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Effect of Exposure Methods and Concentrations on Infectivity of *Beauveria bassiana* in *Atteva sciodoxa* Meyrick (Lepidoptera: Yponomeutidae)

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Abstract: Effect of exposure methods and concentrations on infectivity of *Beauveria bassiana* were assessed in *Atteva sciodoxa* infesting a medicinal plant, *Eurycoma longifolia*. The conidial suspension was used in three exposure methods including inoculation of: both, larvae and leaves, only larvae and only leaves. Five concentrations: 5×10^6, 1×10^7, 5×10^7, 1×10^8 conidia mL^{-1} and a control were applied in a two-factor factorial design. The results indicated highly significant effect of exposure methods on infectivity. Pooled larval infectivity across the inoculating concentrations was ranged between 38.1±3.21% and 94.6±2.40% in three exposure methods. An infectivity of 54.1±1.74% was occurred due to secondary acquisition of conidia from spray residues on the foliage. A hundred percent infectivity was caused by concentration of $5x10^7$ conidia mL^{-1} when either both larvae and leaves or only larvae were inoculated. The difference in infectivity varied significantly among exposure methods at lower concentrations compared to the higher concentrations. The median effective time was found dependent on exposure method and inoculum concentration. An inoculum of 1×10^8 conidia mL^{-1} gave 50% larval infectivity in about three days when both larvae and leaves were inoculated. Based on these findings, it is concluded that infectivity can be enhanced through secondary acquisition from foliage by infecting larvae escaped during direct exposure or by overcoming physical defence mechanism of the larvae adopted momentarily in response to spray stimulus.

Key words: Exposure methods, white muscardine, inoculum, tiger moth, infectivity

INTRODUCTION

The infectivity of an entomopathogenic fungus depends on, *inter alia* its genetic ability to multiply and produce toxic metabolites. The suitable niche for attachment of conidia and subsequently penetration of germ tube via insect cuticle is important for infectivity. Generally, conidia penetrate percutaneous although evidence of gut infection by ingestion has been documented (Jeffs *et al*., 1997). The insect cuticle is one of the major determinants of fungal infection. The physico-chemical composition of the cuticle changes with the location and function of the insect body part. The type and pattern of organization of cuticle composing molecules determine physical properties of insect cuticle which subsequently provide physical defence mechanism again fungal infectivity (Romoser and Stoffolano, 1994; Gillott, 2005).

There are certain insect body parts which are relatively more conducive for attachment and penetration of fungal germ tubes. The main cuticle parts most commonly exposed are inter-segmental regions between the head and thorax, thorax and abdomen, legs, abdominal pro-legs, mouthparts, dorsal and ventral part of the abdomen and anal region (Fernandez *et al*., 2001; Ugine *et al*., 2005a). Among these body parts, legs, thoracic and abdominal segments are hard and heavily sclerotized thus, difficult to penetrate by germ tubes. The inter-segmental regions and ventral abdominal appendages with thin and moist cuticle are suitable for infection. Apart from thin and moist cuticle, conidia can also be trapped in inter-segmental regions, which help to initiate and expedite infection process (Inglis *et al*., 1995).

These cuticle sites can either be inoculated by direct spray or by contact with spray residues on the foliage, known as secondary acquisition. One may assume direct spray to be more effective for inducing infection as compared to acquiring inoculum from spray residues. There is possibility, however, that some of the resident insects can be missed during the direct spray. Conversely,

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the effectiveness of a fungal pathogen might be augmented by reducing the number of missed insects during direct spray through secondary acquisition. Secondary acquisition of *Beauveria bassiana* from exposed foliage has been reported as an important mode of inoculation for several insect pests (Jenkins and Thomas, 1996; Langewald *et al*., 1997; Fernandez *et al*., 2001; Tefera and Pringle, 2003). It is likely that augmentation of direct spray by secondary acquisition depends on combination of pathogen, insect and plant host species.

An isolate of *B. bassiana* (Bba-Pp), isolated from *Pteroma pendula* in 2007, has been found infective against the *Atteva sciodoxa* infesting Asian ginseng, *Eurycoma longifolia* (Abood *et al*., 2010). The effect of exposure methods and concentrations within exposure methods on infectivity of *B. bassiana* in *A. sciodoxa* has not been studied. A better understanding of the exposure methods will help to improve effectiveness of *B. bassiana* against the pest. The present study, therefore, was conducted to assess the effect of (i) exposure methods and (ii) concentrations within exposure methods on infectivity of *B. bassiana* in *A. sciodoxa*.

MATERIALS AND METHODS

This study was conducted at the faculty of Forestry, University Putra Malaysia, Malaysia.

Fungal culture: The isolate Bba-Pp, isolated from *P. pendula* was passaged through *A. sciodoxa*. The fungus was isolated from cadavers on a selective medium of potato dextrose agar (PDA) 3.9% (potato starch 4.0 g L^{-1}, dextrose 20.0 g L^{-1} and agar 15 g L^{-1}) supplemented by 0.5% yeast extract (YE) and added 0.06% streptomycin sulphate and 0.03% chloramphenicol. The culture after purification was maintained on PDA+YE at 27°C in darkness.

A suspension of $1^{-}10^{7}$ conidia mL^{-1} was prepared aseptically from 2 week old culture and 0.1 mL of the suspension was pipetted on the growth medium and spread evenly using cell spreader. The plates were incubated for 2 week at 27°C in darkness. The conidia were harvested with 20 mL of 0.02% aqueous Tween 80, using glass rod. The aliquot was transferred to test tubes and vortexed for 5 min using IKA® MS 3-Digital Vortex at a speed of 3000 rpm. The homogenised suspension was filtered twice using cheesecloth. A dilution of 1:100 was prepared and conidial concentration was determined using Hirschmann® Neubauer improved haemocytometer.

Conidial viability was assessed simultaneously. A 0.1 mL suspension of 1×10^{7} conidia mL^{-1} was pipetted on PDA+YE plate and spread evenly using cell spreader on turn table. The inoculated plates were incubated for 24 h at 27°C in darkness. Three drops of lactophenol cotton blue were added to the incubated medium to fix and stain the conidia. A rectangular piece of PDA (1.5×2 cm) was mounted on microscopic slide and 3x100 conidia were counted using compound microscope (400x). A hundred percent conidial germination was found after 24 h of incubation at 27°C in darkness.

Insect culture: Stock culture of *A. sciodoxa* was maintained in the laboratory at 27±2°C and 75±5% relative humidity with 12 h photoperiod on *E. longifolia*. Full grown larvae were collected from the field and reared in the laboratory for pupation. Pupae were separated sex-wise and kept singly in cylindrical plastic containers (40 mm diameter x 30 mm). Cages were set up for single virgin pairs. Sterilized moistened sand was levelled to a depth of 10 mm in a 10 cm Petri dish and covered with Whatman® filter paper. Fresh *E. longifolia* shoot with 4-5 leaflets was affixed upright through the centre of the filter paper. These leaflets were then placed in cylindrical containers (8 cm diameter x 12 cm). Three drops of 10% honey were placed on the inside base of the container and then inverted. Single pairs of virgin adults in were introduced in each container. The eggs obtained were used to have insect stock culture.

Effect of exposure methods: The 24-36 h old third instar larvae were exposed to *B. bassiana* either inoculating both, larva and leaf (L+Lf), only the larva (L) or only the leaf (Lf). In each exposure method four concentrations 5×10^{6}, 1×10^{7}, 5×10^{7} and 1×10^{8} conidia mL^{-1} as well as a control was used. The concentrations were prepared from stock suspension by adding 0.02% aqueous Tween 80 (dispersant). The conidial suspension was sprayed using Preval® TLC sprayer till runoff point. In the controls 0.02% aqueous Tween 80 was sprayed. The inoculated leaves in exposure method of L+Lf and only Lf were air dried for 20 minutes and transferred to cylindrical containers (11 cm diameter x 8 cm) lined with moist filter paper. The larvae were allowed to feed treated leaves for 24 h. The cleaning of the containers and larval survival was monitored daily. The larva was taken in account as dead when there was no visible movement by prodding. The mortality, however, was determined after appearance of white mycelia on the cadavers.

Statistical designs and analyses: The study was conducted in a two factor factorial design {exposure methods (3) x concentrations (5 including control)} with five replications. The larval mortality over time was corrected according to Abbott's correction formula Abbott (1925). The larval infectivity (mortality) was analysed by

2 Way Analysis of Variance using Minitab 15.1 Statistical Software. The means were analysed by pair-wise contrast using Tukey's HSD test. The median effective concentration (EC_{50}) and the median effective time (ET_{50}) were calculated on the basis of cumulative mortality at day 7, after inoculation. The EC_{50} and ET_{50} were estimated using Probit Programme Version 1.5 (U.S. Environmental Protection Agency). The Intercept and slope values were calculated with linear regression analysis, while χ^2 test was used to find out heterogeneity in larval population. Regression analysis was applied to workout correlation between concentration and larval infectivity.

RESULTS

The results showed a highly significant ($p>0.01$) effect of exposure method, concentration and interaction between exposure method and concentration on *B. bassiana* infectivity. The highest pooled infectivity (mortality) across the tested concentrations was 94.6±2.4%, when both larvae and leaves were inoculated, while the lowest infectivity was 38.1±3.2%, when only leaves were inoculated (Fig. 1). There was a significant (Tukey's HSD; $p < 0.05$; CV=2.75) difference in infectivity among three tested exposure methods. The overall infectivity decreased by 2.5 times when only leaves were inoculated as compared to both larvae and leaves inoculated.

The highest pooled infectivity across the tested exposure methods was found at a concentration of 1×10^8 conidia mL^{-1}, while the lowest infectivity was found at a concentration of 5×10^6 conidia mL^{-1}. The effect of concentration varied significantly (Tukey's HSD; $p<0.05$; CV = 3.49) from one another (Fig. 2). The highest difference between two successive concentrations was 20.5% between concentration of $5x10^6$ conidia ml^{-1} and 1×10^7 conidia mL^{-1}, while the lowest difference was 9.0% between concentration of 5×10^7 conidia mL^{-1} and 1×10^8 conidia mL^{-1}. The overall increase in infectivity was 1.4 times between concentrations of 5×10^6 conidia mL^{-1} and 1×10^8 conidia mL^{-1}.

There was a significant linear correlation between concentration and infectivity in exposure methods including: when both larvae and leaves were inoculated ($Y = 0.162+12.84X$; $R^2 = 0.99$), when only larvae were inoculated ($Y = -1.053+12.43X$; $R^2= 0.98$) and when only leaves were inoculated ($Y = -1.129+10.29X$; $R^2 = 0.76$). The results revealed greater effect of exposure methods on infectivity of *B. bassiana* compared to the concentrations.

There were significant (Tukey's HSD; $p<0.05$; CV = 7.84) differences among interactions between exposure methods and concentrations. The infectivity was cent percent when both, larvae and leaves and only larvae were inoculated at concentrations of 5×10^7 conidia mL^{-1} and 1×10^8 conidia mL^{-1}, while the lowest infectivity was when only leaves were inoculated at a concentration of 5×10^6 conidia mL^{-1} (Table 1). There was not a significant difference between when both larvae and leaves were inoculated and only larvae were inoculated at all tested concentrations except concentration of 1×10^7 conidia mL^{-1}. The infectivity was significantly less, when only leaves were inoculated. The concentration effect within exposure method was significantly linear when both, larvae and leaves and only larvae were inoculated, while this was linear but not significant when only leaves were inoculated (Table 1).

Table 1: Effect of exposure method and concentration on larval infectivity

Concentration (conidia mL^{-1})	Exposure methods		
	L+ Lf±SE	L±SE	Lf ±SE
5×10^6	82.5±1.67^{bc}	75.4±1.58^{c}	18.0±1.54^{f}
1×10^7	95.9±1.52^{a}	84.9±1.28^{b}	38.3±1.38^{e}
5×10^7	100.0±0.0^{a}	100.0±0.0^{a}	41.6±2.89^{e}
1×10^8	100.0±0.0^{a}	100.0±0.0^{a}	54.1±1.74^{d}

Means within columns as well as within rows with same letter are not significantly different ($p>0.05$, Tukey's HSD)

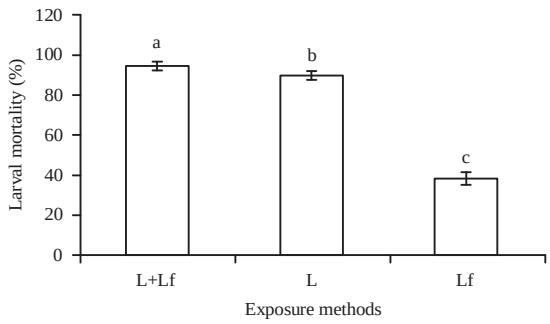

Fig. 1: Pooled infectivity (%) of *A. sciodoxa* in different exposure methods

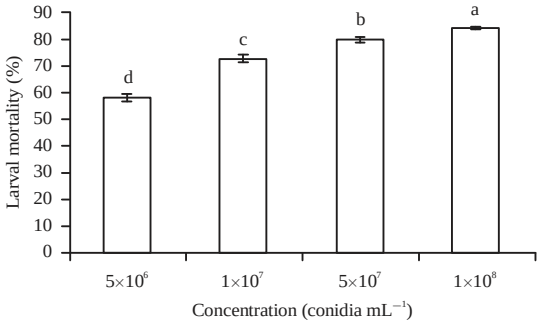

Fig. 2: Pooled infectivity (%) of *A. sciodoxa* caused by different concentrations

Table 2: Median effective concentration in three exposure methods

Exposure methods	EC_{50} conidia mL^{-1}	95% fiducial limits		Slope±SE	χ^2-value
		Lower	Higher		
L+Lf	9.7×10^5	0.0	25.8	1.94±0.97	0.06
L	16.1×10^5	1.55	29.8	1.89±0.59	0.54
Lf	397.2×10^5	200.1	1162.0	0.54±0.15	3.00

Table 3: Median effective time in different exposure methods and concentrations

Exposure method	Concentration (conidia mL^{-1})	ET_{50} (days)	95% fiducial limits		Slope±SE	χ^2-value
			Lower	Higher		
L+Lf	5×10^6	4.9	2.9	5.2	6.02±0.66	4.24
	1×10^7	4.2	4.0	4.5	6.51±0.65	5.25
	5×10^7	3.5	3.3	3.7	7.08±0.74	3.23
	1×10^8	3.3	3.1	3.5	9.16±1.08	3.98
L	5×10^6	5.2	4.8	5.5	5.20±0.66	2.45
	1×10^7	4.4	4.1	4.7	5.57±0.63	0.19
	5×10^7	3.9	3.7	4.2	8.31±0.80	3.24
	1×10^8	3.5	3.3	3.6	9.54±1.05	5.66
Lf	5×10^6	-	-	-	-	-
	1×10^7	7.6	6.9	10.2	6.13±1.80	0.95
	5×10^7	6.8	6.2	7.7	4.91±0.85	4.88
	1×10^8	6.3	5.8	7.1	4.66±0.77	0.84

The median effective concentrations also varied considerably among the three tested exposure methods. The lowest EC_{50} was found when both larvae and leaves were inoculated, while the highest was found when only leaves were inoculated (Table 2).

There was also a highly significant ($p<0.01$) effect of exposure method and concentration on the median effective time. The shortest pooled ET_{50} was observed when both, larvae and leaves were inoculated, while the longest ET_{50} was observed when only leaves were inoculated. There was a significant (Tukey's HSD; $p<0.05$; CV = 0.13) difference in ET_{50} among three tested exposure methods. The shortest pooled ET_{50} among exposure methods was observed at a concentration of 1×10^8 conidia mL^{-1}, while the longest ET_{50} was observed at a concentration of 5×10^6 conidia mL^{-1}. The ET_{50} values were influenced significantly (Tukey's HSD; $p<0.05$; CV = 0.18) by concentrations.

The interaction median effective time between exposure method and concentration varied considerably. The shortest median effective time was when both larvae and leaves were inoculated at a concentration of $1x10^8$ conidia mL^{-1}, while the longest was when only leaves were inoculated at a concentration of 1×10^7 conidia mL^{-1} (Table 3).

DISCUSSION

The present study showed that exposure method has had highly significant effect on infectivity of *B. bassiana*. The most effective exposure method was to inoculate both larvae and leaves. There was an augmentation in loading of conidia through secondary acquisition from spray residues on the foliage. The secondary acquisition was further substantiated by the infectivity recorded when unexposed larvae were exposed to inoculated leaves. Secondary acquisition of conidia enhanced *B. bassiana* infectivity by increasing number of inoculated individuals.

The median effective concentration also indicated considerable effect of secondary inoculation in different exposure methods. When both larvae and leaves were inoculated, the median effective concentration was 0.6 times greater as compared to when only larvae were inoculated. This indicates an acquisition of considerable amount of infective conidia from spray residues. The slope values also indicate early high mortality when both larvae and leaves were inoculated. The chi-square (X^2) test was not significant for the three tested exposure methods, thus indicated a non-significant heterogeneity of *A. sciodoxa* against *B. bassiana*.

The present findings are in corroboration with Fernandez *et al*. (2001), who reported a mortality of 77.8% and 34% in *Leptinotarsa decemlineata*, when larvae were sprayed directly and exposed to inoculated leaves followed by only larvae were sprayed directly and the larvae exposed only to un-inoculated leaves. The difference in present infectivity response and that reported by Fernandez *et al*. (2001), may be explained in terms of different fungus isolate-insect combination, feeding and foraging behaviour of the insect pest and degree of mobility of a particular host stage (Ugine *et al*., 2005a),

physical and chemical characteristics of foliage surface (Inyang *et al*., 1998; Poprawski and Jones, 2001; Kouassi *et al*., 2003), susceptibility of inoculums-recipient body organs (Miranpuri and Khachatourians, 1991) and insect developmental stage (Carruthers *et al*., 1985). Mortality through residual contact or secondary acquisition has also been reported in the Western flower thrips, *Frankliniella occidentalis* (Ugine *et al*., 2005b) and the cabbage looper, *Trichoplusia ni* (Behle, 2006).

The present findings also showed that the median effective time of given isolate-insect combination varied with exposure method. The ET_{50} was shortest when both larvae and leaves were inoculated and the longest when only leaves were inoculated. Apart from exposure methods, an inverse correlation was found between concentration and ET_{50}. The overall trends of median effective times showed that higher the infectivity would have shorter median effective times. These findings are similar to Tefera and Pringle (2003), who obtained significantly different mortality levels and longest ET_{50} in exposure method when only leaves were inoculated in the second instar larvae of the spotted stem borer, *Chilo partellus*, when sprayed directly with conidia, exposed to conidia-treated foliage or dipped into conidial suspension.

The higher loading of infective conidia in larvae inoculated directly, as well as, exposed to inoculated leaves may be due to later-on attachment of conidia when larval body was extended during feeding, whereby inter-segmental regions were exposed. Moreover, during crawling inter-segmental regions and other ventral body parts, like prolegs, crochets and anal region, come into contact with leaf surface. On the other hand, when larvae are inoculated directly their body may shrink thus covers intersegmental regions and also their ventral body surface is not exposed fully to direct spray. Apart from variation in conidial loading in different exposure methods, mode of exposure may also influence conidial attachment and germination on insect cuticle. The conidial lodging within cuticular folds either by direct exposure or by secondary acquisition facilitates attachment, germination and penetration of conidia which enhances the infectivity level.

CONCLUSION

Based on these findings it is concluded that exposure method vis-à-vis conidial concentration have paramount importance for infectivity of *B. bassiana*. More than 95% larval mortality can be achieved when both larvae and leaves are inoculated at a concentration of $1x10^7$ conidia mL^{-1} within 3-4 days. More than 50% larval mortality is possible through secondary acquisition at concentration of $1x10^8$ conidia mL^{-1} which is a promising sign for using *B. bassiana* for control of medicinal plants.

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