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CELL-MEDIATED IMMUNE RESPONSE TO ENTOMOPATHOGENIC FUNGUS *Beauveria bassiana* IN DIFFERENT STRAINS OF SILKWORM *Bombyx mori* L.

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration with the both authors. Authors MSP and GS together designed the study. Author MSP performed the statistical analysis with the help of the statistician. Author MSP wrote the protocol and author GS wrote the first draft of the manuscript. Authors MSP and GS managed the analyses of the study and managed the literature searches. Both the authors read and approved the final manuscript.

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ABSTRACT

Mulberry silkworm *Bombyx mori* is a tiny insect with great economic and scientific significance. Silkworms are delicate and susceptible to different groups of pathogenic microorganisms such as bacterial, protozoan, viral and fungal pathogens due to continuous domestication for centuries. Disease occurrence is the main reason for not achieving the potential yield parameters of silkworm cocoon crops. Silkworm is the best model to assess the immune mechanism in insects and possesses a well-organized and competent immune system that eliminates different types of invading pathogenic microorganisms to protect the body from infection. The immune system in silkworm *Bombyx mori* mainly comprises of cellular immunity and humoural immunity. The cellular immunity is mediated by different types of haemocytes viz., prohaemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids resides in the haemolymph of silkworm *Bombyx mori*. Humoral reactions require several hours for full expression while cellular interactions are immediate and direct between haemocytes and the invading pathogens that include phagocytosis, nodulation and encapsulation. Dynamics of total and differential haemocyte count designate the degree of tolerance/resistance of the host system to infections and can be used as a catalogue for diagnosis of the disease. Therefore, the investigation was carried out with the following objective To examine the cellular immune-potency in silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* by quantifying the density of total and differential haemocytes in three popular silkworm hybrids viz., double hybrid (CSR2 x CSR27) x (CSR6 x CSR 26), crossbreed (PM x CSR2) and bivoltine single hybrid (CSR2 x CSR4) compared to healthy silkworms.

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Keywords: *Bombyx mori*; *Beauveria bassiana*; cellular immunity; total haemocyte count; differential haemocyte count.

1. INTRODUCTION

Silkworm *Bombyx mori* is an economically significant tiny insect and bulk producer of tradable silk, a class of textile fibre of excellence and preferred fabric of high class fashion world. Silkworms are susceptible to various diseases caused by all major groups of micro-organism's viz., protozoan, viral, bacterial and fungal pathogens. Disease incidence in silkworm *Bombyx mori* is fairly common and serious in inflicting silkworm cocoon crop loss. White muscardine caused by *Beauveria bassiana* is a common disease of the silkworm *Bombyx mori* and the disease is prevalent especially during rainy and winter seasons causing great cocoon crop loss to the sericulture farmers. The disease was the first microbial disease described in insects. Agostino Bassi [1] demonstrated the contagious nature of the fungal pathogen *Beauveria bassiana* in silkworms, eventually the fungal pathogen was named as *Beauveria bassiana* in honour of Agostino Bassi. The mode of infection of *Beauveria bassiana* is through integument and occasionally through injury [2]. The germ tubes of the conidia penetrate into the haemocoel and impair the functions of the haemolymph.

Haemolymph is lifesaving fluid that circulates in the body freely that triggers the innate immune response when any foreign particle enters into the silkworm body [3]. Haemocytes are blood cells that circulate within the body cavity of insects that plays a major role in defence mechanism of host organism [4]. Infection stimulates immune response in silkworm *Bombyx mori* and it exhibits through cellular and humoural immune response against the invading pathogen or any other foreign body. Haemocytes in the haemolymph play a vital role in protecting the silkworms against the pathogens that enter the haemocoel [5, 6, 7] Watanabe, 2002. The efficient protective mechanisms of phagocytosis, encapsulation, and other allied defence mechanisms were mainly because of the availability of the circulatory haemocytes. Balavenkatasubbaiah et al., [8] and Balavenkatasubbaih and Nataraju [9] documented five types of haemocytes viz., Prohaemocytes, Plasmatocytes, Granulocytes, Spherulocytes and Oenocytes in the silkworm. Insect's exhibits defence response through cellular and humoral mechanisms (Dunn, 1986). Cellular immune responses are direct and immediate interactions between invading pathogens and haemocytes in the haemolymph [10]. Dynamics of haemocytes indicates degree of tolerance in different silkworm breeds against the diseases [8]. Therefore, the investigation mainly focused to understand the dynamics of total and differential haemocyte count during development of the fungal pathogen in 5th instar silkworms inoculated with *Beauveria bassiana* in different popular silkworm hybrids viz.,bivoltine double hybrid (CSR2 x CSR27) x (CSR6 x CSR 26), crossbreed (PM x CSR2) and bivotine hybrid (CSR2 x CSR4).

2. METHODOLOGY

Selected three silkworm hybrids viz., bivoltine double hybrid (CSR2 ×CSR27) × (CSR6 × CSR 26), crossbreed (PM x CSR2) and bivoltine single hybrid (CSR2 × CSR4) and maintained the stock of silkworms under optimum conditions according to Dandin et al (2003). The rearing house, rearing appliances and surrounding of the rearing house were disinfected thoroughly to maintain pathogenic free environment before rearing and after completion of rearing. Silkworms were fed with a fresh, proteinaceous and correctly matured mulberry leaves in accordance with the age of silkworm and feeding is provided thrice a day.

The optimum temperature of 25°- 26°C for 3rd instar, 23° to 25°C for 4th and 5th instar silkworms was maintained and 75-80% of relative humidity for 3rd instar worms and 70 -75% of relative humidity was maintained for 4th and 5th instar silkworm to obtain healthy growth of silkworm for the experiment.

White powdery material was collected from six days old *Beauveria bassiana* mummified silkworms and transferred onto the Potato Dextrose Agar (PDA) medium and incubated at 25°C for a week. The conidia from a single colony of the fungus were transferred to sterile PDA slants and the pure culture of *Beauveria bassiana* was maintained by repeated transfers for every 15 days under strict aseptic conditions. Using double distilled water spore suspension was prepared from one - week old culture. The concentrated spore suspension was transferred to a sterilized conical flask containing distilled water and was thoroughly shaken for ten minutes. The suspension was then strained through double-layered sterile cheesecloth. The spore count was recorded using Neubauer haemocytometer and expressed as number of spores per ml.

After passing the 4th moult the larvae were taken for experimentation. A hundred larvae were selected randomly per replication from the silkworm stock and inoculated by dipping in the sub-lethal concentration

(2.15×10^5 conidia/ml for crossbreed and Bivoltine single hybrid and 2.15×10^4 conidia/ml for Bivoltine double hybrid) of *Beauveria bassiana* spore suspension @ 50 ml/100 worms for 45 seconds and 4 replications were maintained. The larvae treated with double distilled water have been used as control. One day after the induction of the fungal pathogen, haemolymph was collected from silkworms by clipping the abdominal legs of silkworm into pre-chilled centrifuge tubes with a pinch of phenylthiourea and then the haemolymph was used for enumeration of the total (Tauber and Yeager 1935) and differential haemocytes (Jalali and Salehi 2008).

2.1 Total Haemocyte Count (THC)

For enumeration of total haemocyte count haemolymph of *Bombyx mori* was drawn into clean graduated WBC pipette up to 0.5-mark, excess of the solution was wiped out with the help of tissue and the level of the haemolymph was exactly kept at 0.5 and it diluted up to 101 mark with a diluting fluid (sodium acetate 3.0 g and distilled water 100 ml). Then both the ends of the pipette were held firmly between the forefinger and the thumb and shaken for several minutes. Then the first 2-3 drops were discarded and a drop was transferred to the platform of the counting chamber of improved Neubauer haemocytometer by holding the pipette at 45° to the surface of the chamber then automatically diluted haemolymph runs into the counting chamber due to capillary action. A thick cover slip was applied before transferring the fluid mixture from the pipette and care was taken that no air bubble enters into the counting chamber. The total number of cells in 1 mm3 will be 4000a X 200 = 8, 00,000 × (× = number of cells in each square) and the haemocytes counted in its four corners and one central (1mm3) square. The number of circulating haemocytes per cubic millimetre (mm3) was calculated using the following formula adopted by (Tauber and Yeager, 1935).

$$= \frac{\text{Haemocytes in five 1mm squares x Dilution x Depth factor of the chamber}}{\text{Number of squares counted}}$$

Depth factor of the chamber = 10 (constant)
Number of squares counted = 05

2.2 Differential Haemocyte Count (DHC)

A thin and uniform layer of haemolymph was spread on a clean and grease-free slide then 8-10 drops of the filtered leshmann's stain (leshmann's stain 1.50g was dissolved in methanol 100 ml) solution was poured on the film so that the complete film was covered with the stain and allowed to act for 2 minutes. Then 16-20 drops of the buffer solution (a salt mixture of Di-sodium-hydrogen phosphate - 5.447 g and Potassium-di- hydrogen phosphate - 4.752 was pulverized thoroughly in a mortar and pestle and 1g of this pulverized mixture was dissolved in 1 litre of distilled water to get the required pH of 7.00 Anonymous, 1958) was put over the slide and both stain and buffer solution were mix thoroughly by gentle agitation and the mixture was allowed to act for another 10 minutes.

The excess stain was drained off and the stained smear was washed with the buffer solution and the smear was then blotted dry with filter paper and finally sealed with Dibutylhthalate Polystyrene Xylene (DPX) (Anonymous, 1958). Then the smear was examined under oil immersion phase contrast microscope and 200 cells per slide were differentiated based on their morphological features as described by Yeager's classification as modified after Jones (1959). For each replication, fifteen slides were prepared and 200 cells were counted in each slide. This meets the recommendations of Jones (1962) which states that a minimum of five insects of a given stage should be counted and whenever possible, a minimum of 200 cells should be classified. The total and differential haemocyte count was enumerated in the haemolymph of healthy and inoculated batches.

The data recorded has been analysed by using three-way ANOVA.

3. RESULTS AND DISCUSSION

Total and differential haemocyte count of three breeds viz., bivoltine double hybrid (CSR2 x CSR27) x (CSR6 x CSR 26), crossbreed (PM x CSR2) and bivoltine single hybrid (CSR2 x CSR4) of silkworms is presented in Table - 1 and Graph -1 (Total Haemocyte Count) and Tables 2-6 and Graph 2-6 (Differential Haemocyte Count) during the development of fungal pathogen *Beauveria bassiana.*

3.1 Total Haemocyte Count (THC)

Elevation of total haemocyte count was noticed from 1st to 5th day of 5th instar larvae then reduction of haemocyte count was noticed on 6th day in bivoltine double hybrid. In case of crossbreed and bivoltine single hybrid, gradual increase of Total Haemocyte Count (THC) was a noticed from 1st day to 4th day and reduction of THC was noticed in rest of the instar in both the races viz., crossbreed and bivoltine single hybrid in untreated larvae. In *Beauveria bassiana* inoculated larvae of bivoltine double hybrid, a gradual increase of the total count of haemocytes was recorded up to 4th day and then the reduction of THC was noticed on 5th and 6th day of 5th instar silkworm. In case of crossbreed and bivoltine single hybrid

gradual increase THC was recorded up to 3rd day respectively then substantial decrease of THC was noticed from 4th day to 6th day of the instar in crossbreed and bivoltine single hybrid. Higher levels of THC were observed up to 4th day in bivoltine double hybrid and up to 3rd day in case of crossbreed and bivoltine single hybrid in comparison with control.

Silkworm demonstrates immune response through cellular and humoral defence mechanism and also through cell apoptosis, that provides an effective barrier to infection [11], (Zhu et al 2019). The cellular immune mechanism is a protective immune process in silkworm/insects and is mediated by different types of haemocytes during the progress of pathogen/foreign particles in the host organism. Functionally, haemocytes are motile and phagocytic in nature and involved in the cellular response to pathogenic microorganism as it is a primary line of immune mechanism in insects. Gupta (1986) reported that cellular immune reactions are the interaction between haemocytes and pathogens and the reactions are immediate that include phagocytosis, nodulation and encapsulation. Balavenkatasubbaiah et al. [8] reported that a total number of haemocytes specifies the status of tolerance to diseases. Jones [12] observed a decline of the total haemocyte count in treated worms than the control. The cellular immune response to infection has been conceded out in many insects by earlier researchers [13], Dunn and Drake 1983; Horohov and Dunn 1983).

Nappi [14] and Eslin and Prevost [15] observed the elevation of the haemocyte number in insects in a response to parasitism. According to Brehelin (1982), the population of circulating haemocytes in the haemolymph may indicate whether the host defence mechanism was triggered or not. Chen Wu-guo et al [16] observed a significant increase of total haemocyte count (THC) in *Heliothis armigera* infested with *Ovomermi scinensis*. The findings of the present investigation is in conformity with the previous research studies that the number of haemocytes may increase in silkworm infected with Bm NPV [8] or decrease to encounter the pathogenic micro-organism when infected with Nosema spores [17]. It is very much evident from the present study that the total haemocyte count was increased up to 4th in bivoltine double hybrid and 3rd day in case of crossbreed and bivoltine single hybrid to combat the fungal infection and then reduction of the number of haemocytes was noticed.

Kumar and Singh [18] noticed the enhancement of total haemocyte count in *Antheraea mylitta* infected with cytoplasmic polyhedrosis. Rise of the total haemocyte count was noticed in immunized *Antheraea mylitta* silkworms in comparison with healthy control represents the positive haemocyte mediate response [19]. Several researchers observed the variations in total haemocyte count during the development of fungal pathogen *Beauveria bassiana* in 5th instar silkworm *Bombyx mori* that designate the susceptibility/tolerance level of the organism. The variations in the haemocyte population could be attributed to the stimulation of the immune system by the fungal pathogen *Beauveria bassiana* in the host organism i.e., in silkworm *Bombyx mori*.

3.2 Differential Haemocyte Count (DHC)

Jones [12] classified five types of haemocytes in mulberry silkworm and it was well supported by Balavenkatasubbaiah et al. [8] and Balavenkatasubbaiah and Nataraju [9]. In the present study also five types of haemocytes viz., Prohaemocytes, Plasmatocytes, Granulocytes, Spherulocytes and Oenocytes were observed.

3.3 Prohaemocytes

Elevation of prohaemocyte count was observed from 1stto 4th day in all three breeds viz., bivoltine double hybrid, crossbreed and bivoltine single hybrid then decrease of prohaemocyte count was noticed in the remaining days of the instar in the three breeds selected for the study in healthy control. In *Beauveria bassiana* inoculated silkworms gradual decrease of prohaemocyte count was noticed in three breeds i.e., 1st day to 4th day in bivoltine double hybrid, 1st to 3rd day in case of crossbreed and bivoltine single hybrid but a drastic decrease of prohaemocytes was observed from 5th day onwards in bivoltine double hybrid and 4th day onwards in crossbreed and bivoltine single hybrid. In comparison with control a higher number of prohaemocytes was observed only in 1st day of the instar and subsequently drop in prohaemocyte count was noticed in the rest of the instar of all three breeds of inoculated silkworms.

Prohaemocytes (PR) are thought to be the stem cells from which all kinds of haemocytes arise. These cells account for only a small portion of the total haemocytes. Yamashita and Iwabuchi (2001) reported that from the total dividing stem cells i.e., prohaemocytes 59.2 per cent of daughter cells differentiated into other categories of haemocytes such as plasmatocytes, granulocytes and spherulocytes, the rest of the prohaemocytes divided into new prohaemocytes. The decline of prohaemocyte count was observed in all the three breeds selected for the study but a drastic reduction of prohaemocytes was observed from 4th day onwards in

bivoltine double hybrid and 3rd day onwards in crossbreed and bivoltine single hybrid. The reduction in prohaemocyte population may be due to the transformation of prohaemocytes to other types of haemocytes i.e., plasmatocytes, granulocytes, spherulocytes etc., that are essential to fight against fungal pathogen *Beauveria bassiana* during infestation in silkworm as prohaemocytes are the stem cells from which all haemocytes arise. Similar observations have been made in different strains of silkworms against Bm NPV infection [8]. The result of the investigation is also in compliance with the findings of Gupta and Sutherland [20] and Gupta [21].

3.4 Plasmatocytes

Enumeration of plasmatocytes indicated the elevation of plasmatocytes from 1st to 6th day of 5th instar in all three breeds of untreated silkworms. In inoculated larvae of bivoltine double hybrid and crossbreed the gradual increase of number plasmatocytes was recorded up to 4th day and then the significant decrease was noticed in both the races i.e., bivoltine double hybrid and crossbreed. In the case of bivoltine single hybrid gradual increase of plasmatocytes was recorded up to 3rd day and then a significant reduction of plamatocytes was noticed in the rest of the instar of silkworms. In comparison with healthy larvae, the number of plasmatocyte count was increased up to 5th day in bivoltine double hybrid, up to 4th day in crossbreed and till 3rd day in bivoltine single hybrid.

Plasmatocytes are basophilic and polymorphic in nature and tend to send many pseudopodia around the foreign body and the effective physiological mechanisms of phagocytosis, encapsulation, and other related defence mechanisms were chiefly due to the availability of circulatory immune cells particularly plasmatocytes. In Lepidoptera, plasmatocytes involve in most of the cellular defence responses and these cells have greater adhesive properties that are involved in phagocytosis and also in the encapsulation when any foreign particle enters into the body. Plasmatocytes were gradually increased up to 4th day in bivoltine double hybrid and crossbreed, in case of bivoltine single hybrid it was increased up to 3rd day only then a significant reduction of plasmatocyte count was noticed in the rest of the instar in the three breeds. Phagocytes cells identify pathogenic micro-organisms or any foreign material via a series of receptors on their cell membrane for pathogen related biomolecules. These receptors in turn initiate a series of signalling pathways that instruct the cell to ingest and eventually destroy the non-self material. Similar observations were reported by Ananda Kumar and Ann Sandhya Michael [22] reported a significant increase of plasmatocytes and granulocytes in *Bombyx mori* infected with *Bacillus thuringiensis*. Phagocyte count is known to vary significantly according to the nature and quantity of the substance that entered into the host organism, species, age and biological status of the insect before and after inoculation of a foreign substance, as well as with temperature and time [23, 24]. Enhancement of the plasmatocyte count during the initial stage of *Beauveria bassiana* infection in the silkworm strains selected for the study signifies the primary function of plasmatocytes to fight against the fungal pathogen in the host body. The reduction of plasmatocytes during the later stage of infection may be due to active participation of these cells in phagocytosis, encapsulation and nodule formation against infection.

3.5 Granulocytes

Increase of granulocyte count was noticed in unimmunized silkworms up to 4th day in all three breeds selected for the study then decrease of granulocyte count was noticed in the remaining days of the instar of the three breeds. Gradual increase of granulocytes was observed from 1st day to 6th day in bivoltine double hybrid silkworms, in case of crossbreed and bivoltine single hybrid gradual increase of granulocytes was observed up to 5th day and 4th day respectively and then reduction of granulocyte count was noticed in remaining days of the 5th instar larvae of both the races treated with *Beauveria bassiana.* In comparison with untreated larvae, the high number of granulocytes was observed from 1st to 6th day of the instar in bivoltine double hybrid and bivoltine single hybrid but in case of crossbreed, the elevation of granulocytes was noticed up to 5th day then reduction of granulocytes was noticed on 6th day of the instar.

Granulocytes are immune cells in the haemolymph of insects that help the immune system to fight against the infection. The elevation of granulocytes indicates stimulation of granulocytes to combat against the fungal pathogen *Beauveria bassiana*. After invasion of fungal pathogen granulocytes migrate to the site of infection through adhesion and chemotaxis and then they perform a number of tasks such as phagocytosis, encapsulation, degranulation and release of antimicrobial proteins and cytokines, and finally secrete the neutrophil extracellular traps as granulocytes recognize any foreign particle that invade into the host system. Then the reduction of granulocyte count was noticed in the rest of the days of the 5th instar crossbreed and bivoltine single hybrid, the reduction may be due to significant involvement of these cells in the process cell-mediated immune response such as phagocytosis, encapsulation and

nodule formation to eliminate the foreign materials invading the haemocoel. Granulocytes were found to decrease at 6 h and 24 h which may be due to their involvement in phagocytosis of non-self material. Granulocytes are reported to be involved in phagocytosis in cell-mediated defence of different insects (Wago 1983) [25]. Abir A. Gad [26] reported enhancement of the percentage of two immune-phagocytes, i.e., granulocytes and plasmatocytes in Silkworm *Bombyx mori*, treated with Silver Nanoparticles.

Table - 1 and Graph-1. Dynamics of Total Haemocyte Count (THCx10^3/mm^3) (THC) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instar compared to control

TOTAL HAEMOCYTES COUNT(THCX10^3/MM^3)							
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 26)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	128.4	131.01	121.61	124.41	120.38	121.38
2	Day 2	130.26	133.3	124.6	131.46	123.51	126.17
3	Day 3	138.46	139.23	135.36	141.73	129.47	133.54
4	Day 4	140.36	158.42	147.22	121.52	134.52	119.53
5	Day 5	153.52	153.11	137.18	102.62	124.67	98.23
6	Day 6	149.59	145.37	129.46	91.62	117.58	86.5
Mean		140.1	143.41	133.24	118.83	125.02	114.228
Std. Deviation		10.147	10.333	8.915	17.649	5.864	17.013

Tests of Between-Subjects Effects
Dependent Variable: Total Haemocyte Count

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P value**	**SIG**
Breeds	9333.571	2	4666.786	538.661	0.000	**
Treatments	1438.027	1	1438.027	165.984	0.000	**
Days	3904.293	5	780.859	90.130	0.000	**
Breeds*Treatments	1577.697	2	788.849	91.052	0.000	**
Breeds* Days	5734.292	10	573.429	66.188	0.000	**
Treatments * Days	3527.761	5	705.552	81.438	0.000	**
Breeds *Treatments * Days	1927.840	10	192.784	22.252	0.000	**
Error	623.785	72	8.664			
Corrected Total	28067.267	107				

a R Squared = .978 (Adjusted R Squared = .967)

p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

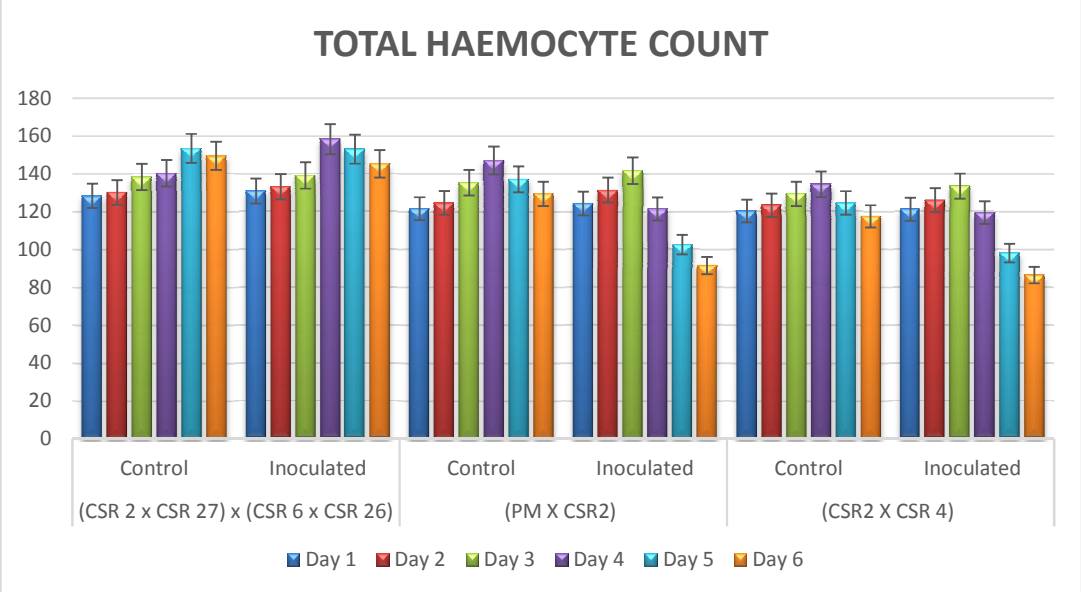


Table -2 and Graph -2. Dynamics of Prohaemocyte Count (DHCx10^3/mm^3) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill. in three breeds selected for the study during fifth instar compared to control

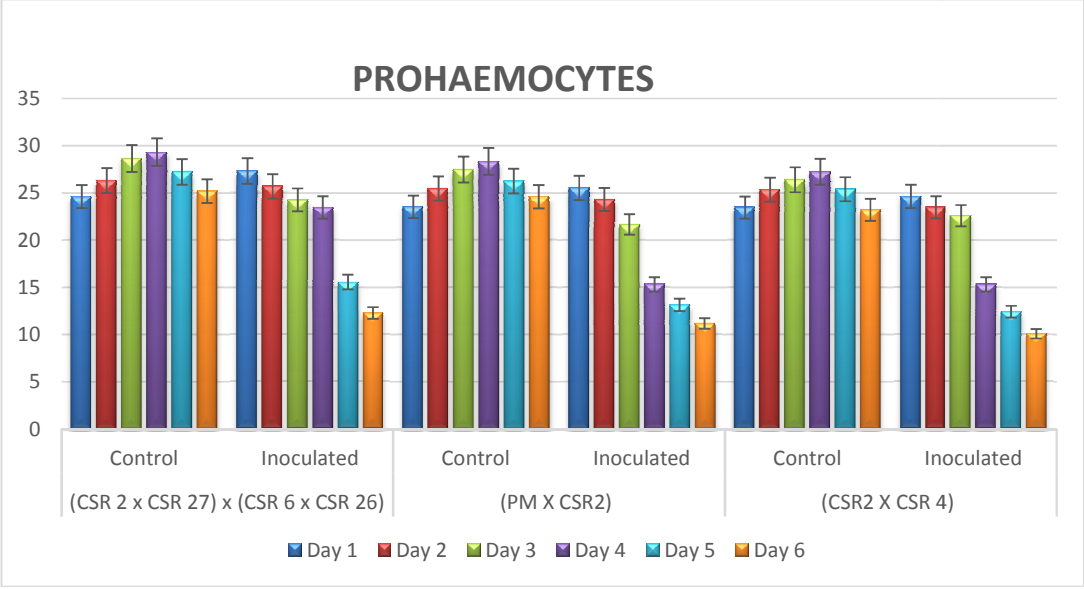
		PROHAEMOCYTES					
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 26)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	24.64	27.35	23.55	25.56	23.48	24.65
2	Day 2	26.33	25.72	25.49	24.34	25.37	23.51
3	Day 3	28.67	24.29	27.51	21.69	26.42	22.61
4	Day 4	29.35	23.49	28.37	15.34	27.27	15.33
5	Day 5	27.25	15.58	26.28	13.17	25.42	12.44
6	Day 6	25.22	12.31	24.63	11.21	23.24	10.11
Mean		27.245	23.792	25.918	18.553	26.901	22.445
Std. Deviation		1.732	2.237	2.157	5.369	1.469	4.0128

Tests of Between-Subjects Effects
Dependent Variable: Prohaemocytes

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P value**	**SIG**
Breeds	209.207	2	104.604	111.412	0.000	**
Treatments	699.926	1	699.926	745.479	0.000	**
Days	169.509	5	33.902	36.108	0.000	**
Breeds*Treatments	74.305	2	37.153	39.570	0.000	**
Breeds* Days	139.040	10	13.904	14.809	0.000	**
Treatments * Days	534.485	5	106.897	113.854	0.000	**
Breeds *Treatments * Days	105.327	10	10.533	11.218	0.000	**
Error	67.600	72	0.939			
Corrected Total	1999.399	107				

a R Squared = .966 (Adjusted R Squared = .950)

p<0.05 Significant at 0.05 levelp<0.01 significant at 0.01 levelp>0.05 Not significant



3.6 Spherulocytes

Increase of spherulocyte count was recorded from 1st to 4th day in all three breeds selected for the study and then decrease in the number of spherulocytes in the rest of the 5th instar was noticed in the three breeds in untreated silkworms. Gradual increase of spherulocyte count was noticed up to 4th day in bivoltine double hybrid and crossbreed and then the significant decrease of spherulocyte count was noticed in remaining days of the instar in both the breeds of *Beauveria bassiana* inoculated larvae. In case of bivoltine single hybrid gradual increase of the number

of spherulocytes was recorded up to 3rd day and then gradual reduction of spherulocytes was noticed i.e., from 4th day to end of the 5th instar. Overall elevation of spherulocyte count was noticed in inoculated bivoltine double hybrid and crossbreed silkworms compared to control in bivoltine single hybrid elevation of the number of spherulocytes was noticed up to 3rd day then decline in the number of spherulocyte count was noticed in the rest of the instar.

Table - 3 and Graph - 3. Dynamics Plasmatocytes Count (DHCx10^3/mm^3) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill. in three breeds selected for the study during fifth instar compared to control

PLASMATOCYTES							
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 26)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	37.5	38.36	35.35	36.86	30.48	30.66
2	Day 2	41.52	45.43	35.45	40.32	31.65	33.49
3	Day 3	43.24	47.38	37.24	45.62	33.56	38.23
4	Day 4	43.29	49.20	38.36	48.54	35.3	32.53
5	Day 5	44.14	46.34	39.24	35.26	35.55	28.38
6	Day 6	45.20	43.45	41.67	32.06	36.17	23.2
Mean		42.48	45.02	37.88	39.78	33.83	31.085
Std. Deviation		2.717	3.685	2.401	6.002	2.0312	4.841

Tests of Between-Subjects Effects
Dependent Variable: PLASMATOCYTES

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P value**	**SIG**
Breeds	2428.070	2	1214.035	1141.289	0.000	**
Treatments	15.008	1	15.008	14.109	0.000	**
Days	650.664	5	130.133	122.335	0.000	**
Breeds*Treatments	119.195	2	59.598	56.026	0.000	**
Breeds* Days	339.611	10	33.961	31.926	0.000	**
Treatments * Days	236.102	5	47.220	44.391	0.000	**
Breeds *Treatments * Days	232.626	10	23.263	21.869	0.000	**
Error	76.589	72	1.064			
Corrected Total	4097.867	107				

a R Squared = .981 (Adjusted R Squared = .972)

p<0.05 Significant at 0.05 levelp<0.01 significant at 0.01 levelp>0.05 Not significant

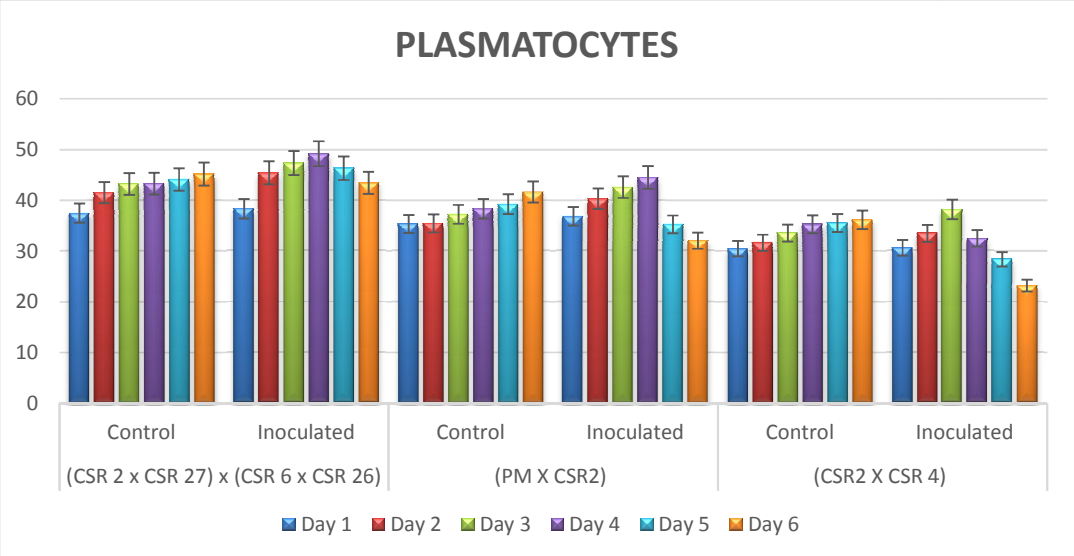


Table - 4 and Graph - 4. Dynamics of Granulocytes Count (DHCx10^3/mm^3) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instarcompared to control

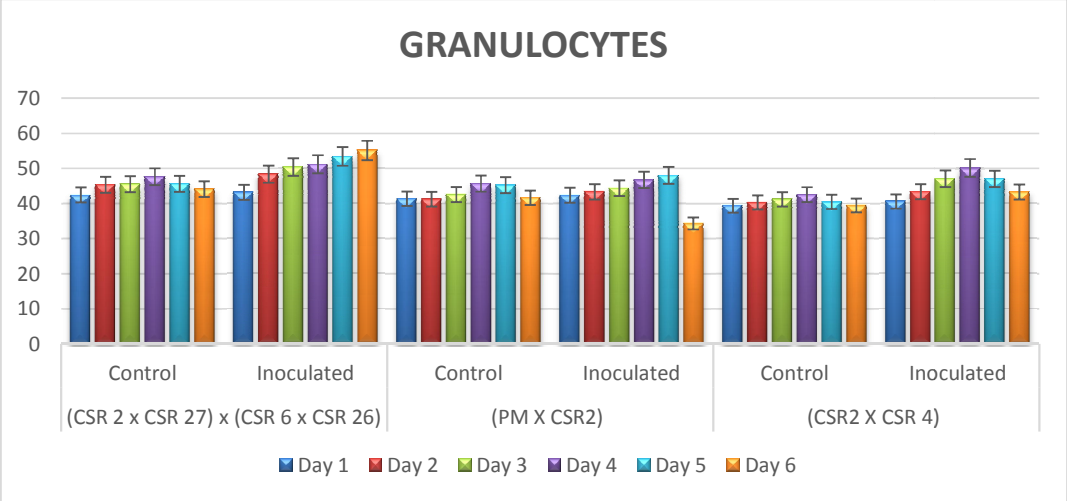
		GRANULOCYTES					
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 26)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	42.54	43.26	41.45	42.45	39.47	40.66
2	Day 2	45.4	48.43	41.32	43.43	40.38	43.46
3	Day 3	45.58	50.5	42.64	44.49	41.27	47.17
4	Day 4	47.73	51.29	45.75	46.81	42.6	50.25
5	Day 5	45.67	53.52	45.35	48.11	40.56	47.1
6	Day 6	44.18	55.2	41.72	34.42	39.52	43.38
Mean		45.187	50.368	43.038	43.285	40.603	45.33
Std. Deviation		1.827	4.053	2.095	4.649	1.382	3.358

Tests of Between-Subjects Effects
Dependent Variable: GRANULOCYTES

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P value**	**SIG**
BREEDS	617.267	2	308.633	297.084	0.000	**
TREATMENTS	228.610	1	228.610	220.055	0.000	**
DAYS	493.807	5	98.761	95.066	0.000	**
BREEDS*TREATMENTS	223.031	2	111.516	107.342	0.000	**
BREEDS* DAYS	197.481	10	19.748	19.009	0.000	**
TREATMENTS * DAYS	90.196	5	18.039	17.364	0.000	**
BREEDS *TREATMENTS * DAYS	146.226	10	14.623	14.075	0.000	**
Error	74.799	72	1.039			
Corrected Total	2071.417	107				

a R Squared = .964 (Adjusted R Squared = .946)

p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant



Spherulocytes (SP) are round or ovoid cells and are characterized by big spherules. Spherulocytes have been suggested to transport cuticular components [27]. Bora and Handique [28] observed an initial increase and then degeneration of spherulocytes following Catharanthus treatment. Begum et al. [29] also reported a similar trend of spherulocyte count and suggested that it may be due to effective utilization of fat reserves during stress condition. Carlos Ribeiro et al. [30] reported that the functions of spherule cells are unknown. For Nittono [31] spherule cells of *Bombyx mori* could be related to silk synthesis.

3.7 Oenocytes

Enumeration of oenocytes indicated the significant increase of oenocyte count from 1st to 4th day in all the three breeds chosen for the study then decrease of oenocyte count was noticed in the rest of the instar of all three breeds of healthy silkworms. Significant

reduction of oenocyte count was noticed up to 5th day in bivoltine double hybrid then no traces of oenocytes were noticed on 6th day of the bivoltine double hybrid but in the case of crossbreed and bivoltine single hybrid reduction of oenocytes count was noticed up to 4th day and then no traces of coenocytes was noticed in *Beauveia bassiana* inoculated silkworm of crossbreed and bivoltine single hybrid.

Table - 5 and Graph - 5. Dynamics of Spherulocytes Count (DHCx10^3/mm^3) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instar compared to control

SPHERULOCYTES							
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 26)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	19.55	23.68	18.52	21.26	18.28	19.15
2	Day 2	20.61	26.18	19.24	23.38	19.36	21.16
3	Day 3	22.43	28.53	19.62	25.48	20.39	23.06
4	Day 4	23.47	29.48	22.27	27.35	20.69	15.34
5	Day 5	21.42	26.36	20.28	23.64	19.66	14.65
6	Day 6	20.49	23.1	19.31	19.31	18.37	13.49
Mean		20.832	27.225	20.253	17.59	19.629	15.98
Std. Deviation		1.914	2.183	1.614	4.653	1.384	1.937

Tests of Between-Subjects Effects
Dependent Variable: SPHERULOCYTES

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P value**	**SIG**
BREEDS	801.790	2	400.895	288.744	0.000	**
TREATMENTS	0.032	1	0.032	0.023	0.880	**
DAYS	154.441	5	30.888	22.247	0.000	**
BREEDS*TREATMENTS	560.658	2	280.329	201.906	0.000	**
BREEDS* DAYS	106.819	10	10.682	7.694	0.000	**
TREATMENTS * DAYS	91.690	5	18.338	13.208	0.000	**
BREEDS *TREATMENTS * DAYS	199.292	10	19.929	14.354	0.000	**
Error	99.966	72	1.388			
Corrected Total	2014.688	107				

a R Squared = .950 (Adjusted R Squared = .926)

p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

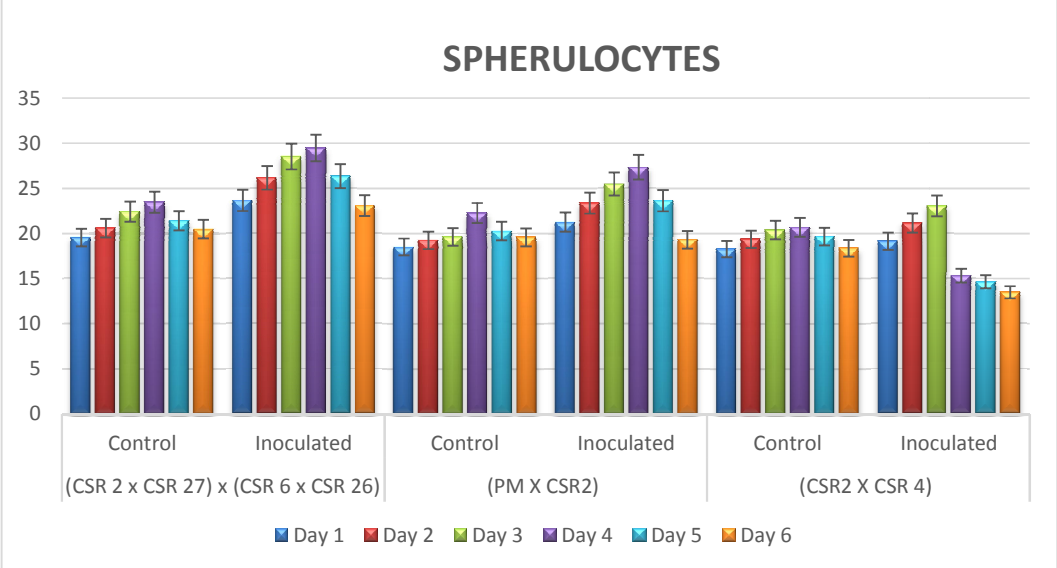


Table - 6 and Graph - 6. Dynamics of Oenocytes count (DHCx10^3/mm^3) in the haemolymph of silkworm *Bombyx mori* L. treated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill.in three breeds selected for the study during fifth instar compared to control

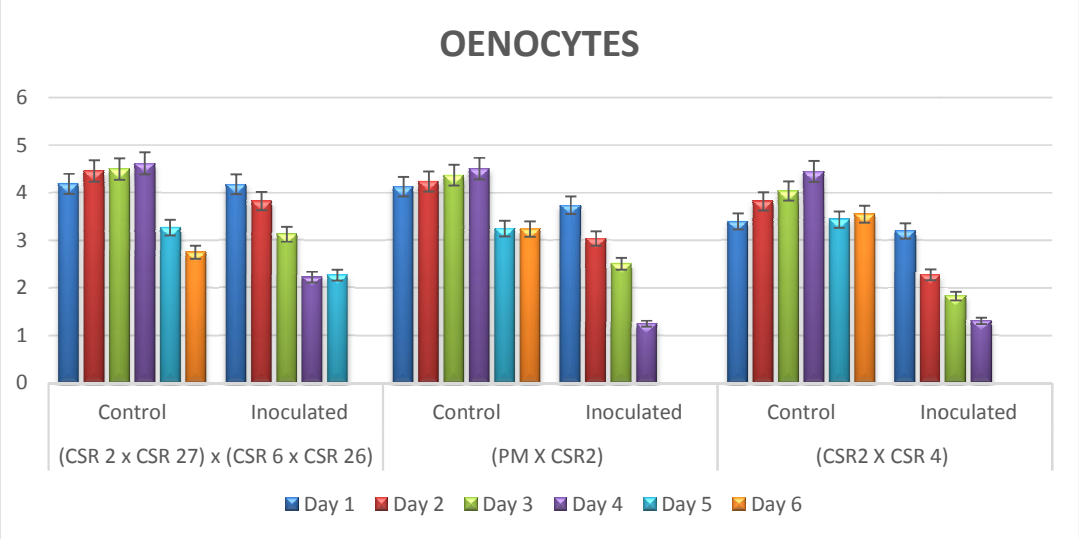
OENOCYTES							
S.NO	**Days**	**(CSR 2 x CSR 27) x (CSR 6 x CSR 6)**		**(PM X CSR2)**		**(CSR2 X CSR 4)**	
		Control	**Inoculated**	**Control**	**Inoculated**	**Control**	**Inoculated**
1	Day 1	4.19	4.18	4.13	3.74	3.4	3.2
2	Day 2	4.46	3.83	4.24	3.04	3.82	2.28
3	Day 3	4.5	3.13	4.37	2.51	4.04	1.83
4	Day 4	4.62	2.23	4.51	1.25	4.45	1.31
5	Day 5	3.27	2.27	3.25	0	3.44	0
6	Day 6	2.75	0	3.24	0	3.55	0
Mean		3.967	2.608	3.958	1.7594	3.786	1.437
Std. Deviation		1.072	1.418	1.573	1.938	0.575	1.198

Tests of Between-Subjects Effects
Dependent Variable: OENOCYTES

Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**P value**	**SIG**
BREEDS	35.237	2	17.619	83.204	0.000	**
TREATMENTS	202.185	1	202.185	954.828	0.000	**
DAYS	57.163	5	11.433	53.991	0.000	**
BREEDS*TREATMENTS	46.528	2	23.264	109.865	0.000	**
BREEDS* DAYS	14.085	10	1.408	6.652	0.000	**
TREATMENTS * DAYS	61.596	5	12.319	58.178	0.000	**
BREEDS *TREATMENTS * DAYS	17.770	10	1.777	8.392	0.000	**
Error	15.246	72	0.212			
Corrected Total	449.810	107				

a R Squared = .966 (Adjusted R Squared = .950)

p<0.05 Significant at 0.05 levelp<0.01 significant at 0.01 level p>0.05 Not significant



Oenocytoids (OE) are of uneven shape and size with more amounts of superficially homogenous cytoplasm. Oenocytoids functions as the site for synthesis of prophenoloxidase and contains cytoplasmic phenoloxidase precursors that likely to play a role in melanization of haemolymph [32]. Reduction of oenocytes count was noticed up to 5th day in bivoltine double hybrid and in crossbreed but in bivoltine single hybrid reduction of oenocytes count was noticed up to 4th day and then no traces of oenocytes was noticed. Bora and Handique [28] also recorded a decreased trend of oenocytes in muga silkworm following Catharanthus treatment. Reduction of oenocytes was noticed during the development of *Beauveria bassiana,* this may be owing to the involvement of oenocytes in synthesis of phenoloxidase enzyme cascade that associated with

melanization process to entrap and kill the invading pathogen.

4. CONCLUSIONS

Haemocytes play a significant role in protecting the organisms against pathogens and react instantly by the entry of any foreign particle or pathogenic organism. The present investigation mainly focused to evaluate the cellular immune-potency in *Bombyx mori* during the development of fungal pathogen *Beauveria bassiana* by enumerating the density of total and differential haemocytes. The investigation obviously provides evidence for the differential influence of different types of haemocytes in three breeds of silkworm *Bombyx mori* inoculated with *Beauveria bassiana*. The degree of variations in the number of haemocytes can be used as a directory for diagnosis of the disease. Increased population of haemocytes can be correlated with efficient defence mechanism in bivoltine double hybrid compared to crossbreed and bivoltine single hybrid during the progress of fungal pathogen *Beauveria bassiana* to protect the host organism. The study may provide a hypothetical basis and reference for the future investigation in insect immunity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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