zare & Gams
4	G4	18.33 ± 0.79	23.94 ± 0.91	11/60	17/71	insecticide
5	G5	8.57 ± 0.23	11.49 ± 0.53	3/35	10/87	distilled water

*Each group represents 18 infested branches with six replicates per experimental treatment. Each replica contains three infested branches treated with five treatments, SE – standard error

P. major was 57.77% (± 1.95 SE) with *B. bassiana*, 46% (± 1.91 SE) with *M. anisopliae* s. l., 23.40% (± 0.75 SE) with *L. lecanii* and 18.33% (± 0.79 SE) with insecticide (positive control). Treatments with *B. bassiana* and *M. anisopliae* s. l. 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* s. l. After twenty days of treatment (Fig. 2b, Table 3), percentage mortality reached a maximum of 98% (± 2.41 SE) with *B. bassiana*, 92.77% (± 2.92 SE) with *M. anisopliae* s. l., 41.17% (± 2.92 SE) with *L. lecanii* and 23.94% (± 0.91 SE) with insecticide (positive control). No significant differences were observed within fungal treatments; however, all treated fungal species caused significantly higher mortality than other treatments. 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. major* adults (Fig. 3). The results also revealed higher efficacy of treatments with *B. bassiana* against *P. major* adults infesting *P. wallichiana*.

Fig. 3. Heavy fungal infections on the branch of *Pinus wallichiana* A.B. Jacks treated with *Beauveria bassiana* (Balsamo) Vuillemin (a), on the branch of *P. wallichiana* treated with *Metarhizium anisopliae* sensu lato (Metchnikoff) Sorokin (b), in the galleries of *Polygraphus major* Stebbing, 1903 with *B. bassiana* (c), in the galleries of *P. major* with *M. anisopliae* s. l. (d), on the body of *P. major* adult with *B. bassiana* (e), on the bodies of *P. major* adults with *M. anisopliae* (f), on the body of *P. major* adult with *Lecanicillium lecanii* (Zimmerman) Zare & Gams (g). Arrows indicate fungal growth

Results obtained in bioassays against *P. major* adults using the petri plate assay are shown in Figs 4a–c. The results revealed that *P. major* adults were highly susceptible to all the fungal species and insecticide tested. After two days of treatment (Fig. 4a), at a spore concentration of 1.0×10^9, the observed percentage mortality of *P. major* was 55% (± 3.51 SE) with *B. bassiana*, 41.66% (± 3.75 SE) with *M. anisopliae* s. l., 13.33% (± 2.33 SE) with *L. lecanii* and 60% (± 4.93 SE) with insecticide (positive control). Treatment with insecticide resulted in significantly higher mortality ($P < 0.05$) than other treatments. There were not any significant differences ($P < 0.05$) between the percentage mortalities caused by *B. bassiana*, *M. anisopliae* s. l. and insecticide. After four days of treatment (Fig. 4b), the observed percentage mortality of *P. major* was 80.83% (± 5.45 SE) with *B. bassiana*, 72.5% (± 5.03 SE) with *M. anisopliae* s. l., 26.66% (± 2.90 SE) with *L. lecanii* and 65% (± 5.17 SE) with insecticide. Treatments with *B. bassiana*, *M. anisopliae* s. l., and insecticide caused significantly higher mortality ($P < 0.05$) than other treatments. No significant differences ($P < 0.05$) were observed between the mortalities caused by *B. bassiana*, *M. anisopliae* s. l. and insecticide but all the three treatments caused significantly higher mortality ($P < 0.05$) than other treatments. After six days of treatment (Fig. 4c), the percentage mortality of *P. major* reached a maximum of 100% (± 0.00 SE) with *B. bassiana*, 91.66% (± 3.33 SE) with *M. anisopliae* s. l., 46.66% (± 3.84 SE) with *L. lecanii* and 68.33% (± 3.92 SE) with insecticide. Treatment with *B. bassiana* and *M. anisopliae* s. l. 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* s. l., however, significant differences were observed when compared with other treatments (Fig. 4c), indicating higher efficacy of these two fungal species against *P. major*.

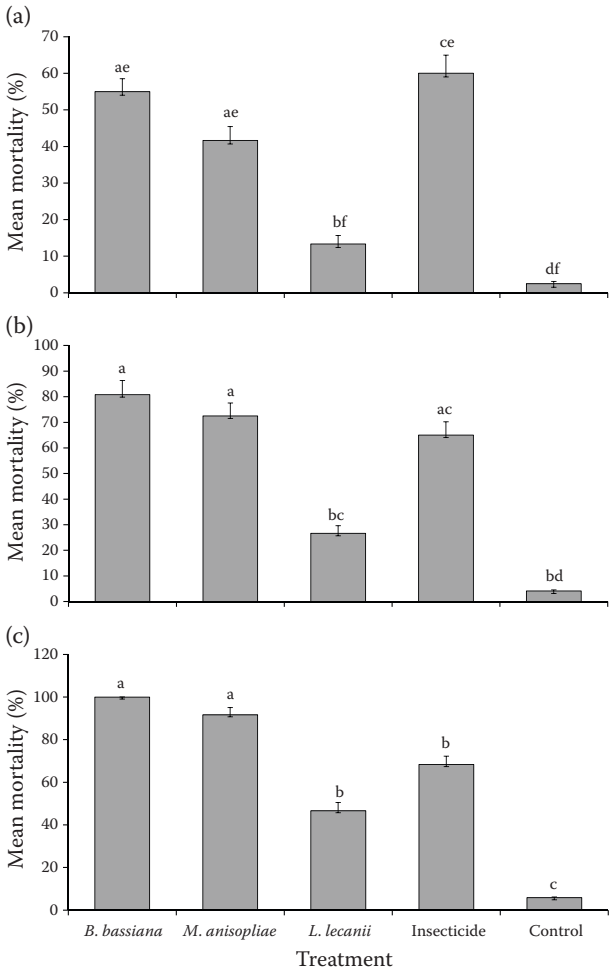

Fig. 4. Mean percentage mortality of *Polygraphus major* Stebbing, 1903 adults after 2 (a), 4 (b), 6 (c) days of treatment using the petri plate assay. Standard error is added on bars and the bars that do not share the same superscript are significantly different at $P < 0.05$ as indicated by Fisher's LSD pairwise multiple comparison test

B. bassiana – *Beauveria bassiana* (Balsamo) Vuillemin, *M. anisopliae* – *Metarhizium anisopliae* (Metchnikoff) Sorokin, *L. lecanii* – *Lecanicillium lecanii* (Zimmerman) Zare & Gams

Incubation of *P. major* adults treated with three fungal species, namely *B. bassiana*, *M. anisopliae* s. l. and *L. lecanii*, in petri dishes under humid conditions at 29.53 ± 2.81°C and 78.15 ± 7.06% relative humidity for one week has revealed the appearance of white mycelium on the bodies of infected individuals of *P. major* with conidiophores and conidia typical of each fungal species and thus the identity of pathogens was confirmed.

DISCUSSION

In the present study it was observed that among the three entomopathogenic fungi, *B. bassiana* and *M. anisopliae* s. l. showed higher efficacy in treated branches infested with *P. major*. Mortality observed in treated branches varies across the different entomopathogenic fungi. Among three fungal treatments, *B. bassiana* and *M. anisopliae* s. l. caused higher percentage mortalities, i.e. 57.77 and 46%, respectively, after 10 days of treatment and 98 and 92.77%, 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. major* and the ability of the conidia borne by this mycelium to infect the beetles inside the galleries. Many species of fungi

have been reported in the galleries of bark beetles from different trees species, even when beetles are no longer present; several of these fungi are entomopathogenic species (Bałazy 1965; Kirschner 2001). Based on the findings of the present study, the increased trend observed in the mortalities caused by *B. bassiana* and *M. anisopliae* s. l. throughout the experiment is considered as a good indication of preserved pathogenicity. Earlier studies on the virulence of *B. bassiana*, *M. anisopliae* s. l. 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* s. l., *Isaria farinose* (Holmskjold) Fries, and *Cordyceps confragosa* (Mains) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora against *Ips typographus* (Linnaeus, 1758) and found that the most aggressive species was *M. anisopliae* s. l. (100% mortality in four days) and the least aggressive was *C. confragosa* (still causing 90% mortality after five days). In another study with *Ips typographus* it was highly susceptible to both *B. bassiana* and *M. anisopliae* s. l.; 90–99% mortality was achieved in inoculated individuals (Mudrončeková et al. 2013). It was also observed that among the three species of entomopathogenic fungi used, *L. lecanii* was found less effective (mortality 41.17%) compared to the other two fungal species, i.e. *B. bassiana* and *M. anisopliae* s. l. against the *P. major* under laboratory conditions.

Fungal treatments using the petri plate assay showed high efficacy against the adults of the bark beetle, *P. major*. Mortality levels varied according to the treatment applied. All treated fungal species, i.e. *B. bassiana*, *M. anisopliae* s. l. and *L. lecanii*, caused higher percentage mortalities after six days of treatment, i.e. 100, 91.66 and 46.66%, respectively. Based on the findings of the plate assay, an increasing trend observed in the mortalities caused by *B. bassiana* and *M. anisopliae* s. l. throughout the experiment confirms that *P. major* appears as a particularly good candidate for biocontrol by entomopathogenic fungi. Promising activity of *B. bassiana* against bark beetles was supported by earlier studies as an isolate of *B. bassiana* from *Ips duplicatus* (Sahlberg, 1836) sprayed onto 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 *Dendroctonus valens* (LeConte, 1860) showed that the fungus caused up to 100% mortality within 4.60 days (Zhang et al. 2011) and inoculation of *Ips sexdentatus* (Boerner, 1767) 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). The only study with an isolate from the *L. lecanii* complex demonstrated that it was found to be infective to *Scolytus scolytus* (Fabricius, 1775) larvae, achieving 100% mortality within five days at a conidial concentration of 4.5×10^6 conidia per millilitre (Bałazy 1963; Barson 1976).

Many biotic and abiotic factors, particularly humidity and temperature, can fluctuate widely in the field and have strong influences on the infectivity and outcomes of bioassays against Scolytine beetles. Bychawska and Swiezynska (1979) sprayed *B. bassiana* conidia onto bait logs in the field conditions but they were unable to infect *Tomicus piniperda* (Linnaeus, 1758); abiotic conditions (rain, snow, humidity and temperature) were identified as the major problems reducing fungal viability. However, when bait logs were sprayed with *B. bassiana* conidia and then covered with polyethylene foil to improve abiotic conditions, 71–100% of the larvae in the logs became infected (Lutyk, Swiezynska 1984). In a field trial, Batta (2007) achieved 80% mortality with *B. bassiana* sprayed onto peach trees against *Scolytus amygdali* (Guerin-Meneville, 1847). These results suggest a great potential of bioassays and also opportunities for improvement.

It was also observed that the applied insecticide (Cyclone) in the petri plate assay also caused higher mortality (68.33%) 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 for preventing *Ips* spp. attacks and brood development on standing, stressed trees and bolt sections of loblolly pine (*Pinus taeda* Linnaeus) in East Texas. Both emamectin benzoate and fipronil significantly reduced *Ips* spp. colonization success 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.

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