International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Determination of Dose Specific Susceptibility of Different Larval Instars and Effect on the Cocoon Economic Traits of *Bombyx mori L.* to *Bacillus thuringienesis* Var *Sotto*

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Abstract: ***Sericulture is one of the promising means of livelihood, especially for rural people. The sericulture is being practiced in indoor condition and the silkworms are prone to many microbial infections cause different diseases. Among Mulberry silkworm diseases, Bacterial disease caused by Bacillus thuringienesis Var Sotto is causing economic loss to sericulturists. Infection of varied concentrations of B. thuringienesis on different larval instars and effect on cocoon economic traits of silkworm, Bombyx mori L. were studied. Fifteen ml of prepared B. thuringienesis inoculums of different concentrations were smeared evenly on mulberry leaves separately, fed to silkworm larvae of different instars and data was collected. The first instar larvae were more susceptible to bacterial disease which is witnessed with high mortality ranged from 38.33% to 92.33%. Whereas, V instar silkworm larvae were relatively less susceptible with low mortality ranged from 7.00% to 47.67% at the concentrations ranged from 1 x 10^{-1} to 1 x 10^{-8} Cells/ml, respectively. Dose specific mortality indicated that the larvae of same age were more susceptible to the higher concentration of the B.thuringienesis. High LC_{50} value (1.2 x 10^{-8} Cells/ml) was observed in case of V instar silkworm followed by IV and III with 1.6 x 10^{-7} Cells/ml and 2.1 x 10^{-4} Cells/ml values, respectively. Low LC_{50} value (3.8 x 10^{-2} Cells/ml) was observed in I instar silkworm. Silkworms infected in lateral stages manage to complete its life cycle but the cocoon characters are affected significantly. The cocoon characters and interaction of IV instar inoculation and V instar inoculation were significantly effected at P<0.01 and P<0.05 level, with 1 x 10^{-7} Cells/ml B. thuringienesis concentration.***

Keywords: Bacillus thuringienesis, Bombyx mori, Susceptibility, LC_{50} value, Mortality

1. Introduction

Sikworm *Bombyx mori, L.* is domestic in nature and reared in door on mulberry leaves and silkworm is most susceptible to a number of diseases caused by different pathogenic agents such as bacteria, viruses, fungus and microsporidia and no race of silkworm can be deemed as totally resistant to either diseases or pests. The major diseases affecting silkworm in India are pebrine, viral, bacterial and fungal diseases. According to Samson et al., 1990 bacterial flacherie accounts for maximum loss followed by viral, protozoan and fungal diseases. *Bacterial flacherie* takes heavy tool of silkworm cocoon crop in India especially during summer season, Chitra et al., (1975) reported 30-40 percent cocoon crop loss in India due to bacterial flacherie. The main causes responsible for bacterial flacherie in *Bombyx mori* are (i) high temperature (ii) high humidity (iii) bad ventilation (iv) soiled Mulberry leaves (v) over crowding etc. The above conditions are very common in southern states like Karnataka, Andhrapradesh and Tamilnadu as they come under temperate climatic zone of the globe.

In this condition, among many measures of silkworm disease control and prevention, utilization of disease resistant/tolerant silkworm breed/hybrid along with the disinfection will be most effective step in the direction of the disease prevention (Kiran et al., 2011). Before initiation of any breeding programme for disease resistance/tolerance, the tolerance level of the each instar at specific dose should be explored. The aim of the most breeding programmes is to improve the yield potential of the breeds/hybrids over the existing, which has played a vital role in increasing the productivity in sericulture (Reddy et al., 2008, 2009a, b, c, 2010; Sivaprakasham and Rabindra, 1995, Kiran et al., 2011). Kodama and Naksuji conducted extensive studies on the bacterial diseases of the silkworm and found that with the increasing number of cells injected, the outset of death is hastened, but the number of cells retrieved for causing 100% mortality varies with the species of bacteria used. They also found that *Streptococcus faecalis, Strepto faecium* intermediate *E.S* can grow luxuriantly in the gut of V instar larvae lowers the gut pH from 8.0-9.5 to7.5-7.6.

Endo toxin of *B. thuringiensis* paralyzed the silkworm when given orally, but no effect when injected in to haemocoel directly Chitra and Vasantha Rajan (1795). Investigation of Specfic toxicity of endotoxins CryIA (a) and CryIA (b) from *B.thuringiensis* subspicies, *aizawai* were used to investigate the specificity in insecticidal activity, Hideshi Ihara,Emi Kuroda, Akira Wadano, and Michino Himeno – Biosci.Biotech.Biochem.,57 (2), 200 -204,1993. B.Nataraju, M.BalaVenatasubbaih, M.Baig etc CSR&TI Mysore 1989 followed standard method of Aizwa et al ., 1961 to isolate *B.thuringiensis* and find out the spore formation Bacillus in the sericulture areas of Karnataka. The symptoms of *B.thuringiensis* infection appeared in 48 hr. after treatment and the larvae died in 72 hr (Majamdar et al., 1955). The inoculation of *B.thurigiensis* to silkworms resulted a change in the metabolic activity in the mid gut just 10 min. after the administration of of endotoxin and even the ionic imbalance in the circulatory system was also noticed (Louloudes and Himpel, 1969; Pendelton, 1970; Fast and Donaghue,1971; Fast and Morison, 1972).

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The crystals of subspecies *B.thuringiensis* viz., *thuringiensis,* sotto and entomocidus when fed to silkworms caused paralysis with in 6 hr at 26, 0.02 and 03µg per g of larvae (Angus 1967). Thirteen isolates of *B.thurigiensis* have been tested per-oraly as washed whole cultures, against *B.mori.* Two of the isolates were not toxic up to 100 µg per g of larva and for other 11 isolates the LD50 (Paralysis in 6 hr) value ranged from 0.1 to 65 µg per g.

The LD50 (death in 48 hr) values ranged from 0.09 to 10 mg per g. There was considerable variation in toxicity within the same serotype (Angus and Noris, 1968). The average LD (µg protein toxin per mg of insect x10-³) values due to B.t Var. Dendrolimus through per os were 4.00, 2.73, 2.33 and 1.15 and 11.09. 11.09, 5.01 and 2.96 through haemocoel for 24, 48, 72 and 96 hr. respectively in *B.mori* (Faust and Dougherty, 1969). According to Angus and Heimpel (1956) the deviation in the blood pH in the fourth instar silkworm larvae at 80 to 65 min. after administration of B. sotto was to the extent of 20.31 percent compared to 19.12 percent in case of fifth instar thus revealing former to be more susceptible.

Therefore, the present experiment was designed to determine the age and dose specific susceptibility of *Bacillus thuringiensis* and its effect on cocoon parameters in silkworm *Bombyx mori*. This study will be absolutely useful for induction and improvement of tolerance in silkworm races.

2. Material and Methods

All the required experiments of the study on determination of Dose Specific Susceptibility of different larval instars and effect on the Cocoon economic traits of Bombyx mori L. to *Bacillus thuringienesis* Var Sotto conducted in, Dept of Sericulture, Sri Krishnadevaraya University, Anantapur, Silkworms CSR 2 XCSR 4 were reared as per the package practices up to the end of the experiment (Krishnaswami et al., 1973). In this experiment 1st to 5th instar larvae are taken to study targeted research aspects.

Purification of Bacteria

The *Bacillus thuringiensis* is isolated from the worms affected from the regular rearings of the Depart of Sericulture , Sri Krishnadevaraya University. The Bacterial spores and crystals are purified with 1.0M NaCl and 0.01percent Triton x-100, vortexed and washed repeatedly with sterile water by centrifugation (Jhonson and McGaghey, 1984) Bacillus infection was made orally to the silkworm larvae on 3 rd day of I, II, III, IV, and V instars for further multiplication and the results are noted and tabulated for further study.

Silkworm hybrid layings (DFL's) of CSR 2 X CSR 4 Layings were surface sterilized with 2.0 % Formalin for 4-5 min. and were incubated under standard conditions for uniform development of embryo. On the day before brushing silkworm eggs, the healthy Mulberry leaves were harvested from Department garden and were washed with distilled water and surface sterilized with 70 percent ethanol with the help of sterile cotton swabbing and leaves were dried under shade conditions before feeding the worms. This is continued throughout the rearing to avoid any contamination of microbes. Silkworms are reared in silkworm physiology research laboratory, Dept of Sericulture, Sri Krishnadevaraya University, Anantapur, under the standard rearing conditions at temperature 26±1°C with 75 ± 5% relative humidity and photoperiod of 16 L: 8D as per the recommended rearing practice (Raja Ram, 2000). In this experiment 4th and 5th instar larvae are taken to study targeted research aspects.

The bacterium, *B.thurigiensis Var.Sotto* is isolated from the infected silkworms. The isolation procedure for bacteria is conducted as per the method followed by Samson (1987). Dilution of the bacterium required for inoculation is prepared in distilled water from the stock solution. Serial diluted concentrations are smeared on to the leaves which are kept ready in rearing room to feed silkworms. The inoculation is done for three replications of worms I to V Instar). Mortality is recorded every 24 hrs and calculations for LC50 were determined according to Finny (1964).

Analysis of data

Larval mortality due to bacterium was recorded daily up to 10th day in each instar and the cumulative mortality was used for Probit analysis (Finney, 1971) to determine the LC_{50} value. In the case of IV and V instars of $1x10^3$, $1x10^5$ and $1x10^7$ Cells/ml concentrations batches were reared up to cocoon formation and the observations were made on cocoon characters. The data on Pupation rate, Single cocoon weight, Single shell weight and Silk ratio was subjected for the Analysis of variance (ANOVA) by employing "INDOSTAT" software.

3. Results

Age and dose specific susceptibility of different instar of silkworm larvae were ascertained through infectivity tests. Infection of Bacillus at each instar was adjudged based on the symptomatology, subsequent larval mortality, microscopic examination and re-isolation of the pathogen from cadavers of the silkworms.

Mortality due to *Bacillus thuringienesis*

Instar wise silkworm *Bombyx mori* larval mortality due to *Bacillus thuringienesis* against different concentrations were represented in Table 1. The 1st instar silkworm when inoculated with the 1 x 10^{-8} Cells/ml, could not survive up to cocooning stage and the mortality rate due to *Bacillus thuringienesis* was observed to be as high as 92.33% with specified duration of observation. The treatments with pathogen load of 1 x 10^{-7} and 1 x 10^{-6} and 1 x 10^{-5} to 1st instar were also equally fatal causing mortality to the tune of 85.00, 80.33 and 72.67%, respectively. Silkworms could survive in treatments having *Bacillus thuringienesis* concentration of 1 x 10^{-4}, 1 x 10^{-3}, 1 x 10^{-2} and 1 x 10^{-1} and the mortality recorded was 59.00, 51.00, 45.67 and 38.33%, respectively. Similarly in the 2nd instar silkworm rearing when inoculated with foresaid eight treatments, the mortality recorded was 85.00, 76.33, 71.00, 67.33, 53.67, 45.33, 39.00 and 32.66%, respectively. However, 3rd, 4th and 5th instar when treated with the above mentioned eight concentrations of *Bacillus thuringienesis* order, the mortality ranged between 25.33 to 80.33%, 18.33 to 54.00% and 7.00 to

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47.67%, respectively. In case of the control, the mortality due to *Bacillus thuringienesis* is zero in all instars.

Lethal Concentration 50% (LC_{50}) of *Bacillus thuringienesis*

Different instars of silkworm when inoculated with the varied concentrations of *Bacillus thuringienesis* showed differences in LC_{50} values (Table 2). The LC_{50} values of *Bacillus thuringienesis* from 1st instar to 5th instar showed continues increasing trend. The LC_{50} value for the 1st instar was the lowest (3.8×10^{-2} Cells/ml) and increased to 2.1×10^{-3} Cells/ml in 2nd instar which further increased in 3rd instar (2.1×10^{-4} Cells/ml) thereafter also the value is promoted in 4th instar (1.6×10^{-7} Cells/ml) and reached highest (1.2×10^{-8} Cells/ml) in 5th instar.

Probit mortality in different instars

Probit graphs (Fig. 1 to Fig. 5) were plotted between probit mortality and log concentration of different instars of silkworm *B.mori*. Probit mortality for 1st instar silkworm larvae was ranged from 4.702 to 6.428 with 0.983 R^2 value (fig. 1). Probit mortality for 2nd instar silkworm larvae was ranged from 4.55 to 6.04 with 0.988 R^2 value (fig. 2). Probit mortality for 3rd instar silkworm larvae was ranged from 4.34 to 5.85 with 0.986 R^2 value (fig. 3). Probit mortality for 4th instar silkworm larvae was ranged from 4.10 to 5.10 with 0.997 R^2 value (fig. 4). Probit mortality for 5th instar silkworm larvae was ranged from 3.52 to 4.94 with 0.977 R^2 value (fig. 5). Probit mortality range was recorded with decreasing trend from 1st instar to 5th instar which is a statistical evidence for the increase of tolerance from young age larvae to the late age silkworm larvae.

Effect of *Bacillus thuringienesis* on the economic traits:

Effect of *Bacillus thuringienesis* inoculation during the later instars on the economic traits of silkworm is represented in Table 3.

Pupation rate

Pupation rate was lower when the dosage was higher and also when the inoculation was earlier. The IV instar larvae were inoculated with lower dosage of inoculum ($1x10^{-3}$ Cells/ml) showed higher percentage of pupation rate of 40.20% and those larve inoculated with higher dosage ($1x10^{-7}$ Cells/ml) showed less pupation rate of 12.80%. The batches of larvae that were administrated with 1×10^{-5} Cells/ml of inoculum showed intermediate level of pupation rate of 23.80%. The highest percentage of good cocoons was obtained in the batches of larvae inoculated with 1×10^{-3} Cells/ml dosage of inoculum during fifth instar (Table 3).

Cocoon characters

The cocoon and shell weights also varied with the dosage of inoculum as well as the stage of administration. Cocoon and shell weights were less in IV stage inoculation, while larvae inoculated at V stage showed comparatively more cocoon and shell weights. The highest and lowest cocoon weights were observed in the larval batches where *Bacillus thuringienesis* dose of $1x10^{-3}$ Cells/ml and $1x10^{-7}$ Cells/ml were administrated V instar larvae and IV instar larvae, respectively.

The highest shell weight was recorded in the batch, inoculated with 1×10^{-3} Cells/ml in V instar (0.284g). Variation has been found in the shell percentage of the cocoons formed by the larvae subjected to different doses of *Bacillus thuringienesis* inoculation in IV and V instars. Shell percentage varied from minimum value of 16.90% to a maximum value of 18.98%. Pupation rate, cocoon weight, shell weight and S.R. % were recorded higher values in the case of control batches in comparison with all treatment batches (Table 3)

Table 1: *Instar wise mulberry silkworm larval mortality due to* Bacillus thuringienesis *Var against different concentrations*

Concentration Cells/ml	Mortality due to *Bacillus thuringienesis* Var				
	I Instar	**II Instar**	**III Instar**	**IV Instar**	**V Instar**
1×10^{-1}	38.33	32.66	25.33	18.33	7.00
1×10^{-2}	45.67	39.00	31.33	21.67	10.67
1×10^{-3}	51.00	45.33	42.67	27.00	19.33
1×10^{-4}	59.00	53.67	46.33	31.00	23.00
1×10^{-5}	72.67	67.33	54.00	38.67	29.33
1×10^{-6}	80.33	71.00	61.67	43.33	35.67
1×10^{-7}	85.00	76.33	69.00	48.67	40.33
1×10^{-8}	92.33	85.00	80.33	54.00	47.67
Control	0.00	0.00	0.00	0.00	0.00

Table 2: *LC_{50} values of* Bacillus thuringienesis *Var to* Bombyx mori L.

Instar	**LC_{50} (Cells/ml)**	**Y mean**	**X mean**	**Chi square**	**SE of b**	**Probability**	**Fiducial Limit**
1st	3.8×10^{-2}	5.363	4.086	2.238	0.022	0.897	0.1866 to 0.2961
2nd	2.1×10^{-3}	5.208	4.307	1.244	0.021	0.975	0.1592 to 0.2625
3rd	2.1×10^{-4}	5.032	4.470	1.395	0.021	0.966	0.1521 to 0.2537
4th	1.6×10^{-7}	4.634	4.719	0.162	0.021	0.999	0.0960 to 0.1971
5th	1.2×10^{-8}	4.412	5.006	1.694	0.023	0.946	0.1356 to 0.2467

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Table 3: Effect of *Bacillus thuringienesis* Var inoculation during the later instars on the economic traits of mulberry silkworm Bombyx mori L.

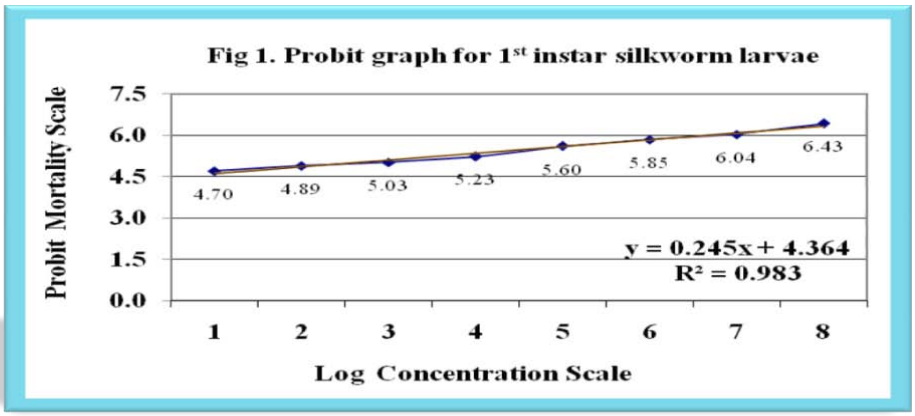
Stage of the inoculation	Bacillus thuringienesis Var dosage (Cells/ml)	Mortality due to B.T (%)		Mortality due to unknown reasons (%)	Pupation rate (%)	Cocoon characters		
		Larval	Pupal			Cocoon.Wt. (g)	Shell.Wt. (g)	S.R (%)
IV Instar	$1x10^{-3}$	31.60	7.00	21.20	40.20	1.496	0.284	18.98
	$1x10^{-5}$	41.40	11.20	23.60	23.80	1.426	0.256	17.92
	$1x10^{-7}$	50.60	12.20	24.40	12.80	1.385	0.234	16.90
	Control	0.00	0.00	10.20	92.92	1.764	0.396	22.45
V Instar	$1x10^{-3}$	19.80	8.80	23.60	47.80	1.506	0.272	18.06
	$1x10^{-5}$	29.40	10.40	25.00	35.20	1.458	0.251	17.22
	$1x10^{-7}$	39.60	15.40	25.40	19.60	1.351	0.240	17.76
	Control	0.00	0.00	08.90	93.02	1.759	0.393	22.28

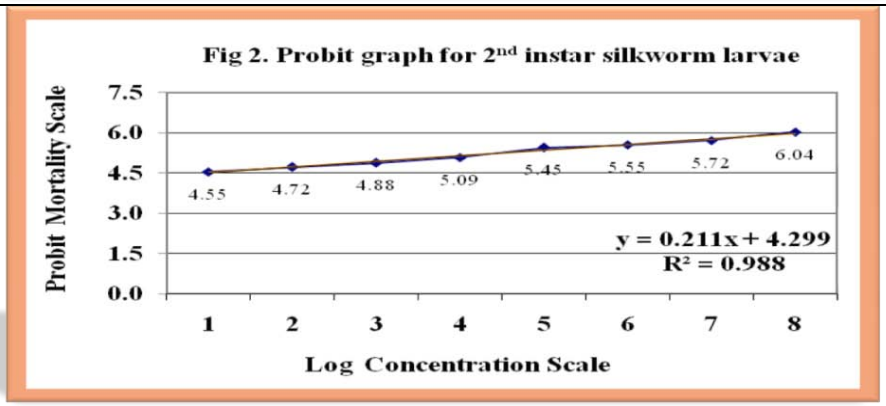
Table 4: Analysis of variance on the economic traits of mulberry silkworm Bombyx mori L.

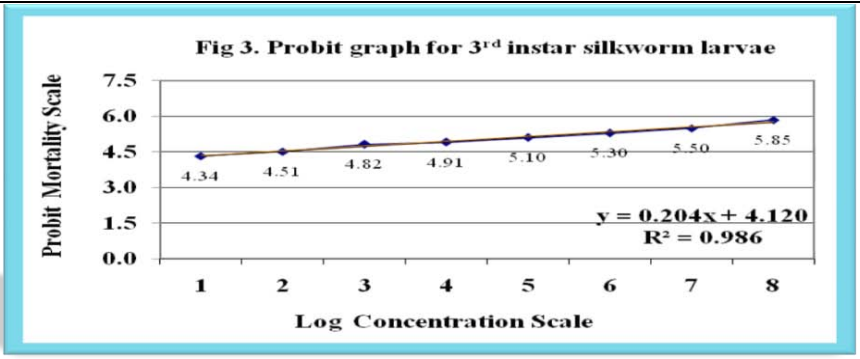
Source of Variations	df	Pupation rate (%)		SCW(g)		SSW(g)		SR (%)	
		Mean Squares	F Ratio	Mean Squares	F Ratio	Mean Squares	F Ratio	Mean Squares	F Ratio
IV instar inoculation	1	554.700**	346.69	1.619**	2199.73	0.022**	897	0.046**	17.44
V instar inoculation	2	1933.300**	1208.31	6.026**	8185.45	0.414**	16562.39	17.195**	6462.47
IV Vs. V instar inoculations	2	15.100*	9.44	0.562*	764.02	0.002*	85.79	1.014*	381.26
Error	32	1.600		0.001		0.000		0.003	

***Significant at (P<0.05); **Significant at (P<0.01)**

Figure







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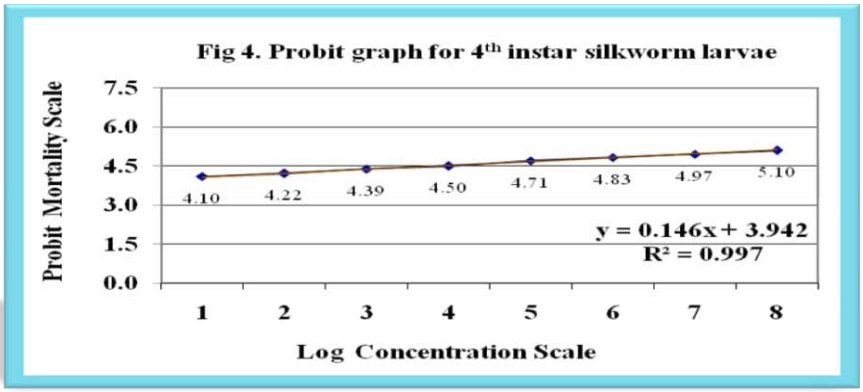

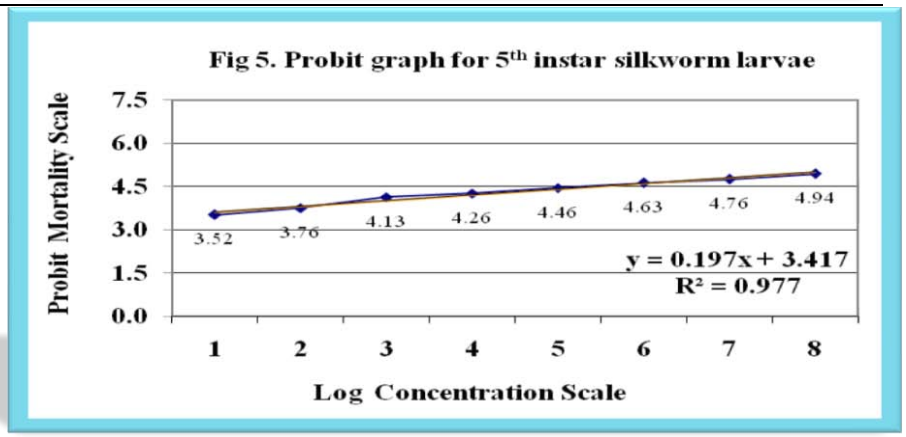

Analysis of variance

Analysis of variance on the economic traits of silkworm *Bombyx mori* was represented in the table 4. The results revealed that IV and V instar inoculation were significantly different at P<0.01 level for the parameters Pupation rate (%), Cocoon weight, Shell weight and S.R% respectively. On the other hand their interaction was significantly different at at P<0.05 level for all the traits under study.

4. Discussion

According to Samson et al., 1990 *Bacterial flacherie* accounts for maximum loss followed by viral, protozoan and fungal diseases. *Bacterial flacherie* takes heavy tool of silkworm cocoon crop in India especially during summer season, Chitra et al., (1975) reported 30-40 percent cocoon crop loss in India due to bacterial flacherie. The estimation of LC_{50} values gives the idea of tolerance or susceptibility and also sensitivity of the different stages of the silkworm *B.mori*.

In the present study, the highest mortality (92.33%) with lowest LC_{50} value (3.8x 10^2 Cells/ml) in 1st instars larvae indicates that the 1st instar larvae are highly susceptible to *B,thuringienesis* infection. This may be due to the longer period of stay of the pathogens after ingestion of endotoxin inside the body of silkworm providing opportunity for development of population within the silkworm.

Since the 1st instar larvae had a longest larval period to reach cocooning stage after inoculation, the *B.thuringiensis* could get maximum period to have higher number of multiplication cycles. *Bacillus* is commonly isolated from soils, the life cycle is characterized by two phases which includes vegetative cell division and spore development.

The 4th and 5th instar larvae were found least susceptible as the LC_{50} values reached upto 1.6 x 10^7 and 1.2 x 10^8, respectively because the *B.thuringiensis* get minimum time for multiplication as the larvae spin cocoon within 10 days after inoculation. This may be the reason of low mortality and high LC_{50} values. The decreasing trend in the survival of silkworm was observed with the increase in pathogen load in each instar.

The effect of pathogencity of *B.thuringiensis* was found severe on the commercial characters of *Bombyx mori*. Silkworms infected in later stages manage to complete its life cycle but the cocoon characters are affected significantly. The high dosage (1×10^7/ml) could cause significantly higher larval mortality when it was inoculated on fourth instar, but when the inoculation was done at advanced larval stage, larval mortality reduced and post cocoon mortality increased significantly. The cocoon weight, shell weight and silk ratio percentage were found significantly different (P<0.05) in cocoons raised from different larval instar with the different concentrations of pathogens load.

5. Conclusion

The first instar silkworm larvae were more susceptible to bacteria whereas, V instar silkworm larvae were relatively less susceptible. Dose specific mortality indicated that the larvae of same age were more susceptible to the higher concentration of the *B.thuringiensis*. Silkworms infected in lateral stages manage to spin the cocoon but they die and decompose in the cocoon by emitting black fluid causing stains on cocoon and made them to unfit for reeling,

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References

[1] **Angus and Heimpel (1956).** The deviation in the blood pH in the fourth instar silkworm larvae at 80 to 65 min. after administration of B. sotto was to the extent of 20.31 percent compared to 19.12 percent in case of fifth instar thus revealing former to be more

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[4] **Chitra and Vasantha Rajan (1795).** Endo toxin of *B. thuringiensis* paralyzed the silkworm when given orally, but no effect when injected in to haemocoel directly.

[5] **Chitra et al., (1975)** reported 30-40 percent cocoon crop loss in India due to bacterial flacherie.

[6] **Faust and Dougherty, 1969**.The average LD (µg protein toxin per mg of insect x10-³) values due to B.t Var. Dendrolimus through per os were 4.00, 2.73, 2.33 and 1.15 and 11.09. 11.09, 5.01 and 2.96 through haemocoel for 24, 48, 72 and 96 hr. respectively in *B.mori.*

[7] **Finny (1964).**Mortality is recorded every 24 hrs and calculations for LC50 were determined according to

[8] **Hideshi Ihara,Emi Kuroda, Akira Wadano, and Michino Himeno – Biosci.Biotech.Biochem.,57 (2), 200 -204,1993**. Investigation of Specfic toxicity of endotoxins CryIA (a) and CryIA (b) from *B.thuringiensis* subspicies, aizawai were used to investigate the specificity in insecticidal activity.

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[11] Kodama and Naksuji conducted extensive studies on the bacterial diseases of the silkworm and found that with the increasing number of cells injected, the outset of death is hastened, but the number of cells retrieved for causing 100% mortality varies with the species of bacteria used. They also found that ***Streptococcus faecalis, Strepto faecium*** intermediate E.S can grow luxuriantly in the gut of V instar larvae lowers the gut pH from 8.0-9.5 to7.5-7.6.

[12] **Krishnaswami et al., 1973**. Silkworms were reared as per the package practices up to the end of the experiment.

[13] **Louloudes and Himpel, 1969; Pendelton, 1970; Fast and Donaghue,1971: Fast and Morison, 1972.** The inoculation of *B.thurigiensis* to silkworms resulted a change in the metabolic activity in the mid gut just 10 min. after the administration of of endotoxin and even the ionic imbalance in the circulatory system was also noticed.

[14] **Majamdar et al., 1955.** The symptoms of *B.thuringiensis* infection appeared in 48 hr. after treatment and the larvae died in 72 hr.

[15] **Raja Ram, 2000.** Under the standard rearing conditions at temperature 26±1°C with 75 ± 5% relative humidity and photoperiod of 16 L: 8D as per the recommended rearing practice.

[16] **Reddy et al., 2008, 2009a, b, c, 2010; Sivaprakasham and Rabindra, 1995, Kiran et al., 2011.** The aim of the most breeding programmes is to improve the yield potential of the breeds/hybrids over the existing, which has played a vital role in increasing the productivity in sericulture.

[17] **Samson (1987).** The bacterium, ***B.thurigiensis Var.Sotto*** is isolated from the infected silkworms. The isolation procedure for bacteria is conducted as per the method followed .

[18] **Samson et al., 1990** *Bacterial flacherie* accounts for maximum loss followed by viral, protozoan and fungal diseases. *Bacterial flacherie* takes heavy tool of silkworm cocoon crop in India especially during summer season.

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