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Pathogenecity of Naturally Isolated Entomopathogens in *Leucinodes orbonalis* and Cross Infectivity on *Helicoverpa armigera*

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Survey was undertaken in six districts of Tamil Nadu to explore the microbial pathogens on *Leucinodes orbonalis* in eggplant. Three entomopathogens including two bacteria *viz., Serratia marcescens* (Bizio) and *Enterobacter* sp. and one fungus, *Aspergillus ochraceus* (Kent) were isolated from *L. orbonalis* larvae. These naturally isolated entomopathogens on *L. orbonalis* were test verified for its cross infectivity on *Helicoverpa armigera.* Since both the bacteria are non sporulating ones, concentration was accounted based on dilution. *Serratia marcescens* at 1/5x dilution produced a mortality of 93.22 and 92.43 per cent on *L. orbonalis* and *H. armigera.* About 24 hours incubation of both the bacteria (*S. marcescens* and *Enterobacter* sp.) favoured high mortality on *L. orbonalis,* while 72 h incubation was essential for both the cultures against *H. armigera.* The fungus, *A. ochraceus* exhibited maximum mortality of *L. orbonalis* second instar (56 %) at a concentration of $1x10^7$ spores per ml.

Key words: Entomopathogens, *Leucinodes orbonalis,* cross infection, *Helicoverpa armigera*

There is considerable pressure on growers to reduce or eliminate the use of pesticides in crop production systems because of concerns about the effects of pesticide residues on human health and on the environment. Occurrence and efficacy of natural enemies in controlling the pest in other Asian countries *viz.,* Bangladesh, Thailand and Sri Lanka have been reported. Scope to exploit such natural enemies in India is enormous as the crop being native to India. Hence, efforts have been made to explore the entomopathogens on *Leucinodes orbonalis.* The naturally isolated pathogen Bt at 10^6 cells per ml resulted in 100 per cent mortality against first, second and third instar larvae of *L. orbonalis* (Sundaramoorthy, 1984).

Materials and Methods

Field survey for the occurrence of entomopathogens from *Leucinodes orbonalis* in Tamilnadu

Regular surveys were undertaken in the egg plant growing tracts of Tamil Nadu to identify and catalogue naturally occurring entomopathogens and their potential role in the regulation of *L. orbonalis* population. During 2003-2004, survey was taken up in the eggplant fields from Coimbatore, Cuddalore, Thiruchirappalli and Pudukkottai districts of Tamil Nadu, India. Ten places of not less than one acre each were surveyed at each site. At every site of survey, 100 plants were observed for diseased *L. orbonalis* larvae.

The diseased cadavers collected in natural condition during survey were transferred in sterile petriplates and glass vials, brought to laboratory and preserved with details on host insect, stage of the host, place and date of collection. Later the specimens on isolation were examined for specific microbial infections by pathogenecity test. Fungal pathogen culture was isolated on potato dextrose agar following standard mycological techniques of CM1 (1983) and the bacterial cultures were isolated by nutrient agar medium (Martin, 2000). The fungi and bacteria

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were mass cultured to assess their pathogenecity and sent for identification to the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, India and Institute of Microbial Technology, Chandigarh respectively.

Microbial infections on L. orbonalis

Two entomopathogenic bacteria *viz., Serratia* and *Enterobacter* and the entomofungus *Aspergellus ochraceus* encountered in the field collected *L. orbonalis* larvae were cultured under laboratory condition for testing the pathogenecity against *L. orbonalis.* Efficacy of these naturally isolated entomopathogens were test verified against the major pest, *H. armigera* in order to check the cross infectivity.

Bacterial infections

Five millilitres of the nutrient agar broth in the test tubes was inoculated with one loop ful of bacterial culture and incubated for 24 h served as mother culture. About one per cent of the mother culture of respective bacteria was inoculated into 25 ml of nutrient broth. After which, the broth was incubated at 30°C for varied periods *viz.,* 24 h, 48 h and 72 h. Bioassay on *L. orbonalis* was made on *L. orbonalis* and *H. armigera* by using three different incubated cultures of bacteria with different dilutions. Since both the bacteria are non sporulating ones, concentration was accounted based on dilution. Test concentrations of respective non-sporulating bacteria were diluted to 1x (without dilution) and 1/5x (five times dilution of 1x) using double distilled water and compared with control.

Bioassay against L. orbonalis

Bioassay was done using surface sterilized (surface sterilizing solution consisting of 250 mg of carbendazim dissolved in 500 ml of sterile water) potato disc (5mmx5mm). Potato discs dipped in the respective test concentrations were shade dried and kept in eppendorf tube. On air-drying for 30 min, a neonate larva per disc was released to each eppendorf tube using a camel hairbrush and tube was closed slightly leaving space for gas exchange. Each treatment was replicated three times and 10 larvae formed a replication. Larval mortality was recorded every 24 h consecutively for seven days. The surviving larvae were weighed on the final day of experiment. The ratio between the weight of treated larvae and the untreated control was taken as criteria for computing the effect on larval development.

Bioassay against H. armigera

Bioassay against *H. armigera* was done by using surface diet contamination (Gujar *et al.,* 2000). All the components of the semi synthetic diet except formalin were used during bioassays. Approximately, 1 ml of the diet was dispensed into 1.8 ml cryovial (Tarson®; 1 cm dia) and allowed to dry for an hour. After the diet got solidified, the bacterial dilution was coated on the diet surface at 10µl per vial using a sterile blunt glass rod. On air-drying for 30 min, one neonate larva per vial was released using a camel hairbrush and closed halfway with a screw cap leaving space for gas exchange. Entire bioassay was carried out in laminar flow chamber. A diet vial without bacterial suspension served as control. Each treatment was replicated three times and 10 larvae formed a replication. Larval mortality was recorded as done for *L. orbonalis*

Fungal pathogen

The naturally occurred entomofungal pathogen *A. ochraceus,* was isolated using the potato dextrose agar. The healthy pure culture was maintained in the laboratory condition by sub culturing at every fortnight interval. This pathogen was subjected to pathogenecity test against *L. orbonalis* alone. The eggplant shoot of the size (3 x 0.7 cm) was cut using the sterile blade and dipped at the fungal concentrations. Four concentrations tested were $1x10^7$, $6.2x10^6$, $5.7x10^6$ and $4.5x10^6$ spores/ml containing 0.01 per cent of Tween 20®. Shoot treated with distilled water having 0.01 per cent of Tween 20® served as control. One larva per shoot was released to a covered plastic cups lined with moist filter paper and three replications were maintained with ten larvae per replication for each treatment. The

Table 1. Pathogenecity of two bacterial cultures against *L. orbonalis* neonate larvae at different incubation periods

S. No.	Treatment & Concentration	Per cent mortality after 2 days 24 h culture	48 h culture	72 h culture	Mean	Per cent mortality after 4 days 24 h culture	48 h culture	72 h culture	Mean	Per cent mortality after 7 days 24 h culture	48 h culture	72 h culture	Mean	Grand Mean
1.	*Serratia marcescens* 1x	53.33	40.00	46.70	46.67	73.26	73.73	40.00	62.33	93.33	100.00	86.33	93.22	67.41
		(46.91)	(39.23)	(43.10)	(43.08)	(58.66)	(59.17)	(39.23)	(52.42)	(75.03)	(89.26)	(68.30)	(74.90)	(55.19)[a]
2.	*Serratia marcescens* 1/5x	40.40	40.00	13.31	31.10	46.17	53.66	33.66	44.50	93.33	60.00	93.00	82.11	52.57
		(39.23)	(39.23)	(21.39)	(33.28)	(42.80)	(47.10)	(35.46)	(41.78)	(75.03)	(50.77)	(74.66)	(66.82)	(47.29)[b]
3.	*Enterobacter* 1x	20.00	11.33	26.74	19.36	73.33	20.00	53.66	49.00	93.33	93.15	73.66	86.71	51.69
		(26.56)	(19.67)	(31.13)	(25.79)	(58.90)	(26.56)	(47.10)	(44.19)	(75.03)	(74.83)	(56.12)	(69.66)	(46.54)[b]
4.	*Enterobacter* 1/5x	20.00	26.70	20.00	22.23	33.36	33.66	27.60	31.64	66.37	73.67	20.00	53.34	35.73
		(26.56)	(31.11)	(26.56)	(28.08)	(35.46)	(35.46)	(31.69)	(34.20)	(54.21)	(59.12)	(26.56)	(46.75)	(36.34)[c]
5.	Control	0.00	0.00	6.66	2.28	0.00	13.66	13.60	9.09	6.36	13.00	13.66	11.01	7.44
		(0.57)	(0.57)	(14.95)	(6.08)	(0.57)	(21.69)	(21.64)	(14.99)	(21.13)	(21.13)	(21.69)	(19.14)	(13.40)[d]
	Mean	**26.75**	**23.60**	**21.80**	**24.32**	**45.22**	**38.94**	**33.72**	**39.31**	**70.54**	**67.96**	**57.33**	**58.61**	**40.74**
		(28.18)	**(26.17)**	**(27.43)**	**(27.26)**	**(39.53)**	**(37.99)**	**(35.02)**	**(37.52)**	**(58.85)**	**(55.52)**	**(50.06)**	**(50.14)**	**(40.95)**

	D (Days after mortality)	H (Incubation hours)	T (Treatment)	D x H	H x T	D x T	D x H x T
CD	0.12833	0.12933	0.16696	0.22400	0.28919	0.28919	0.50089

Mean of 30 replications
Figures within parenthesis are arc sine transformed values
Means followed by common alphabets are not significantly different at 5% level by LSD

Table 2. Pathogenecity of two bacterial cultures against *H. armigera* neonate larvae at different incubation periods

S. No.	Treatment & Concentration	Per cent mortality after 2 days 24 h culture	48 h culture	72 h culture	Mean	Per cent mortality after 4 days 24 h culture	48 h culture	72 h culture	Mean	Per cent mortality after 7 days 24 h culture	48 h culture	72 h culture	Mean	Grand Mean
1.	*Serratia marcescens* 1x	41.61	20.80	56.62	39.64	66.76	33.33	81.60	60.56	79.20	56.52	87.00	74.24	58.14
		(40.20)	(27.04)	(48.75)	(39.68)	(54.82)	(35.25)	(64.60)	(51.55)	(62.86)	(48.74)	(68.86)	(60.16)	(50.13)[b]
2.	*Serratia marcescens* 1/5x	52.18	20.80	60.90	44.62	65.21	63.63	81.81	70.21	86.40	100.00	90.90	92.43	69.08
		(46.25)	(27.09)	(51.30)	(41.56)	(52.86)	(52.92)	(64.79)	(57.17)	(68.36)	(88.35)	(72.44)	(76.38)	(56.88)[a]
3.	*Enterobacter* 1x	83.33	33.23	41.70	52.78	86.94	62.50	54.55	67.99	91.30	91.70	77.30	86.76	69.17
		(65.92)	(35.26)	(40.21)	(47.13)	(69.00)	(52.24)	(97.61)	(56.22)	(64.37)	(73.25)	(61.54)	(66.39)	(58.13)[a]
4.	*Enterobacter* 1/5x	56.00	12.50	16.00	28.16	54.13	72.72	30.76	52.53	62.50	52.40	81.81	65.57	48.75
		(48.45)	(20.71)	(23.55)	(30.91)	(47.37)	(28.39)	(33.67)	(46.52)	(52.24)	(46.37)	(64.75)	(54.45)	(43.96)[c]
5.	Control	0.00	3.45	3.38	2.27	0.00	3.84	17.23	7.02	7.70	4.00	20.70	10.80	6.69
		(1.39)	(10.69)	(10.35)	(7.56)	(0.19)	(11.12)	(24.50)	(12.49)	(16.11)	(11.53)	(27.08)	(18.24)	(12.76)[d]
	Mean	**46.62**	**18.17**	**35.70**	**33.49**	**54.60**	**46.43**	**51.66**	**51.66**	**65.42**	**60.92**	**71.54**	**65.96**	**50.36**
		(40.43)	**(24.18)**	**(34.88)**	**(33.17)**	**(45.29)**	**(42.04)**	**(44.79)**	**(44.79)**	**(52.79)**	**(53.65)**	**(58.94)**	**(55.12)**	**(44.36)**

	D (Days after mortality)	H (Incubation hours)	T (Treatment)	D x H	H x T	D x T	D x H x T
CD:	0.10493	0.10493	0.13456	0.18174	0.23463	0.23463	0.40639

Mean of 30 replications

Figures within parenthesis are arc sine transformed values

Means followed by common alphabets are not significantly different at 5% level by LSD

observation on the larval mortality was taken at three different intervals *viz.,* 24, 48 and 72 h after treatment. Each treatment was replicated three times and 10 larvae formed a replication.

Data of various results of laboratory experiments were statiscally analysed using completely randomised design. The data obtained in percentages were transformed to corresponding angles (Arcsine "percentage). Data pertained with weight of the host insects were transformed into square root values " $\sqrt{x+0.5}$). Larval mortality in control was corrected using Abbotts correction (Abbott's, 1925).

Results and Discussion

Pathogenecity of bacterial culture against L. orbonalis

The mortality effected by bacterial cultures indicated that *S. marcescens* at 1x gave a maximum overall mean mortality of 67.41 per cent, while the mortality in control was 7.44 per cent. *Enterobacter* sp. at 1x resulted in 51.69 per cent mortality which was found on par with *S. marcescens* at 1/5x having 52.57 per cent. After seven days of treatment, the culture incubated for 24 h produced maximum mortality of 70.54 per cent (Table 1). Of all the four tested concentrations of bacteria evaluated against *L. orbonalis* and *H. armigera* neonates, *S. marcescens* at 1x was found to be effective in causing maximum mortality of 56.29 per cent. Rangarajan *et al*. (1971) have also reported the successful use of *S. marcescens* against *L. orbonalis*. Among the three incubated cultures used for the study, there was no survival of larvae treated with the bacterial cultures incubated for 48 hours. Hence, incubation for 48 hours would be sufficient to cause maximum mortality of *L. orbonalis*.

Mortality was the highest on seven days after treatment, while it was significantly low at two and four days after. At the maximum pathogenecity stage of 24 h incubated cultures, when inoculated for seven days, there was no difference due to *S. marcescens* at 1x and 1/5x and *Enterobacter* sp. culture at 1x, as all of them produced 93.30 per cent mortality and found superior to *Enterobacter* sp. culture at 1/5x (66.33%) and control (6.36%). Though *Serratia marcescens* at 1x had maximum mortality of 53.33 per cent on two days after inoculation, similar pathogenecity was caused by both concentrations of *S. marcescens* (1x and 1/5x) and *Enterobacter* sp. (1x).

S. marcesens was isolated from eggs of insectary reared *H. zea* (Bell, 1982) and *H. virescens* (Sikorowski, 1985) and also from field collected egg masses of *Ostrinia nubilalis* (Lynch *et al*., 1976). *S. marcescens* at 1x and 1/5x dilution incubated for 24h required seven days to cause maximum mortality of *L. orbonalis*. However, the incubation for 48 h / 72 h reduced the pathogenecity.

Pathogenecity of bacteria against H. armigera

The mortality due to the two concentrations of bacterial cultures showed that the *Enterobacter* sp. at 1x and *S. marcescens* at 1x produced a maximum mean mortality of 69.08 and 69.17 per cent, when the mortality in control was just 6.69 per cent (Table 2). Seven days after inoculation, the cultures incubated for 72 h produced a maximum mortality of 71.54 per cent than 24 and 48 h incubation. Dilution of 1/5x *S. marcescens* and 1x dilution of *Enterobacter* sp. caused maximum mortality in *H. armigera* than other dilutions.

The time required for expressing its pathogenecity was found to be seven days as the mortality was significantly more (92.42%) than two and four days after inoculation. The maximum pathogenecity stage of 72 h when inoculated for seven days, *S. marcescens* 1/5 x dilution produced the maximum mortality of 90.90 per cent while the mortality in control was 20.70 per cent. *Enterobacter* sp. at 1x dilution incubated for 48 h produced maximum mortality (91.70%) after seven days of inoculation than the 24 (91.30%) and 72 h (77.30%) incubated culture. Seven days after inoculation, no larva of *L. orbonalis* and *H. armigera* larvae survived when treated with 48 h incubated *S. marcescens* at 1x and 1/5x dilution, respectively.

Table 3. Pathogenecity of two bacterial cultures on growth inhibition of *L. orbonalis* and *H. armigera larvae*

S. No.	Treatment & Concentration	Mean weight of surviving larva (mg) *						Mean* (mg)	Larval growth inhibition over control (%)						Mean ** (%)	
		24 h culture		48 h culture		72 h culture			24 h culture		48 h culture		72 h culture			
		L. orbonalis	*H. armigera*	*L. orbonalis*	*H. armigera*	*L. orbonalis*	*H. armigera*		*L. orbonalis*	*H. armigera*	*L. orbonalis*	*H. armigera*	*L. orbonalis*	*H. armigera*		
1.	*Serratia marcescens* 1x	11.40	37.90	0.00	44.05	27.14	40.55	12.84	40.83	78.12	41.95	100.00	46.75	44.90	48.00	74.34 45.60
		(3.44)	(6.19)	(0.70)	(6.67)	(5.16)	(6.40)	(13.34)[a]	(41.33)[b]	(62.11)	(40.36)	(0.57)	(41.44)	(42.07)	(43.85)	(68.53)[a](42.47)[a]
2.	*Serratia marcescens* 1/5x	6.51	56.40	23.35	0.00	16.85	63.10	15.57	39.83	87.50	13.49	65.00	100.00	65.77	19.00	72.15 44.16
		(2.64)	(7.54)	(4.88)	(0.71)	(4.16)	(7.97)	(16.07)[b]	(40.33)[a]	(69.08)	(14.94)	(65.90)	(0.57)	(54.19)	(17.81)	(58.53)[b](41.80)[b]
3.	*Enterobacter* 1x	9.77	48.65	8.65	47.70	33.70	45.10	17.37	47.15	81.25	25.48	83.33	42.34	31.55	42.00	68.97 36.60
		(3.20)	(7.01)	(3.02)	(6.94)	(5.84)	(6.75)	(17.87)[c]	(47.65)[c]	(64.34)	(30.31)	(46.91)	(41.44)	(34.17)	(40.39)	(56.14)[c](37.22)[c]
4.	*Enterobacter* 1/5x	32.24	53.40	24.2	57.70	30.51	31.55	28.98	47.55	38.12	18.21	53.00	30.24	38.02	60.00	43.04 36.15
		(5.70)	(7.34)	(4.96)	(7.60)	(5.56)	(5.66)	(29.48)[d]	(48.05)[d]	(38.12)	(25.26)	(43.15)	(33.36)	(38.06)	(50.77)	(41.00)[d](36.96)[c]
5.	Control	52.12	65.29	51.90	32.70	49.23	78.00	51.08	75.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00 0.00
		(7.25)	(8.11)	(7.23)	(5.71)	(7.05)	(8.85)	(51.58)[e]	(75.83)[e]	(0.57)	(0.57)	(0.57)	(0.57)	(0.57)	(0.57)	(1.28)[e] (1.28)d
	Mean	**22.50**	**33.60**	**21.62**	**31.49**	**1.68**	**51.66**	**25.16**	**50.13**	**56.99**	**19.82**	**38.36**	**23.96**	**36.04**	**33.80**	**48.14 25.83**
		(4.45)	**(7.24)**	**(4.16)**	**(5.53)**	**(5.55)**	**(7.13)**	**(29.48)**	**(50.68)**	**(46.80)**	**(22.30)**	**(40.98)**	**(27.73)**	**(33.88)**	**(33.72)**	**(43.93) (30.54)**

	H	L	T	HL	LT	HT	L x H x T		H	L	T	HL	LT	HT	L x H x T
CD:	0.111	0.090	0.143	0.209	0.203	0.249	0.352	CD:	1.706	1.393	2.203	2.41	3.11	3.42	5.396

Means followed by common alphabets are not significantly different at 5% level by LSD

**Figures within parenthesis are arc sine transformed values except column ** are $\sqrt{x+0.5}$ arc sine transformed values

H = Incubation hours; L = Insect larva ; T - Treatment

Table 4. Infectivity of *Aspergillus ochraceus* against second instar larvae of *L. orbonalis*

S.No.	Conc. spores / ml	Per cent mortality			
		After 24 h	After 48 h	After 72 h	Mean
1.	1 x 10^7	35.88	56.06	76.06	56.00
		(36.79)	(48.48)	(60.70)	(48.66)[a]
2.	6.2 x 10^6	26.88	40.00	53.06	39.98
		(31.22)	(39.23)	(46.75)	(39.07)[b]
3.	5.7 x 10^6	10.00	15.00	23.06	16.02
		(18.43)	(22.78)	(28.69)	(23.30)[c]
4.	4.5 x 10^6	2.06	8.06	10.06	6.72
		(8.25)	(16.49)	(18.49)	(14.41)[d]
5.	Control	0.16	0.16	0.16	0.16
		(2.20)	(2.20)	(2.20)	(2.20)[e]
	Mean	**14.99**	**23.85**	**32.48**	**23.77**
		(19.38)	**(25.84)**	**(31.37)**	**(25.53)**

	h	t	h x t
CD :	0.26	0.33	0.58

Mean of thirty replications.
Means followed by common alphabets are not significantly different at 5% level by DSD
Figures within parenthesis are arc sine transformed values.

Maximum seven days was required for both the pathogens to express its pathogenecity. Considering the mean weight of the surviving larva, *S. marcescens* 1/5x treated *H. armigera* weighed (39.33 mg) very less after seven days of inoculation, while the highest mean weight was (75.33 mg) in control. Surviving *L. orbonalis* larvae treated with *S. marcescens* at 1x dilution resulted in mean weight of 0.71 mg while the maximum mean weight of 1.43 mg was in control (Table 3). *S. marcescens* at 1/5x incubated for 24 and 72 h had a minimum mean weight of 6.51 and 16.85 mg of *L. orbonalis* respectively. Considering the overall effect, 48 h incubated *S. marcescens* at 1x and 1/5x dilution significantly reduced the larval weight of *L. orbonalis* and *H. armigera,* respectively.

Larval growth was significantly inhibited over control in the surviving larva of *L. orbonalis* and *H. armigera* due to inoculation of new bacterial cultures. Maximum larval growth inhibition (100%) was observed in larvae of *L. orbonalis* and *H. armigera* treated with *S. marcescens* at 1x and 1/5x culture incubated for 48 h respectively. To achieve a maximum growth inhibition of 33.80 per cent in *H. armigera*, a minimum of 72 h incubation period of bacterial culture was essential while, in *L. orbonalis* even the 24 h incubation was sufficient to cause the maximum growth inhibition of 56.99 per cent (Table 3). Considering *H. armigera*, minimum growth inhibition (13.49%) was observed due to *S. marcescens* at 1/5x culture incubated for 24 h, while for *L. orbonalis, Enterobacter* sp. at 1/5x incubated for 48 h exhibited 30.24% of mortality. Comparing the different periods of incubation, bacteria incubated for 72 h produced maximum inhibition (33.8%) of growth in *H. armigera* larvae, while 24 h incubated culture produced a maximum growth inhibition of (56.99%) on *L. orbonalis*.

Overall higher pathogenecity was exhibited on both the larvae by *Enterobacter* sp. without dilution than *S. marcescens*. Comparatively, *H.*

armigera was found more susceptible to the pathogens than *L. orbonalis*. *S. marcescens* without dilution was required to reduce the mass of surviving *L. orbonalis* larvae while, on *H. armigera* even at 1/5x dilution had maximum mortality. Concerned with safety, human being is reported to be affected by only non-pigmented biotype of *S. marcescens* (Neter, 1974) but the broader significance of pigmented biotypes in human is uncertain (Tanada and Kaya, 2004).

Infectivity of Aspergillus ochraceus against L. orbonalis

Fungal pathogen infecting *L. orbonalis* was identified as *A. ochraceus.* On evaluation of *A. ochraceus* against second instar larvae under laboratory conditions revealed that with a highest concentration of 1 x 10^7 spores ml^{-1} excelled the other concentration with maximum mean mortality of 56.00 per cent, whereas the mortality in control without entomofungi was 0.16 per cent (Table 4). Laboratory evaluation showed that a concentration of 1x10^7 spores/ml of *A. ochraceus* brought in highest mortality of 56.00 per cent in *L. orbonalis. A. ochraceus* was recovered as the native entomopathogen of *Hieroglyphus banian* SW. (Parthasarathy, 1997) in rice ecosystem.

The lowest concentration of 4.5 x 10^6 spores ml^{-1} produced 6.72 per cent mortality (76.06 %). Pertaining to the pathogenecity on three different days of observations, the mortality was highest after 72 h of application, while lowered 24 and 48 h. The highest concentration of 1 x 10^7 spores per ml. required 72 h to produce maximum mortality on the tested second instar larvae of *L. orbonalis*.

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