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Effect of Fungal Extracts on *Bacillus thuringiensis* Serotypes Causing Flacherie Disease in Mulberry Silkworm, *Bombyx mori* L.

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A B S T R A C T

Antibacterial activity of six different species of fungal extracts viz., *Aspergillus ochraceous, Paecilomyces variotii, Schizophyllum sp., Epicoccum sp., Metarhizium* sp., *Aspergillus niger* were evaluated against ten serotypes of *Bacillus thuringiensis* to develop new bioactive secondary metabolites from the microbial sources. Fungal crude extracts were isolated by ethyl acetate solvent extraction method and product yields varied from 0.150g to 0.538g. Thin Layer Chromatography was carried out for detecting the small molecule compounds present in the crude extracts. Antibacterial activities of fungal extracts were analyzed by disc diffusion method. All ten B.t serotypes were inhibited by six fungal extracts with zone of inhibition ranging from 12 ± 1.73 to 36 ± 1.53. Among the six extracts *Paecilomyces variotii* showed highest inhibition of 36 mm against *B.t sotto*. In vitro and in vivo studies conducted for the effect of extracts with different concentration and exposure duration reveals that effective against all serotypes. The results suggested that the Microbial derived extracts could be a new source of alternative in developing an effective bioactive molecule for the management of silkworm bacterial diseases.

Introduction

Diseases are the most important limiting factor affecting silkworm, *Bombyx mori* L., known for the production of silk cocoons are generally affected by viral, fungal and protozoan pathogens among which bacterial pathogens alone cause cocoon loss about 7.5 to 20.0 percent (Selvakumar, 2013). The major fact responsible for bacterial flacherie was the rearing conditions, rise in temperature and humidity in rearing place, insufficient bed spacing leads to dysfunction of alimentary canal which encourages flacherie (Nataraju *et al.*, 2005). *Bacillus thuringiensis* (*Bt)* is one of the causative agents for flacherie disease and the impact was high in silkworm larvae (Nishiit sutsuj *et al.*, 1979). *Bt* is a Gram positive spore forming bacterium characterized by the formation of parasporal inclusions during sporulation (Ohba, 1996). After being ingested by the larvae, the parasporal inclusions are dissolved in larval midgut juice and release protoxins. The

activated toxins interact with the larval epithelial membrane and induce pore formation in the membrane, which ultimately leads to insect death (Gill *et al.*, 1992). Larvae affected by *Bt* lose their appetite, undergo convulsions and their bodies become stretched and cracked. The cadavers gradually become brown to black-brown and finally when rotting they turn black with fowling smell (Aruga, 1994). Researchers have come out with different sources of bioactive compounds/crude extracts from plants to prevent the silkworm bacterial pathogens (Selvakumar *et al.,* 2001; Manimegalai and Chandramohan, 2005; Priyadharshini, 2008) but limited attempts were made for the use of microbial metabolites. In the present study, the efficacy of fungal extracts controlling the ten serotypes of *Bt* causing flacherie disease in mulberry silkworm was evaluated to develop a effective bioactive molecules for the control of disease.

Materials and Methods

Microbial materials

Six different fungal cultures *viz*., *Aspergillus ochraceous, Paecilomyces variotii* (Fig.1), *Schizophyllum sp., Epicoccum sp., Metarhizium sp., Aspergillus niger* were procured from culture collection centre, NCL, Pune, India for extraction of fungal product. These cultures were maintained at 4 °C and sub cultured in Potato dextrose agar slants. Ten *Bacillus thuringiensis* serotypes *viz*., *tolworthi, aizawai, japonensis, sotto, kenyae, israelensis, morrisoni, kurstaki, thuringiensis, alesti* were collected and maintained for the experiments.

Preparation of fungal extract

Fungal strains were grown in PDA culture medium at 28 °C for 5 days after that cultures were cut into solid discs ($1cm^2$) and inoculated into 1000 ml Potato dextrose broth in Erlenmeyer flasks. These cultures were grown with continuous shaking of 200 rpm at 25 ± 2 °C in shaking incubator for fifteen days. At the end of incubation, the fungal mycelium and the culture medium were separated by filtration using Whatmann filter No.3. The filtered supernatant was extracted thrice with equal volume of ethyl acetate. The combined organic phase was dried with anhydrous sodium sulphate and concentrated in reduced vacuum evaporator at 45°C. The crude extract dissolved in 10% dimethylsulfoxide (DMSO) and kept in 4 °C for further use.

Identification of small molecules

Thin Layer Chromatography technique was used for detecting the small molecule compounds present in the fungal crude extracts. The extract samples were spotted on silica plates and eluted in a TLC chamber with ethyl acetate and hexane as the mobile phase in the ratio 8:2. The plates were then air dried and visualized by exposing to iodine vapor.

In vitro antibacterial activity by disc diffusion

Antimicrobial susceptibility test was carried out by the disc diffusion method. Overnight incubated *Bt* cultures ($1x10^8$spores/ml) spread on a Muller Hinton Agar plate. Whatman No.1 filter paper discs (6 mm diameter) dipped in the extract concentration of 50mg/ml solution and kept on inoculated plates. Ampicillin (10 mcg) was used as positive control. The plates inoculated with bacteria were made in triplicate and incubated at 32±1°C for 48 hours. The development of zone around the disc diameter was measured in millimeters. All data were analyzed through Microsoft Excel for Windows.

Efficacy of minimum antibacterial concentration

The effect of different concentrations of fungal product for minimum germicidal activity was tested *in vitro* against *B. thuringiensis* serotypes ($1x10^8$cells/ml). These pathogens were suspended separately in different concentrations of fungal product (0.25 to 4%) for different durations 30 min, 1 hour and 2 hour at room temperature. After this incubation, samples were inoculated in Louria Bertani agar plate and incubated at 32±1°C for 48 hours. The experiment conducted in triplicate. The minimum bactericidal activity was determined by the lowest concentration of fungal product incubated with pathogens were completely killed.

In vivo activity of fungal extract

The antibacterial activity of the product with different concentrations tested *in vivo* against *Bacillus thuringiensis* serotypes ($1x10^8$ cells/ml). These pathogens suspended to the various concentrations of product and incubated at 25±2°C for different duration (30 min, 1 hour and 2 hour). After the treatment, samples centrifuged, washed and used for inoculation to determine the infectivity of the pathogens. 0.5 ml of this sample orally inoculated to third instar larvae.

In positive control, viable pathogens inoculated without treatment to the larvae and in negative control, without inoculation larvae reared for comparative study in three replications. All the batches were reared till spinning and allowed to spin the cocoons. Observations recorded for disease development and mortality. Treatment effects were analyzed to determine the differences between the treatment means of extract concentration.

Results and Discussion

Screening of small molecules

Antibacterial activities of the fungal extracts isolated from six different fungi were analyzed to derive high active biomolecules for treatment of *Bt* serotypes. The crude extract of fungal product yields varied from 0.150g to 0.538g. A range of yields among extracts was observed depending on the fungal material used.

The maximum extraction yield was obtained from *Schizophyllum sp.* with 0.538g and *P. variotii* exhibited the lowest extraction yield of 0.150g. Thin Layer Chromatography was carried out for detecting the small molecule compounds present in the crude extracts and 2 to 5 product spots were appeared in all fungal extract in the range of 0.2 to 0.84 retention factor *(Rf)* with ethyl acetate as mobile phase for the extract.

In vitro antibacterial activity

In vitro studies results showed that all six fungal extracts exhibited antibacterial activity in the range of 12 ± 1.73 to 36 ± 1.53 against *Bt serotypes* (Table 1), (Fig.2 & 3). The *maximum* activity of 36± 1.53 mm observed in *P. variotii* and 29 ± 1.00 in *A. ochraceus* against *sotto* and followed by 28 ± 0.58 mm in diameter against *japonensis* by *Epicoccum sp.* In particular, *P. variotii* and *Epicoccum sp.* showed above 20 mm inhibition against all serotypes of *Bt* except *kenyae* and *tolworthi* respectively. Among the six extracts, *Metarhizium sp.* exhibited lowest activities observed in all *Bt* bacteria except against *sotto* which was 25 ± 1.00 mm in diameter of growth inhibition. Ampicillin (10 mg) was used as positive control and results indicated that the extracts showed almost equivalent activity at low concentrations.

Table.1 Antibacterial activity of the fungal extract against *BT* serotypes. Zone of inhibition measured in diameter (mm) mean± SD

BT serotypes	*Aspergillus ochraceus*	*Paecilomyces variotii*	*Schizophyllum sp.*	*Epicoccum sp.*	*Metarhizium sp.*	*Aspergillus niger*
tolworthi	12 ± 1.73	22 ± 2.08	16 ± 3.21	17 ± 1.00	13 ± 1.73	13 ± 1.15
aizawai	22 ± 1.53	20 ± 1.00	18 ± 1.15	20 ± 0.00	16 ± 0.58	16 ± 2.00
japonensis	21 ± 1.15	20 ± 1.00	23 ± 0.58	28 ± 0.58	15 ± 0.58	24 ± 0.58
sotto	29 ± 1.00	36 ± 1.53	22 ± 1.15	24 ± 2.00	25 ± 1.00	24 ± 0.58
kenyae	26 ± 1.15	17 ± 1.15	25 ± 1.73	26 ± 1.15	18 ± 1.15	23 ± 1.73
israelensis	14 ± 3.61	23 ± 1.00	19 ± 1.15	20 ± 0.58	20 ± 1.73	19 ± 2.31
morrisoni	21 ± 1.15	22 ± 1.53	21 ± 1.53	21 ± 2.31	17 ± 1.73	18 ± 2.52
kurstaki	13 ± 2.52	22 ± 1.15	21 ± 1.73	20 ± 1.73	23 ± 1.15	21 ± 1.15
thurigiensis	17 ± 1.53	22 ± 3.00	23 ± 2.08	21 ± 2.00	21 ± 2.65	19 ± 2.00
alesti	20 ± 1.53	25 ± 1.15	21 ± 2.31	24 ± 1.53	18 ± 2.65	23 ± 3.51

Table.2 *In vitro* testing of *P. variotii* extracts with different concentrations and treatment

BT serotypes	30 min Treatment						1 hour Treatment						2 hour Treatment					
	0.25	0.5	1	2	4	C+	0.25	0.5	1	2	4	C+	0.25	0.5	1	2	4	C+
tolworthi	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
aizawai	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
japonensis	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
sotto	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
kenyae	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
israelensis	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
kurstaki	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
morrisoni	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
thurigiensis	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+
alesti	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	+

(-) effective (+) not effective

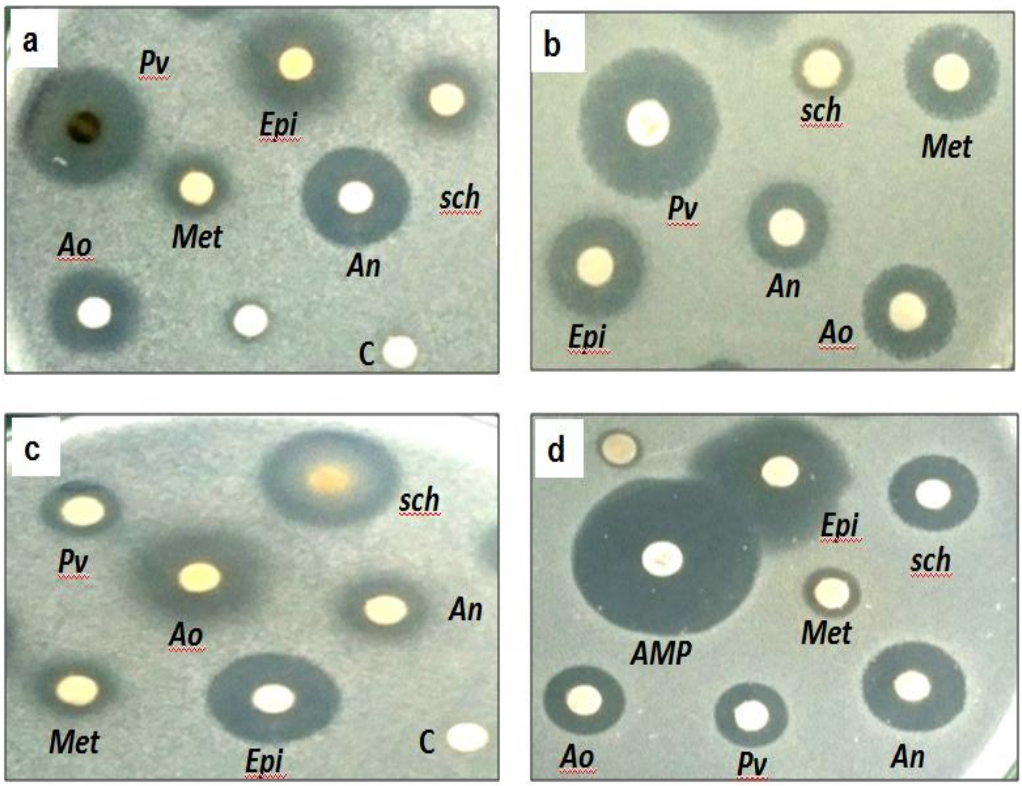
Table.3 *In vivo* activity of fungal extract *P. variotii* with different exposure time

BT serotypes	30 min Treatment					1 hour Treatment					2 hour Treatment				
	1	2	4	C+	C -	1	2	4	C+	C -	1	2	4	C+	C -
tolworthi	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
aizawai	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
japonensis	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
sotto	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
kenyae	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
israelensis	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
kurstaki	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
morrisoni	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
thurigiensis	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-
alesti	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-

(-) effective (+) not effective C+ positive control (Inoculated) C- Negative control (No inoculation)

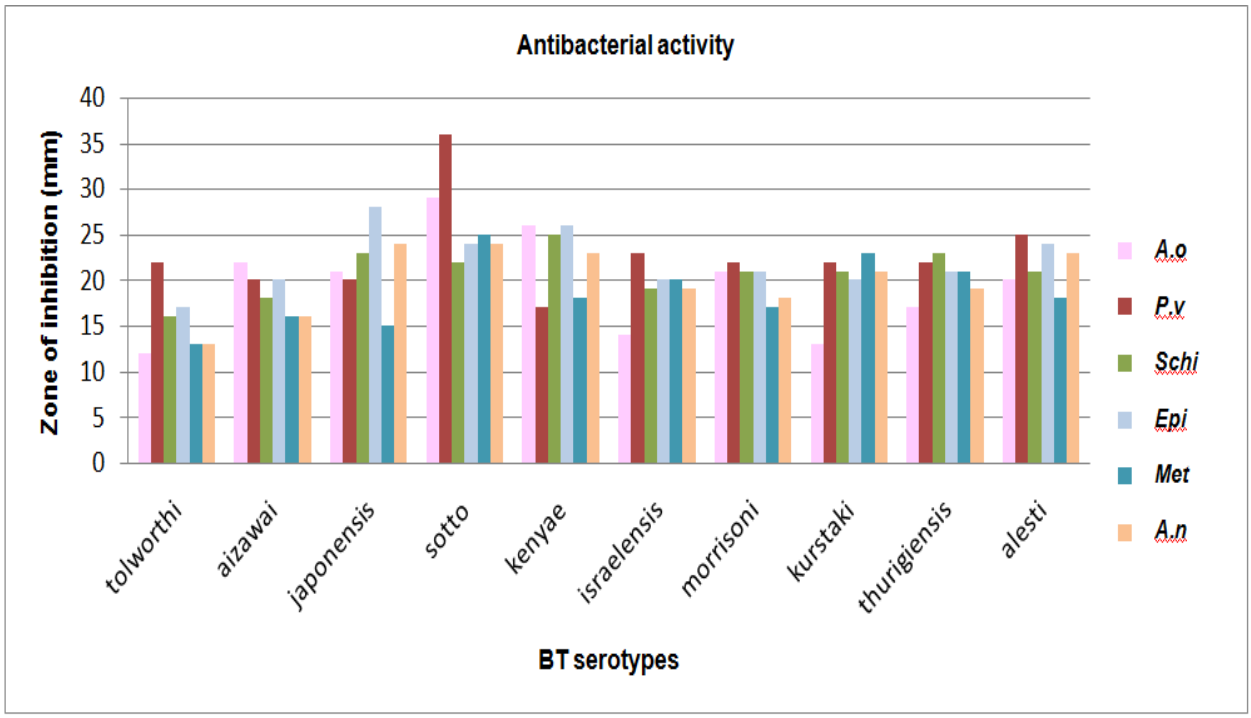
Fig.1 Culture growth of *P. variotii* on PDA medium

Fig.2 Zone of inhibition formed against the *Bt* serotypes by fungal extracts



a- *alesti*, b - *sotto*, c- *kenyae*, d - *japonensis*. *A.o - Aspergillus ochraceus, P.v- Paecilomyces variotii, schi- Schizophyllum sp., Epi- Epicoccum sp., Met- Metarhizium sp., A.n- Aspergillus niger*, C- control, AMP- Ampicillin

Fig.3 Antibacterial activity of the Secondary metabolites extracted from the fungi against *BT* serotypes



A.o - Aspergillus ochraceus, P.v- Paecilomyces variotii, schi- Schizophyllum sp., Epi- Epicoccum sp., Met- Metarhizium sp., A.n- Aspergillus niger

Efficacy of minimum antibacterial concentration

P. variotii performed well in all serotypes and considered for minimum efficiency of concentration *in vitro* and *in vivo* studies. The extract was prepared in various percentage of concentration with different exposure duration (Table 2). In that 30 min exposure duration with the extract, 2 and 4% concentrations are effective and observed devoid of colonies. Up to 1% concentration was not effective and 10 to 50 bacterial colonies observed in 0.5, 1% and more than 300 colonies was observed in 0.025 % extract on the LB medium incubated for 48 hours. In 1 hour treatment similar trend was observed but number of colonies was less than the 30 min duration. In 2 hour duration, 1% extract onwards effective in all serotypes.

In vivo activity of extracts

An *in vivo* study reveals that 1% concentration of *P. variotii* extract effective for only 2 hour incubation, 2% and 4% extract was effective for all three exposed durations against *Bt* serotypes (Table 3). In positive control, without treatment larvae were infected by the pathogens but mortality rate was varied for all *Bt* serotypes. *Bt morrsoni* infected larvae died within 24 hours after inoculation of pathogen followed by *japonensis, tolworthi, aizawai, sotto, alesti* and less mortality rate was observed in *kenyae, israelensis, kurstaki, thuringiensis* with the influence of toxicity, temperature and/or food quality. Similar results reported by Selvakumar, (2013) stated that environmental factors have little impact on the mortality through toxicity and not infection by the bacteria.

Bacillus thuringiensis is a widely distributed facultative entomogenous bacterium well known for bio pesticide and employed extensively against insect pests in agriculture but they are pathogenic to the silkworm (Selvakumar *et al.,* 1999). Mahmoud *et al.,* (2019) reported that black seed and basil leaves extracts treated with *Bacillus thuriengiensis* infected larvae significantly decreased larval mortality in *B. mori*.

Similarly, aqueous extract of *Thuja orientalis* (Manimegalai and Chandramohan, 2005), leaf extract of *Aegle marmelos* and bark extracts of *T. orientalis* (Manimegalai *et al.,* 2010) were effective in managing *Bt* infected *B. mori.* Similar observation was indicated by Mohanta *et al.,* (2013) extracts of *C. zeylenicum*, *C. longa*, *Z. officinale* showed a strong antibacterial activity against Flacherie pathogens. The secondary metabolites have no apparent metabolic, physiologic and structural role in the producer, but have effects on other organisms function as biochemical defence (Jain *et al.,* 2004). Zhang *et al.,* (2011) isolated four active secondary metabolite (Cerebroside C, Cerebroside D, 2-Hydroxybenzyl alcohol, and 2-(4-Hydrroxypheny) ethanol) from the *Paecilomyces sp*. In the present study P. *Variotii* showed highest activity against all *Bt* pathogens and this might be presence of these compounds in the extract. Similar results reported by Oliveira *et al.,* (2009) *P. variotii* extracts inhibited eight clinical isolates of *entericococcs* bacterial in the ranging from 25 to 35.25mm.

These results indicated that the possibility of the presence of highly active bioactive compounds/ secondary metabolites in the fungal crude extract. It is concluded that further studies are required to find out the bioactive compounds/ secondary metabolite responsible for the activity. Microbial derived products may be a source of alternative active compounds for control of silkworm diseases and it is a new approach in silkworm management.

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