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Full Length Article

Effectiveness of Isolates of *Metarhizium anisopliae* against *Aphis gossypii* (Hemiptera: Aphididae) on *Capsicum annum* and *Solanum melongena*

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Abstract

The virulent of five *Metarhizium anisopliae* isolates (PR1, GT2, TFFH3, GJ4 and HSAH5) at the concentration of 1 x 10^7 conidia mL^{-1} against *Aphis gossypii* reared on *Capsicum annum* (chilli) and *Solanum melongena* (brinjal) was evaluated *in vitro* by leaf dipping method. There was a significant different between isolates on the virulent against first nymphal instar of *Aphis gossypii* for both crops ($F = 341.38$, $P < 0.01$). The PR1 and GJ4 isolates of *M. anisopliae* were the most virulent with PR1 showing mortality of 99 and 100% on *C. annum* and *S. melongena* respectively. The LC_{50} of PR1 isolate on the aphid reared on chilli (3.76×10^5) was lower than aphid reared on brinjal (9.68 x 10^5). Among the selected isolates, PR1 treatment on chilli had 18.6 times lower LC_{50} (3.76×10^5 conidia/mL) compared to GJ4 on chilli (7.00×10^6 conidia/mL). The virulent of PR1 assessed against all life stages of aphid showed significant difference between life stages of *A. gossypii* (1st, 2nd, 3rd, 4th and adult) ($F = 163.52$, $P < 0.01$) with the most susceptible was first instar follow by its subsequent stages and the least susceptible was adult stage. The result indicates that the isolate PR1 has potential as biological control agent of the aphid and application at the earlier stages should be utilized in controlling the aphid.

Keywords: Virulent; *Metarhizium anisopliae*; *Aphis gossypii*; Brinjal; Chilli

Introduction

Aphis gossypii Glover (Hemiptera: Aphididae) is a cosmopolitan and a polyphagous sap sucking insect pest infesting numerous economic important crops worldwide includes tropical, subtropical and temperate areas (Singh and Singh 2015; Wang *et al.* 2016; Liang *et al.* 2019). The aphid is a serious pest of brinjal and chilli causing injury by direct feeding, indirectly produce sooty mould and serves as a vector of many plant viral diseases (Satar *et al.* 1999; Singh and Singh 2015; Jaharlal *et al.* 2016). Controlling the aphid using insecticides is the most popular method and the neonicotinoids have proven to be more reliable than the older chemicals for controlling aphid pests of brinjal, as with other Solanaceae (Dewar 2007). However, a total reliance on chemical control shows there was a limit to its effectiveness and profitability because *A. gossypii* developed resistance to the insecticides (Matthews 2000). The alternative is to use the microbes. Entomopathogenic fungi (EPF) causing diseases in insects by infesting through the insect cuticle and do not require to be ingested, display a great potential for controlling sap sucking insects such as aphids and whiteflies (Pedrini *et al.* 2007). Most EPF used for controlling of sap sucking insect whitefly, *Bemisia tabaci* and aphid, *A. gossypii* based on *Verticillium lecanii*, *Paecilomyces fumosoroseus* and *Beauveria bassiana* (Faria and Wraight 2001; González-Mas *et al.* 2019). In Malaysia, most EPF research focuses on economic important pest for industrial crop palm oil and only some significant vegetable pest including Lepidoptera, Isoptera and Coleoptera (Nugroho and Ibrahim 2007; Lin *et al.* 2017). Among all Hyphomycete fungus, only *Lecanicillium lecanii* was reported naturally occur on aphid and a few studies were confirmed *L. lecanii* highly virulent against three aphids' species *M. persicae*, *A. gossypii* and *Brevicoryne brassicae* (Milner 1997; Alavo *et al.* 2001).

The development of EPF is a strategy in Integrated Pest Management (IPM) to utilize eco-friendly alternative to reduce our reliance on chemical pesticides that mainly used against sap sucking insect and to secure food safety at the same time as conserving nature (Sani *et al.* 2020). Research of using *M. anisopliae* against *A. gossypii* and other sap-sucking insect are still limited in Malaysia. As such, to the best of found knowledge, this is the first research of using *M. anisopliae* against *A. gossypii* on family of Solanaceae in Malaysia. It is postulated that *M. anisopliae* isolate was

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effective against *A. gossypii* on *S. melongena* and *C. annum*. The objectives of this study were: 1) to determine effectiveness of selected *M. anisopliae* isolates against *A. gossypii* 2) to determine the concentration response of the most promising among the isolates and 3) to compare the susceptibility of first, second, third, fourth instars nymph and adult stages of *A. gossypii* on chilli and brinjal.

Materials and Methods

This study was conducted in Insectary and Forest Entomology Laboratory at Faculty of Forestry, University Putra Malaysia. The plants were brinjal, *S. melongena* and chilli, *C. annum*. During the colony establishment to the period of laboratory test *A. gossypii* was continuously provided with pesticide free new plants every two weeks.

The *A. gossypii* population originated from Agro technology Sepang was collected from the field by taking the infected foliar, plant part or whole plant from several different areas of crop of brinjal and chilli. The varieties of the sources of plant were recorded. The collections were brought back to the Insectary Room and transferred to the newly prepare fresh plant. The *A. gossypii* population was transferred and mass-reared in separate insect cages accordingly to the plant they were collected to establish new colony of brinjal and chilli host-plant. In order to obtain a uniform age population of *A. gossypii*, 50 to 100 adults from insect cage were transferred to young green brinjal and chilli plants for 24 to 36 h followed by the removal of all adults afterward. The newly infected plant with first instar remained in the transfer area for further development of homogeneous populations under control environment at 26 ± 2℃ temperature, 65% RH with a 14:10 (L:D) photoperiod.

The isolates were obtained from stock cultures held at the Forest Entomology Laboratory, Faculty Forestry of Universiti Putra Malaysia and Table 1 shows the locations where they were collected. Cultures were maintained on Sabouraud Dextrose Agar (SDA). In order to obtain conidial, fungal isolates were cultured on cooked rice with 0.1% yeast extract for at least ten days at 25℃. The conidia were harvested by sieving through a 125-μm particle size laboratory test sieve (Endecotts, London, England) to remove mycelial mats. The concentration of spores in the final suspension was determined by Neubauer Haemocytometry. Viability of conidia was assessed before preparation of suspensions by germinating tests. Serial dilutions of the suspension were prepared and the conidial suspension used was adjusted by diluting conidia with 0.05% Tween 80 (Oliveira *et al.* 2015). In all experiments, germination rates higher than 80% after 24 h at 25℃.

Virulent evaluation of isolates against *A. gossypii*

The immersion bioassay procedure for screening test assay and dosage/concentration response assay known as leaf dip method was modified from (Cahill *et al.* 1995; Quesada-Moraga *et al.* 2006). The first instar of *A. gossypii* was used for all screening bioassay procedures. The individual *S. melongena* leaf discs (≈40-mm diameter) with 20 nymphs each were used immediately upon excision. These leaf discs with nymphs were immersed for 10s in 20 mL of 1.0×10^7 conidia/mL conidial suspension added with 0.02% Tween 80 (Pham *et al.* 2010). Treated leaves were placed on filter paper for 15 ± 5 min to remove excess moisture. The leaves were then placed in 5% water agar 90 × 15 mm diameter petri plates, which were sealed with Parafilm and incubated in a growth chamber under control environment at 25 ± 2℃ and 14:10 (L:D) photoperiod. Relative humidity was maintained close to 100% by using water agar in each petri plate than using wet filter paper. For aeration purposes, each plate was opened daily for 15 ± 5 min. This procedure was necessary to minimize development of saprophytic fungi on *A. gossypii* honeydew. The screening test on *C. annum* leaf follows the same procedure as *S. melongena* except that the individual *C. annum* leaf discs were about 20 mm diameter and the *A. gossypii* were transferred to the leaf discs following dipping into the conidial suspension.

The total numbers of dead *A. gossypii* nymphs was counted and recorded daily for seven days based on (Quesada-Moraga *et al.* 2006) as follows:

a) Nymphs desiccated and/or discolored and mycosed individuals
b) Nymph become moribund and were gradually covered by mycelia
c) Symptomatic nymph pale yellowish/white mycelia/ light to darker green coloration (Sajap and Kaur 1990)
d) Fungal outgrowth or conidiation on the cadavers
e) Nymphal meet above condition and unresponsive after provoked using brush or fine forceps.

The percentage mortality data of diseased insects were corrected by Schneider-Orelli's formula (Püntener 1981). Two-way Analysis of variance (ANOVA) was performed using the statistical package SAS for comparison of isolates on two crops. The test was arranged in Completely Randomized Design (RCD) with four replicates. All isolates were assayed at one time, using randomized groups of insects from a single batch. Four replicate infested leaf pieces were used for each isolate, and the whole experiment was repeated with a new batch of insects and new conidial suspensions. In the virulence bioassays, isolate that causing the greatest mortality against *A. gossypii* was selected for further test.

Concentration response bioassay

Two isolates that gave the highest percentage of mortality against first nymphal instar of *A. gossypii* was used in determination of the LC_{50}. Eight concentrations of conidia 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 1×10 conidia/mL plus one control were used to estimate LC_{50}. Each concentration consisted of four

replicates and each leaf pieces contain 30 individuals of *A. gossypii* nymphs. Control leaf pieces were treated with a solution of 0.02% Tween 80 (Pham *et al.* 2010). The total number of dead and infected *A. gossypii* was recorded daily for seven days. The percentage of mortality data were corrected using Schneider-Orelli's formula (Püntener 1981) and LC_{50} value was calculated by Probit Analysis (statistical package Polo Plus version1), based on (Finney 1971). Relative median potencies and their 95% confidence intervals were calculated for different treatments when their slopes did not differ significantly.

Toxicity of *M. anisopliae* against life stage of *A. gossypii*

The method was similar to the screening test except different life stages of *A. gossypii* was used in this study. The life-stage of *A. gossypii* tested were first, second, third, fourth instar and adult. The concentration used for the test was from the lowest LC_{50} of the concentration response for both isolates.

Statistical Analysis

There were four replicates for each bioassay. The mortality of diseased insect was recorded daily up to seven days treatment. The percentage mortality data of diseased insects were corrected by Schneider-Orelli's formula. Two-way Analysis of variance (ANOVA) was performed using the statistical package SAS for comparison of two factor which were isolates and life stages.

Results

Virulent evaluation of isolates against *A. gossypii*

Isolate PR1 and GJ4 caused the highest mortality of *A. gossypii* on both of host plant *S. melongena* and *C. annum* (Fig. 1). The result showed that there was significant difference between isolates (PR1, GT2, TFFH3, GJ4 and HSAH5) with (F = 341.38, $P < 0.01$), which indicated the isolate was associated with the mortality rate. Similarly, there was significantly different between two crops (*S. melongena* and *C. annum*) with (F = 43.47, $P < 0.01$), which indicates that the crop was associated with the mortality rate of the *A. gossypii*. However, the interaction between isolates and crops was not significantly different. Therefore, the most effective isolates were PR1 and GJ4 on both crops (*S. melongena* and *C. annum*) while the lowest isolates were GT2, TFFH3 and HSAH5. All the isolates applied caused > 80% mortality rate on *S. melongena* indicates that the crop may possibly cause the pest susceptible to infection of fungal compared to *C. annum*.

Concentration response bioassay

The most effective isolate PR1 and GJ4 were selected

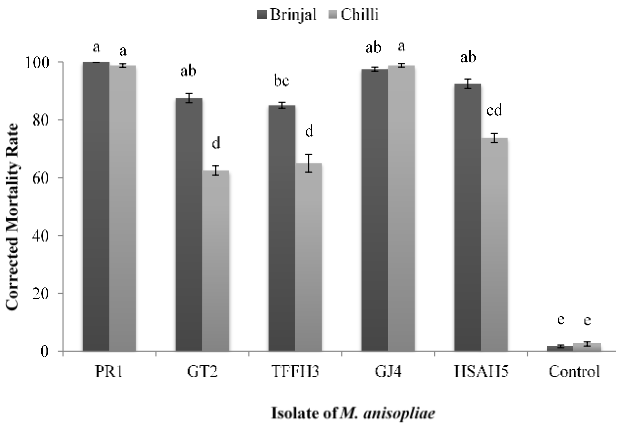

Fig. 1: Percentage Mortality (± SEM) of first instars nymphs of *A. gossypii* on *S. melongena* (black bars) and of on *C. annum* (grey bars) by conidial suspensions several isolates of *M. anisopliae* (1 x 10^7 conidia/mL). For each aphid nymph, bars with the same letter are not significant difference

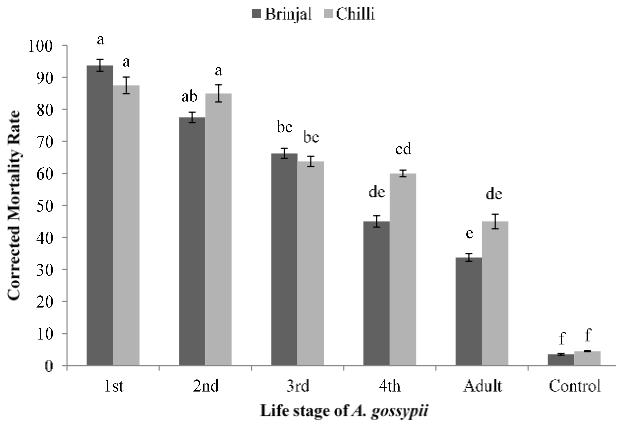

Fig. 2: Percentage Mortality (± SEM) of life stages of *A. gossypii* with brinjal (black bars) and of chilli (grey bars) by conidial suspensions of *M. anisopliae*. For each *A. gossypii* nymph, bars with the same letter are not significant difference

from the screening test for the concentration response bioassay. The standard selection for most effective isolate was confirmed of more than 97% of mortality rate after seven days application of the fungus. Among the selected isolates, PR1 treatment on chilli had 18.6 times lower LC_{50} (3.76×10^5 conidia/mL) compared to GJ4 on chilli (7.00×10^6 conidia/mL) however no significant difference was observed (Table 2). The LC_{50} of PR1 and GJ4 against *A. gossypii* reared on chilli and brinjal showed no significant difference due to overlapping of 95% fiducial limit.

Toxicity of *M. anisopliae* against life stage of *A. gossypii*

The result showed that there was significant difference between life stages of *A. gossypii* (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and adult)

Table 1: *M. anisopliae* isolates obtained from the Faculty of Forestry, Universiti Putra Malaysia stock culture and the locations where they were collected

Code of Isolates	Location	GPS
PR1	Pantai Remis (Isolate 1), Perak	4°26' 59.99" N, 100°37' 59.99" E
GT2	Gunung Tahan (Isolate 2), Pahang	4°37' 59.99" N, 102°13' 60" E
TFFH3	Tranum Forest Fraser Hill (Isolate 3), Pahang	3°43' 60" N, 101°49' 1" E
GJ4	Gunung Jerai (Isolate 4), Kedah	5°34' 59.99" N, 100°22' 59.99" E
HSAH5	Hutan Simpan Ayer Hitam (Isolate 5), Selangor	3°1' 28.87" N, 101°37' 47.08" E

Table 2: Analysis of probit mortality and log-concentration of bioassay with the most effective *M. anisopliae* against first instar nymphs of *A. gossypii*

Isolate [a]	No. of insects	Slope (SE)	LC_{50} [b]	95% FL	Heterogeneity
PR1 (B)	720	6.65 (± 1.67)	9.68×10^5	9.51×10^4 - 2.88×10^6	0.72
PR1 (C)	720	4.19 (± 0.40)	3.76×10^5	1.19×10^5 - 1.29×10^6	1.99
GJ4 (B)	720	8.73 (± 1.51)	3.56×10^6	1.21×10^6 - 8.07×10^6	0.73
GJ4 (C)	720	8.50 (± 1.56)	7.00×10^6	2.21×10^6 - 1.97×10^7	1.10

[a] Mortality of the controls ranged between 0.8 to 4.2% and 4.4 to 6.7% in virulence assays with aphid species respectively
[b] LC_{50} values and their 95% fiducial limits are expressed in conidia per milliliter

($F = 163.52$, $P < 0.01$), indicating that the life stages affect the mortality rate (Fig. 2). In contrast, no significant difference between crops indicating that the two crops did not affect the mortality rate of the *A. gossypii*. Based on the result, the relationship between life stages and mortality rates do not depends on the crops.

Discussion

In this study, the nymph and adult cadaver of *A. gossypii* infected by *M. anisopliae* (all isolates) showed symptom of dehydrated, fall, and occasionally flip from the leaves turned into distinctly darker and covered by white to pale green fungus. Correspondingly with (Batta 2003) after three days of treatment using *M. anisopliae*, infected larvae and pupae of *B. tabaci* turned into black-greenish. The screening test showed positive response of the five isolates of *M. anisopliae* and was highly pathogenic against first instar of *A. gossypii* with more than 97% mortality rate at the concentration 1×10^7 conidia/mL seven DAT. This result showed mortality obtained in this study was at the lower concentration compared to Nazir *et al.* (2019) that found 93% mortality of adult green peach aphid *Myzus persicae* at concentrations 1×10^8 conidia/mL of *B. bassiana* at 10 DAT. Another study by Ashouri *et al.* (2004) also found that the highest mortality 100% reported at 10^7 and 10^8 conidia/mL of *L. lecanii* against third instar nymphs of *M. persicae* at 12DAT.

Based on result, the lowest LC_{50} of PR1 for *A. gossypii* reared on chilli was 3.76×10^5 conidia/mL. This result is in correspondence with Eidy *et al.* (2016) found that LC_{50} value of *B. bassiana* on aphid, *M. rosae* was 2.67×10^5 conidia/mL. Similarly, another study by Nirmala *et al.* (2006) showed that 6.57×10^5 conidia/mL was the LC_{50} value for a *B. bassiana* strain against *A. gossypii* nymphs. Contrarily, Sayed *et al.* (2019) found that the concentration of 4.6×10^6 conidia/mL of *B. bassiana* reduced aphid *M. rosae* infestation on rose plant. Anderson *et al.* (2011) indicated that virulence of EPF against a particular host insect influence by biological properties of the specific isolate-host combination and fungal concentration. The speed of kill is related to the number of conidia received by the individual pest (Bateman *et al.* 1993). Kershaw *et al.* (1999) speculated that the differences in isolate virulence (speed of kill) from the same species due to two reasons: a) an isolate produce immense extent of toxins or b) an isolate center most of their energy into vegetative growth.

The most susceptible life stages of *A. gossypii* reared on *C. annum* was the first stages while the invulnerable life stage was adult stage with the lowest mortality rate. Corresponds, Basit *et al.* (2011) stated that the resistance or vulnerability to any treatment at any given concentration may depend on the life stage of sap sucking insect with nymphs infected with EPF was more susceptible than the adult stage. The first instar was faster to be infected possibly for the reason that the first instar cuticle may vary than cuticle from subsequent instar. These results correspond with Boucias and Pendland (1991) that stated production of appressoria on intersegmental areas or direct hyphal penetration may vary depending on the thickness of the insect's cuticle. Lacey *et al.* (1996) indicated that although later stage resisted the infection yet adults emerging could also die later and presented symptoms of fungal infection.

This study also shows that *A. gossypii* with various life stages were susceptible to fungal treatment irrespective to the host plant the aphid growth. Samih *et al.* (2014) corresponds that the type of host plant indeed had significantly affect on the development, survival and reproduction of sap sucking insect. The mortality of *A. gossypii* on two plants (*S. melongena* and *C. annum*) showed that *A. gossypii* was significantly invulnerable when reared on chilli than on brinjal. This result was comparable

with finding by (Zafar *et al.* 2016) that the mortality of nymphs differed significantly among all the host plant species tested. Comparably, study on other sap-sucking insect known as *Bemisia tabaci* treated with *B. bassiana* showed that percentage mortality was influenced by both host plant and concentration (Santiago-Alvarez *et al*. 2006). However, this study showed that the fungal treatment applied effectively cause mortality to the aphid regardless of the host plant. This indirectly agreed that the effectiveness of treatment based solely on the virulence of the fungal treatment itself than the host-plant the pests grow.

Conclusion

EPF *M. anisopliae* as biological control to suppress and control the infestation of sap sucking insect on two most commonly grown vegetables in Malaysia is an interesting and important approach. All of the isolates used in this study proved to be pathogenic to *A. gossypii*. However, they vary between isolates depend on the concentration the insect received in initial exposure. Two isolates PR1 and GJ4 of *M. anisopliae* showed equivalent high virulence. The finding shows that *A. gossypii* most susceptible stage to PR1 isolate was first instar follow by their subsequent stage and the least susceptible was adult stage. The isolate PR1 has the potential to control sap-sucking pest on chilli and brinjal.

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Author Contribution

N.L. wrote the major part of the article. D.O. constructed and made critical corrections in this paper. A.S.S and R.M.A provided supportive information on methodology and taxonomy. R.R., M.M and T.L.P. provided correction, review and editing

Conflicts of Interest

The authors declare no conflict of interest

Data Availability

All relevant data are within the paper

Ethics Approvals

The research does not involve human or animal subjects and ethics approval is not applicable

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