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Biocontrol efficiency of *Trichoderma atroviride* against larvae of *Culex quinquefasciatus* Say and *Anopheles stephensi* Liston

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ABSTRACT

Conidial suspension of *Trichoderma atroviride* isolated from larvae of *Anopheles* sp., at a concentrations of 6.9×10^6 spores/ml 11.6×10^6 spores/ml, killed 65-70% *Culex quinquefasciatus* and 25% *Anopheles stephensi* 3rd instar larvae on 72 h exposure. Thin sections of infected larvae respectively of *Cx. quinquefasciatus* showed accumulation of conidia in the gut. Tests conducted revealed that the conidia were viable even after passage through the gut of *Cx. quinquefasciatus* and in the faecal pellets of *An. stephensi*. The LC_{50} value of metabolites from cell free extract of *T. atroviride* against *Cx. quinquefasciatus* larvae was 26.36 µl /ml at 24 h. Mortality difference was significant within the 5 replicates/dose ($F = 6.227$, $p < 0.002$), while it was highly significant between the 3 doses ($F = 244.342$, $p < 0.001$). It is, therefore, projected that *T. atroviride* could be a potential biolarvicidal material.

Keywords: *Trichoderma atroviride*; entomopathogenic fungi; *Culex quinquefasciatus; Anopheles stephensi; Trichoderma* metabolites

INTRODUCTION

Bancroftian filariasis and malaria are world's most predominant tropical infectious diseases. Of the 128 million cases of lymphatic filariasis estimated globally, 91% are caused by *Wuchereria bancrofti* (Michael and Bundy, 1997). *Cx. quinquefasciatus* is the principal vector of bancroftian filariasis in India breeding in polluted waters. *An. stephensi*, the malaria vector in urban areas, is responsible for serious malaria outbreaks in Goa (Dash *et al.*, 2008). In the adult *Culex* mosquitoes rapid build up of resistance to synthetic pyrethroids and emergence of insecticide resistance in species of *Anopheles* has led to the search for alternate vector control strategies.

Entomopathogenic fungi are considered as potential alternatives for chemical insecticides. Reports are available on effective use of entomopathogenic hyphomycetes and their development as microbial control agents (Ferron, 1978; Tanada and Kaya, 1993; Hajek and St. Leger, 1994; Scholte *et al.*, 2004; Keshava Prasad and Bhat, 2007).

Many entomopathogenic fungi produce insecticidal toxins in liquid culture (Charnley, 2003). Tolypin from *Tolypocladium niveum* was reported to be larvicidal to the larvae of mosquitoes and blackflies (Matha *et al.*, 1988). The depsipeptide beauvericin from some *Fusarium* spp. was toxic to Colorado potato beetle (Gupta *et al.*, 1991) and from *Beauveria bassiana* caused ultrastructural changes especially in the midgut epithelium and mortality in the *Culex pipiens autogenicus* larvae (Zizka and Weiser, 1993). Extracellular metabolites of fungi active against mosquito larvae have been screened in India (Vijayan and Balaraman, 1991; Mohanty and Prakash, 2004).

Trichoderma atroviride, a filamentous soil fungus, is known to function as biocontrol agent for a wide range of economically important aerial and soil borne plant pathogens (Papavizas, 1985; Chet, 1987). The present study describes activity of *T. atroviride* and its metabolites against the mosquito vectors.

MATERIALS AND METHODS

Test Organism

Cx. quinquefasciatus larvae cultured and maintained by the National Institute of Malaria Research, Field Station, Goa, at a temperature 25 ± 2°C, relative humidity 70 ± 5% and photoperiod : scotoperiod 14:10 h (light : dark) were used in the present study.

Mycopathogen

An isolate of *Trichoderma* was recovered from 3rd instar larva of *Anopheles* sp. inhabiting curing water at a construction site in Cuncolim, Goa, by direct isolation method and maintained at Goa University Fungal Culture Collection (GUFCC No. 5103). The isolate was maintained on Malt Extract Agar (MEA) medium (Himedia Laboratories Pvt. Ltd.: 2.5 g dehydrated malt extract and 20 g agar in 1000 ml distilled water). For all experiments, the isolate was

subcultured on Corn Meal Agar (CMA) medium (Himedia Laboratories Pvt. Ltd.: 17g cornmeal agar in 1000 ml distilled water). Following Gams *et al.* (1987), the isolate was identified as *Trichoderma atroviride* P. Karsten.

Cultural Characters of *T. atroviride*:

Colonies of the fungus on CMA (Fig.1a) were circular, with rhizoidal margin, attaining a diam of 8-9 cm in 7 days, slimy conidia in patches, initially white, rapidly turning to green, with watery exudates on the surface, conidiation appeared granular with age, initially glaucous, reverse uncoloured. The mycelium was composed of smooth, hyaline, septate, freely branched thin-walled hyphae, 4-7 µm wide. Unicellular, subglobose chlamydospores were present within the hyphal cells. Conidiophores were mononematous, relatively narrow and flexous with usually paired short primary branches arising at regular interval, which extensively rebranched, coarse with tubercles, septate, thin walled hyaline, $30-40 \times 2-3$ µm (Figure 1b). The conidiophores terminated with few phialides and with their branches at right angles assumed a pyramidal shape. The conidiogenous cells were phialidic, discrete, rough, hyaline, $5-7 \times 3-3.5$ µm, lageniform, with a very narrow beak like distal tip; length/width ratio of phialides was 1.7 – 2.3 µm (Fig.1c) Conidia were globose to subglobose, smooth forming sticky balls of 10 – 15 on each conidiogenous cell, green, 2 – 3 µm diam (Fig.1d).

Preparation of Conidial Suspension:

For obtaining conidia in large quantity, the isolate was grown on CMA. The culture plates were flooded with sterile 0.05% Tween 80 and spores harvested. The suspension centrifuged at 6000 rpm for 10 min yielded a pellet which was washed in sterile distilled water, re-suspended in minimum amount of water and vortexed. The spore concentration was determined using improved Neubauer haemocytometer (Goettel and Inglis, 1997).

Preparation of Cell Free Extract (CFE):

Two 250 ml conical flasks containing sterilized 100 ml of Glucose Peptone Yeast Medium (glucose 10 g, peptone 10 g, yeast extract 4 g, soluble starch 10 g, distilled water 1000 ml, pH 5.5) were inoculated with conidia of *T. atroviride* from 14 d old culture and incubated at 25.8 ± 2°C for 10 d at 150 rpm on an Orbitek rotary shaker. The buff coloured culture obtained when sonicated using electronic sonicator for 2 min yielded a homogenous brown coloured liquid. The liquid was centrifuged for 20 min at 5500 rpm, the residual pellet discarded and the CFE used for bioassay. An uninoculated culture medium served as control.

Bioassays

Larvicidal activity of *T. atroviride* against *Cx. quinquefasciatus* 3rd instar larvae was assessed using a standard method recommended by the World Health Organization (WHO, 1996). Bioassays were set up in 500 ml plastic bowls with a wide surface area in a final volume of 50 ml sterile distilled water with the desired active dose of 6.9×10^6 spores/ml. In all, 5 replicates each with 20 healthy 3rd instar larvae were set. Three controls, without spore suspension, were maintained. The bowls were covered with nylon net secured by a rubber band. Larvae were fed with 20 – 25 mg baby food Farex™. Assays were terminated at 72 h and mortality counts made based on the number of live larvae remaining in each bowl. Moribund and dead larvae were examined individually under a microscope.

Similar assay with a dose of 11.6×10^6 spores/ml was carried out against *An. stephensi* 3rd instar. Bioassay of conidial suspension with dose of 11.6×10^6 spores/ml from 28 d culture, was carried out against *Cx. quinquefasciatus*

Studies on mode of action:

Stereomicroscopic studies showed presence of green matter within the exposed larva like a tube. The larvae were dissected with a sterilized blade to expose the contents of the gut lumen.

Light microscopy of *Culex* larvae:

The location of conidia in the gut of *Cx. quinquefasciatus* after ingestion was studied under a light microscope. After 48 h exposure the treated and control larvae were fixed in Altmann's fixative for 2 h (Gatenby and Beams, 1950) and sectioned in a Leica M-800 cryostat at −14°C to obtain 20 µm thick longitudinal sections. They were air dried, stained with lactophenol mixed with cotton blue and observed under a binocular microscope.

Viability of *T. atroviride* conidia in the gut of *Cx. quinquefasciatus*:

The viability of *T. atroviride* conidia in the gut of *Cx. quinquefasciatus* following their ingestion and excretion by the larvae was studied by glutting with India ink (Goettel, 1988 b).

Three replicates with 10 larvae per replicate were used.

Bioassays with metabolites of *T. atroviride*:

Screening crude metabolites in the CFE of *T. atroviride* against *Cx. quinquefasciatus* larvae resulted in mortality above 60% in 24 h. Based on these results main assays were set with three different concentrations of metabolites 1 µl/ml, 10 µl/ml and 100 µl/ml. Five replicates of 10 *Cx. quinquefasciatus* larvae per replicate per dose were set in 500 ml plastic bowls with total volume made to 50 ml with deionised water. Concurrently three controls with respective concentration of media were maintained. In each bowl 15 mg baby food Farex ™ was provided as food. The bowls were covered with nylon net secured with a rubber band. Larval mortality was recorded at 24 h and 48 h. Experimental tests that demonstrated more than 20% control mortality were discarded and repeated. When control mortality reached between 5-20%, the mortality observed was corrected by Abbott's formula (Abbott, 1925).

STATISTICAL ANALYSIS

Results of efficacy of *T. atroviride* metabolites assessed against *Cx. quinquefasciatus* were analyzed by probit analysis (Finney, 1971). The concentrations caused 14 – 100% mortalities in the larvae and LC_{50} values were calculated with their fiducial limits at 95% confidence level (Table 1). The relation between probit and log concentration was established as probit equations. Percentages of larval mortality were determined and subjected to analysis of variance (ANOVA) (Table 2) using SPSS version 16 software.

RESULTS AND DISCUSSION

The bioassay of *T. atroviride* conidial suspension against 3rd instar larvae of *Cx. quinquefasciatus* resulted in 65% – 70% mortality and in *An. stephensi* 25% were killed. In a previous study (Keshava Prasad *et al.*, 2000) 65% mortality was seen in 2nd instar *Cx. quinquefasciatus* on 120 h exposure to *T. atroviride* (GUFCC 5103). Comparatively the susceptibility of the 3rd instar *Cx. quinquefasciatus* seems to be more in the present as mortality was achieved on 72 h exposure. *An. stephensi* appears less susceptible though the dose was almost two-fold higher. Under stereomicroscope the alimentary canal in both *Culex* and *Anopheles* larvae appeared packed with green coloured matter probably green spores of *T. atroviride*. Dead larvae had distorted body with head separated from the thorax. The surviving larvae entered the 4th instar but breadth wise they were thinner than the control larvae. The larvae in the control however were active, feeding voraciously, healthier and developed normally.

Table1. Larvicidal activity of secondary metabolites of *Trichoderma atroviride* against the larvae of *Culex quinquefasciatus*

Time (h)	Probit equation	LC_{50} (µl/ml)	95% Confidence Interval LBUB	x^2
24	$Y = -1.600 + 1.126\ X$	26.36	.408 1.844	1.205
48	$Y = -1.057 + 1.373\ X$	5.886	.551 2.195	1.375

LB = Lower Bound; UB = Upper Bound

Table2: ANOVA table to see the significance of larvicidal activity of metabolites of *Trichoderma atroviride* against *Culex quinquefasciatus larvae*

Source of variation	Degrees of freedom df	Sum of squares ss	Mean sum of squares ss	F cal	F prob
Replications	4	430.048	107.512	6.227	0.002*
Treatments	5	16531.740	3306.348	191.502	0.000**
Factor A	2	8437.281	4218.640	244.342	0.000**
Factor B	1	346.363	346.363	20.061	0.000**
A X B	2	7748.097	3874.048	224.383	0.000**
Error	20	345.307	17.265	–	–
Total	29	–	–	–	–

Coefficient of Variation = 9.185

Factor A = Dose; Factor B =Time; df = degrees of freedom; SS = Sum of squares; Mean SS = Mean sum of squares; F cal = F calculated value; F prob = F probability ;* = significant, ** = highly significant.

Al-Aidroos and Roberts (1978) observed gut invasion of larvae of *A. aegypti* by *M. anisopliae*, confirming that mosquitoes are vulnerable to this mode of attack. Reports of insect mortality due to the toxic activities of large number of ingested but ungerminated conidia in the mosquito larvae are available (Roberts, 1970; Crisan 1971). In the present study the dissected gut showed the conidia in the chyme, light microscopy revealed the presence of the conidia throughout the alimentary canal (Figure 1.d) in the longitudinal sections (L.S) of *Cx. quinquefasciatus* larvae, it was also noted that the tissue had taken up blue colour but the conidia were unstained (Figure 1.e) right from foregut uptil the hindgut, probably they were coated with some secretions, many had assumed crescent shape, slight flattening of the conidia observed maybe due to mechanical pressure, a tendency of the conidia to remain more towards the gut epithelium was seen (Figure 1.f). Though the conidia had remained for 48 h in the gut germination or hyphal formation was not seen. The viability experiments showed that after being held for more than two hours in the gut, the conidia were viable. They were viable prior to ingestion. The conidia retained their viability ensuing ingestion and excretion. After holding in distilled water for 24 h when plated on CMA the conidia were viable.

Similarly in *T. cylindrosporum* (Goettel 1988a, b) the viability of spores was not reduced after a 2.5 h passage through the larval gut of the 2nd instar *A. aegypti* and at the time of death in the larvae exposed to higher concentrations of conidia, less extensive mycoses was observed. In concurrence with Soares (1982) in the present study mortality in larvae maybe attributed to nutritional stress or it maybe due to production of toxic metabolites. Bioassay with a higher dose of conidial suspension from 28 d culture against *Cx. quinquefasciatus* did not cause any mortality, showing that higher concentrations may not be causing death. Green faecal pellets were observed showing that the spores were ingested and egested. The results of bioassays with metabolites from CFE of 10 d submerged culture indicate production of larvicidal toxins. Similar toxins might be produced by the conidia in the gut of the larvae and the age of the conidia seems to play an important role. In *Anopheles* larvae the conidia from the faecal pellets plated on antibiotic loaded CMA germinated.

Bioassays with metabolites from the CFE of *T. atroviride* against *Cx. quinquefasciatus* larvae with doses of 1 µl/ml, 10 µl/ml and 100 µl/ml caused percent mortalities of 14%, 22% and 76% respectively after 24 h exposure and 24%, 46% and 99%, respectively after 48 h exposure. Control mortality ranged from 0 – 6.66%. LC_{50} for 24 h = 26.36 µl/ml and LC_{50} for 48 h = 5.886 µl/ml. Mortality difference was significant within the 5 replicates/dose ($F = 6.227$, $p = 0.002$), while it was highly significant between the 3 doses ($F = 244.342$, $p < 0.001$). The significance in mortality was high with increase in exposure time from 24 h to 48 h ($F = 20.061$, $p = 0.000$). In their studies Vijayan and Balaraman (1991) obtained the LC_{50} values of fungal metabolites from 17 species in the range of 3 – 24 µl/ml for *Cx. quinquefasciatus* 3rd instar larvae on 48 h exposure whereas in the present LC_{50} value was achieved on 24 h exposure with slightly higher dose of 26.36 µl/ml. The LC_{50} value of *Chrysosporium tropicum* (Priyanka and Prakash, 2003) evaluated against 3rd instar *Cx. quinquefasciatus* larvae was 79 µl/ml which is three-fold higher than *T. atroviride*. A crude extract of tolypin caused 100% mortality in the larvae of *Cx. pipiens* and *An. maculipennis* at a concentration of 100 µl /ml (Weiser and Matha, 1988).

Our results show that *T. atroviride* (GUFCC 5103) and its metabolites are highly efficacious against the *Cx. quinquefasciatus* larvae and has potential which needs to be exploited against other vector larvae.

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REFERENCES

ABBOT, W.S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. **18:** 265-267.

AL-AIDROOS, K., AND ROBERTS, D.W. 1978. Mutants of *Metarhizium anisopliae* with increased virulence towards mosquito larvae. Can. J. Genet. Cytol. **20:** 211-219.

CHARNLEY, K.A. 2003. Fungal pathogens of insects: cuticle degrading enzymes and toxins. Advances in Botanical Research **40:** 241-321.

CRISAN, E.V. 1971. Mechanism responsible for release of toxin by *Metarhizium* spores in mosquito larvae. J. Invertebr. Pathol. **17:** 260-264.

DASH, A. P., VALECHA, N., ANVIKAR, A. R. AND KUMAR, A. 2008. Malaria in India: Challenges and opportunities. J. Biosci. **33(4):** 583–592.

DILLON, R. J., AND CHARNLEY, A.K.1991.The fate of fungal spores in the insect gut. In: *The Fungal Spore and Disease Initiation in Plants and Animals*. (Eds. G.T.Cole and H.C. Hoch). Plenum press, New York and London. 129-156 pp.

FEDERICI, B.A. 1981. Mosquito control by fungus *Culicinomyces, Lagenidium* and *Coelomomyces*. In: *Microbial Control of Pests and Plant Diseases* 1970-1980. (Ed. H.D.Burges). Academic press, London. 555-572 pp.

FEDERICI, B.A. 1995. The future of microbial insecticides as vector control agents. J. Am. Mosq. Control Assoc. **11:** 260-268.

FERRON, P. 1978. Biological control of insect pests by entomogenous fungi. Annu. Rev. Entomol. **23:** 409-442.

GATENBY, J.B AND BEAMS, H.W. 1950. The Microtomist's Vade-Mecum: A handbook of the Methods of Animal and Plant Microscopic Anatomy. (Bolles lee) J. and A. Churchill Ltd. London.

GOETTEL, M.S. 1988a. Pathogenesis of the hyphomycete *Tolypocladium cylindrosporum* in the mosquito *Aedes aegypti*. J. Invertebr. Pathol. **51:** *259-274.*

GOETTEL, M.S. 1988b. Viability of *Tolypocladium cylindrosporum* conidia following ingestion and excretion by larval *Aedes aegypti*. J. Invertebr. Pathol. **51:** 275-277.

GOETTEL, M.S. AND INGLIS, G.D. 1997. Fungi: Hyphomycetes. In: *Manual of techniques in insect Pathology* (Ed. L.A.Lacey.). Academic Press, San Deigo. 213-249 pp.

HAJEK, A.E. AND ST.LEGER, R.J. 1994. Interactions between fungal pathogens and insect host. Annu. Rev. Entomol. **39:** 293-322.

KESHAVAPRASAD, T.S., KUMAR, A. AND BHAT, D.J. 2000. Mosquito larvicidal and pathogenic fungi from Goa. In: *Prospects and Problems of Environment Across the Millennium*. (Eds. M.N. Madhyastha, K.R.Shridhar and Dr. Ahana Lakshmi) Daya Publishing House, New Delhi, India. 143-149 pp.

LACEY, M.C., LACEY, L.A. AND ROBERTS, D.W. 1988. Route of invasion of *Metarhizium anisopliae* in *Culex quinquefasciatus*. J. Invertebr. Pathol. 52: 108-118.

MICHAEL, E., AND BUNDY, D.A. 1997. Global mapping of lymphatic filariasis. Parasitol Today **13:** 472-476.

MOHANTY, S.S., AND PRAKASH, S. 2004. Extracellular metabolites of *Trichophyton ajelloi* against *Anopheles stephensi* and *Culex quinquefasciatus* larvae. Curr. Sci. **86(2):** 323-325.

ROBERTS, D. W. 1967. Some effects of *Metarhizium anisopliae* and its toxins on mosquito larvae. In: *Insect Pathology and Microbial Entomology* (ed. Van der Laan, P. A.), North-Holland, Amsterdam, 1-360 pp.

ROBERTS, D.W. 1970. *Coelomomyces, Entomophthora, Beauveria* and *Metarhizium* as parasites of mosquitoes. *Miscellaneous Publications of the Entomological Society of America* **7:** 140-155.

SCHOLTE, E-J., KNOLS, B.G.J., SAMSON, R.A., AND TAKKEN, W. 2004. Entomopathogenic fungi for mosquito control: A review. J. Insect Sci. **4(19):** 1-24.

TANADA, Y., AND KAYA, H.K. 1993. *Insect Pathology*. Academic Press, London.

VIJAYAN, V., AND BALRAMAN, K. 1991. Metabolites of fungi and actinomycetes active against mosquito larvae. Indian J. Med. Res. [A] **93:** 115-117.

WEISER, J. AND MATHA, V. 1998. The insecticidal activity of cyclosporins on mosquito larvae. J. Invertebr. Pathol. **51:** 92-93.

WEISER, J., AND MATHA, V.1988. Tolypin a new insecticidal metabolite of fungi of the genus *Tolypocladium*. J. Invertebr. Pathol. **51:** 94-96.

WHO. 1996. Report of the WHO Informal consultation on the evaluation and testing of Insecticides, CTD/WHOPES/IC/. 96.1, p. 69.