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Effects of *Metarhizium brunneum* and *Aspergillus* spp. on the emergence of coconut leaf beetle parasitoid, *Tetrastichus brontispae*

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Abstract. Integrated pest management (IPM) for *Brontispa longissima*, have regarded the discrete utilization of *Tetrastichus brontispae* and *Metarhizium* spp. as the most effective control means for the said pest. Combined or additive applications of these two different biocontrol agents have not been documented, although many parasitoids have been found compatible with entomopathogenic fungus (EPF), which resulted to a more efficient pest management. Yet, information is lacking on the susceptibility of *T. brontispae*, to the commonly used Green Muscardine Fungi (*Metarhizium* spp.). To fill in this gap, we assessed the susceptibility of parasitoid, *T. brontispae* to an exotic obligate entomopathogen, *Metarhizium brunneum* (*M.b*) and ubiquitous opportunistic *Aspergillus* spp. representing sections *Fumigati* (*Asp01*) and *Flavi* (*Asp02*), previously observed to infect *B. longissima* at $1x10^5$ conidia mL^{-1} concentration. By evaluating the emergence and survival of *T. brontispae*, the three fungal isolates were found to be pathogenic to the parasitoid, where a significant decline in number of emerging parasitoids was observed. This negative effect on parasitoids by fungal species pathogenic to *B. longissima* may complicate or suggestively prevent simultaneous use of EPF with parasitoids as biocontrol agents.
Key Words: biocontrol, *Brontispa longissima*, entomopathogen, integrated pest management.

Introduction. Suppression and control of *Brontispa longissima* (Coleoptera: Chrysomelidae) population by applying pesticides brought unsatisfactory results (Pimentel 2000; Hosang et al 2004), as chemical control practices have caused development of pest resistance, toxic effects to natural enemies, and threats to crops and non-target organisms, public health and the environment (Nguyen et al 2004). In the latter years of managing *B. longissima* - locally known as coconut leaf beatle (CLB), introduction of entomopathogenic fungi (EPF) particularly *Metarhizium anisopliae* and *Beauveria bassiana*, provided satisfactory solution, and has consequently been used in many affected coconut (*Cocos nucifera*) producing countries (Zimmermann 1993; Nguyen et al 2004). Biological control agents (BCA) other than microorganisms are also used as alternative means to minimize pest populations (Dean et al 2012). Eulophid parasitoids are used to attack the larval and/or pupal stages of CLB (Nakamura et al 2006; Chen et al 2010). *Asecodes hispinarum* is a valuable parasitoid used in parasitizing larva of pests (Pundee 2009) and *Tetrastichus brontispae*, a pupal parasitoid, which originated from Java, Indonesia, has been successfully imported to many countries to effectively curb CLB infestation (Cochereau 1969; Boheman et al 1979; Stapley 1979; Muniappan et al 1980; Stechmann & Semisi 1984; Halfpapp 2001).

Integrated pest management (IPM) for *B. longissima*, has regarded the discrete utilization of *Metarhizium* spp. or *T. brontispae* as the most effective control means for the said pest (Rethinam & Singh 2004; Hosang et al 2004; Chen et al 2010). Combined or additive application of *T. brontispae* and *Metarhizium* spp. has not been documented, although many parasitoids and predators have been found compatible with the EPF and the combination of BCA resulted to a more efficient pest management (Labbé et al 2009;

Gao et al 2012; Aiuchi et al 2012; Wu et al 2015). However, few studies have also shown the susceptibility of many parasitoid species to fungal infection (Furlong & Pell 1996; Danfa & van der Valk 1999; de la Rosa et al 2000; Lord 2001), characterized by reduced parasitoid survival, additional sublethal effects such as behavioral changes, reduced feeding, or decreased fecundity have also been observed (Madelin 1963; Tanada & Kaya 1993; Hajek & St. Leger 1994). Yet, information is lacking on the susceptibility of *T. brontispae*, to the commonly used Green Muscardine Fungi (*Metarhizium* spp.). Aside from the commonly utilized EPF, the presence of other opportunistic fungal pathogens such as the previously described effects of *Aspergillus* species on CLB (Pajar et al unpublished data) may pose similar problem for parasitoid-assisted pest management considering acknowledged natural interactions.

In this study we evaluated the susceptibility of parasitoid *T. brontispae* to three fungal parasites of *B. longissima* – an exotic obligate entomopathogen, *Metarhizium brunneum* (M.b), usually introduced by aerial spray for foliar insect pests, and ubiquitous opportunistic *Aspergillus* species representing sections Flavi (Asp02) and Fumigati (Asp01). We hypothesize that as members of the phylum Arthropoda, *T. brontispae* may also be susceptible to the fungal pathogens at conidial concentration that efficiently kill *B. longissima*.

Material and Method. The experiment was conducted at the Microbiology laboratory, Department of Biological Sciences, Mindanao State University-Iligan Institute of Technology, Philippines from August 2014 to January 2015.

Fungal cultures. *Aspergillus* spp. previously identified to be representatives of sections Fumigati (Asp01) and Flavi (Asp02) were cultured on Saboraud Dextrose Agar in 90 mm Petri dishes at 25±1°C. The commercially available EPF strain *Metarhizium brunneum* was cultured from ®Met52, Novozymes South Asia Pvt Ltd. All pure isolates were incubated in the dark at 25±1°C for 15 days (Goettel & Inglis 1997) and were then stored at 4°C until used.

Preparation of conidial suspension. After growth and sporulation, conidia and hyphae were harvested by washing the culture with 10-15 mL of 0.05% Triton X-100 solution to produce fungal suspension. Fungal suspensions were filtered from mycelium and other substrates using filter paper fitted on a glass funnel and placed on 100 mL beaker. Conidial concentrations were determined by standard count using Neubauer haemocytometer and suspensions were adjusted to obtain a concentration of $1x10^5$conidia mL^{-1}, which was considered as lethal dose to the target organism (Ugine et al 2005).

Bioassay. Mummified pupae of *B. longgisima* containing the pupal stage of *T. brontispae* were obtained from Philippine Coconut Authority – Davao Research Center. Rounded plastic containers with a meshed hole in the lid were contained with damped filter paper to provide moisture. Three mummified pupae containing approximately 50 adult parasitoids were immersed simultaneously for 5 seconds in 750 µL of conidial suspension (*M. brunneum*, Asp01 and Asp02), including a negative control of 0.05% Triton X-100 solution. This step was replicated thrice for each of the treatments. After immersion, pupae were transferred to the sterile plastic containers. All set-ups were incubated for seven days at room temperature and maintained with moisture by damping the filter paper with sterilized distilled water. Emergence and survival of parasitoids were checked daily.

Statistical analyses. Data were subjected to analysis of variance (ANOVA) and were further analyzed in Tukey-Kramer HSD to test for significant differences on pairs of treatments using JMP 7.0 (Windows, SAS Institute, Inc. 2007).

Results and Discussion. The bioassay was performed two days before the expected time of emergence, where approximately 50 adult *T. brontispae* were expected to

emerge from a mummified *B. longissima* pupa. We observed a delay in the emergence of the parasitoids (Figure 1), and further delay was exhibited by parasitoids administered with fungal treatments, particularly that of Asp01 and Asp02-treated set-ups.

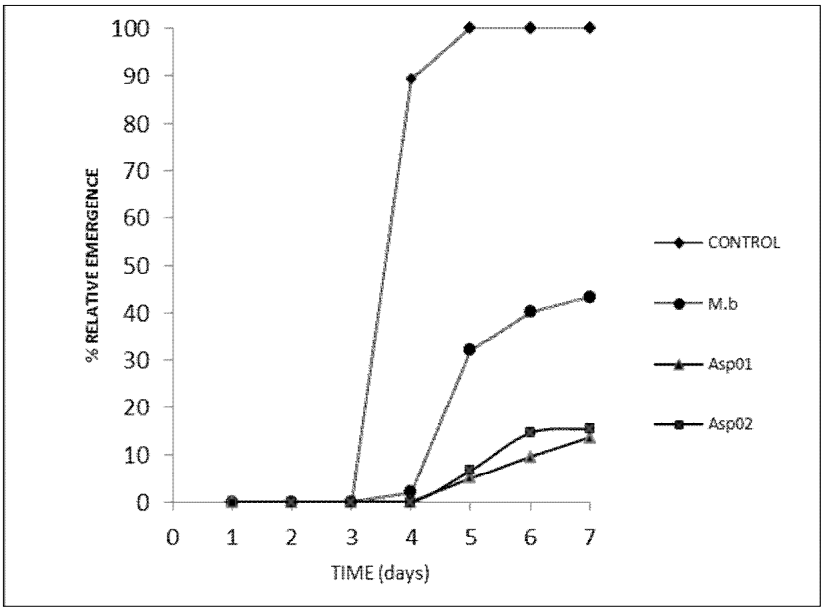

Figure 1. Relative percentage of emerging *T. brontispae*, 7 days after inoculation (DAI) (5 days after expected time of emergence).

Relative emergence was comparatively referred to the control set-up to get an estimate count of the total parasitoids expected to emerge. A hundred percent emergence was estimated to occur five days after the estimated day of first emergence. Reduced count of emerging parasitoids was perceived from mummies treated with Asp01 and Asp02, while only 43% of the parasitoids emerged from mummies treated with the EPF *M. brunneum* relative to the control.

A two-way ANOVA was conducted to examine the effect of treatment and time on the emergence of *T. brontispae*, two days after inoculation (DAI). There was no statistically significant interaction between the effects of treatment and time on the emergence of parasitoid [F (6, 24) = 0.063, p = 0.999]. Simple main effects analysis showed that the number of emerging parasitoids were significantly more influenced by treatment – the presence or absence of fungal species in the inoculum [F (3, 24) = 19.5, p < 0.001] (Table 1).

Table 1

Results of the two-way ANOVA of time and treatment on the emergence of *T. brontispae*

Effect	*Sum of squares*	*Degrees of freedom*	*Mean squares*	*F-statistic*	*P-value*
Treatment	385	3	128	19.5	0.000001*
Time (days)	6.49	2	3.25	0.4922	0.617315
Treatment x Time	2.48	6	0.413	0.0627	0.998824

*p ≤ 0.001.

Since treatments posed significant effect on parasitoid emergence, a one-way between subjects ANOVA was conducted to compare the effect of each fungal treatment with that of 0.05% Triton X-100 solution (control). There was a significant effect of the different fungal parasites at p < 0.01 [F(3, 8) = 10.887, p = 0.003]. Post hoc comparison using the Tukey-Kramer HSD test indicated that the mean score for the control (M = 100, SD =

12.2) was significantly different than the *M. brunneum*-treated (M = 43.3, SD = 12.2), Asp01-treated (M = 13.8, SD = 12.2) and Asp02-treated (M = 15.4, SD = 12.2) set-ups (Figure 2).

Our results show susceptibility of *T. brontispae* to infection by *M. brunneum* and high susceptibility to Asp01 and Asp02 at concentrations pathogenic to *B. longissima* under laboratory conditions. Although EPF is said to be precise in infecting and targeting only certain type of host (Butt et al 1994; Thungrabeab & Tongma 2007) and despite the many studies reporting compatibility of *Metarhizium* spp. and parasitoids (de la Rosa et al 2000; Labbé et al 2009; Gao et al 2012; Aiuchi et al 2012; Wu et al 2015), our results depicted otherwise. In a study conducted by Thungrabeab & Tongma (2007), some species of parasitoids were found to be infected by certain isolates of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. It has been suggested that fungi could be the reason for drastic reductions in numbers of parasitoids of insect pests (Falcon 1974).

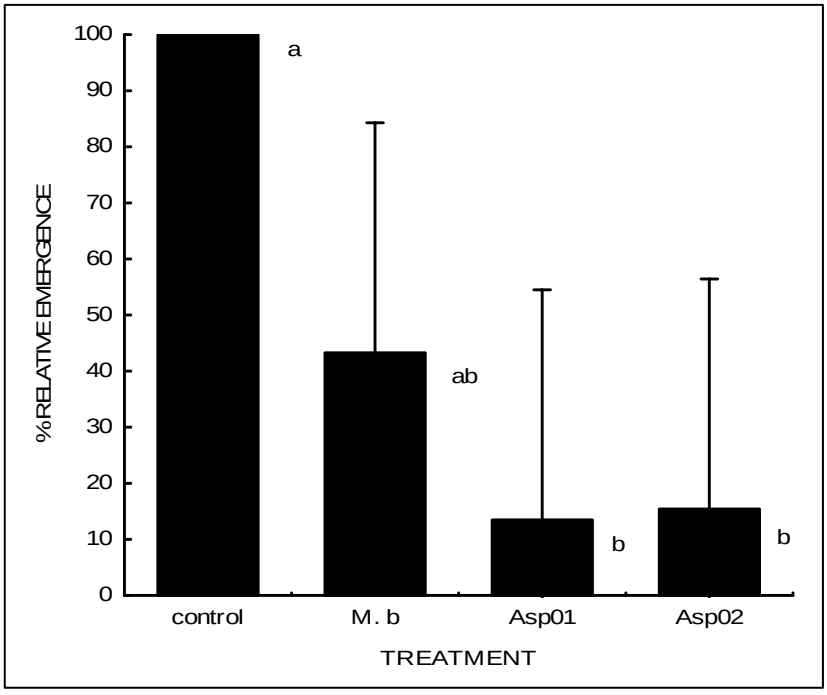

Figure 2. Relative emergence of *T. brontispae*, 7 DAI. Levels not connected by the same letter are significantly different [F(3, 8) = 10.887, p = 0.003]. Vertical lines denote 0.99 confidence intervals.

The significant decline on the percentage of emerging parasitoids from Asp01- and Asp02-treated set-ups shows susceptibility of *T. brontispae* towards ubiquitous fungal pathogens, which may naturally occur alongside with, or as a consequence of infection by introduced microbial BCA (Hughes & Boomsma 2004). Negative effects of naturally occurring fungal species on parasitoids may complicate, or possibly prevent simultaneous use of *M. brunneum* with *T. brontispae* as biocontrol agents.

The results suggest the use of *M. brunneum* in *B. longissima* IPM, but it is necessary to pay attention to its impact on the parasitoids by applying the fungus at a time favourable for the survival of the parasitoids, and by using techniques which minimise parasitoid exposure to the pathogen. Thus, the reduced emergence of the parasitoid *T. brontispae* could suggest that combination of biocontrol agents may further be subjected to modification in pest management taking into consideration the time at which the parasitoids are exposed to the pathogen which can be of great importance as far as a possible infection is concerned.

Conclusions. *T. brontispae* was noted to be highly susceptible to the EPF *M. brunneum* as well as to ubiquitous fungal parasites Asp01 and Asp02. Our results suggest that the release of *T. brontispae* is not to be accompanied by, or follow after EPF application.

Moreover, presence of *Aspergillus* spp. also appeared to be a threat to *T. brontispae*. The experimental setup was simplified by optimum laboratory conditions and does not necessarily reflect the complexities of the multidimensional environment of a field situation. In the field, the parasitoids may not acquire infections until later in their reproductive life and thus might be less affected by fungal exposure than these laboratory results would indicate.

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