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STUDIES ON THE BACTERIA ASSOCIATED WITH THE MORTALITY OF LARVAL INSTAR OF ANTHEREAE MYLITTA DRURY.

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Abstract: Tasar silkworm is a subtropical, polyphagus and sercigenous insect generally known for their valuable product known as silk. The insect is wild in nature and cannot be domesticated completely. Therefore the insect is directly exposed to the external environment and under the continuous threat of microbial infections. The different life stages of the silk insect are infected by no of pathogens including bacteria. The bacterial diseases cause significant loss in cocoon crop and thus hamper the silk economy. In the present study the local Daba TV ecorace were used for the isolation of bacterial pathogens and their subsequent study to identify them along with their potential to impart death in 4^{th} and 5^{th} larval instars. A total of seven bacterial isolates were purified from Anal Lip (AL) and Rectal Protution (RP) showing infected instars. Upon identification, five different bacterial genus were verified using online verification tool, Microrao. The severity of the different bacterial isolates was found different, *Salmonella spp. & Shigella spp.* with % Death average (45.0) and (40.0)were identified as most severe, while *E. coli* cause moderate(30.0) *and Enterobacter and Enterococcus* were found to have least pathogenecity (20.0) for 4^{th} and 5^{th} larval instars.

Index Terms **- Component,formatting,style,styling,insert.**

INTRODUCTION:

The tropical silkworm *Anthereae mylitta* Drudy is a polyphagous sericigenous insect that belongs to superfamily Saternaide and produces a kind of unique silk fibre generally known as tasar silk. Tasar silkworm is spreaded throughout the different states of India including Andhra Pradesh, Chhattisgarh, Orissa, Jharkhand, Bihar, West Bengal etc. The insect is currently facing the threat of natural habitat loss. Tasar silkworm is a wild insect and cannot be domesticated yet. Due to their direct exposure to the environment the insect is highly prone to microbial diseases such as bacterial, fungal, protozoon etc. Different microbial diseases are devastating for the tasar silk production as mass crop loss due to microbial infections at different stages of the silkworm. Bacterial diseases are one of the most common disease of silk crop. The disease caused by bacteria is commonly known as bacteriosis and it accounts for the pronounced loos in tasar silk industry (Sahay et al., 2000). Number of bacterial species is associated with the bacteriosis and generally affects the larval stages of the silkworm.

Symptoms includes darkening of the body colour, non feeding behaviour, lack of response towards external stimuli ,loses weight, fall off and ultimately die (Singh *et al*., 2011). Different investigations had been made in order to isolate and

characterise the bacteria pest of silk insect to develop suitable control measures (Kalpana *et al.*, 1994; Nahar, 1995; Ramesh *et al.*, 2009; Jing *et al.*, 2008).

REVIEW OF LITERATURE:

Tasar silkworm, Antheraea mylitta Drury is a tropical silkworm of India (Thangavelu, 1991, 1992). Tasar silk is also known as vanya silk and produced by subtropical serecigenous insect Anthereae mylitta belongs to Sturnidae super family. A. mylitta is endemic and distributed in different geographical regions of India in the form of different ecological races (Mahendran et al. 2006). It shows variation in phenotypic traits such as fecundity, voltinism, cocoon weight, and also in its host plant preference (Sinha et al. 1994). The population of A. mylitta is declining due to varied reasons that include deforestation, pathogenic infection and socioeconomic problems. Infection due to pathogens like microsporidia, virus, bacteria and fungus leads to 40–50% death of larvae every year (Singh et al. 2011). Sen et al., (1969) reported the susceptibility o f tasar silkworm (Antheraea mylitta D.)to all the tour kinds of infections, viz., bacteria, viral, sporozoan and fungal.

MATERIALS AND METHODS:

COLLECTION OF SAMPLES:

The third instar laval were collected from the Silk Seed Multiplication Centre, Barari, Bhagalpur, and they were raised indoor on hanging fresh arjuna twigs. The experiments were conducted during the second crop season (September-October) at Post Graduate Department of Biotechnology, Tilka Manjhi Bhagalpur University Bhagalpur, Bihar. The larval instars possessing the symptoms of anal lip sealing and rectal protrusion were collected from the group of feeding silkworms.

ISOLATION OF BACTERIA FROM INFECTED LARVAL INSTARS:

Larval instars showing anal lip sealing and rectal protrution were separately used for the isolation of hind gut. The collected hind guts were homogenized separately in normal saline in order to prevent any osmotic shock. Each homogenised samples were serial diluted in sterile normal saline. The solution having dilution factor (DF) 10^{-5} were used for the isolation of bacterial colonies using Agar plating Method (Nataraju *et al.*, 2005). The pure colonies were picked and maintained on Nutrient Agar for the preparation of stock bacterial culture for their further estimation.

WORKING CULTURE OF BACTERIA:

Bacterial working cultures were prepared in broth for this a loop full of pure bacterial sample from their respectivstock were sub-cultured in Nutrient Broth and incubated for 24 hours.

MORPHOLOGICAL CHARACTERIZATION:

Shape, Size and colour of individual bacterial colony and culture were made using compound microscope (Olumpus) and Stereomicroscope. Gram Staining were performed to identify the associated bacteria.

BIOCHEMICAL CHARACTERIZATION:

Various Biochemical testing of each bacterial culture were done including the IMVIC test in order to characterize the bacteria (Anitha *et al.*, 1994; Amini *et al.*, 2011). The results obtained after biochemical testing were fed to the online identification for their genus identification and verification.

MORTALITY/PATHOGENESIS TESTING :

A total of twenty 4^{th} larval instars were taken for each test group to evaluate the pathogenecity of different isolated bacteria and compared with a standard control group. Individual bacterial suspension having $1X10^{6}$ bacterial cells/ml were orally inoculated within each test group and observed for the development of bacteriosis and larval deaths within 4^{th} and 5^{th} larval instars.

RESULTS AND DISCUSSION:

ISOLATION OF BACTERIA FROM DISEASED SILKWORM:

Total eight different bacteria were isolated from silkworm suffered with anal lip sealing diseased and seven bacteria from silkworm suffered with rectal protrusion on the basis of shape and color of colony appeared in agar plates. The bacteria isolated were coded as shown in Table 1.0.

IDENTIFICATION OF BACTERIAL ISOLATES:

The cultural characters of the bacterial isolates are presented in Table 2.0 In cultural method of identification the bacterial isolates were characterized by the colour (white or yellow), shape (circular, irregular, filamentous and spindle), of growth in slant (beaded, filliform, echinulate and effuse) and habitat (aerobic, anaerobic and facultative) (Fedhila *et al*., 2006).

Table no 2.0 shows the morphological characters of the different isolates first 5 bacterial isolates (AL1 to AL5) were collected from the sealed anal lips of infected larval instars and two from protruded rectum and coded with AL and RP for Anal Lip and Protruded Rectum. Isolates AL1, AL2, AL4. AL5 and RP2 were of white colony appearance. Circular growth were observed within the isolates AL1, AL2, AL5 and RP2 while AL3 shows diffused colony growth, AL4 had Irregular and RP2 showed filamentous colony growth. AL2, AL3 and AL5 showed beaded growth pattern while AL1 and RP2 showed filiform, AL4 had effuse while RP1 showed Echinulate colony growth pattern. Dependence upon oxygen for growth was observed within the isolates AL1, AL2, AL3, AL5 and RP2. Anaerobic growths were observed within the isolates AL4 and RP1.

Table no 3.0 shows the morphological characteristic of isolated bacterial cultures. AL1, AL2, AL3,AL4, RP1 and RP2 were rod shaped along with gram negative in their cell wall characteristic while AL5 were coccoid and gram positive in nature. Among different isolated rod bacteria RP1 was found to be largest in shape and size (4.15±0.35, 1.22±0.22) while AL3 isolates was found to be least in size among rods (3.34±0.71, 0.54±0.22). For the assessment of bacterial size more than 30 bacterial cells of each isolates were examined through micrometry.

BIOCHEMICAL CHARACTERIZATION OF DIFFERENT ISOLATES:

The purified bacterial samples were analyzed biochemically for their identification. A total of 10 different biochemical tests were performed thrice in order to verify results and summarised in tabular form. The biochemical charecterization were done according to the standard Bergey's Manualans suggested by many workers (Selvakumar et al., 1998, 1999 and Patil,1990). The different morphological and biochemical test results was online tested by using Microrao free domin web service for their identification and validation of different bacterial isolates. According to the results shown by the software the isolates were identified and listed in table number 5.0.

ASSESSMENT OF LARVAL MORTALITY:

The table no 6.0 and Fig 6.0 shows the mortality data within the 7 different test groups containing twenty larval instars of 4^{th} stage. Each test group were inoculated by bacterial suspension prepared in normal saline in a concentration of 1×10^6 bacterial cells / ml twice with a total volume of 2 ml per test group. The bacterial inoculation were done by using spray method directly on fresh leaves and fed to the caterpillars. Number of deaths was observed upto 5^{th} instar larval condition before their transformation in cocoon. The data was compared with the test and the level of significance was calculated by using statistical tool, ANOVA to validate the results obtained.

Fig 1.0 Different Bacterial Isolates

Table 1.0 Bacterial isolates and their assigned Codes

Sample Isolates	1	2	3	4	5	6	7
Code of bacterial isolates	AL1	AL2	AL3	AL4	AL5	RP1	RP2

AL- Anal lip and RP- Rectal Protrusion.

Table2.0 Cultural Characteristics of different isolate colonies

Bacterial code	Colour	Morphology	Growth Pattern	Aerobic/Anaerobic
AL1	White	Circular	Filiform	Aerobic
AL2	White	Circular	Beaded	Aerobic
AL3	Yellow	Diffused	Beaded	Aerobic
AL4	White	Irregular	Effuse	Anaerobic
AL5	White	Circular	Beaded	Aerobic
RP1	Yellow	Circular	Echinulate	Anaerobic
RP2	White	Filamentous	Filiform	Aerobic

Table 3.0 Morphological characterization of different isolates

Bacterial Isolates	Gram Stain	Shape	Size(µm)	
			Length	Width
AL1	-	Rod	1.62±0.14	0.85±0.21
AL2	-	Rod	2.11±0.33	0.68±0.25
AL3	-	Rod	1.33±0.27	0.54±0.22
AL4	-	Rod	3.34±0.71	1.05±0.42
AL5	+	Coccoid	1.31±0.41	
RP1	-	Rod	4.15±0.35	1.22±0.22
RP2	-	Rod	1.48±0.24	0.65±0.34

Table 4.0 : Biochemical charecterization of different isolates

Isolate	Catasae Prod.	Amylase Activity	Proteolytic Activity	Citrate Utilization	MR Red.	VP Reac^n	Indole Prod.	Nitrate Prod.	Cellulase activity	H_2S Prod.
AL1	+	+	+	+	+	+	+	+	-	-
AL2	+	+	+	+	+	+	-	-	+	-
AL3	+	+	+	+	-	+	-	+	-	-
AL4	+	+	+	+	-	+	-	+	-	-
AL5	+	-	+	+	-	+	-	+	+	-
RP1	+	+	+	+	+	+	+	+	-	-
RP2	+	+	+	+	-	+	-	+	-	-

Table 5.0 Different isolates and their Identity varified

S.No	Isolates	Identified As
1.	AL1	*E. coli*
2.	AL2	*Salmonella spp.*
3.	AL3	*Enterobacter spp.*
4.	AL4	*Enterobacter spp.*
5.	AL5	*Enterococcus spp.*
6.	RP1	*E. coli*
7.	RP2	*Shigella spp.*

Table 6.0 Deaths in 4th and 5th instars and overall % Death average

Test Groups	Isolate Inoculated	No of samples	Death in 4th Instars	Deaths in 5th Instars	Total Deaths	%Death Average
T1	AL1	20	03	03	06/20	30.0*
T2	AL2	20	05	04	09/20	45.0*
T3	AL3	20	03	01	04/20	20.0*
T4	AL4	20	02	00	02/20	10.0
T5	AL5	20	03	01	04/20	20.0*
T6	RP1	20	04	02	06/20	30.0*
T7	RP2	20	05	03	08/20	40.0*

(* Highly significant)

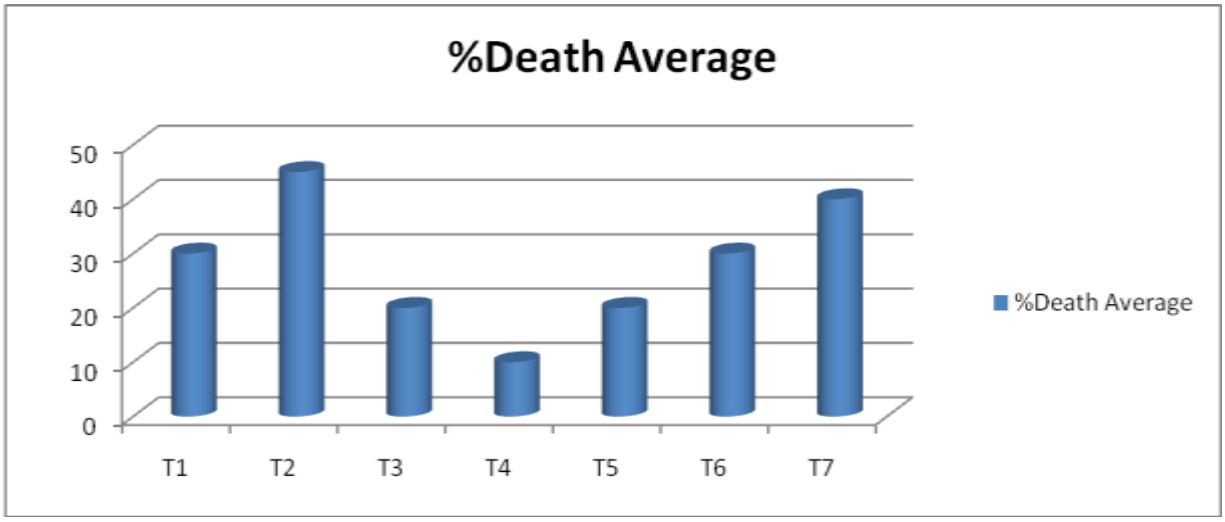

Fig 2.0 Graphical depiction between % Death Rate among different Test groups

CONCLUSION:

Bacterial disease is very common among silkworms and cause severe loss in their cultivation ultimately reducing the overall productivity of silk threads. As estimated bacterial disease accounts for 10-15% loss in cocoon crop (Baig *et al*., 1990; Patil, 1990). In the present piece of work the 7 bacterial species were isolated and further tested for their pathogenisity and potential to impart death within larval stages. It was found that all bacterial isolates has the potential to cause disease within larval instars, the isolate AL4 and RP2 were most severe , AL1 and RP1 were moderate while AL3 and AL5 were least infective for 4^{th} and 5^{th} larval instars.

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