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Pathogenicity of symbiotic bacteria associated with entomopathogenic nematodes on larvae of *Galleria mellonella*

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Abstract
The investigation was carried out to study the effect of different population of symbiotic bacteria associated with entomopathogenic nematodes isolated from agroclimatic zone 5 of Karnataka on second instar larvae of greater wax moth, *Galleria mellonella* by artificial diet method. Entomopathogenic nematodes were isolated by insect bait method and symbiotic bacteria were isolated and identified by morphological and biochemical tests. Second instar larvae of *G. mellonella* were fed with artificial diet containing different populations of bacterial cells and mortality data of larvae was recorded after 48hrs. The cumulative mortality of larvae has increased with increase in the concentration of bacterial cells. The median lethal concentration varied among the bacterial isolates against *G.mellonella* larvae. Among the twenty isolates used in the study, isolate KPR1 was found to be highly pathogenic with a lower median lethal concentration of $0.018x10^5$ cells/ml followed by HEB2 ($0.084x10^5$ cells/ml), KPR4 ($0.12x10^5$ cells/ml), CHK1($0.13x10^5$ cells/ml), KPR3($0.16x10^5$ cells/ml), EXP2 ($0.19x10^5$ cells/ml) CHK2 ($0.19x10^5$ cells/ml), RMG2($0.20x10^5$ cells/ml). The remaining twelve bacterial isolates showed higher median lethal concentration with isolate BGR showing the highest LD_{50} of $26x10^5$ cells/ml. These results suggest that the toxic activity to *G. mellonella* varied among the Symbiotic bacteria isolated from different locations.

Keywords: *Galleria mellonella*, entomopathogenic nematodes, bioassay, LD_{50} and symbiotic bacteria.

INTRODUCTION

Soil is the natural habitat for EPNs where they are associated with various insects. They can be extracted from soil by baiting with susceptible insects or from infected insects. Entomopathogenic nematodes (EPNs) are soil-inhabiting, lethal insect parasitoids that belong to the Phylum Nematoda, commonly called roundworms. EPNs live inside the body of their host, and so they are designated endoparasitic. They infect many different types of soil insects, including the larval forms of butterflies, moths, beetles, flies, as well as adult crickets and grasshoppers. EPNs have been found in all inhabited continents and a range of ecologically diverse habitats, from cultivated fields to deserts. The most commonly studied genera are those that are useful in the biological control of insect pests, the Steinernematidae and Heterorhabditidae [1]. Bacteria of the genera *Photorhabdus* and *Xenorhabdus* form a mutually beneficial symbiotic complex with the Entomopathogenic nematodes (EPNs), which are able to infect, kill and reproduce in many insect species. It would be easy to consider the nematode as little more than a biological syringe for the bacterium, but the relationship between these two organisms is one of the classical mutualism. All of the *Xenorhabdus* isolates studied so far, and almost all of the *Photorhabdus* isolates have been obtained from nematodes harvested from soil samples. Free-living form of the bacteria has not yet been isolated from soil or water source. These finding suggest that, the bacterium requires the nematode for protection from the environment, penetrating into the host haemocoel, and inhibition of the immune proteins. According to Heidi and coworkers these bacteria are found to be effective biocontrol agents against many insect pests [2]. In order to be able to infect its host and survive, *P. luminescens* must be capable of producing a wide range of proteins, including toxins. The complete genomic analysis of this organism done by Duchaud and coworkers revealed that it indeed possesses a lot of genes encoding for toxins, proteases and lipases [3].

In the present study, twenty Symbiotic Bacteria were isolated from EPNs of agro climatic zone 5 of Karnataka. In addition an analysis was carried out to study the effect of different population of symbiotic bacteria associated with entomopathogenic nematodes isolated from agroclimatic zone 5 of Karnataka on second instar larvae of greater wax moth, *Galleria mellonella* by artificial diet method.

MATERIAL AND METHODS

Isolation and identification of symbiotic bacteria

Isolation of EPNs was done using *Galleria mellonella,* a host susceptible to EPNs by baiting method [4] from different locations of agroclimatic zone 5 of Karnataka. Twenty symbiotic bacteria were isolated from these nematodes and named based on the source place (Table 1). These bacteria were identified based on microscopic observation, biochemical and physiological characters like Gelatin liquefaction, Catalase test, Lactose fermentation test, Urease test,

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Motility test and Colony morphology studies on different media viz,. Nutrient bromothymol agar, Nutrient agar and Macconkey agar [5-6].

Table 1. List of symbiotic bacteria isolated from EPNs of agro climatic zone 5 of Karnataka

Sl.No	Isolates	Location	Crop
1	HORT1	Horticulture	Grapes
2	HORT2	Horticulture	Grapes
3	KPR1	Kanakapura	Banana
4	KPR2	Kanakapura	Banana
5	KPR3	Kanakapura	Fodder
6	KPR4	Kanakapura	Fodder
7	RMG1	Ramanagara	Paddy
8	RMG2	Ramanagara	Fodder
9	RMG3	Ramanagara	Mulberry
10	EXP1	Experimental plots, GKVK	Groundnut
11	EXP2	Experimental plots, GKVK	Groundnut
12	EXP3	Experimental plots, GKVK	Green gram
13	HEB1	Hebbal	Maize
14	HEB2	Hebbal	Maize
15	HEB3	Hebbal	Maize
16	HEB4	Hebbal	Maize
17	BGR	Botanical garden	-
18	CHK1	Chikballapur	Maize
19	CHK2	Chikballapur	Paddy
20	TUM1	Tumkur	Paddy

Bioassay

The toxic activity of symbiotic bacterial isolates was determined on *G. mellonella* larva by artificial diet method. Second instar larvae of *Galleria* were fed with artificial diet containing different concentrations of bacterial cells as described by Mahar and coworkers [7].

Culture and counting of bacteria

The symbiotic bacterial colony from NBTA plate was inoculated into 10 ml of LB broth and incubated at 28^0C overnight. The bacterial culture was pelleted by centrifuging at 10,000 rpm for 10min and the pellets were re-suspended in 10ml of sterile water. The concentration of cells was estimated by use a counting slide. This bacterial suspension was further diluted to obtain different concentrations of bacteria ranging from $1x10^1$ to $1x10^8$ cells per ml.

Treatment for bioassay

For each concentration, 10ml of the bacterial suspension was mixed with 50gm of artificial diet and placed in a plastic container. Then, for each container 10 second instar galleria larvae were added and kept for 2 days. Each treatment was maintained in duplicate. Larval mortality was assessed and recorded at 48hr after treatment. For each treatment a control is maintained treated with sterile water only. Bioassay treatment details is given below.

Treatments	Bacterial Population (cells/ml)
Control	0
T_1	$1x\ 10^1$
T_2	$1x\ 10^2$
T_3	$1x\ 10^3$
T_4	$1x\ 10^4$
T_5	$1x\ 10^5$
T_6	$1x\ 10^6$
T_7	$1x\ 10^7$
T_8	$1x\ 10^8$

Statistical analysis

The larval mortality data was subjected to probit analysis using SPSS software to estimate the median lethal dose (LD_{50}) of the symbiotic bacteria to *G.mellonella*.

RESULTS AND DISCUSSION

Isolation and Identification of Symbiotic bacteria from EPNs

Twenty isolates were isolated from EPNs and named based on the source place (Table 1). The cultures so isolated were characterized by a number of morphological and physiological tests for identification of symbiotic bacteria (Table 2).

Table 2. Biochemical characters of symbiotic bacterial isolates

Sl.No	Biochemical tests	Result
1	Lactose fermentation	+
2	Gelatin liquefaction	+
3	Catalase	-
4	Urease	+
5	Motility	+

Bioassay

Different concentrations of symbiotic bacterial isolates were evaluated against second instar larvae of *G. mellonella* to study the toxic activity of these bacteria under laboratory conditions. The cumulative mortality of larvae was increased with increase in the concentration of bacterial cells. The median lethal concentration varied among the bacterial isolates against *G. mellonella.*

Table 3. Probit analysis of dosage mortality response of symbiotic bacteria to the *G.mellonella* larvae

Sl.No	Isolate	Chi^2 (d.f.6)	Regression equation (Y=bx+a)	LD_{50} (cells/ml)	Fiducial limit (cells/ml)
1	HORT1	0.410	0.171X-0.456	$3.68X10^5$	(112658.12- 1730979.0)
2	HORT2	7.811	0.180X-0.390	$0.85\ X10^5$	(28267.39- 307629.00)
3	KPR1	2.814	0.152X+0.004	$0.018\ X10^5$	(432.45- 6139.72)
4	KPR2	10.178	0.188X-0.676	$17.9\ X10^5$	(605925.89- 6773231.44)
5	KPR3	12.311	0.114X-0.108	$0.16\ X10^5$	(1139.27-17654.31)

6	KPR4	3.372	0.114X+0.029	0.12 $X10^5$	(2585.52-57858.57)
7	RMG1	4.650	0.125X-0.162	1.84 $X10^5$	(46454.51-883497.60)
8	RMG2	2.221	0.094X+0.088	0.20 $X10^5$	(2958.37-126881.79)
9	RMG3	2.808	0.123X-0.202	5.12 $X10^5$	(124017.03-286666.70)
10	EXP1	8.193	0.147X-0.215	0.73 $X10^5$	(21130.97-264807.29)
11	EXP2	2.961	0.132X-0.069	0.19 $X10^5$	(5004.27-77012.39)
12	EXP3	2.629	0.121X-0.101	0.89 $X10^5$	(21514.02-422971.87)
13	HEB1	2.169	0.146X-0.201	0.62 $X10^5$	(17897.04-225802.82)
14	HEB2	9.120	0.143X-0.063	0.084 $X10^5$	(2144.86-29463.45)
15	HEB3	2.500	0.138X-0.113	0.26 $X10^5$	(7146.52-99964.82)
16	HEB4	1.781	0.176X-0.526	6.63 $X10^5$	(220066.80-2333489.73)
17	BGR	1.287	0.164X-0.556	26.1 $X10^5$	(785016.48-1216000)
18	CHK1	5.464	0.106X-0.056	0.13 $X10^5$	(2347.53-70447.40)
19	CHK2	0.735	0.148X-0.136	0.19 $X10^5$	(5527.24-68267.56)
20	TUM1	2.930	0.160X-0.357	2.23 $X10^5$	(70034.87-803190.54)

Among the twenty isolates used in the study isolate KPR1 was found to be highly pathogenic with a lower median lethal concentration of $0.018X10^5$ cells/ml followed by HEB2 ($0.084x10^5$ cells/ml), KPR4 ($0.12x10^5$ cells/ml), CHK1($0.13x10^5$ cells/ml), KPR3($0.16x10^5$ cells/ml), EXP2 ($0.19x10^5$ cells/ml) CHK2 ($0.19x10^5$ cells/ml), RMG2($0.20x10^5$ cells/ml).The remaining twelve bacterial isolates showed higher median lethal concentration with isolate BGR showing the highest LD_{50} of $26x10^5$ cells/ml (Table 3). These results suggest that the toxic activity to *G.mellonella* varied among the symbiotic bacteria isolated from different locations.

Sun and coworkers [8] reported similar results on pathogenicity studies conducted against *Galleria mellonella* larvae by direct injection method. Bacterial concentration of 60 to 80 cells per larvae was found to be pathogenic against *G. mellonella* larvae. Mahar and coworkers [9] conducted studies on different application methods of *Xenorhabdus* and *Photorhabdus* cells and their toxin to control locust (*Schistocerca gregaria*) and found that mortality percentage significantly increased with increase in concentration of the bacterial cell.

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