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PER ORAL INOCULATION OF LYSINIBACILLUS SPHAERICUS WITH PATHOGENIC MIBROBES ON REARING AND COCOON PARAMETERS OF SILKWORM, *Bombyx mori* L.

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ABSTRACT
Mixed infections are complex interactions between pathogens and silkworm which cause diseases and reduces cocoon crop loss. The per oral inoculation of pathogenic bacterium Lysinibacillus sphaericus along with *viz*., A. faecalis (A), B. subtilis (B) and NPV (N) to third instar PM×CSR_2 (50 silkworms/ replication) resulted minimum of 7.87 and 9.85 days for ET_{50} symptom expression and mortality in treatment T_6- L. sphaericus + A. faecalis +NPV. The remaining treatments T_5 triple inoculation (L+A+B) (9.89 and 12.36 days) and dual inoculation T_2 (L+A) (20.89 and 23.29 days) ,T_3 (L+B) (10.10 and 12.48 days) and T_4 (L+N) (10.18 and 11.18 days) recorded different days for symptom expression and mortality. Further, number of larvae entered to fourth instar was noticed minimum in T_6-L. sphaericus + A. faecalis + NPV (30) and maximum in T_2-L. sphaericus + A. faecalis (44). Further, the cocoon parameters *viz*., single cocoon weight (1.00 g), shell weight (0.08 g), shell ratio (8.00) and silk productivity (0.69 cg/ day) was noticed minimum in T6. The interaction effect of Lysinibacillus sphaericus with other pathogenic bacteria *viz*.,Bacillus subtilis, Alcaligenes faecalis and NPV had synergistic effect compared to Lysinibacillus sphaericus alone.

INTRODUCTION

Among different diseases, the bacterial Flacherie is considered to be a major disease in silkworm and flaccidity of larva is the major symptom. The disease is very common during summer and rainy seasons in all the sericultural areas of India which accounts to crop loss of 27 – 35% with cocoon yield loss of 11 – 15kg /100 dfls. It is reported that, flacherie is caused by different species of bacteria, viruses and their mixed infections (Selvakumar, 2013). In most of the cases of silkworm rearing, the bacterial flacherie disease is caused by involvement of different groups of pathogenic bacteria and virus. Therefore, these bacteria many a times have mutualistic effect and very rarely they are pathogenic during their occurrence in silkworm larva and affect the cocoon parameters (Anusha and Bhaskar *et al*., 2016). In this view, flacherie disease samples were collected from farmers rearing house in Kolar and Chikkaballapur districts of Karnataka and among isolated strains, commonly found three strains of bacteria (B1, B2 and B3) were identified through molecular techniques as *Lysinibacillus sphaericus* (L), Alcaligenes faecalis (A), Bacillus subtilis (B) (Anusha *et al*., 2016) and experiment was carried out to know the 'Interaction effect of Lysinibacillus sphaericus with pathogenic mibrobes on rearing and cocoon parameters of silkworm, *Bombyx mori* L.

MATERIALS AND METHODS

To know the interaction effect of per oral inoculation of Alcaligenes faecalis, Lysinibacillus sphericus, Bacillus subtilis and NPV was carried out. Immediately after the third moult of Pure Mysore ×CSR2, silkworms were inoculated with the bacteria (at the rate of 0.25 ml per 25 larvae with 10^{-7} dilution to each replication at the beginning of the third instar) by smearing the bacterial solution onto the leaf surface, dried in shade. While inoculating two micro-organisms, first micro-organism was inoculated at the beginning of the third instar and the second organism (isolate) was inoculated during middle of the third instar. In case of three Micro-organism administration, first one micro-organism was administered at the beginning and the other two Micro-organisms were inoculated at the middle of the third instar larvae of PM×CSR2 (Govindan *et al*., 1998). Same number of larvae were fed with distilled water smeared mulberry leaves and normal mulberry leaf which was considered as a control. Silkworm larvae were reared at 27ºC room temperature with realative humidity 70 - 80%(Suparna *et al*., 2011).

Treatments details:Lysinibacillus sphaericus with other organisms

T_1- Lysinibacillus sphaericus

T_2- Lysinibacillus sphaericus+ Alcaligenes faecalis

T_3- Lysinibacillus sphaericus+ Bacillus subtilis

T_4- Lysinibacillus sphaericus+ NPV

T_5- Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis

T_6- Lysinibacillus sphaericus + Alcaligenes faecalis + NPV

T_7- Normal Leaf

T_8- Sterile water

The rearing and cocoon parameters were recorded for

evaluation *viz.*,larval weight reduction, larval duration, moulting duration, ET_{50} for symptom expression and mortality, number of silkworms entering to subsequent instar and spinning . ERR, larval mortality, single cocoon weight, single shell weight, Shell ratio and silk productivity and one way anova data analysis was used for statistical analysis (Sundarraj *et al*., 1972).

RESULTS AND DISCUSSION

The experimental data on combined inoculation of Lysinibacillus sphaericus with other organisms registered significant results on per cent weight reduction over control was noticed in third instar inoculated larvae of PM × CSR_2.

The maximum per cent larval weight reduction of 23.56, 41.06, 23.94 were observed at the end of third, fourth and fifth instar larvae of PM × CSR_2 administered with T_6-Lysinibacillu s sphaericus + Alcaligenes faecalis + NPV and minimum (16.10, 16.06 and 15.48 %) was found during the inoculation of single pathogen (T_1-Lysinibacillus sphaericus). Further, among dual inoculation of bacterial isolates more larval weight reduction was found in T_4-Lysinibacillus sphaericus + NPV (20.14, 29.96 and 19.55 %) followed by T_3-Lysinibacillus sphaericus + Bacillus subtilis (17.09, 26.44, 19.15 %) and T_2-Lysinibacillus sphaericus + Alcaligenes faecalis(15.12,23 and11.50 %) over sterile control (Table 1).

The experimental data, on total larval duration as well as moulting duration registered significant variation. However, maximum (5.38, 6.98 and 11.85 days) and minimum of 4.80 5.98 and 9.98 days were recorded during third, fourth and fifth instars of inoculated batches of T_5-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis and T_1-

Table 1: Influence of combined inoculation of Lysinibacillus sphaericus with other different bacterial isolates on rearing parameters of PM × CSR2

Treatments	Larval weight reduction (%)			Larval duration (Days)				Moulting duration (h)			ET_{50}	ET_{50}
	III instar End	IV instar End	V instar End	III	IV	V	Total	III	IV	Total	Symptoms (Days)	Mortality (Days)
T_1- L	16.1 (23.53)	16.06 (23.6)	15.48 (23.14)	4.8	5.98	9.98	20.76	30.96	31.1	62.06	11.57	14.1
T_2 -L + A	15.12 (22.74)	23 (28.64)	11.5 (19.78)	4.9	6.1	10.29	21.29	32.28	33.22	65.5	20.89	23.29
T_3- L + B	17.09 (24.33)	26.44 (30.51)	19.15 (25.95)	5.18	6.24	10.56	21.98	34.18	35.96	70.14	10.1	12.48
T_4-L + N	20.14 (26.62)	29.96 (34.23)	19.55 (26.24)	5	6.18	10.98	22.16	31.68	32.18	63.86	10.18	11.18
T_5-L + A + B	17.13 (24.36)	37.38 (33.79)	19.11 (25.92)	5.38	6.98	11.85	24.21	39.96	40	79.96	9.89	12.36
T_6-L + A + N	23.56 (28.96)	41.06 (39.85)	23.94 (29.29)	5.21	6.64	11.45	23.3	36.8	37.1	73.9	7.87	9.85
T_7-Control	3.03 (10.02)	2.2 (7.07)	0.8 (5.13)	3.16	4.28	6.98	14.42	26.1	26.2	52.3	0	0
T8-SW	0 (0.58)	0 (0.58)	0 (0.58)	3.16	4.28	6.98	14.42	26.1	26.2	52.3	0	0
F-test	*	*	*	*	*	*		*	*		*	*
SEm ±	2.77	1.5	0.85	0.04	0.02	0.07		0.04	0.3		0.58	1
CD at 5 %	5.88	3.18	1.8	0.08	0.05	0.16		0.09	0.63		1.23	2.11

* - Significant at 5 %;T_1-Lysinibacillus sphaericus(L), T_2-Lysinibacillus sphaericus + Alcaligenes faecalis (L + A), T_3- Lysinibacillus sphaericus + Bacillus subtilis (L + B), T_4-Lysinibacillus sphaericus + NPV (L + N), T_5-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (L + A + B), T_6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (L + A + N), T_7-Normal Leaf (Control), T_8-Sterile water (SW)

Table 2 : Rearing parameters of PM × CSR2 as influenced by Lysinibacillus sphaericus with other bacterial isolates causing flacherie disease of silkworm, Bombyx mori(Third instar inoculated batches)

Treatments	III instar inoculated batches					
	No. of worms entering into subsequent instars			No. of worms entering to spinning (%)	Effective rate of rearing (%)	Larval mortality (%)
	III	IV	V			
T_1- L	50	40	28	42.04	37.97	62.03
T_2 -L + A	50	44	34	56.1	47.95	52.05
T_3- L + B	50	38	26	36.03	31.8	68.2
T_4-L + N	50	32	24	32.2	29.75	70.25
T_5-L + A + B	50	34	25	38.03	27.93	72.07
T_6-L + A + N	50	30	21	30.04	19.71	80.29
T_7-Control	50	49	49	96.05	91.7	8.3
T_8-SW	50	49	49	96.08	93.97	6.03
F-test	NS	*	*	*	*	*
SEm ±	2.76	1.38	1.08	1.68	0.95	1.36
CD at 5 %	5.86	2.93	2.28	3.56	2.02	2.9

* - Significant at 5 %, NS - Non significant;T_1-Lysinibacillus sphaericus(L), T_2-Lysinibacillus sphaericus + Alcaligenes faecalis(L + A), T_3-Lysinibacillus sphaericus + Bacillus subtilis (L + B), T_4-Lysinibacillus sphaericus + NPV (L + N), T_5-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (L + A + B), T_6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (L + A + N), T_7-Normal Leaf (Control), T_8- Sterile water (SW)

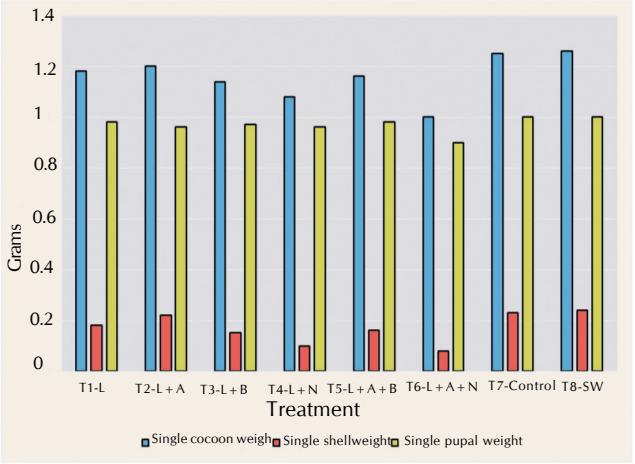

Figure 1: Deterioration of cocoon parameters as influenced by Lysinibacillus sphaericus with other bacterial isolates causing flacherie disease of silkworm, Bombyx mori

Table 3:Deterioration of cocoon parameters as influenced by Lysinibacillus sphaericus with other bacterial isolates causing flacherie disease of silkworm, Bombyx mori(Third instar inoculated batch)

Treatments	III Instar inoculated batch				
	Single cocoon weight (g)	Single shell weight (g)	Single pupal weight (g)	Shell ratio (%)	Silk productivity (cg/day)
T_1-L	1.18	0.18	0.98	15.25	1.74
T_2-L+A	1.2	0.22	0.96	18.33	1.2
T_3-L+B	1.14	0.15	0.97	13.15	1.42
T_4-L+N	1.08	0.1	0.96	9.25	0.9
T_5-L+A+B	1.16	0.16	0.98	13.79	1.35
T_6-L+A+N	1	0.08	0.9	8	0.69
T_7-Control	1.25	0.23	1	18.4	3.29
T_8-SW	1.26	0.24	1	19.05	3.43
F-test	*	*	*	*	*
SEm±	0.03	0.02	0.05	0.91	0.06
CD at 5 %	0.06	0.06	0.11	1.93	0.13

* - Significant at 5 %, NS - Non significant;T_1-Lysinibacillus sphaericus(L), T_2-Lysinibacillus sphaericus + Alcaligenes faecalis(L + A), T_3-Lysinibacillus sphaericus + Bacillus subtilis (L + B), T_4-Lysinibacillus sphaericus + NPV (L + N), T_5-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (L + A + B), T_6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (L + A + N), T_7-Normal Leaf (Control), T_8- Sterile water (SW)

Lysinibacillus sphaericus respectively. It is very clearly indicated that, administration of T_1-Lysinibacillus sphaericus alone recorded less larval duration compared to T_2- Bacillus subtilis + Lysinibacillus sphaericus (4.90, 6.10 and 10.29), T_3- Lysinibacillus sphaericus + Bacillus subtilis (5.18, 6.24 and 10.56) and T_4- Lysinibacillus sphaericus + NPV (5, 6.18, and 10.98) days which differed significantly compared to inoculation of T_6- Bacillus subtilis + Lysinibacillus sphaericus + NPV (5.21, 6.64 and 11.45 days). Further, the maximum of 39.96 and 40.00 hours and minimum of 30.96 and 31.10 hours of moulting duration was recorded for third and fourth moult of third instar inoculated batch (Table 1).

The inoculated batch with Lysinibacillus sphaericus registered non significant results with T_3-Lysinibacillus sphaericus + Bacillus subtilis (10.10), T4-Lysinibacillus sphaericus + NPV (10.18) and T_5-Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (9.89) days where as significant difference in expression of disease symptom with T_1-Lysinibacillus sphaericus (11.57) alone, T_2-Lysinibacillus sphaericus + Alcaligenes faecalis (20.89), T3-Lysinibacillus sphaericus + Bacillus subtilis (10.10), T5- Lysinibacillus sphaericus + Alcaligenes faecalis + Bacillus subtilis (9.89) and T6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (7.87) days for ET50 symptom expression value which are found to be highly pathogenic compared to dual administration. The bacterial combination with virus, there was decrease in ET50 value for mortality the minimum was 9.85 days in T6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (Table 1).

In addition significant variation existed with respect to number of worms entering into subsequent instar, among dual administration of Lysinibacillus sphaericus with Alcaligenes faecalis more number of silkworms entered to next instars (50 to 44, 44 to 34) followed by with Bacillus subtilis (50- 38, 38-26) and with NPV (50-32, 32-24), whereas during triple inoulation of Lysinibacillus sphaericus with Alcaligenes faecalis + Bacillus subtilis (50-34, 34-25) and Alcaligenes faecalis + NPV (50-30, 30-21) resulted less compared to control (50-49, 49-49). The trend was same with respect to number of worms entering to spinning was same (Table 2).

Further, effective rate of rearing was recorded less in treatment T_6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV(19.71%) and maximum in T_2-Lysinibacillus sphaericus + Alcaligenes faecalis (47.95%) where as per cent larval mortality more in T_6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (80.29 %) and minimum in T_2-Lysinibacillus sphaericus + Alcaligenes faecalis (52.05 %) because of interaction of two different micro organisms resulted more larval mortality due to negative effect (Table 2).

The interaction effect of Lysinibacillus sphaericus with other organisms resulted variation in single cocoon weight, shell weight, shell ratio and silk productivity. The maximum and minimum cocoon weight, shell weight, shell ratio and silk productivity were noticed in T_2-Lysinibacillus sphaericus + Alcaligenes faecalis (1.20, 0.22, 18.33 % and 1.2 cg/day) and T_6-Lysinibacillus sphaericus + Alcaligenes faecalis + NPV (1.00 g., 0.08 g., 8.00 % and 0.69 cg/day) compared to control (1.26, .24 g , 19.05 % and 3.43 cg/day) (Table 3)(Figure 1).

These findings are in confirmative with Chitra *et al*. (1974) different bacterial isolates *viz*., Aerobacter cloacae, Achromobacter superficialis, Achromobacter delmarvae, Staphylococcus albus, Escherichia freundii and Pseudomonas ovalis varied in their pathogenicity to silkworms and stage of growth of larvae affected their virulence. Invasion of pathogens into larval body caused considerable reduction in larval weight from 10.10 to 27.50 per cent in V instar of silkworm, B. mori and deleterious effect increased with age of infection. It is further attributed that, per oral inoculation of Bacillus species to PM×CSR2 resulted in decreased larval weight from the beginning and end of the fourth instar (1.95-13.40 g/ 10 in Pure Mysore, 5.40-21.5 g/10 in CSR2) compared to control(2.55-13.80 and 6.38-23.5) (Anusha and Bhaskar, 2016).

Further, Doreswamy (2002) observed that, when Bacillus sp., Streptococcus faecalis and Staphylococcus aureus were administered to fourth instar larvae of PM×NB4D2 hybrid significantly longer duration of 114.88, 119.66, 123.05 h was noticed compared to control 102.00 h. and Manoj et al. (2013)

reported that, moulting was delayed by nearly 24 h between inoculated groups and control, when the bacterial suspensions of 1.0×106 CFU and 4.0×106 CFU was inoculated to the silkworm larvae.

The oral administration of Staphylococcus sp. And Serratia marcescenswere cause variation in worms entering to spinning, when third, fourth and fifth instar larvae of PM×C. Nichi were fed at the rate of 10×10^6, 1×10^6 and 10^6 cell/ larvae revealed 45/50, 38/50 50/50 entered to spinning (Vasantharajan and Muniratnamma, 1978).

Selvakumar *et al*. (2009) studied on the synergism between the pathogen *viz*.,Streptococcus sp. and BmDNV1where Streptococcus sp. bacteria caused 6.00 (low) and 31.67 per cent (medium) mortality in silkworm. The mortality was initiated on 11th and 10th day post inoculation with low and medium pathogenic bacteria, respectively. However, BmDNV1 alone in different concentrations ($IC_{50}\times10^2$, $IC_{50}\times10^1$, IC_{50} and $IC_{50}\times10$) did not cause any mortality. BmDNV1 in synergistic association with low pathogenic Streptococcus led to increased mortality from 6.00 to 39.33-64.76 per cent. The mortality was also advanced from 11th day to 6th day. BmDNV1 in synergistic association with medium pathogenic Streptococcus also led to increase in mortality from 31.67 to 85.33-95.33 per cent. The mortality was advanced from 10th to 5th day post inoculation.

In addition, Rosa Estela Quiroz *et al*. (2015) reported, the supernatant extract (1µg) of A. faecalis MOR02 killed more than 70 per cent G. mellonella larvae 96 h after infection. Further, when the larvae were injected with 240 CFU and 2,400 CFU had 3.33 per cent mortality at 72 h after injection and 6.67per cent at 48 h after injection, respectively. With 2,400 injection CFU, 33.33 per cent mortality was observed at 96 h after injection. Conversely, larvae injected with 24,000 CFU had 96.67 per cent mortality at 24 h after injection, while 100 per cent mortality was observed using 2,40,000 CFU.

The effect of synergism between Streptococcus sp. bacteria and BmDNV1 on single cocoon weight, single shell weight and shell ratio percentage were elucidated by (Selvakumar *et al*., 2009). The lowest cocoon weight (1.102 g), single shell weight (0.123 g) and shell ratio percentage (11.16 per cent) were noticed in BmDNV1 (IC_{50}) + medium pathogenic bacteria Streptococcus sp. (1×107) followed by medium pathogenic bacteria Streptococcus sp. (1×107) + BmDNV1 (IC_{50})(1.181g, 0.170 g and 14.39 %) respectively, whereas control batches recorded highest for all the above mentioned parameters.

Shorter filament length was obtained in inoculation with BmIFV + BmDNV (420.27 m), BmIFV + BmDNV + Bacillus sp. (424.44 m), BmIFV + BmDNV + S. faecalis(426.21), BmIFV + BmDNV + S. aureus (445.55 m), BmIFV + S. aureus (447.16 m), BmIFV + S. faecalis(450.44 m), BmIFV + Bacillus sp. (446.10 m), BmDNV + Bacillus sp. (442.99 m) and BmDNV + S. faecalis (452.21m) whereas, significantly longer filament length was recorded in distilled water control (730.00m) and in untreated control (725.00m). Significantly thinner denier was obtained in inoculation with BmIFV + BmDNV + Bacillus sp. (1.75) and BmIFV + BmDNV (1.80) whereas thicker denier was obtained in distilled water control (2.40) and untreated control (2.30) (Doreswamy *et al*., 2004).

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