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SHORT COMMUNICATION

Efficacy of culture filtrates of *Metarhizium anisopliae* against larvae of *Anopheles stephensi* and *Culex quinquefasciatus*

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Abstract Efficacy of culture filtrates of five strains of *Metarhizium anisopliae* isolated from insects were evaluated against *Anopheles stephensi* and *Culex quinquefasciatus*. The culture filtrates released from the strains of *M. anisopliae* in the YpSs and chitin broths were filtered and used for the bioassays after a growth of 7 days. Among the culture filtrates of five strains, *M. anisopliae* 892 was found to be more effective against both the mosquitoes. The LC_{50} values of culture filtrates of *M. anisopliae* 892 in chitin broth was lower than the LC_{50} of culture filtrates in YpSs broth against first and fourth instars of both the mosquitoes. The LC_{50} values of culture filtrates were significantly different between first and fourth instars of *A. stephensi* (t test; $P = 0.0001$) and *C. quinquefasciatus* (t test; $P = 0.02$). The larvae of *A. stephensi* were more susceptible than *C. quinquefasciatus* except in two cases. This is the first report of efficacy of culture filtrates produced by *M. anisopliae* in chitin broth against mosquitoes and have potential as a biological control agent of mosquitoes.

Keywords Chitin · Efficacy · Larvae · Metabolites · *Metarhizium anisopliae* · Mosquitoes · Nutrition

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Introduction

Mosquito vector control is an integral part of controlling malaria [1]. In India, *Anopheles stephensi* and *Culex quinquefasciatus* are two major vectors of malaria and filariasis. The former is a well established vector of urban malaria whereas the later is a vector of filariasis [2, 3]. In India, vector control is exclusively based on the use of chemical insecticides for the adults as well as for the larvae. The drawbacks associated with the chemical insecticides, e.g., resistances in vector population, environmental pollution and costs have led to the search for alternative control agents. In recent years, efforts have been made on the search for natural products derived from plants and micro-organisms as an alternative to conventional chemical insecticides for insect control [4]. A common feature of some micro-organisms, principally bacteria and fungi, is their natural ability to produce metabolic compounds that may be toxic against insect pests. Fungi pathogenic to insects are serious contenders for use as biological control agents because they secrete different types of bioactive compounds when infect their insect hosts [5, 6]. The toxicogenic activity of insecticidal metabolites of *Beauveria bassiana* was increased after two passages through Malt agar whereas two passages through Sabouraud's dextrose agar decreased its toxicity [7]. Growth medium has a major contribution in the enhancement of toxicogenic activity of the culture filtrates, therefore in the present study we evaluate whether or not culture filtrates from the different culture medium of *Metarhizium anisopliae* are or not active against the selected host.

Materials and methods

Fungi

Five strains of *M. anisopliae* (892, 3210, 3943, 4100 and 4102) were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. All the strains were isolated from insects by various workers and were maintained in the above deposition centre. These five strains were routinely maintained in our laboratory on YpSs agar medium at 25 ± 2 °C.

Preparation of broth and culture of *M. anisopliae*

Two types of broths were prepared for the culture of the *M. anisopliae*. Six 250-ml conical flasks, each containing 100 ml YpSs (g/l: Soluble starch 15, Yeast extracts 4, K_2HPO_4 1, $MgSO_4$ 0.5, distilled water 1,000 ml) was autoclaved at 20 psi for 20 min. The sterilized broth was supplemented with 25 µg/ml of chloramphenicol as a bacteriostatic agent. The chitin broth (CB) contained g/l: K_2HPO_4 1, KCl 0.5, $MgS0_4$ 0.5, $FeS0_4$ 0.01, colloidal chitin 10 and 0.02% yeast extract as a source of vitamin. Colloidal chitin was the only source of carbon and nitrogen in CB. Colloidal chitin was prepared according to the methods described by Shimahara and Takiguchi [8]. Like YpSs, CB was sterilized at 20 psi for 20 min in six 250-ml conical flasks containing 100 ml broth and bacteriostatic agent was supplemented. Conidial suspensions of *M. anisopliae* were prepared by scraping conidia from well-sporulated 15-days-old fresh slant cultures into sterile distilled water and their concentration estimated by the colony forming unit method. To prepare a primary culture, 1 ml of a suspension of conidia (adjusted to 1×10^7 spores/ml) was inoculated into 25 ml of YpSs liquid medium in 100 ml Erlenmeyer flasks and cultured at 25 ± 2 °C on a rotary shaker at 110 rpm for 4 days. To inoculate secondary cultures for large-scale growth of the isolates, 2 ml of the primary cultures were transferred into 250 ml of YpSs and CB medium in one-litre Erlenmeyer flasks, and cultured on the above shaker at 25 ± 2 °C and 110 rpm for 7 days, before removing the mycelial material by filtration through Whatman No. 3chr filter paper. The serial dilutions of cell free culture filtrates in YpSs and CB were made separately. The amount of culture filtrates required for the mortality of the 50% larvae in 1 ml assay water was expressed as LC_{50}.

Mosquito rearing

Mosquito larvae were maintained in the laboratory at a temperature of 27 ± 2 °C, relative humidity of 70 ± 5% and a photoperiod of 14:10 h (Light: Dark). Different instars of *A. stephensi* and *C. quinquefasciatus* were maintained in separate enamel container (25 × 15 × 5 cm). Larvae were provided a mixture of yeast powder and dog biscuit as food in each 24 h. Food was weighted and distributed in the ratio of 1.0 mg/larva in the containers. Food amount and distribution frequency were depending on the amount of food still available in the containers. Larvae were reared in double-distilled water at pH 7.0. To counteract evaporation, water was added daily.

Efficacy

Twenty-five larvae of first and fourth instar of *A. stephensi* and *C. quinquefasciatus* were placed separately in 500 ml beakers containing 250 ml test medium (water + culture filtrates). Six dilutions of culture filtrates of both the broths were made of the culture filtrates concentrate. First and fourth instars of both the species were exposed separately to six different concentrations. Bioassays and controls were set up in triplicates and performed three times on different days. All dead larvae were counted and discarded from the test medium in every 12 h. Assays were terminated at 48 h and mortality counts were made based in the number of live larvae remained in each beaker. Culture filtrates of five fungi were tested separately. In control, 25 larvae of first and fourth instar of each species were put in separate beakers with 250 ml sterile double-distilled water. The control beakers were supplemented with respective concentrations of filtrate medium, which were not used for the fungus culture. The pH of the test medium was recorded at a concentration of 100 µl/ml of culture filtrates.

Statistics

The LC_{50}s with 95% fiducial limits were calculated by probit analysis using SPSS. Differences among mean values were determined by t test at $P = 0.05$ using the software M. S. Excel. Control mortalities were corrected by the Abbott's formula and replicates were discarded when control mortality exceed 5% [9]. Abbott's formula for the calculation of the corrected percentage of mortality is given below:

$$\text{Corrected percentage of mortality} = \frac{\text{Test mortality} - \text{Control mortality}}{100 - \text{Control mortality}} \times 100.$$

Results and discussion

When the efficacies of the culture filtrates of five strains of *M. anisopliae* were compared against *A. stephensi* and *C. quinquefasciatus*, *M. anisopliae* 892 was found to be more

effective against both the mosquitoes. The LC_{50}s of culture filtrates in chitin broth of *M. anisopliae* strains were lower than the LC_{50}s of culture filtrates in YpSs broth against first and fourth instars of both the mosquitoes (Table 1). The culture filtrates in chitin broth of *M. anisopliae* strains were significantly more efficacious than the culture filtrates in YpSs broth against *A. stephensi* (t test; $P < 0.00001$) and *C. quinquefasciatus* (t test; $P < 0.001$). The LC_{50}s of culture filtrates of all the strains were significantly different between first and fourth instars of *A. stephensi* (t test; $P = 0.0001$) and *C. quinquefasciatus* (t test; $P = 0.00001$). The fourth instars of *A. stephensi* were more susceptible than the first instars against the culture filtrates in both the broths. However, first instars of *C. quinquefasciatus* were more susceptible than the fourth instars against all the strains and are reverse to the results of *A. stephensi*. The larvae of *A. stephensi* were more susceptible than *C. quinquefasciatus* except in two cases (LC_{50} of culture filtrates in chitin broth of *M. anisopliae* 892 and 4100). The LC_{50} values of culture filtrates of *M. anisopliae* 892 in CB for first and fourth instars of *A. stephensi* were 1.84- and 3.26-fold less than that of in YpSs broth. Moreover, LC_{50} values of culture filtrates in CB for first and fourth instar of *C. quinquefasciatus* were 2.79- and 5.85-fold less than that of in YpSs broth. The pH of the final test medium at a concentration of 100 µl/ml was 6.72 ± 0.4 and 6.84 ± 0.59 for the culture filtrates of YpSs and chitin broth, respectively. Thus the pH of the test medium was not the cause of the efficacy of the mosquito larvae.

A major hindrance to the development of entomopathogenic fungi as mycopesticides has been that 5–10 days are required after application to kill an insect pest. Thus, a key aim of most recent work has been to increase the speed of kill and thus improve commercial efficacy, mainly by optimising production, stability and application of the inoculum [10]. *M. anisopliae* is amongst those fungi, which kill before extensive invasion of organs takes place [11], and two toxins (destruxins A and B) have been isolated from culture filtrates and mycelia of this fungus [11]. Quesada-Moraga et al. [4] screened the efficacy of culture filtrates of 25 fungal isolates of four fungal species against *Spodoptera littoralis* and isolated one effective strain *M. anisopliae* 01/58-Su like in the present investigation. Dose of 1.8 mg crude protein extract of *M. anisopliae* 01/58-Su with an artificial diet of 1 g caused 82.5% mortality of second instar *S. littoralis* larvae after 7 days of feeding. However, 100% mortality of second instar *S. littoralis* larvae was recorded after 7 days of feeding on the 5 mm alfalfa disc treated with 30 µg of extracted protein. The lethal doses of *M. anisopliae* 01/58-Su for both the above assays were lower than the present investigation. Moreover, the methods of application in both the cases were

Table 1 LC_{50} of culture filtrates of *Metarhizium anisopliae* strains in µl/ml for the first and fourth instars of *Anopheles stephensi* and *Culex quinquefasciatus* after 48 h of inoculation (95% fiducial limits in parentheses)

MTCC number of *M. anisopliae* strains	*Anopheles stephensi*				*Culex quinquefasciatus*			
	First instar		Fourth instar		First instar		Fourth instar	
	YpSs	Chitin	YpSs	Chitin	YpSs	Chitin	YpSs	Chitin
892	7.23 (6.26–8.15)	3.94 (2.69–5.10)	5.23 (4.46–6.07)	1.62 (1.33–2.08)	8.15 (7.20–9.06)	2.92 (1.49–4.32)	41.83 (31.91–59.22)	7.15 (5.49–8.76)
3210	18.07 (16.34–21.46)	15.42 (13.78–17.26)	13.08 (12.23–15.95)	12.33 (11.81–13.04)	36.89 (29.27–40.61)	28.14 (22.86–31.8)	92.75 (86.28–99.16)	72.1 (68.18–79.45)
3943	13.25 (10.73–16.58)	11.87 (9.84–14.05)	10.44 (9.13–12.28)	9.3 (8.1–10.82)	25.74 (22.04–29.68)	19.44 (16.77–25.41)	62.87 (55.19–69.57)	44.28 (39.92–50.64)
4100	20.85 (18.07–22.35)	18.64 (16.58–21.46)	16.82 (15.03–19.38)	14.01 (12.69–16.08)	22.96 (18.82–26.90)	18.02 (16.04–21.09)	78.54 (70.08–85.93)	50.24 (42.67–56.81)
4102	11.62 (10.07–13.11)	9.72 (9.18–10.65)	10.95 (9.85–12.15)	8.32 (7.35–9.02)	13.18 (10.05–15.86)	10.22 (9.26–11.39)	62.81 (57.18–67.82)	44.82 (40.42–49.21)

different and it was difficult to calculate the feeding rate of mosquito larvae. In case of *S. littoralis* only food has been treated but for mosquito larvae the whole habitat has been altered. Therefore, ecosystem of the pest plays an important role in the requirement of insecticide.

Little is known about the impact of nutrition on the toxicity of culture filtrates of *M. anisopliae* against mosquitoes. Production of toxins in two strains of *M. anisopliae* was recorded in 0.5 and 1% peptone added Czpapek Dox broth and the 0.5% peptone added broth was found to be more effective [11]. The toxicity of *M. anisopliae* culture filtrate was increased when grown in medium with organic nitrogen than inorganic nitrogen [11]. The ED_{50}s of culture filtrates of both the sources (>150 µl/g) of *M. anisopliae* against *Galleria mellonella* were higher than the present investigation. The efficacy of culture filtrates of submerged culture methods was more effective than the surface culture method. In this study, the culture filtrates of submerged methods were used.

Most of the works in nutrition were done only on the nitrogen energy source. Recently the activity of chymoelastase (Pr1) of *M. anisopliae* was studied in medium with variable carbon source as energy. However, this is the first report regarding the effect of chitin (present in the nutrition as the only energy source) on the culture filtrates of *M. anisopliae*. Vijayan and Balaraman [12] evaluated the efficacies of culture filtrates of 17 fungi against third instars of *A. stephensi* and *C. quinquefasciatus* and the LC_{50}s were recorded in the range of 7–83 and 3–24 µl/ml, respectively. The LC_{50}s of culture filtrates of *M. anisopliae* 892 for fourth instars of *A. stephensi* (YpSs—5.23 µl/ml, CB—1.62 µl/ml) were lower than the LC_{50}s (7–83 µl/ml) recorded by Vijayan and Balaraman [12]. However, the LC_{50}s of culture filtrates of *M. anisopliae* 892 recorded for *C. quinquefasciatus* were higher than the LC_{50}s recorded by Vijayan and Balaraman [12]. The LC_{50} values of culture filtrates of *Trichophyton ajelloi* for first instars of *A. stephensi* and *C. quinquefasciatus* were higher than the LC_{50}s of culture filtrates of *M. anisopliae* 892 in CB and YpSs broth [13]. The secondary metabolites of *Lagenidium giganteum* were highly toxic to mosquitoes and were less toxic to non-target organisms [14]. However the toxicity of secondary metabolites of *L. giganteum* is difficult to compare with the present investigation because of variation in growth time and filtration procedure.

Conclusion

It can be concluded from the observation that the culture filtrates of *M. anisopliae* 892 are better equipped against *A. stephensi*. This is the first report of efficacy of culture filtrates produced by *M. anisopliae* in chitin broth against mosquitoes. These culture filtrates have potential as a biological control agent of mosquitoes. This information will be helpful in the selection of medium for the mass production of metabolites against mosquitoes.

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