ISOLATION, INSECT BIO-CONTROL ACTIVITY AND MOLECULAR DIVERSITY OF *Photorhabdus luminescens*

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2014

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Thesis submitted to the

UNIVERSITY OF AGRICULTURAL SCIENCES, BANGALORE

In partial fulfillment of the requirements

For the award of the degree of

MASTER OF SCIENCE (AGRICULTURE)

in

PLANT BIOTECHNOLOGY

BANGALORE **SEPTEMBER, 2014**

Affectionately Dedicated to

my family

&

Dr. C. K. Suresh

DEPARTMENT OF PLANT BIOTECHNOLOGY
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CERTIFICATE

This is to certify that the thesis entitled **"Isolation, insect bio-control activity and molecular diversity of *Photorhabdus luminescens*"** submitted by **Mr. RAJEN CHOWDHURY, I.D. No. PALB 2250**, for the award of degree of **MASTER OF SCIENCE (AGRICULTURE) IN PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, GKVK, Bangalore, is a record of research work done by him during the period of his study in this University under my guidance and supervision, and the thesis has not previously formed the basis for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bangalore
September, 2014

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ACKNOWLEDGEMENT

This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well-wishers, my friends and colleagues. At the concluding stages of my thesis, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

I express my profound sense of reverence to my Guide ***Dr. C. K. Suresh*** *for his constant guidance, support, motivation and untiring help during my research. I have been amazingly fortunate to have an advisor who gave me the freedom to explore on my own and at the same time the guidance to recover when my steps faltered. His valuable suggestions about the life were authentically unforgettable and I will try to follow these suggestions for the betterment of my life.*

In wish to express my profound sense of gratitude to ***Dr. N. Eranna,*** *Professor, Department of Plant Biotechnology, UAS, GKVK, Bangalore,* ***Dr. Anitha Peter*** *Associate Professor, Department of Plant Biotechnology, UAS, GKVK, Bangalore,* ***Dr. V. V. Belavadi*** *Professor, Dept. of Agricultural Entomology, UAS, GKVK, Bangalore and* ***Dr. B. C. Mallesha*** *Professor, Dept. of Agricultural Microbiology, UAS, GKVK, Bangalore for their unceasing interest and inspiring guidance as the members of my advisory committee.*

I express my deepest gratitude to the ***Dr. H. V. Vijay Kumar Swamy, P****rofessor and head for boosting my morale throughout the course of research and helping to complete the formalities as ad hoc chairman.*

I also thank profoundly ***Dr. K. M. Harinikumar,*** *Professor, Post-Graduation Studies co-coordinator Department of Plant Biotechnology, UAS, GKVK, Bangalore, who has been helpful to serve as nominated member of the Advisory committee.*

I sincerely thank ***Dr. K. Srinivasa Murthy****, Principal Scientist, Division of Molecular Entomology, NBAII, Bangalore and* ***The Director of NBAII****, Bangalore for providing me lab space and guided through the research work. I also want to thank*

Chaube *sir for his valuable suggestions and guidance regarding research work and* ***Dr. Jagadish Patil,*** *Scientist NBAII, Bangalore for helping me through statistical analysis.*

My diction is too poor to translate the gratitude into so many words, the help received from my classmates ***Sukanta, Jameel, Ashutosh, Laxman, Rohini, Shiv, Akash, Ashwat*** *and* ***Vinay*** *in whose company I never felt my work as burden. My special thanks extended to all my seniors* ***Swetha madam, Prakash sir, Vimarsh sir, Pavan sir, Shyam sir, Swapnil sir, Kheta sir, Raghu sir, Sumanth sir, Ningaraj sir, Sujeet sir, Meena sir, Narendra sir, Amitendra sir, Shraddha madam,*** *and junior* ***Swapnil, Saurav, Manoj, Abinash,*** *for their cheerful company and help. I cannot miss to express heartfelt thanks to my lab mates* ***Santaveera sir, Rameya madam*** *for their joyful company during my research work specially* ***Dr. Julia Rani Francis and Swetha H.G.***

My friends, ***Paresh Pal, Tanmoy Ghosh*** *and* ***Chiranjit Ghosh*** *have helped me to stay sane through these difficult years. Their support and care helped me to overcome setbacks and stay focused on research. I greatly value their friendship and I deeply appreciate their belief in me. I am obliged to all the teaching and non-teaching staff member of this Department for their immense help and suggestion.*

I would like to express my cordial gratitude to my family specially my sister ***Tumpa Chowdhury****. My family has aided and encouraged me throughout this endeavor.*

At last, I take this opportunity to sincerely acknowledge the ***DBT-HRD****, By Government of India, New Delhi, for providing financial assistance in the form of Fellowship and grants for consumables which buttressed me to perform my work comfortably.*

Any omission in this brief acknowledgement does not mean lack of gratitude.

Bangalore

September, 2014 ***Rajen Chowdhury***

"Isolation, insect bio-control activity and molecular diversity of *Photorhabdus luminescens*"

RAJEN CHOWDHURY

ABSTRACT

In the present study insect biocontrol activity and molecular diversity of *Photorhabdus luminescens* strains isolated from the Mandya locality of agro climatic zone 06 of Karnataka. Thirty soil samples were collected from different crop fields of Maize, Ragi, Aerobic Rice, Cowpea and Sugarcane. Isolation of entomopathogenic nematodes was done using *Galleria*-bait method. *In vitro* mass multiplication of the nematode was done using White's trap method. Eleven symbiotic bacteria were isolated from the insect haemolymph and identified under microscope using Gram staining. Colony morphology was studied on differential media *viz*., Nutrient bromothymol agar and MacConkey agar. Gelatin liquefaction, Lactose fermentation test, Urease test and Motility test were carried out to study biochemical and physiological characters. Biocontrol activity was tested against 2^{nd} instar larvae of *Galleria mellonella* and *Plutella xylostella* (Diamond Black Moth) using different concentrations of *P. luminescens viz.*, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 cells/ml. Mortality of both the insects was increased with increase in concentration. Median lethal dose was varied among strains. Strain 10 showed least LD_{50} value of 0.24×10^6 cells/ml for *G. mellonella* and 0.25×10^6 cells/ml for *P. xylostella*. Molecular diversity of *P. luminescens* was analyzed using RAPD marker. A total of 33 bands were taken for cluster analysis in UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method using NTSYSpc (Ver. 2.02). All the 11 strains formed a major cluster in dendogram. Sub cluster 1 was further subdivided into 2 sub-clusters at similarity coefficient of 0.70. Strain 3 was found distinct with a similarity coefficient of 1.00.

UAS, GKVK, Bangalore-65

September, 2014

(**Dr. Vijaya Kumara Swamy H. V.)**
Major Advisor

ಫೋಟೋರಾಬ್ಡಿಡಸ್ ಲೂಮಿನಸೆನ್ಸ್‌ನಲ್ಲಿ ಕೀಟ ಜೈವಿಕ ಪ್ರತ್ಯೇಕತೆಯ ನಿಯಂತ್ರಣದ ಚಟುವಟಿಕೆ ಮತ್ತು ಅಣ್ವಿಕ ವೈವಿಧ್ಯತೆಯ ಅಧ್ಯಯನ

ರಾಜನ್ ಚೌದ್ರಿ

ಪ್ರಭಂದದ ಸಾರಾಂಶ

ಪ್ರಸ್ತುತ ಅಧ್ಯಯನವು ಕರ್ನಾಟಕದಲ್ಲಿನ ಮಂಡ್ಯದ ಸ್ಥಳೀಯ ಕೃಷಿ ಹವಮಾನದ ವಲಯದಲ್ಲಿ *ಫೋಟೋರಾಬ್ಡಿಡಸ್ ಲೂಮಿನಸೆನ್ಸ್* ಎಂಬ ಕೀಟ ಜೈವಿಕ ನಿಯಂತ್ರಣ ತಳಿಯನ್ನು ಅಭಿವೃದ್ದಿ ಪಡಿಸಿ ಅಣುವೈವಿದ್ಯತೆಯನ್ನು ಅಭ್ಯಾಸಿಲಾಗಿದೆ. 30 ಮಣ್ಣಿನ ಮಾದರಿಯನ್ನು ವಿವಿಧ ಕಬ್ಬು, ಅಲಸಂಡೆ, ಮೆಕ್ಕೆ ಜೋಳ, ಏರೋಭಿಕ್ ಭತ್ತ ಮತ್ತು ರಾಗಿ ಬೆಳೆಯುವ ಕ್ಷೇತ್ರದಿಂದ ಸಂಗ್ರಹಿಸಲಾಗಿದೆ. ಎಂಟೋಮೋಪೆತೋಜನಿಕ್ ನೆಮೆಟೋಡ್ ಅನ್ನು ಕೀಟದ ಆಹಾರ ವಿಧಾನದ ಮೂಲಕ ಪ್ರತ್ಯೇಕಿಸಲಾಗಿದೆ. ವೈಟ್ಸ್ ಟ್ರಾಪ್ ಎಂಬ ವಿಧಾನದಿಂದ ನೆಮೆಟೋಡ್ ಅನ್ನು ಪ್ರಯೋಗಾಲಯದಲ್ಲಿ ಸಾಮೂಹಿಕವಾಗಿ ಬೆಳೆಸಲಾಗಿದೆ. 11 ಬ್ಯಾಕ್ಟೀರಿಯ ಸಹಜೀವಿಗಳನ್ನು ಕೀಟ ಹಿಮೋಲಿಂಫ್‌ನಿಂದ ಪ್ರತ್ಯೇಕಿಸಿ ಅದನ್ನು ಗ್ರಾಂ ಸ್ಟೈನಿಂಗ್ ವಿಧಾನದ ಮೂಲಕ ಸೂಕ್ಷ್ಮಾಣೂ ದರ್ಶಕದಿಂದ ದೃಢಿಕರಿಸಲಾಗಿದೆ. ಬ್ರೋಮೋತೈಮೋಲ್ ಅಗರ್ ಮತ್ತು ಮ್ಯಾಕೋಂಕಿ ಅಗರ್ ನಂತಹ ವಿವಿಧ ಮಾಧ್ಯಮಗಳ ಮೇಲೆ ಕಾಲೋನಿ ರೂಪವಿಜ್ಞಾನದ ಅಧ್ಯಾಯನ ಮಾಡಲಾಯಿತು. ಶೀತದ ಘಡ್ಡೆ ಕಟ್ಟುವಿಕೆಯ ದ್ರವೀಕರಣ, ಕೆಟಲೇಸ್, ಲ್ಯಾಕ್ಟೋಸ್‌ನ ಹುಳಿಬರಿಸುವಿಕೆ, ಯುರಿಯೇಸ್, ಮೊಟಿಲಿಟಿ ಪರೀಕ್ಷೆಯ ಅಧ್ಯಯನ ಮಾಡಲಾಯಿತು. ವಿವಿಧ ಸಾಂದ್ರತೆಯ *ಫೋಟೊರಾಬಿಡ್ಡಸ್ ಲ್ಯುಮಿನಸೆನ್ಸ್* 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 ಜೀವಕೋಶಗಳು/ಮಿಲಿ ಬಳಸಿ *ಗೆಲೇರಿಯ ಮೆಲೊನೆಲ್ಲಾ* ಮತ್ತು *ಫ್ಲುಟೆಲ್ಲಾ ಕ್ಸೈಲೋಸ್ಟೆಲ್ಲಾ* ಎರಡನೇ ಹಂತದ ಮರಿಗಳ ಮೇಲೆ ಜೈವಿಕ ನಿಯಂತ್ರಣ ಚಟುವಟಿಕೆಗಳನ್ನು ಪರೀಕ್ಷೀಸಲಾಗಿದೆ. ಸಾಂದ್ರತೆಯು ಹೆಚ್ಚಿದಂತೆ ಎರಡೂ ಕೀಟಗಳ ಮರಣವು ಹೆಚ್ಚಿರುತ್ತದೆ. ಒಟ್ಟು ತಳಿಗಳ ನಡುವೆ, ಮಧ್ಯೆ ನಂಜಿನ ಪ್ರಮಾಣ ಬದಲಾಗುತಿತ್ತು. *ಗೆಲೇರಿಯ ಮೆಲೊನೆಲ್ಲಾದ* ಒಟ್ಟು ತಳಿಗಳಲ್ಲಿ 10 ನೇ ತಳಿಯು ಅತಿ ಕಡಿಮೆ ಎಲ್‌ಡಿ$_{50}$ ಮೌಲ್ಯದ $0.24\text{x}10^6$ ಜೀವಕೋಶಗಳು/ಮಿಲಿ ತೋರಿಸಿದೆ ಹಾಗು *ಫ್ಲುಟೆಲ್ಲಾ ಕ್ಸೈಲೋಸ್ಟೆಲ್ಲಾದ* 10ನೇ ತಳಿಯು $0.25\text{x}10^6$ ಜೀವಕೋಶಗಳು/ಮಿಲಿ ತೋರಿಸಿದೆ. ಆರ್‌ಎಪಿಡಿ ಗುರುತುಕಾರಕಗಳನ್ನು ಬಳಸಿ *ಫೋಟೊರಾಬಿಡ್ಡಸ್ ಲ್ಯುಮಿನೋಸೆನ್ಸ* ನ ಅಣುವೈವಿಧ್ಯತೆಯನ್ನು ವಿಶ್ಲೇಷಿಸಲಾಗಿದೆ. ಯೂಪಿಜಿಎಂಎ ವಿಧಾನದಲ್ಲಿ NTSYSpc (version 2.02) ಅನ್ನು ಬಳಸಿ ಕ್ಲಸ್ಟರ್ ವಿಶ್ಲೇಷಣೆಯಲ್ಲಿ ಒಟ್ಟು 33 ಬ್ಯಾಂಡ್‌ಗಳನ್ನು ವಿನಿಯೋಗಿಸಲಾಗಿದೆ. ಒಟ್ಟು 11 ತಳಿಗಳು ಪ್ರಮುಖವಾಗಿ ಡೆಂಟ್ರೋಗ್ರಾಂನಲ್ಲಿ ಕ್ಲಸ್ಟರ್‌ಗಳಾಗಿ ಸೇರುತ್ತದೆ. 0.70 ಹೋಲಿಕೆ ಗುಣಾಂಶದಲ್ಲಿ ಉಪಕ್ಲಸ್ಟರ್ ಒಂದನ್ನು ಮತ್ತೇ ಉಪವಿಭಾಗಿಸಿ 2 ಉಪಕ್ಲಸ್ಟರ್ ಗಳನ್ನಾಗಿ ವಿಂಗಡಿಸಲಾಗಿದೆ. ಹೋಲಿಕೆ ಗುಣಾಂಶ 1.00 ರಲ್ಲಿ, ತಳಿ 3 ವಿಶಿಷ್ಟವಾಗಿ ಕಂಡುಬಂದಿದೆ.

(ಡಾ॥ ಹೆಚ್.ವಿ ವಿಜಿಯಕುಮಾರಸ್ವಾಮಿ)
ಮುಖ್ಯ ಸಲಹೆಗಾರರು

ಸೆಪ್ಟೆಂಬರ 2014
ಜೈವಿಕ ತಂತ್ರಜ್ಞಾನ ವಿಭಾಗ
ಕೃಷಿ ವಿಶ್ವವಿದ್ಯಾನಿಲಯ, ಜಿ.ಕೆ.ವಿ.ಕೆ., ಬೆಂಗಳೂರು–65

ISOLATION, INSECT BIO-CONTROL ACTIVITY AND MOLECULAR DIVERSITY OF *Photorhabdus luminescens*

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INTRODUCTION

✓The demand for biological control of insect pest is increasing all over the world.

✓Photorhabdus is entomopathogenic bacteria that are symbiotically associated with the entomopathogenic nematode of the family *Heterorhabditidae*.

✓*Photorhabdus luminescens* plays a role in the elimination of competitors, host colonization, invasion and bioconversion of the insect cadaver, making it a effective biocontrol agent against many insect pests.

✓The bioassay study of different strains will help to identify promising strains which can be useful to control insect pests in enviornment sound way.

OBJECTIVES

1. Isolation and identification of *Photorhabdus luminescens* from soils collected from Agroclimatic Zone-6 Southern Dry Zone.
2. Bio-control activity of *Photorhabdus luminescens* against insect crop pests.
3. Molecular diversity of *Photorhabdus luminescens* isolates using RAPD technique.

MATERIALS & METHODS

Galleria mellonella baited trap for isolation of EPNs from soil sample

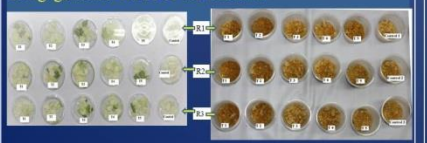
✓With the objective of isolation of *Photorhabdus luminescens* bacteria from karnataka agroclimatic Zone-6 (Southern Dry Zone). Soil samples were collected from V.C.Farm, Mandya. Isolation of the EPNs was done using *Galleria mellonella* bait.

✓ Bacteria have isolated from haemolymph of infected larvae and grown in sterile plated NBTA medium. Sub culturing was done till uniform size and colony morphology were obtained.

✓Identification was done by checking colony morphology on media such as NBTA media, Mac Conkey agar, and Nutrient agar. Microscopic observation was done for cell morphology and gram reaction. The biochemical studies viz., Gelatin liquefaction test, Catalase test, Lactose fermentation test, Urease test and Motility test were conducted.

✓The toxic activity of symbiotic bacterial isolates was determined on second instar larvae of Diamond back moth (*Plutella xylostella*) by feeding with treated cabbage leaf of uniform size and greater wax moth (*G. mellonella)* larva by feeding with artificial diet containing different concentrations of bacterial cells starting from 1×10^3, 1×10^4,1×10^5,1×10^6 and 1×10^7.

✓To check molecular diversity RAPD was carried out using genomic DNA of the bacteria.



RESULTS

All 11 isolates formed typical bluish green colonies in NBTA media, in Mac Conkey agar characteristic pinkish red colony and in Nutrient agar cream colored colonies were obtained.

characteristic pinkish red colony in Mac Conkey agar

Typoical bluish green colonies in NBTA media

Biochemical and physiological characters of symbiotic bacterial isolates are shown in the table:

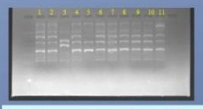
Biochemical tests	Result
Lactose fermentation	+
Gelatin liquefaction	+
Catalase	-
Urease	+
Motility	+

Gram negetive, rod shaped *Photorhabdus luminescens*

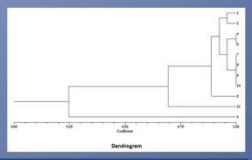
Motility Test_dark red is positive

Urease test positive(red)

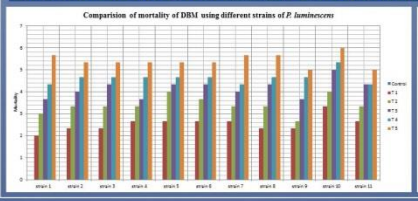
10 decamer random primers were used for amplication. Out of that 4 primers giving good polymorphism were selected for analysis. To check molecular diversity cluster analysis was done using NTSYS software.

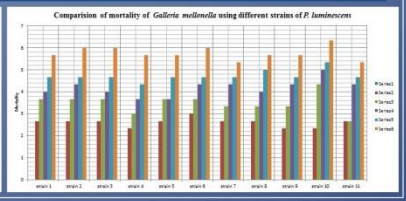


RAPD agarose gel profile using OPA18 primer



Probit analysis of dosage mortality response of symbiotic bacteria was done using SAS to get LD50 (cells/ml). Isolate no. 10 showed very good mortality response against *Plutella xylostella* as well as *G. mellonella* LD50 is 0.25×10^6 and 0.24×10^6 respectively.





DISCUSSION

✓ RAPD analysis shows significant variability among different strains.

✓Bioassay of DBM and wax moth using different strains of *P.luminescens* shows significant death to be used as biocontrol agent. Out of these strain no. 10 showed promising result in relation to control insect pests used in this study.

SUMMARY

- ✓ *Photorhabdus luminescens* plays an important role in controlling insect pests.
- ✓ In this study *P.luminescens* bacterium was isolated from different crop fields of mandya.
- ✓ Identification was done based on morphological and biochemical properties.
- ✓ 11 strains isolated were used for RAPD analysis.
- ✓ Biocontrol activity of these strains were checked against DBM and Wax moth. All of these strains showed significant death response.
- ✓ Out of 11 strains, strain no 10 showed maximum death at lowest concentration.
- ✓ I have presented this research work in national conference on Productivity and sustainability : Role of agriculturally important microorganisms April 10-12, 2014.

ADVISORY COMMITTEE

1. Dr. C. K. SURESH (Guide)
2. Dr. Dr. N. ERANNA
3. Dr. ANITHA PETER
4. Dr. V. V. BELAVADI
5. Dr. B. C. MALLESHA

Member's

ACKNOWLEDGEMNET

- Authors are highly greateful to DBT-HRD, New Delhi for providing fellowship and grants to carry out the research and DBT project for providing consumeables.
- Authors want to acknowledge members of the advisory committee Dr. N. Eranna, Dr. Anitha Peter, Dr. V. V. Belavadi, Dr. B. C. Mallesha for their great support and valuable advice regarding research work.

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I. INTRODUCTION

Since, 1960 the food production has increased in several folds because of the green revolution and technological development in agriculture and allied fields. However, demand for food is increasing and will continue to increase due to increasing population and change in lifestyle. The world population will increase to 9306 million in 2050, in 2080 it will be 9969 million and in 2100 it will be 10125 million (Alexandratos and Bruinsma, 2012). Most of the high yielding cultivars used in green revolution era were easily susceptible to pests; hence there was drastic lift in the pest population in agriculture field. To control the newly introduced pests and diseases in agriculture, chemical pesticides were used indiscriminately. Subsequently, along with the use of high yielding cultivars and hybrids the use of chemical pesticides was increased drastically and it is being continued.

Synthetic pesticides have been extensively used in developing countries mostly after the adoption of green revolution and for the control of vector borne diseases. By the early 1980's the developing countries were thought to use 10-25% of the world pesticide production. However, about 1/3rd of the crops were still lost to pests each year (Misra, 2011). Among different factors insect pests cause 15 percent of global crop production loss (Maxmen, 2013). The drawbacks with injudicious and indiscriminate use of pesticides includes development of pest resistance to pesticides, destruction of natural enemies, poisoning of man and animals, environmental pollution, minor pest assuming major status and increasing costs. (Kumar *et al.,* 2008; Dhaliwal and Koul, 2010). To overcome detrimental impact of chemical pesticides, the biological method of insect pest control has proved beneficial.

Modern era is witnessing versatile application and utilization of processes and products of sustainable agriculture that pose minimum or negligible negative impacts on environment. Use of microorganisms for curbing the attack of plant pathogenic organisms/pests forms the foremost limb of integrated pest management and is responsible for either imparting induced systemic resistance or improving the general health of the inoculated plant due to enhanced nutrient acquisition/nutrient availability, thereby increasing the chances of survival, growth, and development of plant. Conservation, augmentation, and classical biological control (also called importation) are tactics for harnessing the effects of natural enemies. Biological control utilizes insect predators, parasitoids and parasites such as viruses, rickettsia, nematode, fungi and bacteria deriving its energy directly from the pests themselves (Owuama, 2001). Biological control agents of plant diseases are most often referred to as antagonists. Biological control methods can replace, at least in part, some hazardous chemical pesticides (Kumar *et al*., 2008). It also reduces expenses and health hazards associated with chemical pesticides (Kouser and Qaim, 2011). It would be a useful technology that could suffice the existing arsenal of chemical pesticides in the coming years.

Microorganism as the active ingredient can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest. For example, there are fungi that control certain weeds, and other fungi that kill specific

insects. One bacterial species like *Bacillus thuringiensis* may be more effective on *Aedes aegypti* while another *B. sphaericus* strain can be effective on different types of mosquito like *Culex quinquefasciatus*. Some exclusive examples of biological control include entomopathogenic nematodes (EPNs) which 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, and 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. Bacteria of the genera *Photorhabdus* and *Xenorhabdus* form a mutually beneficial symbiotic complex with the EPNs in the family Heterorhabditidae and Steinernematidae respectively, which are able to infect, kill and reproduce in many insect species. The life cycle of *Photorhabdus* and *Xenorhabdus* begins and ends with the colonization of the intestinal tract of a soil dwelling and non-feeding stage of the nematode known as the infective juvenile. During this life cycle, *Photorhabdus* and *Xenorhabdus* must successfully accomplish three distinct roles: (i) rapid killing of insects, (ii) production of nutrients from the insect cadaver to facilitate growth and development of the nematode, and (iii) colonization and growth within the infective juvenile (IJ) stage of the nematode (Heidi and David, 2007).

Photorhabdus luminescens is a gram-negative gamma proteobacterium belonging to the family Enterobacteriaceae. Based on molecular analysis, the *Photorhabdus* genus is divided into three bacterial species: *Photorhabdus luminescens*, *P. temperata*, and *P. asymbiotica* (Saux, *et al.,* 1999; Boemare, 2002). It has symbiotic relationship with soil entomopathogenic nematodes of the family Heterorhabditidae and pathogenic to a wide range of insects (Park *et al*., 2013).

Photorhabdus luminescens promotes its own transmission among susceptible insect populations using its nematode host. Upon invasion of the host the bacteria are released directly into the open blood system of the insect. Its life cycle comprises a symbiotic stage in the nematode's gut and a virulent stage in the insect larvae, which it kills through toxemia and septicemia. After the nematode attacks a prey insect and *P. luminescens* is released, the bacterium produces a wide variety of virulence factors ensuring rapid insect killing. Bioconversion of the insect cadaver by exoenzymes produced by the bacteria allows it to multiply and the nematode to reproduce (Bowen and Ensign, 1998; Ffrench-Constant and Bowen, 2000). During this process *P. luminescens* produces antibiotics to prevent invasion of the insect cadaver by bacterial or fungal competitors and the carcass becomes visibly luminescent due to the bioluminescence of *P. luminescens*. After infection the insect cadaver is bioconverted into a source of nutrients for both the bacteria and the nematodes. Infective juvenile nematodes subsequently re-acquire the bacteria and leave the insect to infect new hosts (Ghazal *et al*., 2014).

Molecular diversity analysis will give valuable information about similarity and dissimilarity among the strains isolated from different regions. Genetic diversity can be

estimated at molecular level. Randomly amplified polymorphic DNA (RAPD) technique has been used for typing and identification of number of closely related species of bacteria and assessment of genetic relationships. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing methods like restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP). RAPD results are usually consistent with those of DNA-DNA homology studies and can be used to estimate the genetic distances (Bert *et al*., 1996; Kumar *et al*., 2005).

Looking at the importance of *Photorhabdus luminescens* in the field of agriculture in controlling insect pests biologically, the investigations were conducted with the following objectives.

The specific objectives of the present study are:

1. Isolation and identification of *Photorhabdus luminescens* from soils collected from V C farm Mandya (Karnataka agroclimatic Zone-6).
2. Bio-control activity of *Photorhabdus luminescens* against insect pest *Galleria mellonella* (greater wax moth) and *Plutella xylostella* (diamondback moth).
3. Molecular diversity analysis of *Photorhabdus luminescens* isolates using RAPD marker.

II. REVIEW OF LITERATURE

The action of parasites, predators, or pathogens in maintaining another organism's population density at a longer average than would occur in their absence is called biological control (DeBach, 1964). Demand for the biological control agent is increasing all over the world. In many cases, however, research cannot keep up with the expectations of growers.

Biological control was discovered by trial and error and then practiced in agriculture long before the term itself came into use (Baker and Cook, 1974). To elaborate a new biological control technique, many species and strains of potential control organisms have to be evaluated.

2.1 Biological Control of insect pests in India

Systematic biological control research began with the establishment of the Indian station of Commonwealth Institute of Biological Control (CIBC) at Bangalore in 1957 (Singh, 2004). The All-India Co-ordinated Research Project on Biological Control of Crop Pests and Weeds (AICRP) was established in 1977 with 10 centres under the aegis of the Indian Council of Agricultural Research (ICAR) for carrying out biological control research in different parts of the country. The AICRP was elevated to Project Directorate of Biological Control (PDBC) in 1993. The PDBC is the nodal agency in India for organizing research on biological control of pests of agricultural importance (During XI plan, PDBC was upgraded as National Bureau of Agriculturally Important Insects (NBAII) in 2005). It coordinates 16 centres spread across the country. Indian Council of Agricultural Research (ICAR), state Agricultural Universities and traditional universities are also involved in biological control research.

The production of food grain should increase to 250 million tonnes by the year 2020 in order to meet the needs of the growing population (Sharma *et al.*, 2013). Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. A concomitant increase in the proportion of pests and diseases resulted in the increased use of toxic chemical for their management. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fear mongering by some opponents of pesticides, has led to considerable changes in people’s attitudes towards the use of pesticides in agriculture. Hence, general agreement on trade and tariff of world trade organization has given more emphasis on use of ecofriendly pesticide for crop production in view of their least toxic nature, low levels of disease resistance and low residue problems.

Natural enemies play an important role in pest management and can be used to specifically target unwanted host in an ecosystem hopefully without having adverse outcomes such as destroying crops themselves. These enemies can be parasites or predators both with the mission to attack and kill pests. Bacteria, fungi and nematodes can be used as natural enemies. Several nematode species have been identified to have the potential to successfully kill specific insect host in a few days after invasion, and examples include *Heterorhabditis* and *Steinernema* species (Jagdale *et al*., 2006).

2.2 Entomopathogenic Nematodes (EPNs)

Entomopathogenic nematodes are non-segmented roundworms also known as threadworms or eelworms. They range in size from 0.3 mm to 10 mm long and they can be more or less cylindrical. They have an excretory, nervous, digestive and reproductive system but lack the circulatory and respiratory system. The alimentary tract consists of the mouth, buccal cavity or stoma, oesophagus, intestines, rectum and the anus respectively (Flint and Dreistadt, 1998). EPNs are found in various locations and can survive externally on insect exoskeleton or internally in the reproductive, respiratory, digestive or excretory system. Generally some nematodes reside in very dry areas including deserts and most have the ability to tolerate environmental stresses which include anoxybiosis, thermobiosis and desiccation (Grewal, 2000).

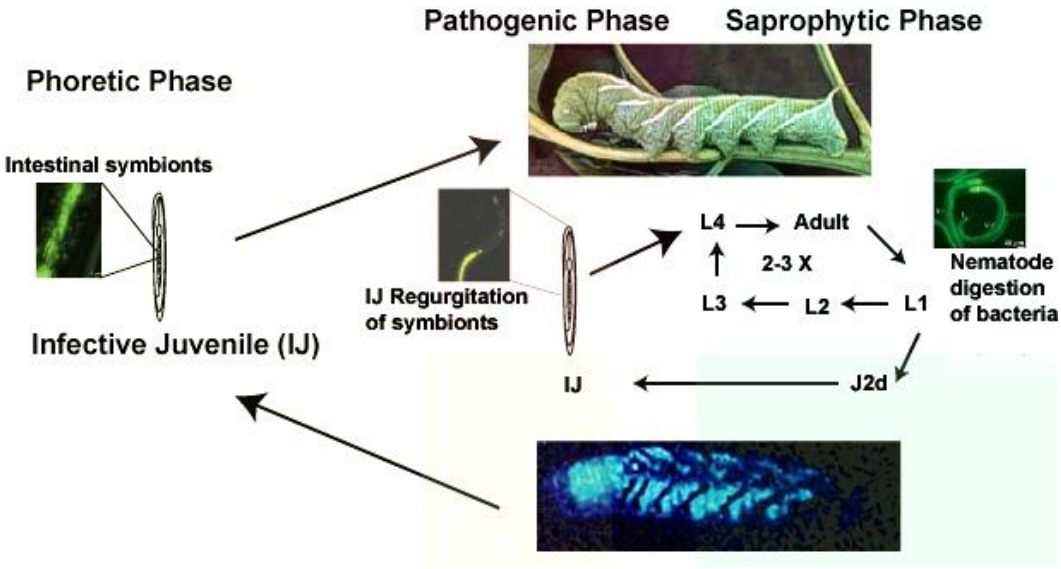
Entomopathogenic nematodes (EPNs) are excellent candidates for managing a wide range of insect pests. They are safe for human health and environment. But, India has yet to exploit this area of high biological control potential. EPNs belonging to the families Steinernematidae and Heterorhabditidae are soil dwelling lethal parasites of insects that can be used for managing a wide range of foliar and soil insect pests used for inundative, augmentative or inoculative biological control (Gaugler and Kaya, 1990; Kaya and Gaugler, 1993; Gaugler *et al*., 2002).

2.3 Entomopathogenic nematode (EPNs)

2.3.1 Life cycle of Entomopathogenic nematode (EPNs)

The non-feeding infective juveniles (IJs) start the life cycle of EPNs, as they can survive for longer duration in the soil and search insect hosts to infect. The 3rd stage IJ of *Heterorhabditis* enters the insect by penetrating the cuticle. Generally EPNs can enter their host through all openings such as the mouth, spiracles and anus. Once the IJs are inside the host, they mainly attack the hemocoel where they expel the infectious symbiotic bacteria which carry powerful toxins capable of weakening the insect and swiftly killing it within 24-48 hours. Once the insect host dies and favourable conditions are created as IJs start feeding on the bacteria and cadaver. Then proceeding to reproduce, giving rise to 2-3 generations of EPNs inside the insect cadaver. The 3rd stage IJs released can survive for longer duration in the soil environment while other EPNs in other stages of the life cycle may die when nutrients are depleted (Grewal *et al.*, 2001).

The non-feeding developmentally arrested dauer or infective juvenile (IJ) transmits a monoculture of symbiotic *P. luminescens* (green fluorescent protein (GFP) labelled *P. luminescens* are shown) to the haemocoel of an insect host, where it regurgitates the bacteria. The bacteria rapidly kills the insect (usually <48h.) and grow to high densities allowing nematode growth and reproduction. The nematodes grow for 2–3 generations, feeding on *P. luminescence*, after which (~10 days at 28°C) IJs are formed in masses, most transmitting symbiotic *P. luminescens* (Ciche and Sternberg, 2007).



Life-cycle of *Heterorhabditis bacteriophora*.

2.3.2 Distribution of Entomopathogenic nematodes:

Some entomopathogenic nematodes have been isolated from insects naturally infected in the field, but they are most commonly recovered from soil by baiting with susceptible insects such as wax moth larva *Galleria mellonella* (L.) (Bedding and Akhurst 1974). *Steinernematids* and *Heterorhabditids* are ubiquitous in distribution and have been recovered from soils throughout the world (Hominick *et al.,* 1996). List of species of *Steinernema* and *Heterorhabditis* with their original localities and sources of isolation is given in Table 1 (Grewal *et al.,* 2001).

2.3.3 Isolation of Entomopathogenic nematodes (EPNs)

Entomopathogenic nematodes reside naturally in the soil in association with various insects. Most commonly used method to recover infective juveniles of EPNs from soil is baiting technique with the larvae of greater wax moth, *Galleria melonella (L.*), (Bedding and Akhurst, 1975). Efficiency of this method had been increased using several consecutive baiting rounds (Fan and Hominick, 1991) and by baiting at two temperatures (Mráček and Bečvář, 2000).

Tóth (2006) isolated *Heterorhabditis* and *Steinernema* by using late instar larvae of *Galleria mellonella* and *Tenebrio molitor* as bait. He collected 89 soil samples in 2003 and 200 soil samples in 2005 from different parts of Hungary, five samples randomly from each site. He put three late instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) in a porous Eppendorf vial into each sample bag in 2003. In 2005, three larvae of *G. mellonella* and two larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae) were put into each sample. He recorded larval mortality after seven days and cultured dead larvae in a water trap to obtain infective juvenile nematodes, which he stored, suspended in water in a refrigerator at 5 to 7°C and found one *steinernematid* and three *heterorhabditid* species. Most common species was *S. feltiae*, which made up 79.5% of the nematodes isolated.

Table 1: List of valid species of *Steinernema* and *Heterorhabditis* with their original localities and sources of isolation (Grewal *et al.*, 2001)

Nematode species	Original locality	Original Source
***Heterorhabditis Poinar* 1976**		*Graphognathus sp*. Buchnan
***H. argentinensis* Stock 1993**	Rafaela, Argentina	*Heliothis punctigera*
***H. bacteriophora* Poinar 1976**	Brecon, South Australia	*Heliothis punctigera Wallengren*
***H. brevicaudis* Liu 1994**	Fujian Province, China	Soil
***H. hawaiiensis* Gardener *et al.* 1994**	Hwaii, USA	Soil
***H. indica* Poinar *et al.* 1992**	Coimbatore, India	Soil*
***H. marelata* Liu And Berry 1996**	Seaside, Oregon, USA	Soil
***H. megidis* Poinar *et al.* 1987**	Jeromesville, Ohio, USA	*Popillia japonica* Newman
***H. zealandica* Poinar 1990**	Auckland, New Zealand	*Heteronychus arator* F.
***Steinernema Travassos* 1927**		
***S. abbasi* Elawad *et al.* 1997**	Sultanate of Oman	Soil
***S. affine* (bovine 1937) Wouts *et al.* 1982**	Denmark	*Bibio sp.*
***S. areanarium* (Artyukhovsky 1967) Wouts *et al.* 1982**	Central Russia	Soil
***S. bicornutum* Tallosi *et al.* 1982**	Strazilovo, Yugoslavia	Soil
***S. carpocapsae* (Weiser 1955) Wouts *et al.* 1982**	Czechoslovakia	*Cydia pomonella* (L.)
***S. caudatum* Xu *et al.* 1991**	China	Soil
***S. ceratophorum* Jian *et al.* 1997**	Jining Province, China	Soil
S. Cubanum Mracek *et al.*	Western Cuba	Soil
***S. feltiae* (Filipjev 1934) Wouts *et al.* 1982**	Denmark	*Agrotis feltiae*
***S. glaseri* (steiner 1929) Wouts *et al.* 1982**	New Jersey. USA	*Popilia japonica* Newman
***S. intermedium* (poinar 1985) Mamiya 1988**	South Carolina, USA	Soil
***S. karii* waturu *et al.* 1997**	Central Province, Kenya	soil
***S. krausei* (Steiner 1923) Travassos 1927=Type species**	Germany	Cephaleia abietis (L.)
***S. kushidai* Mamiya 1988**	Hamikita, Japan	*Anomala cuprea* Hope
***S. longicaudatum* Shen and Wang 1991**	Guangdong, China	Soil
S. montricolum Stock *et al.*1997	Republic of Korea	Soil
***S. neocurtillae* Nguyen and Smart**	LaCrosse, Florida, USA	*Neocurtilla hexadactylla* (perty)
***S. orgonense* Liu and Berry 1996**	Oregon, USA	Soil
***S. puertoricense* Roman and Figueroa 1994**	Puerto Rico	Soil
S. rarum (de Doucet 1986) Mamiya 1988	Cordoba, Argentina	Soil
***S. riobrave* Cabanillas *et al.* 1994**	Waslaco, Texas, USA	Soil
S. ritteri de Doucet and Doucet 1990	Cordoba, Argentina	Soil
S. scapterisci Ngyuen and Smart 1990	Rivera, Uruguay	*Scapterisus vicinus*, Scudder
***S. siamkayai* Stock *et al.* 1988**	Petchabum Province, Thailand	

*Soil baited with *Scirpophaga excerptalis* Walker, larvae

Emelianoff *et al*. (2008) isolated EPNs directly from the soil using a modified Galleria-trap technique by placing a last instar larva of *Galleria mellonella (Lepidoptera, Galleriidae)* into a 0.5 ml Eppendorf tube pierced with 1 mm diameter holes. They placed 50 Galleria- traps at each location, at three depths in the soil: 0, 10 and 20 cm. They left the traps for 72 h. in the soil before removal. Once in the laboratory, dead insects were placed into White's traps at 24°C in the dark to collect emerging infective juveniles (IJs). They isolated two different strains of *H. bacteriophora* assigned by ITS sequences. They also isolated three different species of *Steinernema* and identified as *S. feltiae*, *S. affine* and one undescribed *Steinernema sp.* which assigned to the ''glaseri-group" on the basis of morphological criteria.

Hsieh *et al*. (2009) isolated EPNs from sandy coastal soils from moist bamboo forest in southern Taiwan. They sampled 25 locations each consisting of 100 m^2 area with 30 sample points containing 500 g of soils each. They recovered EPNs from soil using five last-instar *G. mellonella* (L.) larvae in 250-ml plastic containers (five containers for each sample) with moistened soil sample and kept at room temperature (20 ± 3°C). They checked larvae every 2–3 days and replaced dead larvae by fresh ones. After 7 days, dead insects were thoroughly rinsed in distilled water and placed in modified White traps until emergence of third-stage IJs.

2.3.4 Identification of isolated nematodes

Morphological characteristics for grouping species according to taxonomic criteria were suggested by Stock and Kaya (1996), Hominick *et al.* (1997) and protocols were described by Kaya and Stock (1997).

Uribe-Lorío *et al.* (2005) reported morphological studies of live and heat-killed (60°C) nematodes in Ringer's solution. The heat- killed nematodes were placed in triethanolamine–formalin (TAF) waxative and processed to anhydrous glycerine for mounting Observations were made from live and mounted specimens using an Olympus BX81 microscope.

Emelianoff *et al.* (2008) sorted EPNs according to the color of the infected *G. mellonella* cadavers, as *Steinernematids* (cream, ocre colour) or *Heterorhabditids* (red, burgundy colour). The morphology is characterised by morphometrics of the IJ with a thick or double epiptygmata in first generation females, which may support that the species isolated may be indeed similar to *S. australe* isolated from sandy loamy soil collected from Chile, Island Magdalena (Edgington *et al.*, 2009). The IJs are also characterised by a very long body ranging between 1116 to 1484 μm and thus classified under the glaseri-group. *S. australe* IJ exhibits a long tail and possesses an excretory pore towards the anterior far from the posterior region. First generation males have long spicules (55-78 μm) and long gubernaculums (36-51 μm).

Table 2: General morphology of entomopathogenic nematodes, *Heterorhabditis bacteriophora* (Nguyen and Smart, 1996) and *Steinernema* species (Edgington *et al.*, 2009).

Steinernema austral		***Heterorhabditis bacteriophora***	
Adult Females	**Adult males**	**Adult Female and hermaphodites**	**Adult males**
Anterior region (Head)		**Anterior region (Head)**	
6 Labial papilla, 4 cephalic papillae, 2 amphids and a mouth.	6 Labial papilla, 4 cephalic papillae and raising perioral disc	Oesophagus without metacarpus, and enlarged basal bulb	Single testis; Busa peloderan usually has 9 pairs of genital papillae
Posterior region (Tail)		**Posterior region (Tail)**	
Pointed tail and phasmid present before the end of the tail. Vulval flap	Blunt tail Specule present	Pointed tail, females have ovaries, hermaphrodites have ovotestis and amphidelphic have a vulva median	Specules present sometimes curved, paired or separated. Blunt tail in males
Additional characteristics		**Additional characteristics**	
Cheilorhabdions present Epiptygma Fat bodies		Gubernaculum present Fat bodiejs	
Infective Juvenile (3rd stage)		**Infective Juvenile (3rd stage)**	
Head has a mouth, amphid, 4 cephalic papillae and one line of lateral field.		Pointed tail, 2 cuticular sheaths covering the IJ, mouth and anus closed, immature oesophagus. Symbiotic bacteria in intestine present	

Heterorhabditis bacteriophora IJs have a sheath protecting the entire body of the nematode, a tessellate on the anterior region and is characterized by longitudinal ridges throughout almost the entire body length. The IJ, and the third stage which develops from it, have a dorsal tooth on the labial region. The fourth stage has low lips and labial papillae, and a rounded oral aperture. IJ having huge lips with prominent labial papillae at the apex of the lips are considered to be hermaphrodite (Malan *et al.*, 2011).

Erbaş *et al.* (2014) reported morphometric analysis of IJs of EPN. They have randomly selected 20 IJs and 20 first-generation males for *Steinernema* and males from the first generation hermaphrodite for *Heterorhabditis* from different infected *G. mellonella* larvae. The males and IJs were killed and fixed using hot 4% formalin (60°C)

for 2 min and kept in this solution for 12 h at room temperature. They transferred fixed nematodes to anhydrous glycerin and mounted on slides using cover glass supports to avoid flattening them and took morphological observations according to the taxonomic criteria of Hominick *et al.* (1997). However, molecular characterization of the isolates by analysis of the ITS rDNA sequences data are needed to confirm distinctness. They evaluated evolutionary relationships of the isolates and other closely related species. They used approximately 700–850 bp segments of ITS rDNA for the phylogenetic analysis by molecular evolutionary genetics analysis (MEGA) software. The phylogenetic analysis of the ITS rDNA sequence data placed *Heterorhabditis* and *Steinernema* isolates in a clade with other isolates of *Heterorhabditis* and *Steinernema* species.

2.3.5 Safety of EPNs to non-target organisms

Brown (1974) reported *S. feltiae* to be non-pathogenic to rodents. The non-susceptibility of mice and chicks to nematodes was reported by Poinar *et al.* (1982). Capinera *et al.* (1982) showed non-significant infection of earthworms and snails by entomopathogenic nematodes. Plants also are not affected by these nematodes. After initial application of the nematodes there were no detrimental effects on the natural enemies of the target pests.

Larvae and adults of honey-bees were shown to be susceptible to nematodes but this did not adversely affect the colony (Kaya *et al.*, 1982). Poinar and Thomas, (1985a, 1985b) reported infection of spiders, harvestmen, and pseudoscorpions when exposed to very high concentrations of *Steinernema* and *Heterorhabditis*. Infection of predators occurs when they eat a nematode-infected insect was observed by Ishibashi *et al.* (1987).

Poinar and Thomas (1988) also reported infection of tadpoles. Akhurst and Boemare, (1990) reported infection of parasitoids by nematodes when parasitoids and entomopathogenic nematodes co-infect an insect in laboratory trials.

Boemare *et al.* (1996) concluded that safety tests demonstrated that neither of the symbionts in the partnership is potentially hazardous to human health. The production of entomopathogenic nematodes should, however, avoid contamination by non-symbiotic bacteria, through usual good laboratory practice; this will avoid any possible hazard and also will ensure good production of commercial inoculum.

2.3.6 EPNs as bio control agents

Work of Glaser and Fox (1930) in controlling grubs of the Japanese beetle, *Popillia japonica* with the use of EPNs started the interest in research on bio control of insect pests through EPN. Uses of EPNs have innumerable advantage in controlling insect pests. Selection of an EPN for control of a particular pest insect is based on several factors that include the nematode's host range, host finding or foraging strategy, tolerance of environmental factors and their effects on survival and efficacy. The four most critical factors are moisture, temperature, pathogenicity for the targeted insect and foraging strategy (Kung *et al.*, 1991, Kaya and Gaugler, 1993, Campbell *et al.*, 2003, Grewal *et al.*, 2005).

Bruck and Walton (2007) reported the susceptibility of the two primary insect pests of hazelnuts to three species of entomopathogenic nematodes viz. *Heterorhabditis marelatus* Pt. Reyes, *Steinernema carpocapsae* and *Steinernema kraussei* L137. The entomopathogenic nematodes were used in laboratory and soil bioassays to determine their virulence against filbertworm (*Cydia latiferreana*) and filbert weevil (*Curculio occidentalis*). All three nematode species were infective in laboratory bioassays. Infectivity ranged from 73–100% and 23–85% for filbertworm and filbert weevil respectively. Field results were similar to those found in the laboratory with filbertworm larvae being more susceptible to nematode infection.

The susceptibility of the Mediterranean flat headed root borer (*Capnodis tenebrionis*) to 13 isolates of entomopathogenic nematodes was examined using GF-677 potted trees (peach almond hybrid) as the host plant. The nematode strains tested included nine *Steinernema feltiae,* one *S. affine,* one *S. carpocapsae* and two *Heterorhabditis bacteriophora*. Nematodes showed the ability to locate and kill larvae of *C. tenebrionis* just after they enter into the roots of the tree. *S. feltiae* strains provided an efficacy ranging from 79.68% to 88.24%. *H. bacteriophora* strains resulted in control of 71.66% –76.47%. *S. carpocapsae* (B14) and *S. affine* (Gspe3) caused lower control of *C. tenebrionis* larvae, 62.03% and 34.76% respectively (Morton and delPino, 2008).

Biological control potential of nine entomopathogenic nematodes, *Heterorhabditis bacteriophora* CLO51 strain (HbCLO51), *H. megidis* VBM30 strain (HmVBM30), *H. indica*, *Steinernema scarabaei*, *S. feltiae*, *S. arenarium*, *S. carpocapsae* Belgian strain (ScBE), *S. glaseri* Belgian strain (SgBE) and *S. glaseri* NC strain (SgNC) were tested against second, third-instar larvae and pupae of the white grub (*Hoplia philanthus*) in laboratory and greenhouse experiments. In the laboratory experiments, SgBE, SgNC, HbCLO51 and HmVBM30 were highly virulent to third-instar larvae and pupae, while SgBE was virulent only to second- instar larvae. Pupae were highly susceptible to HbCLO51, HmVBM30, SgBE and SgNC (57% –100%) followed by *H. indica* and *S. scarabaei* (57% –76%). In pot experiments, HbCLO51, SgBE and *S. scarabaei* were highly virulent to the third-instar larvae compared to the second-instar larvae (Ansari *et al.*, 2008).

Infectivity of entomopathogenic nematode species against olive fruit fly (*Bactrocera oleae*) was compared by exposing third-instar larvae to infective juveniles on a sand-potting soil substrate. When IJs were sprayed over naturally infested fallen olives, many larvae died within treated olives as well as in the soil, *Steinernema feltiae* caused the highest overall mortality of 67.9 percent. *B. oleae* mature larvae were susceptible to EPN infection both in the soil and within infested olives (Sirjani *et al.*, 2009).

Campos-Herrera and Gutiérrez (2009) performed virulence screening of 17 native Mediterranean EPN strains to select the most promising strain for regional insect pest control. *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae) Rioja strain produced 7%, 91% and 33% larval mortality for the insects *Agriotes sordidus* (Illiger) (Coleoptera: Elateridae), *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), respectively, and was selected as

the most promising strain. The *S. feltiae* Rioja strain – *S. littoralis* combination was considered the most suitable to develop the Rioja strain as a bio control agent for soil applications. The effect of soil texture on the virulence of the Rioja strain against *S. littoralis* was determined through dose–response experiments. The estimated LC_{90} to kill larvae in two days was 220, 753 and 4178 IJs/cm^2 for soils with a clay content of 5%, 14% and 24%, respectively, which indicates that heavy soils produced negative effects on the virulence of the Rioja strain. The nematode dose corresponding to the LC_{90} for soils with a 5% and 14% clay content reduced insect damage to *Capsicum annuum* Linnaeus (Solanales: Solanaceae) plants under greenhouse microcosm conditions.

Laznik *et al*. (2010) tested three new strains (B30, B49 and 3162) of the entomopathogenic nematode *Steinernema feltiae* in controlling rice weevil (*Sitophilus oryzae*) in a laboratory bioassay. They studied the pathogenicity of biological agents at four different temperatures (15, 20, 25 and 30ºC) and for five concentrations of nematode suspension (125, 250, 500, 1000 and 2000 IJs per adult). Beetle mortality was determined on 4, 6 and 8 days after treatment. The results showed that all studied strains were most pathogenic (42-72% mortality) at 25ºC and the highest concentration of the nematode suspension, while the lowest pathogenicity (from 6 to 11%) was found at 30ºC and the lowest concentration of the nematode suspension. Besides, at higher concentrations the suspension of entomopathogenic nematodes can be an effective biological agent in controlling adult rice weevils. The lowest LC_{50} value (1165 IJs/adult after an 8-day exposure) was obtained for the Hungarian strain 3162 at 25ºC, while the highest (2533 IJs/adult after an 8-day exposure) was obtained for the Slovenian strain B30 at 30ºC.

Kulkarni *et al*. (2011) reported infectivity and virulence of EPN, *H. indica* on teak skeletonizer, *Eutectona machaeralis*. They have done bioassay studies by exposure of early last instar larvae of the teak skeletonizer to two bioassay conditions; filter paper bioassay (3, 5, 10, 20 and 30 IJs larvaG1) and leaf treatment (30, 60, 100, 200 and 300 IJs larva) to determine susceptibility and critical doses. *E. machaeralis* larvae showed 35.29% mortality at lowest dose 3 IJs larvae and 100% mortality was obtained at the highest dose of 30 IJs larvae. They also showed ten times more doses, i.e. above 30 IJs larva were required to cause larval mortality when larvae were exposed to leaf treatment experiment using Potter's Tower. LC_{50}, LC_{90}, LT_{50} and LT_{90} values for *H. indica* in filter paper bioassay (4.57, 50 90 50 90 12.02 and 30.20, 54.95, respectively) and leaf treatment method (54.37, 114.50 and 40.62, 122.70, respectively) were calculated. Production of IJs in progeny was maximum in 30 IJs larva (1, 07, 067 IJs larva). They have concluded that doses above 100 IJs larva may be required for managing the pest by leaf treatment.

Le Vieux and Malan (2013) reported efficacy of killing adult female *Planococcus ficus* (Signoret) by *Heterorhabditis zealandica* and *Steinernema yirgalemense*, which were responsible for 96% and 65% mortality respectively. Bioassays indicated a concentration-dependent susceptibility of *P. ficus* to *H. zealandica*, *S. yirgalemense* and commercially produced *H. bacteriophora*, with LC_{50} and LC_{90} values of 19, 82; 13, 80; and 36, 555 respectively. Both *H. zealandica* and *S. yirgalemense* were able to move 15 cm vertically downward and infect *P. ficus* with a respective mortality of 82% and 95%.

Kepenekci *et al*. (2013) reported biocontrol efficiency of three Turkish isolates of the entomopathogenic nematodes *Steinernema carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora* when evaluated against the last instar of potato tuber moth (PTM) *Phthorimaea operculella* under laboratory conditions. To determine optimum nematode application rate and temperature, they have conducted the experiments with 100, 500 and 1000 IJs at 10, 15 and 25°C. Temperature and nematode concentration had a significant effect on *P. operculella* larval mortality. *S. carpocapsae* and *H. bacteriophora* species displayed increased virulence in parallel with rising temperature and the number of infective juveniles applied. At 25°C and 1000 IJs concentration, the larval mortality was 96% and 80% for *S. carpocapsae* and *H. bacteriophora*, respectively. However, *S. feltiae* did not exhibit more than 40% mortality at any temperature or concentration, except when the nematodes were applied in infected insect host cadavers. At 25°C, infected cadaver applications showed 97, 83 and 67% mortality for *S. carpocapsae*, *H. bacteriophora* and *S. feltiae*, respectively. They have concluded that *S. carpocapsae* blacksea strain has a high level of potential to control *P. operculella* larvae.

Kamali *et al*. (2013) reported biocontrol potential of the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* on cucurbit fly, *Dacus ciliates*. Bioassay using EPN species were applied to naturally infested fruit (150 and 300 IJs/cm^2), the mortality rates of *D. ciliatus* larvae were 28% for *S. carpocapsae* and 12% for *H. bacteriophora*. In green house experiment, EPNs had similar effects on fly larvae. They showed higher rates of larval mortality in sandy loam and sandy soils than in clay loam. Efficacy of both the EPNs was higher at 25°C and 30°C than at 19°C. They concluded that *S. carpocapsae* had the best potential as a bio control agent of *D. ciliatus*, based on its higher virulence and better ability to locate the fly larvae within infected fruits.

Shahina *et al*. (2014) showed infectivity of EPN isolates viz., *Steinernema pakistanense, S. asiaticum, S. feltiae* and *Heterorhabditis indica* against the cotton bollworm complex in field. They have cultured EPNs on *Galleria mellonella L.*, stored in distilled water at 5°C and kept at room temperature for 24 h. before use. They have assessed the number of bollworms on plants before and 24 h. after EPN spray @ 1000 and 2000 juveniles/ml water to calculate mortality percentage. They found all four species of insects, viz., *Helicoverpa armigera*, *Earias insulana*, *E. vitella* and *Pectinophora gossypiella* were susceptible to infective juveniles of the four EPN species. Among them *S. pakistanense* was the most virulent EPN species.

2.4 Nematode – Bacterial Association

Photorhabdus and *Xenorhabdus* species are symbiotically associated with *Heterorhabditis* and *Steinernema* EPNs species respectively. These bacteria belong to the family Enterobacteriaceae (Boemare and Akhurst, 1988). Once the nematodes invade the insect's hemocoel, they release symbiotic bacteria which carry immune depressors which are involved in blocking the activity of phospholipase A2 of the insect and thus protecting the nematodes and the bacteria themselves from attack and degradation (Ji *et al.,* 2004). The infected insect becomes more susceptible to saphrophytic pathogens and it

becomes weaker. Previous studies have shown that gram negative *Xenorhabdus nematophila* is symbiotically associated with *Steinernema carpocapsae*. These enterobacteria are capable of protecting themselves and the IJs which have invaded the insect by inducing immunodepression of the invaded insect host. *X. nematophila* secretes antibiotics and hydrolytic enzymes into the hemolymph of the insect, killing the host within 16 hours after invasion. *X. nematophila* also secretes active immunodepressive agents which protect the association of the nematodes-bacteria from insect's immune response upon invasion (Park *et al.,* 2004). These species of bacteria are both involved in insect pathogenicity and thus enabling nematodes they live in to be potential candidates for biological control of insects (Ciche *et al.,* 2006). Bacterial symbionts live inside the intestine of IJs which provide them with shelter and transport.

2.5 The genus *Photorhabdus*

The genus *Photorhabdus* comprises of three species, *Photorhabdus luminescens, P. temperata* and *P. asymbiotica*, which all include several further subspecies and are always, associated with nematodes of the genus *Heterorhabditis*. *Photorhabdus* forms gram-negative, asporogenous, rod-shaped (2-6 × 1-1.4 μm) peritrichious cells. All strains are mesophilic bacteria with an optimal growth temperature of 28-30°C, only a few strains exhibiting an even broader temperature range between 16-38°C (Fischer-Le Saux *et al.,* 1999; Peel *et al.,* 1999). The fact that *Photorhabdus* cells cannot reduce nitrate but can produce iso-branched fatty acids is very uncommon to Enterobacteriaceae and keeps them detached from other genera in this family (Janse and Smits 1990; Suzuki *et al*., 1996). Another uncommon and remarkable feature is their capability to produce bioluminescence. The bioluminescence is obtained by a set of *lux* genes, which share high synteny to *lux* genes from the phylogenetically distant genera *Vibrio* and *Photobacterium*, suggesting a horizontal gene transfer (Gaugler and Kaya 1990). Nevertheless, *Photorhabdus* is currently the only known non-marine luminous bacterium, but the biological function of the produced bioluminescence is still elusive. *Photorhabdus* is also marked by another laboratory relevant trait. One can observe a variation in colony forms after prolonged sub culturing, a primary and a secondary form which are also referred to as phase 1 and phase 2 forms. The primary form is displayed by pigmentation, the adsorption of dyes like bromothymol blue or neutral red, the ability to constitute inclusion bodies and to bioluminesce, but also to produce lipases, phospholipases and proteases. The secondary form has lost these characteristics and can be further discriminated by a decreased support of nematode growth and antibiotic production. It has been shown that isolates from nematodes are always in primary form and the conversion is unidirectional from primary to secondary form (Gerritsen *et al.,* 1992; Wang *et al.,* 2007). In principle all *Photorhabdus* isolates are obtained from infected insects or their associated nematode host and no free living strains have been detected yet. However, some isolates of human wounds draw attention as clinical relevant strains causing invasive soft tissue and disseminated bacteremic infections. It was the first time that the bacterium was not recognized to be associated with a nematode and according to this the strain was given the name *P. asymbiotica*. But the epithet turned out to be a misnomer, as Gerrard *et al.* (2006) succeeded in identifying the nematode symbiont. For all that, the reports of *Photorhabdus asymbiotica* isolates from human wounds revealed

that this strain represents an opportunistic human pathogen, in which the nematode's part during infection is still unclear (Farmer *et al.,* 1989; Gerrard *et al.,* 2003).

A very important part in the pathogenicity of *Photorhabdus* is provided by the production of insecticidal toxins, where as one family is the toxin complex (Tc) proteins, which consist of high molecular weight proteins. These toxins mediate an oral activity against many insects and represent an alternative to the entomopathogenic *Bacillus thuringiensis* toxins. As one protein expressed in transgenic plants conferring insect resistance. (Waterfield *et al.,* 2001; Waterfield *et al.,* 2005) Another effective protein makes the caterpillar floppy (*Mcf*) toxin, which derived its name from the phenotype, which is induced by *Mcf* treatment that leads to a rapid loss of body turgor making the caterpillar floppy. *Mcf* is a potential toxin inducing apoptosis on the insect haemocytes and midgut epithelium. The extraordinary potency of *Mcf* was evinced by the conclusion that non-entomopathogenic *E. coli* harbouring the *mcf* gene were able to persist within the insect and furthermore were even able to kill the insect (Daborn *et al.,* 2002; Dowling *et al.,* 2007). However, small molecules also play an important part in pathogenicity and symbiosis.

Watson *et al*. (2005) succeeded in showing that an *exbD* homologue, a component of the *TonB* complex, is needed for the uptake of small iron scavenging siderophore molecules in *Photorhabdus temperata*. A mutation in this gene generated a less virulent phenotype that was unable to support nematode development. Further evidence of the involvement of small molecules in symbiosis was gained from a mutation in the gene *ngrA*. This gene encodes a phosphopantetheinyl transferase that is required to activate polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), which are responsible for the production of secondary metabolites. The mutation resulted in a phenotype unable to support IJ recovery and let presume that small molecules also act as signalling molecules (Ciche *et al.,* 2003). A detailed overview on secondary metabolites of *Photorhabdus* is given in the section of secondary metabolites. The life cycle of *Photorhabdus* implicates a switch between mutualism in the nematode and pathogenicity in the insect as well. This change is subject to regulatory networks and some regulatory proteins have been described. A mutation in the gene *pgbE1* that is part of the seven gene *pgbPE* operon rendered *Photorhabdus* to an attenuated virulence. It could also be shown that pgbE1 is required for a proper colonisation of the IJ (Bennett and Clarke 2005). The already alluded phase variation is also discussed as an adaptation to the respective virulent or symbiotic phenotype. A mutation in the *hexA* gene, a transcriptional regulator, was sufficient to restore most of primary-specific characteristics in a secondary form, whereas overexpression of *hexA* in the primary form triggered a conditional phenotypic variation towards secondary form (Joyce and Clarke 2003; Joyce *et al.,* 2006). But phase variation is also influenced by the AstR-AstS two component signal transduction system (adaptation to stationary-phase regulator and sensor), which positively regulates the universal stress proteins UspA, UspB and UspC. These stress proteins help to respond and protect on changes in the environment. Mutants in the *astR* gene were shown to undergo phase variation much earlier than the wildtype (Derzelle *et al.,* 2004).

2.6 Antibiotics of symbiotic bacteria

Development and commercialization of biological control using a nematode bacterium combination (*Steinernema- Xenorhabdus spp.*) against insect pests, is harmless to non-target animals and plants. A crucial feature of this bio control agent is that the bacterium symbiont (*Xenorhabdus spp*.) of the nematode (*Stenernema spp*.) produces a wide range of bioactive metabolites including antimicrobial substances that inhibit the growth of bacteria, fungi and yeasts.

Dutky (1974) first suggested that one of the entomopathogenic bacteria, namely the one from DD136 strain of *Neoplectana* (*Steinernema feltiae*) produced antibiotics. Antibiotic system of *Xenorhabdus* and *Photorhabdus* species act in two broad ways. One broad spectrum chemical antimicrobials inhibit microbial species and the second broad spectrum antibiotics are bacteriocins that inhibit closely related species or strains of the same species.

Paul *et al.* (1981) reported that nine strains of *Xenorhabdus nematophilus* and *P. luminescens* produce antibiotics the inhibit growth of the luminous bacteria, *Vibrio spp.* and *Photobacterium spp*. They isolated four indole derivatives from *X. bovienii* and hydroxystilbene derivatives from *P. luminescens* all of which have antibiotic activity. Richardson *et al.* (1988) purified the same hydroxystilbene derivative from *P. luminescens*.

McInerney *et al*. (1991) reported seven antibiotic compounds from *Xenorhabdus spp.* Out of these, five dithiolopyrrolone derivatives, named Xenorhabdins are organic soluble. The other two, benzopyran-1-one derivatives, named Xenocoumacin1 and 2 are water soluble.

Sundar and Chang (1992; 1993) investigated the activity and mechanism of action of the Indole and Stilbene derivatives were found effective against both gram-positive and gram-negative bacteria, causing a severe inhibition of RNA synthesis by inducing an accumulation of the regulatory nucleotide guanosine-3',5'-bis-pyrophosphate in susceptible bacteria.

Sztaricskai *et al*. (1992) isolated anthraquinone pigments with antibacterial activity from an unspecified strain of *P. luminescens*. Li *et al.* (1995) isolated the same anthraquinone from strain C9 of *P. luminescens*.

2.7 Toxins of *Photorhabdus luminescens*

In order to infect its host and survive *P. luminescens* must be capable of producing a wide range of proteins including toxins. In *P. luminescens*, toxins are classified into four major groups: the toxin complexes (Tcs) with a high similarity to the ones found in *Yersinia pestis* genome (Parkhill *et al.,* 2001). Four pathogenicity islands were identified in the *P. luminescens* genome (Waterfield *et al.,* 2002) three of them contain genes that encode for toxins, while the last one encodes for a type III secretion system.

The complete genomic analysis of this organism has revealed that it indeed possesses a lot of genes encoding for toxins, proteases and lipases (Duchaud *et al.*, 2003). The *Photorhabdus* insect related (Pir) proteins, the "makes caterpillars floppy" (Mcf) toxins and the *Photorhabdus* virulence cassettes (PVC) (Yang *et al.,* 2006; Ffrench-Constant *et al.,* 2007) were also reported.

2.7.1 Toxin Complexes (Tcs)

The toxin complexes (*Tcs*) are encoded by the pathogenicity island I (PAI I) (Waterfield *et al.,* 2002) and have been identified as high molecular weight insecticidal toxins comprising of multiple subunits (Ffrench-Constant *et al.,* 2007). There are four such complexes, namely *tca, tcb, tcc* and *tcd* found in different loci. However, *tca* and *tcc* loci encode for several open reading frames (ORFs), thus producing multiple components per locus; *tcb* and *tcd* are comprised of a single long ORF. *tca, tcb* and *tcc* showed no overall similarities with other sequences in GenBank, however *tccA* (one of the three different ORFs of the *tcc* locus) shows similarity to a *Salmonella* protein (*SpvA*), while *tcaC* showed similarity to *SpvB* (Ffrench-Constant and Bowen, 1999). The three complexes show significant similarity to one another. Therefore three basic types of genetic elements have been identified: the *tcdA*-like element, equivalent to the combination of *tcaA* and *tcaB*, the *tcdB*-like element, equivalent to the *tcaC* and the *tccC*-like element. *tcdA*-like elements are responsible for establishing primary toxicity, while the *tcdB*/*tccC*-like elements are potentially toxic (Pinheiro and Ellar, 2007).

Genomic analysis of *P. luminescens* W14 has also revealed the presence of cytolytic RTX-like toxins, similar to those secreted by the two-partner system of *Vibrio cholerae* (RtxA-RtxB), *Serratia marcescens* (ShA-ShB), *Erwinia tarda* (EthA-EthB) and *Erwinia chrysanthemi* (HecA-HecB), as well as the presence of novel tc loci, based on homology and BLAST search (Ffrench-Constant and Bowen, 2000). The tcb and tcd loci encode for proteins of similar sizes as the A and B toxins of *Clostridium difficile*, suggesting similar modes of action between the proteins of the two loci and the ones of the *Clostridium* (Bowen and Ensign, 1998), tca and tcd encode for orally active toxins, responsible for the majority of the insecticidal activity in *Manduca sexta* (Blackburn *et al.,* 1998). The tc genes are encoded alongside other genes with putative virulence functions (Waterfield *et al.,* 2001; Yang *et al.,* 2006). The tc-like genes have been identified in both other insect-associated bacteria such as *Serratia entomophila* and non-insect-associated bacteria like some *Pseudomonas* species (Waterfield *et al.,* 2001; Ffrench-Constant *et al.,* 2007). The insecticidal activity of these complexes is of proteinaceous nature and toxicity is achieved through oral administration (Bowen *et al.,* 1998).

2.7.2 Photorhabdus Insect Related (Pir) Toxins

The *Photorhabdus* insect related (Pir) toxins act as binary proteins. They are encoded by the PirAB genes, located at plu4093-4092 (pirA) and plu4437-4436 (pirB) loci of *P. luminescens* TT01 genome, both proteins are necessary for injectable but not oral activity (Yang *et al.,* 2006). These proteins show similarities to the δ-endotoxins of *Bacillus thuringiensis*, thus making them a putative substitute for Bt (*Bacillus thuringiensis* toxin) recombinant crops (Ffrench-Constant *et al.,* 2007). PirA shows little

similarity to known proteins, but its partner PirB shows high homology with the N-terminal region of the pore-forming domain of the Cry2A insecticidal toxin, suggestive of the existence of a similar motif in these binary proteins. PirB also has similarities with a developmentally regulated protein from the beetle (*Leptinotarsa decemlineata*), this beetle protein is presumed to have a juvenile hormone esterase (JHE) activity due to the relation of its expression profile during insect development and the levels of juvenile hormone (JH) produced, thus leading to the assumption that PirB may display the same kind of activity. However, such activity is not demonstrated by PirB (Waterfield *et al.,* 2005; Wilkinson *et al.,* 2009). Within the DNA sequences of the encoding genes, enterobacterial repetitive intergenic consensus (ERIC) sequences have been identified (Waterfield *et al.,* 2002), suggested to be important for mRNA stability.

2.7.3 Makes Caterpillars Floppy

The "makes caterpillars floppy" toxins 1 (Mcf1) and 2 (Mcf2) act upon injection and they are encoded by PAI II (Waterfield *et al.,* 2002), along with other hemagglutinin-like proteins. Mcf1 has been shown to promote apoptosis in the midgut, producing a characteristic "floppy" phenotype in the infected insect (Dowling *et al.,* 2004) it mimics BH3 domain proteins that are found in mitochondria and have proapoptotic actions as in its N-terminal domain, this protein has a Bcl2-homology 3-like domain (BH3 domain). Its central domain is of hydrophobic character with high similarity of the translocation domain of the Clostridium difficile toxin B, while the C-terminal domain of Mcf1 resembles the repeats-in toxin (RTX) like toxins of another bacterium (*Actinobacillus pleuropneumoniae*) (Dowling *et al.,* 2004).

2.7.4 Photorhabdus Virulence Cassettes (PVC)

Yang *et al*. (2006) reported that plenty of other toxins should be encoded by the *P. luminescens* genome, in order to make it more efficient in its survival; another group of putative toxins has been identified through homology to antifeeding genes in *Serratia entomophila.* These genes in *Serratia* are encoded in a plasmid carrying *tc* homologues and are located in a locus encoding proteins of high similarity to phage proteins (Hurst and Williams 2000; Hurst *et al*., 2004).

In *P. luminescens*, multiple copies of those genes have been recognized and are known as "Photorhabdus virulence cassettes" (PVCs). These genes show sequence similarities to known toxins such as the Mcf of *P. luminescens* or the toxin A of *C. difficile* (Ffrench-Constant *et al.,* 2007). Each of these cassettes encodes for 15-20 proteins, about 30 nm wide, that resemble R-type pyocins, a type of bacteriocin. The protein products of the PVCs have no direct antibacterial activity, but do destruct insect hemocytes. These proteins also show similarities to phage tail and base plate assembly proteins, fimbrial usher and proteins from other pathogenic bacteria (Yang *et al*., 2006). Their loci can be found clustered between a type IV conjugation pilus and the mukB locus, a locus involved in plasmid stability. Furthermore, their effector proteins are located always downstream of the PVCs and are flanked by transposon sequences, indicative of a possible mechanism of insertion in the PVC or even their movement among different PVCs (Yang *et al.,* 2006).

2.7.5 Mode of Action of the Toxin Complexes (Tc's)

The mode of action of the Tc's as with so many of their other aspects is poorly understood. There are many reports of their toxicity and their effects on insect models or cultured cells, but still there is no real insight into how they mediate these effects. It should be noted at this stage that the mode of action for all the Tc's is likely to be similar despite any differences in host specificity or indeed between the insecticidal and mammalian toxins. The real differences between them are more likely to be due to specificity in receptor-binding and how and when they are expressed. Thus, this section will relate the observations of toxicity in action and discuss the hypotheses as to how the toxicity is achieved.

The first reported toxicity to specific tissues and cell types rather than whole organisms was by Bowen *et al*. (1998), who showed that ingestion of purified *P. luminescens tca* by *M. sexta* led to complete destruction of the midgut epithelium leading to cessation of feeding and eventual starvation of the insect host. This was followed by a more comprehensive study into the histopathological effects of tca on *M. sexta* (Blackburn *et al*., 1998). Within 3 hours of administration, ingestion of tca resulted in apical swelling of the columnar cells in the epithelium of the anterior midgut and blebbing of vesicles into the gut lumen. By six hours the blebs contained nuclei and large vacuoles and the lumen contains debris from both columnar and goblet cells. By twelve hours the destruction was essentially complete and the basal membrane was exposed. This pathology progressed within the midgut from anterior to posterior.

Injection of tca directly into the hemocoel produced similar effects on the midgut epithelium, but to a lesser extent and over a greater time period. It is likely that Tc expression only occurs once *P. luminescens* has colonised the space between the extracellular matrix on the basal side of the midgut epithelium. Thus the toxic effects are likely to be as rapid and effective as those associated with ingestion of the toxin. There is no effect on undifferentiated regenerative cells and no pathological effects on any other tissues indicate a definite specificity to the toxin. This is presumably due to the expression of a specific receptor on the target cells and differential receptor expression between the anterior and posterior regions of the midgut could also explain the differences in toxic effects along the length of the midgut (Bowen *et al*., 1998).

2.8 Isolation of *P. luminescens* from the haemolymph of infected hosts

Akhurst (1980) reported isolation of symbiotic bacteria *P. luminescens* from infected host. He placed five last instar *Galleria mellonella* larvae in petri plates lined with moist filter paper and inoculated with 100 IJs of *H. bacteriophora* larvae. Larval cadavers were surface sterilized after 72 h. of infection by dipping in 70% ethanol and dissected the larval cadavers with the help of a pair of sterile scissors and needle after passing over the flame for few seconds. A drop of haemolymph streaked on to NBTA (Nutrient Agar with Bromothymol Blue) media and subcultured in yeast salt broth and incubated in dark for 3 days at 25°C.

Babic *et al.* (2000) isolated bacteria associated with infective juveniles by the hanging-drop method. A sterile drop of insect haemolymph was seeded with surface-disinfected infective juvenile nematodes (10 min in 10%, v/v, fresh commercial sodium hypochlorite solution) and rinsed three times in sterile water. The microorganisms developing in the drop after 48 h. at 28°C were streaked on MacConkey agar media. Isolates were cultured on nutrient agar plates and incubated at 28°C and 37°C.

Han and Ehlers (2001) reported isolation of *P. luminescens* from the hemocoel of *G. mellonella* larvae 48 h. after infection with nematode DJs. cultured at 25°C and 200 rpm on a rotary shaker in 100 ml flasks filled with 40 ml sterile liquid culture medium (LCM) containing 10 g trypcase soy broth (bioMerieux), 10 g nutrient broth (Difco), 5g yeast extract, 5 g casein peptone, 0.35 g KCl, 0.21 g $CaCl_2$, 5.0 g NaCl (Merck) and 30 ml vegetable oil (Azco) in 1000 ml tap water.

Mahar *et al*. (2005) reported isolation of symbiotic bacteria from infected *G. mellonella* with IJs of *S. carpocapsae* that died after 24-48 h. were surface sterilized in 70 % alcohol for 10 minutes, passed over the flame and allowed to dry in a laminar air flow cabinet for 2 minutes. Larvae were cut opened with sterile needles and scissors and care being taken not to damage the gut epithelium. A drop of the oozing haemolymph and streaked with a needle on to nutrient agar (37 g nutrient agar; 25 mg Bromothymol Blue powder; 4 ml of 1% 2, 3, 5 Triphenyl-tetrazolium chloride; 1000 ml distilled water) in Petri dishes which were sealed with Para film and incubated at 28°C in the dark for 24 h.

2.9 Bioassay

G. mellonella larvae have been used extensively for pathogenic studies, since it is highly susceptible to bacterial infection (Forst and Nealson, 1996).

Sun and Yeon (1999) reported pathogenicity of symbiotic bacteria *Xenorhabdus nematophilus* on *Galleria mallonella* larvae. They made different concentrations of cell suspension by bacterial colony on NBTA plate with saline solution. Then they injected 5 µl of cell suspension into the hemolymph of larvae of *G. mellonella* and incubated at 25°C. Number of bacteria injected per larvae was varied from 0 to 2×10^5. They showed injection of 60-80 cells of bacterial isolates was sufficient to kill the larvae within 40 h. The mean lethal time was decreased to 20 h. as the bacterial concentration was increased up to $6\text{-}8\times10^5$ cells/larvae.

Abdel-Razek (2003) reported pathogenicity of *P. luminescens* and *X. nematophilus* against *Plutella xylostella* (Diamondback moth) and influence of number of bacterial cells to the rate of killing of *P. xylostella* pupae. He also reported pathogenic capability of P. luminescens over that of *X. nematophilus* i.e. they gave 60 % and 40 % mortality with LD_{50} values of 5×10^4 and 5.5×10^5 cells/ml respectively.

Brown *et al*. (2005) reported toxic effect of a Ubiquitous Insecticidal Toxin Protein Txp40, from *Xenorhabdus* and *Photorhabdus* Bacteria. They found lethal dose after testing against larvae of insects such as *Galleria mellonella* and *Helicoverpa armigera* at doses of 30 to 40 ng/g larvae.

Mahar *et al*. (2005) reported biocontrol of *Galleria mellonella* by entomopathogenic bacterium *Photorhabdus luminescens*. He conducted bioassay in the sand media in which the bacterium penetrated into haemocoel on contact with insect body he concluded that the bacterium or its toxic secretions can be used for insect control and can be important component of integrated pest management.

Blackburn *et al*. (2006) reported toxic effect of Pir toxins A and B expressed in *Escherichia coli* when feed to larvae of *Plutella xylostella L*. (Lepidoptera: Plutellidae). They concluded *P. xylostella* is at least 300-fold more susceptible to Pir toxins than other insect species tested.

The toxic activity of bacteria against insect larva can be determined either by artificial diet method or direct injection method. In case of injection method, a known volume of bacterial cell suspension or known concentration of protein will be directly injected into the larvae and larval mortality will be assessed after a certain period of time. While, in artificial diet method, a known volume of bacterial cell suspension or known concentration of protein will be mixed with artificial diet and insect larvae will be allowed to feed this diet and mortality rate will be recorded and subjected to statistical analysis for determination of LD_{50} (Li *et al*., 2009).

2.10 Random Amplified Polymorphic DNA (RAPD)

RAPD markers are DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams *et al*., 1990). 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers sequence. RAPDs also have the advantage that no prior knowledge of the genome is necessary for successful application (Welsh and McClelland, 1990; Williams *et al.*, 1990; Fischer *et al.*, 2000; Klinbunga *et al*., 2000). It has been used increasingly to distinguish closely related organisms (Bassom *et al*., 1992).

Marcelo *et al*. (1998) reported genetic diversity analysis among *Beauveria bassiana* isolates from Argentina and Brazil by the use of RAPD marker. They used fluorescent deoxynucleotide in PCR reaction in order to achieve automated laser fluorescens analysis of amplified products. High resolution was achieved by polyacrylamide gel electrophoresis of amplified products. After cluster analysis they got a high level of variability among the genotypes. A phonetic group of 80% similarity represented mainly the isolates exhibiting high virulence against the sugar cane borer, *Diatraea saccharalis*.

Kumar *et al*. (2010) reported use of RAPD markers to determine genetic diversity pattern of seventy isolates of *Bacillus thuringiensis* isolated from soil samples collected from cotton fields. They used a total of 1935 RAPD fragments for the diversity analysis out these 1865 were polymorphic and 68 monomorphic. When they subjected the RAPD banding pattern data to construct dendrogram, the 70 isolates fell into two separate clusters, cluster I and cluster II, which includes 26 and 44 *B. thuringiensis* isolates,

respectively. These two main clusters were further divided into four subclusters at Eucledian distance of 150 and 80% similarity index.

Suardana *et al*. (2013) reported genetic diversity analysis of *Escherichia coli* O157:H7 local isolates with the help of random amplified polymorphic DNA (RAPD) technique. They used ten random decamer primers (OPA-02, OP-03, OPA-04, OPA- 07, OPA-08, OPA-09, OPA-10, OPA-13, and OPA-19) to differentiate isolates originated from humans and animals. Primers showed a good differentiation pattern of *E. coli* O157:H7 isolated from human, cattle and chicken feces, as well as from beef. The primers constantly produced 16, 16, 11, 14, 8, 24, 7, 11, 7, and 12 polymorphic bands, respectively. Cluster analysis by Simple Matching Coefficient (Ssm) of similarity and algorithm Unweighted Pair-Group Method using Arithmetic Average (UPGMA) of various human isolates from clinically ill and healthy patients shared common genetic clusters with animal isolates from beef, as well as cattle and chicken feces with genetic similarity coefficients greater than 70%. Based on the genetic similarity generated by cluster analysis they concluded the transmission of E. coli O157:H7 local isolates from animals to humans is potential occur.

Singh *et al*. (2014) reported molecular characterization of six different samples of *Staphylococcus aureus* isolated from Urinary tract infection, knee wound and sputum using random amplified polymorphic DNA (RAPD). They tested a total of five primers (D-18, D-20, T- 7, W-2 and X-6), among which, primer D-20 produced maximum number of bands. From the result obtained by cluster analysis they showed that clinical isolates of *S. aureus* from knee wound (SA-1) and sputum sample (SA-6) were closely related. They also reported close relation between sputum sample (SA-4) and UTI sample (SA-5). They concluded that *S. aureus* strains were not specific for infection based on the cluster analysis of RAPD profile generated.

III. MATERIAL AND METHODS

The present investigation was carried out in the Department of Plant Biotechnology, University of Agricultural Sciences G.K.V.K. Campus, Bangalore-560065 during the years 2012 to 2014. The details regarding the materials chosen and the methods followed for each of the experiments are given below.

The following experiments were carried out for the present study.

1) Isolation and identification of *Photorhabdus luminescens* from soils collected from V C farm Mandya (Karnataka Agroclimatic Zone-6).
2) Bio-control activity of *Photorhabdus luminescens* against insect crop pests.
3) Molecular diversity of *Photorhabdus luminescens* isolates using RAPD technique.

3.1 Collection of Soil from V C farm Mandya (Karnataka Agroclimatic Zone-6)

Soil collection was done from Zonal Agricultural Research Station (ZARS), V.C. Farm, Mandya, University of Agricultural Sciences, Bangalore which is geographically located at 12° 30' latitude north and longitude of 76° 50' east and altitude of 694.65 meters above mean sea level. The farm represents the agro climatic zone 6 of Karnataka. Soil samples were collected from different crop fields and fallow land and each sampling field was 100m away from the nearest. From each crop field five soil samples at a depth of 10 to 15 cm were taken randomly at a distance of about 1m and about 500g of soils were placed in plastic bags and transported to the laboratory. A total of 30 soil samples were collected.

3.2 Isolation of Entomopathogenic Nematodes (EPNs) from soil

Entomopathogenic nematodes were recovered from collected soil samples using modified Galleria-trap technique (Plate 3, Plate 4) consisting of placing a last instar larva of *Galleria mellonella* (Lepidoptera, Galleriidae) into a 2.0 ml Eppendorf tube pierced to make holes as described by Kehres *et al.* (2001). At least 10 *G. mellonella* traps were installed in each soil sample. Water was sprinkled on the soil each day or second in order to keep the soils moisten. Galleria traps were harvested at 4, 7 and 12 day intervals. The larvae were washed with distilled water and kept on White's trap (White, 1927) to collect released nematodes (Plate 5).

3.3 *In vivo* mass multiplication of nematodes

Galleria larvae that were infected with EPNs were kept on White's trap to collect nematode culture suspensions and sterilized with 0.1 percent hyamine to get rid of all contaminants associated with infective juveniles and also to kill non-infective juveniles. A small suspension of nematode culture was added to a clean petri plate using moist filter paper. Later, few last instar *G. mellonella* larvae were placed and incubated at 28°C. After two days the larvae were observed for mortality and then surface sterilized and again kept on White's trap (Plate 5).

3.4 Isolation of symbiotic bacteria from infected larvae

Dead larvae were surface sterilized with 70 percent ethanol and rinsed 3-4 times with sterilized distilled water. Then the larvae were passed over a flame using a sterile forceps for few seconds. Then, they were dissected and a drop of haemoceal from insect cadaver was streaked on NBTA medium. i.e., (37g Nutrient Agar (NA) in 1000ml distilled water to prepare NA medium. NA medium was supplemented with 25mg Bromothymol blue and 4ml of 1 percent 2,3,5 Triphenyl-tetrazolium chloride before adding to petriplates). Later petri plates were sealed with para film and incubated at 28°C in the dark for 24-48 h. The primary form of bacteria absorbs Bromothymol blue dye and appears blue. Single colony of the bacterium staining blue was selected and streaked on to new NBTA medium and subcultured continuously until colonies of uniform size and morphology were obtained (Plate 10A).

3.5 Identification of the symbiotic bacterial isolates

Cultures isolated were characterized through a number of microbiological and biochemical tests for identification of symbiotic bacteria (Boemere and Akhurst, 1988; Thomas and Poinar, 1983).

3.5.1 Morphological Characterization

Colony Morphology

The symbiotic bacteria form characteristic colonies on NBTA media (Appendix-I), which is a tool for preliminary identification. Each isolate was streaked on sterile petri plate containing NBTA medium and incubated for 4 days. Typical bluish green colonies were observed (Plate 10A).

On MacConkey agar (Appendix-I) - the symbiotic bacteria absorb neutral red from MacConkey and form characteristic red colonies. Each isolate was streaked on petri plates containing MacConkey media and incubated for 6 days (Plate 10B).

On Nutrient agar (Appendix-I) - the symbiotic bacteria form characteristic buff or cream coloured colonies. Each isolate was streaked on petri plates containing Nutrient agar medium and incubated for 4 days (Plate 10C).

Microscopic observation

The symbiotic bacterial isolates were studied for cell morphology and Gram reaction. The Gram staining was done using 24 h. old cultures by the standard procedure (Peel *et al.,* 1999). The stained cells were observed under a microscope with oil immersion lens. The Gram reaction and the cell morphology were recorded (Plate 11).

3.5.2 Biochemical observation

All the biochemical and physiological tests mentioned were conducted in duplicate for each isolate (Albert *et al.,* 1992).

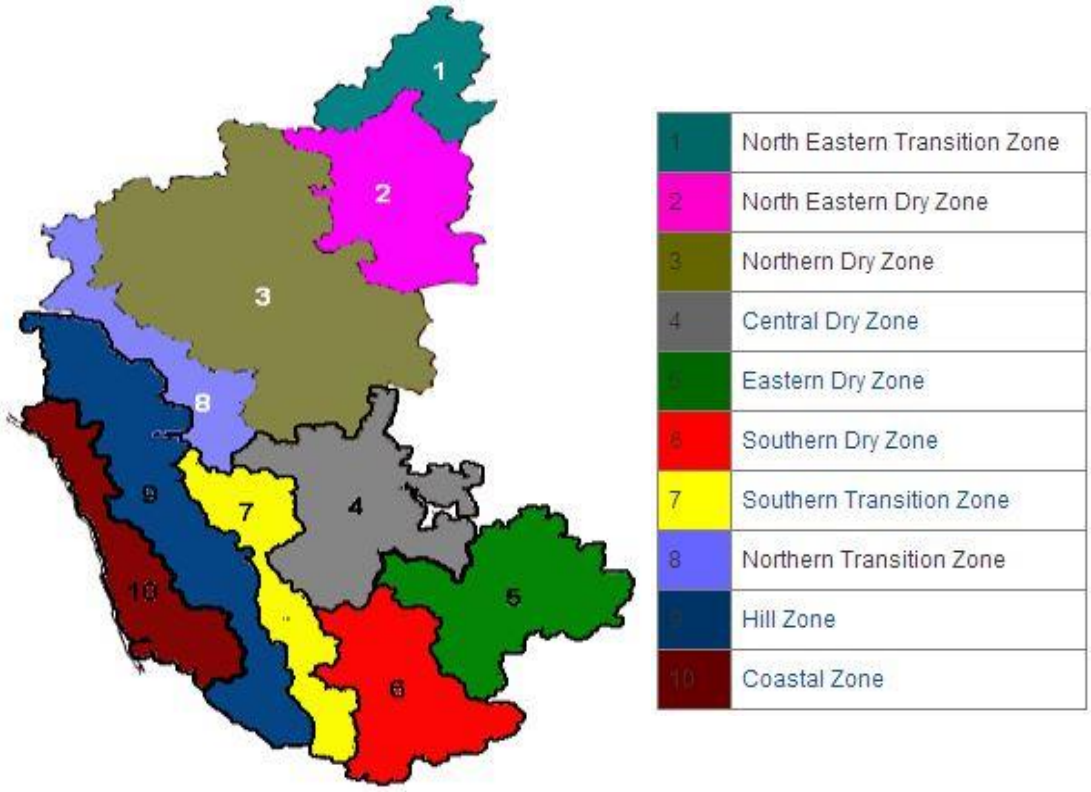


Plate 1: Map showing location of agro climatic Zone 06 of Karnataka

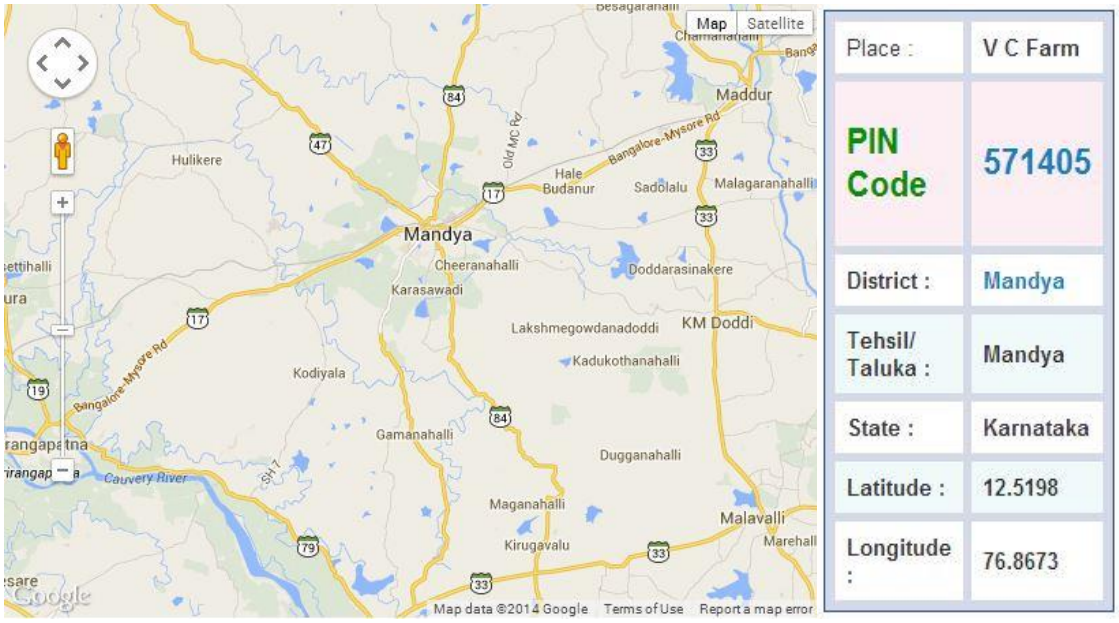


Plate 2: Map showing V C Farm, Mandya, Karnataka

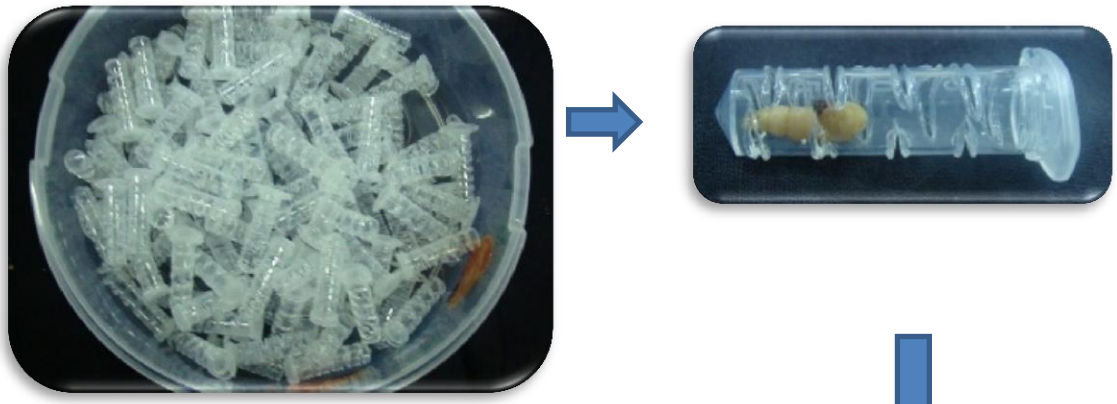


Plate 3: *Galleria mellonella* baited trap for isolation of EPNs from soil

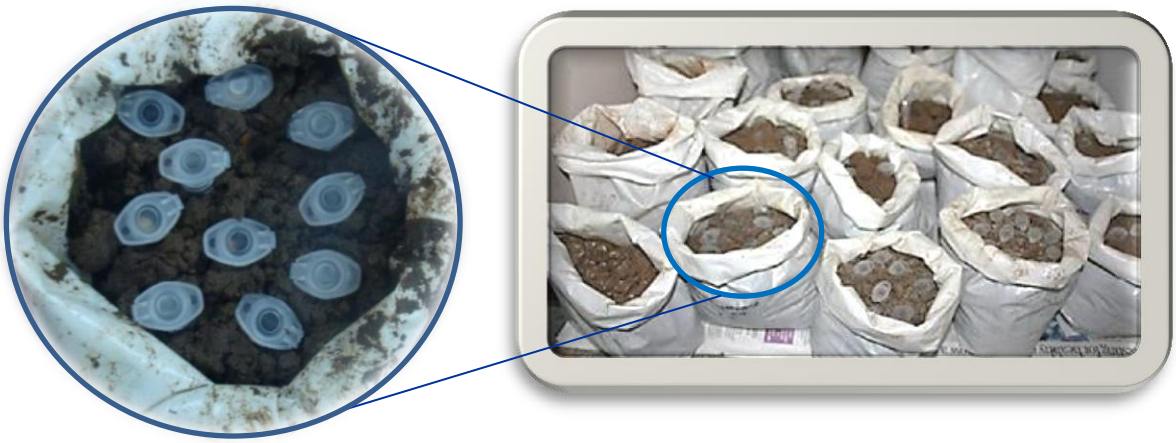


Plate 4: Installation of baited trap for isolation of EPNs from soil

Dead *Galleria mellonella* larvae infected with entomopathogenic nematode (EPN)

Fresh and alive last instar larvae of *Galleria mellonella* with EPN infected dead *G. mellonella* larvae in the White's trap

Plate 5: White's trap for *in vivo* mass multiplication of EPNs

Gelatin liquefaction test

Petriplates containing gelatin agar were spotted with overnight grown bacterial culture at 30ºC and incubated for 3 days. The plates were then flooded with 12 percent $HgCl_2$ solution and allowed to stand for 20 minutes. The plates were observed for clear zones around the growth of bacterium to indicate gelatin liquefaction.

Urease test

Test tubes containing urease broth were inoculated with bacterial cultures and incubated at 30°C for 24 h. Appearance of red colour is positive for urease test.

Motility test

Motilty test medium were inoculated with bacterial cultures and incubated at 37ºC for 24-48 h. Appearance of dispersed red coloured growth is positive for motility test.

Lactose fermentation test (Acid gas production)

Bacterial isolates were tested for acid and gas production by inoculating 5 ml of the sterile lactose broth in test tubes containing Durham's tube. The tubes were incubated for 7 days at 30ºC. Accumulation of gas in these Durham's tube was taken positive for gas production and change in colour of the medium to yellow was taken as positive for acid production.

3.6 Storing and culturing of bacteria

Glycerol stocks were prepared for each strain. Multiple stocks of the same *P. luminescens* strain were prepared at a time as the bacteria lose viability very quickly if they are repeatedly frozen and thawed. A 0.5 ml aliquot of an exponentially growing culture was added to 0.5 ml of sterile 80 percent (v/v) glycerol in a cryogenic tube. Stocks were stored at -80°C.

In order to culture *P. luminescens* an inoculating loop was used to transfer culture from a glycerol stock to an Luria Broth (LB) or nutrient agar plate. *P. luminescens* plates were incubated at 30°C for 24-48 hours. Working stocks were stored on agar plates at 4°C for up to three days for *P. luminescens*. For liquid cultures one single colony from a plate was transferred to 5 ml of LB or nutrient broth in a 25 ml tube and tubes were incubated at 30°C for *P. luminescens* in a shaker incubator at 200 rpm overnight. For larger cultures, 1 ml of a 5 ml overnight culture was added to 100 ml of broth in a 250 ml flask or 5 ml of overnight culture was added to 500ml of broth in a 1L flask and incubated in the same way as 5 ml cultures.

3.7 Insect bio-control activity of *Photorhabdus luminescens*

Insect bio-control activity of the *P. luminescens* strains were carried out using *Galleria mellonella* (Greater Wax Moth) and *Plutella Xyllostella*, Diamond Back Moth (DBM). *P. xyllostella* which is a severe pest of most of crucifereae crops. This pest is found throughout the America and in Europe, Southeast Asia, Australia, and New Zealand and cause devastating damage to the crops. Greater Wax Moth is a pest of honey

bees and causes severe loss to the apiary cultivation by damaging the honeycomb. These two insect pests were used for the bio control study. Culturing of these two insect pests was done and bioassay studies were performed with different treatments along with the control using all the isolates.

3.7.1 Culturing of Greater Wax Moth (*Galleria mellonella*)

The *Galleria mellonella* larvae were collected from dept. of Entomology, UAS GKVK, Bangalore and culturing was done at Department of Plant Biotechnology, UAS GKVK, Bangalore-560065. The larvae were kept in capped plastic boxes having small holes in it for aeration. Artificial diet was provided in the boxes.

Composition of the artificial diet is given below (Anonymous, 2007).

Wheat flour (100mg)

Wheat bran (100g)

Milk Powder (100g)

Maize flour (200g)

Dried Yeast (50g)

Honey (175ml)

Glycerine (175ml)

Folded paper strips with *G. mellonella* eggs were placed in plastic boxes containing the artificial diet. Eggs begin to hatch in about 10 days. Larvae completed development in about 20 days, spun cocoon and pupated. The pupae were transferred to a reperch box. Adults that started emerging in 8 to 10 days were transferred to a cage provided with a mixture of honey and water as food. Folded paper strips were placed in the cage for egg laying. After egg laying the paper strips were transferred to a new box with artificial diet.

3.7.2 Culturing of Diamond Back Moth (DBM) *Plutella xylostella*

Culturing of Diamond Back Moth (DBM), *Plutella xylostella* (Linnaeus) (Insecta: Lepidoptera: Plutellidae) was done at NBAII (National Bureau of Agriculturally Important Insect), Bangalore in the laboratory of Dr. K. Srinivasa Murthy. DBM was cultured in cages covered with white cloth. Mustard (*Brassica nigra*) seedling was used as food material for the DBM as it is a crop naturally infested by this pest. DBM larvae was collected from cabbage field at the Agricultural College Farm, University of Agricultural Sciences, GKVK Bangalore, Karnataka which is geographically located at 12°58' latitude north and 77° 35' longitude east. Collected DBM larvae were put on the mustard seedling cups inside the cage. 1st instar larvae took 14-18 days to become pupae and emergence of adult moth occurred after 4-8 days. In between time from pupae to adult fresh mustard seedlings were supplied inside the cage. The adult female lays 70-100 numbers of eggs on an average. The eggs were hatched within 4-7 days and the larvae started feeding upon the freshly grown mustard seedlings.

Plate 6: Mass multiplication of *Galleria mellonella* (Greater wax moth)

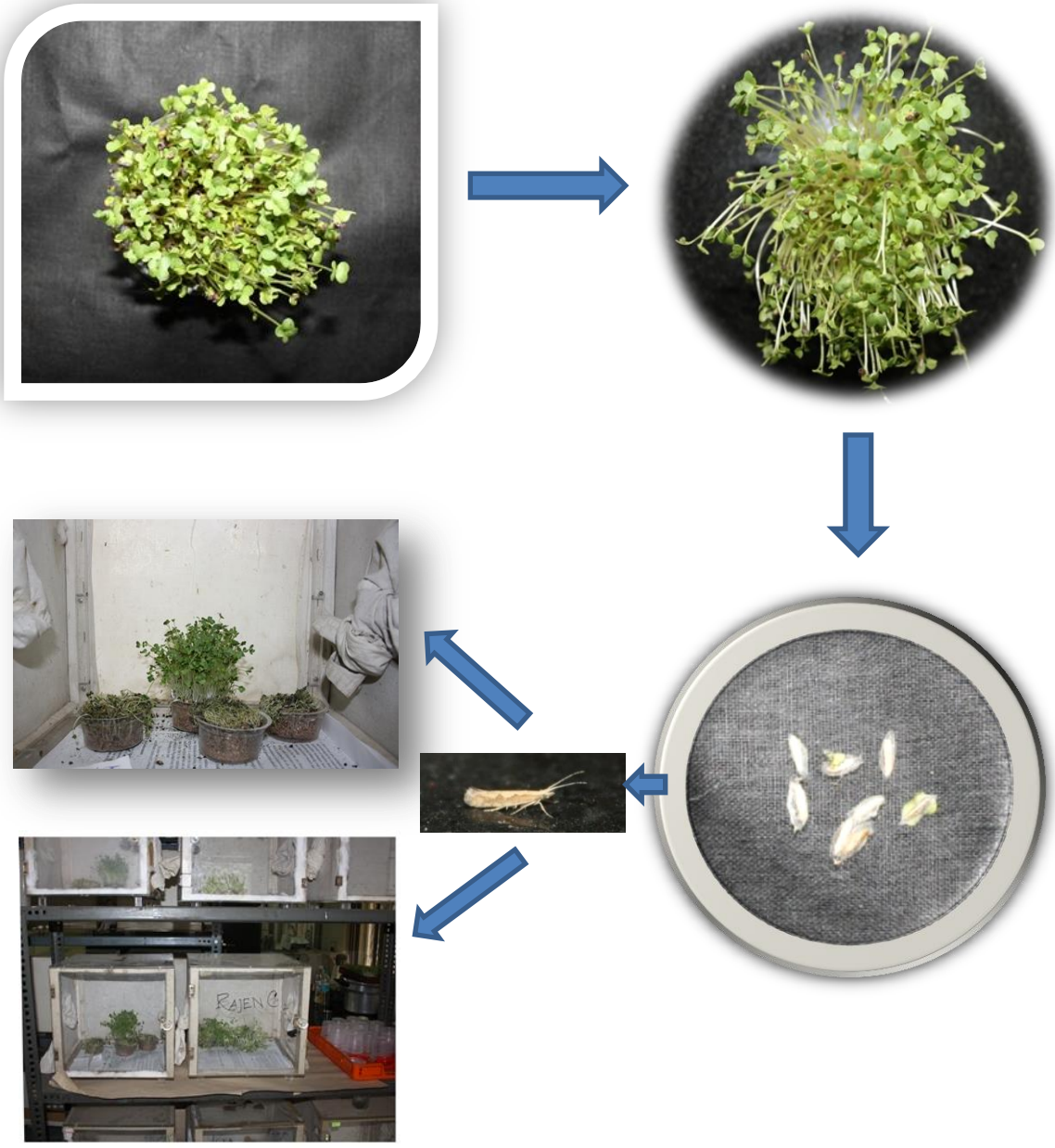


Plate 7: Mass multiplication of *Plutella xyllostella* (Diamond Back Moth)

3.7.3 Bioassay set up

Isolates of *P. luminescens* strains were sub-cultured in Luria broth and incubated at 30°C for 48h. in a shaker incubator at 200 rpm. 1ml culture suspension of 48 h. old freshly cultured *P. luminescens* strains were taken and centrifuged at 5000 rpm at 4°C for 2 min. Then supernatant was discarded and the cell pellet was taken and cells were dissolved in sterilized distilled water. Cell count was done with the help of a haemocytometer. Dilutions were made as 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 cells per mililitre used as treatment 1 (T_1), treatment 2 (T_2), treatment 3 (T_3), treatment 4 (T_4) and treatment 5 (T_5) respectively in bioassay of both *G. mellonella* as well as DBM.

3.7.4 Bioassay set up for *G. mellonella*

Bioassay set up of *G. mellonella* was done using all isolates, from each isolate five treatments (T_1, T_2, T_3, T_4 and T_5) was set up along with control (C) using sterile distilled water. Each treatment and the control were done in three replicates. In each replicate a total of 10 second instar larvae were used. In this bioassay completely randomized design (CRD) design was used. Treatment was done using 50g of artificial diet. According to the treatments different concentration of bacterial cells (20ml) were mixed with the food in separate treatment. Mortality of *G mellonella* was recorded at every 8 h.

3.7.5 Bioassay set up of DBM

Bioassay set up of DBM was done same as the *G. mellonella* except food material. All isolates each with five treatments (T1, T2, T3, T4 and T5) along with control (C) using sterile distilled water was used in this set up. Three replicates were maintained for all the treatments and control. In each replicate a total of 10 second instar larvae were used. In this bioassay completely randomized design (CRD) design was followed. Treatment was done using 20g of cabbage leaf with approximately $1cm^2$ area. Different concentrations of bacterial cells (10ml) were spread on the cabbage leaf according to the treatments. Mortality of *P. xylostella* was recorded at every 8 h.

3.7.6 Statistical analysis

The larval mortality data was subjected to probit analysis as prescribed by Finney (1970) using SPSS software version 17.0 to estimate the median lethal dose (LD_{50}) of the *Photorhabdus luminiscens* strains to *G. mellonella* and *P. xylostella*.

3.8 Molecular diversity of *Photorhabdus luminescens* isolates using RAPD technique

3.8.1 Isolation of *P. luminescens* genomic DNA

DNA extraction protocol (Reagents and preparation of reagents Appendix II).

DNA extraction protocol was followed according to Sambrook *et al*. (1989). Bacterial isolates were grown in Luria broth and incubated at 32°C overnight under shaking. About 1.5 ml of culture was taken in micro centrifuge tube, spun for 7 minutes at 8,000 rpm and supernatant was decanted. To the pellet 567µl of TE Buffer, 3µl of 20

mg/ml Proteinase-K, 30µl of 10 % SDS were added and incubated for one hour at 37°C. Again 100µl of 5 M NaCl and 80 µl of CTAB solution were added and incubated for 10 minutes at 65ºC. Further it was extracted with equal volume of Chloroform: Isoamyl alcohol and the aqueous phase was transferred to the fresh tube and to this equal volume of Phenol: Chloroform: Isoamyl alcohol was added and subjected to centrifugation at 8,000 rpm for 5 min at 4ºC. It was washed with chloroform: Isoamyl alcohol until the clear supernatant was obtained. Then equal volume of chilled Propanol was added, mixed gently and kept at -20ºC overnight for precipitation of DNA. Later centrifuged at 10,000 rpm for 20 min at 4ºC to pellet the DNA. The pellet was washed with 70 per cent ethanol and air-dried. The DNA was dissolved in TE buffer and stored at 4ºC. Total DNA isolated was quantified by following the Ethidium bromide spotting method as given by Sambrook and Russel (2001).

3.8.2 DNA quantification

UV spectrophotometer (Varian, Australia) was used to measure the absorbance of isolated genomic DNA at A260 and A280 nm. While the purity of extracted DNA was determined based on the ratio of A260/A280. The yield was measured according to the formula (DNA (µg) = A260 × 50 × Dilution factor). A sample run on 0.8% agarose gel was utilized to have a visible test of quantity and quality of extracted DNA. The gel was run in 1x TAE buffer (for 250ml 50x TAE -60.5 gm of trisbase, 14.25 ml glacial acetic acid and 25ml of 0.5 M EDTA) for ~45 minutes and stained in 0.5 µg/ml ethidium bromide solution. The isolated DNA was compared with low range DNA ruler (GeNei^{TM}, Bangalore) as a DNA marker. The gel was photographed using the gel documentation unit (Alpha Innotech, U.S.A.).

3.8.3 Selection of primers

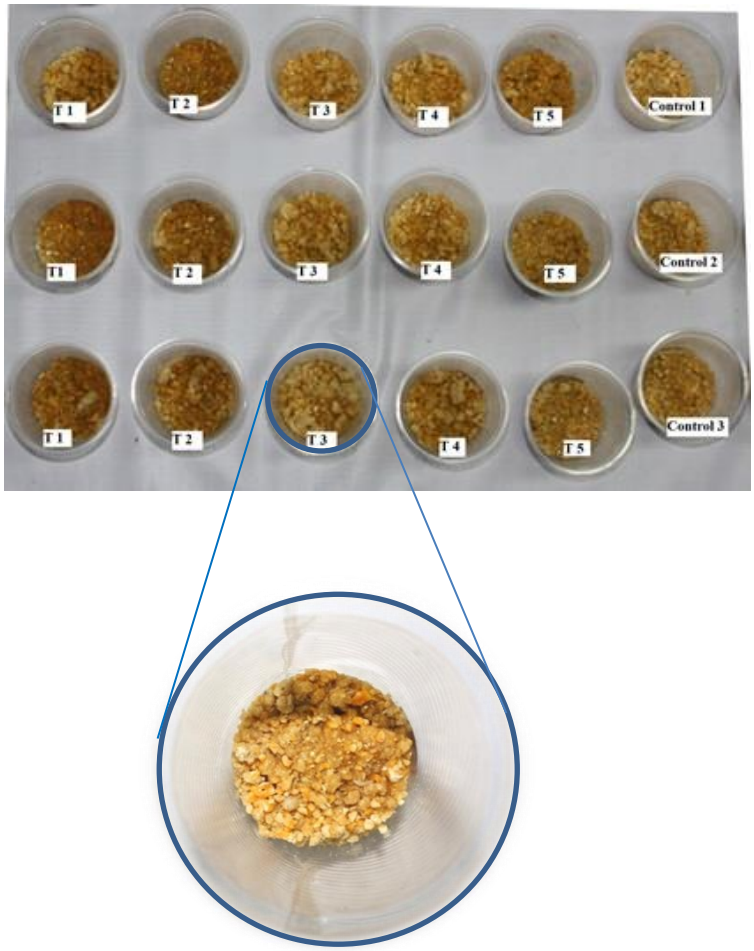
To choose the RAPD primers that can amplify informative sequences, Primer screening was carried out using DNA obtained from the Symbiotic bacterial isolates (Table 3).

Table 3: List of oligonucleotide Primers used in RAPD diversity analysis

Sl. No.	Primer Name	Primer Sequence
1.	OPA- 03	AGTCAGCCAC
2.	OPA- 13	CAGCACCCAC
3.	OPA- 17	GACCGCTTGT
4.	OPA- 18	AGGTGACCGT

3.8.4 PCR amplification conditions

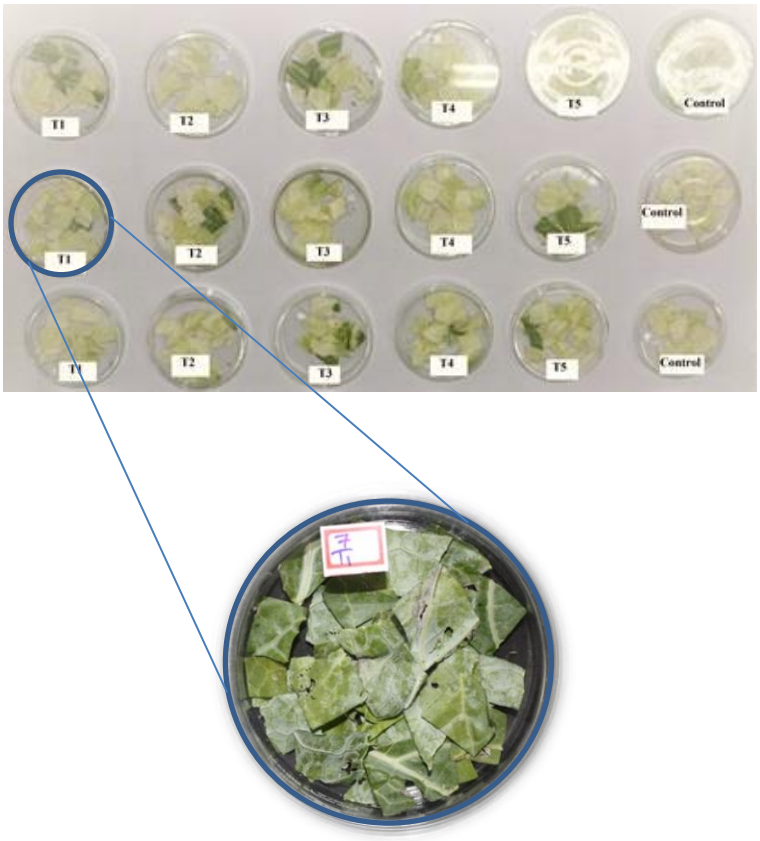
PCR reactions were performed in a final volume of 25 µl containing 30 ng of template DNA, 0.75 µl of 2mM dNTPs each, 2.5 µl of 10X taq buffer, 0.36µl 1 unit of Taq DNA polymerase, 3 µl of 10 pico mole Primer. Amplifications were achieved in Bio-Rad primus thermocycler with the program consisting initial denaturation of 94°C



Non infected **Infected**

Plate 8: Bioassay set up of *Galleria mellonella* using one control and five treatments of *Photorhabdus luminescens* strains

Treatments: Control= 0.0 cells/ml, T1= 10^3cells/ml, T2=10^4cells/ml, T3=10^5cells/ml, T4=10^6cells/ml, T5=10^7cells/ml



Non infected **Infected**

Plate 9: Bioassay set up of *Plutella xylostella* using one control and five treatments of *Photorhabdus luminescens* strains

Treatments: Control= 0.0 cells/ml, T1= 10^3cells/ml, T2=10^4cells/ml, T3=10^5cells/ml, T4=10^6cells/ml, T5=10^7cells/ml

for 3 min followed by 45 cycles each consisting of denaturation at 94°C for 1 min, primer annealing temperature at 35.5°C for 1 min, primer extension at 72°C for 3 min, and a final extension of 72°C for 10 min. These reactions were repeated to check the reproducibility of the amplification (Table 4).

Table 4: Preparation of master mix for PCR

Sl. No.	Reaction reagents	Volume
1.	DNA template (50-75 ng/µl)	1.2 µl
2.	Taq polymerase (3U/µl)	0.4 µl
3.	Taq buffer (10X)	2.0 µl
4.	dNTPs (2.5 mM)	0.8 µl
5.	Primer (10 pm)	2.0 µl
6.	Sterile distilled water	13.6 µl
	Total	20 µl

3.8.5 Agarose gel electrophoresis

Amplification products were resolved on 2% agarose gel containing ethidium bromide (0.5µg/ml) using 1 X TAE. Totally 10 µl of PCR product with 4 µl of loading buffer was loaded into the wells. 100bp ladder was used as molecular weight markers. Electrophoresis was carried out at a constant voltage of 80 V for 2 h. The gels were visualized under UV light and documented by using gel doc unit (Alpha Innotech, U.S.A.) in the computer.

3.8.6 Scoring of bands

Banding pattern of agarose gels were used for scoring. The bacterial strain profiles produced by RAPD markers were scored manually for further analysis. During scoring, only intense and clearly resolved amplification products were considered for further analysis. The presence of band is scored as (1) while absence of band is scored as (0) and the binary data were used for statistical analysis.

3.8.7 Cluster analysis for molecular data

Pairwise genetic similarity (GS) was calculated among all the *P. luminescens* strains using Jaccard's similarity coefficient (Jaccard, 1908) which is given by J= N11/ (N1+N10+N01), (where N11 is the number of bands present in both individuals; N10 is the number of bands present only in the individual i; N01 is the number of bands present only in the individual j; N represent the total number of bands). The values of GS may range from '1' (identical profiles for all marker in the two strains) to '0' (no common bands).

The binary data generated for all the varieties for the polymorphic markers was entered in the NTedit program of NTSYSpc version 2.02 softwere. This data was further analysed to generate a similarity matrix using SimQual module for qualitative data. The similarity matrix used to generate dendogram using the SAHN module for cluster analysis.

IV. EXPERIMENTAL RESULTS

The experiment was conducted to isolate and identification of symbiotic bacteria *Photorhabdus luminescens* associated with entomopathogenic nematodes from VC farm, Mandya (Karnataka agro climatic zone-06) (Plate 1), bio-control activity of insect pests *Galleria mellonella* and *Plutella xylostella* and molecular diversity analysis using RAPD technique, presented here.

4.1 Entomopathogenic nematodes: Source of symbiotic bacteria

4.1.1 Collection of EPNs

Isolation of entomopathogenic nematode done using modified Galleria-trap technique and data on infestation of *G. mellonella* larvae in each trap was recorded. The results of the experiment are presented in Table 5.

Table 5: Per cent infection of *Galleria mellonella* larvae by entomopathogenic nematodes in soil, across different crop fields of V C farm, Mandya (agro climatic Zone 6 of Karnataka).

Crop field (Location of soil collection)	Total number of soil sample	Total number of trap	Total number infected	Total per cent infection
Maize	5	50	3	6
Ragi	5	50	4	8
Upland Rice	5	50	7	14
Cowpea	5	50	3	6
Non-cultivated land	5	50	2	4
Sugarcane	5	50	1	2

Out of 300 traps a total of twenty nematode cultures were collected. Total percentage mortality of *G. mellonella* larvae to EPNs was recorded as follows- maize field 6%, ragi field 8%, upland rice 14%, cowpea field 6%, non-cultivated land 4% and sugarcane 2% (Table 5).

4.1.2 *In vivo* mass multiplication of nematodes

Galleria larvae that were infected with EPNs were kept on White's trap (Plate-5). Sterilization of EPNs was done with 0.1 percent hyamine to get rid of all contaminants and to kill non-infective juveniles. Later few last instar *G. mellonella* larvae were placed and incubated at 28°C (Plate 5). After two days the larvae were observed for mortality then they were surface sterilized and again kept on White's trap (Plate 5).

4.2 Isolation of symbiotic bacteria from EPNs

Collected nematode cultures were used to isolate symbiotic bacteria using *G. mellonella* larvae haemolymph of dead larvae was streaked on sterile petri plate containing solidified sterile NBTA medium under aseptic condition (Plate 10A). After incubation colony characters were observed. Upon confirmation these cultures were preserved as a glycerol stock. 11 isolates of symbiotic bacteria were obtained from 20 nematode cultures from 300 traps baited in 30 soil samples.

4.2.1 Identification

4.2.1.1 Colony morphology on different media

a) NBTA media

All bacterial isolates absorbed bromothymol blue dye from the media and formed characteristic bluish green colonies on NBTA media (Plate 10A).

b) Mac Conkey agar

All bacterial isolates absorbed neutral red from MacConkey agar media and formed characteristic pinkish red colonies (Plate 10B).

c) Neutral Agar

All bacterial isolates formed characteristic buff or cream coloured colonies on nutrient agar media (Plate 10C).

4.2.1.2 Microscopic observation

Symbiotic bacterial isolates were further examined for their Gram's reaction and shape. All the isolates were found Gram negative and rod shaped (Table 6, Plate 11).

4.2.1.3 Biochemical and physiological characters

4.2.1.3.1 Gelatin liquefaction

All isolates were found positive to gelatin liquefaction as indicated by the production of yellowish or bluish green fluid on the surface of gelatin agar medium (Table 7).

4.2.1.3.2 Motility test

All isolates were found positive for motility test indicated by the appearance of dispersed red colour growth in the motility test medium (Table 7, Plate 12).

4.2.1.3.3 Urease test

All isolates were found positive for urease test as indicated by the change in colour from yellow to red (Table 7, Plate 13).

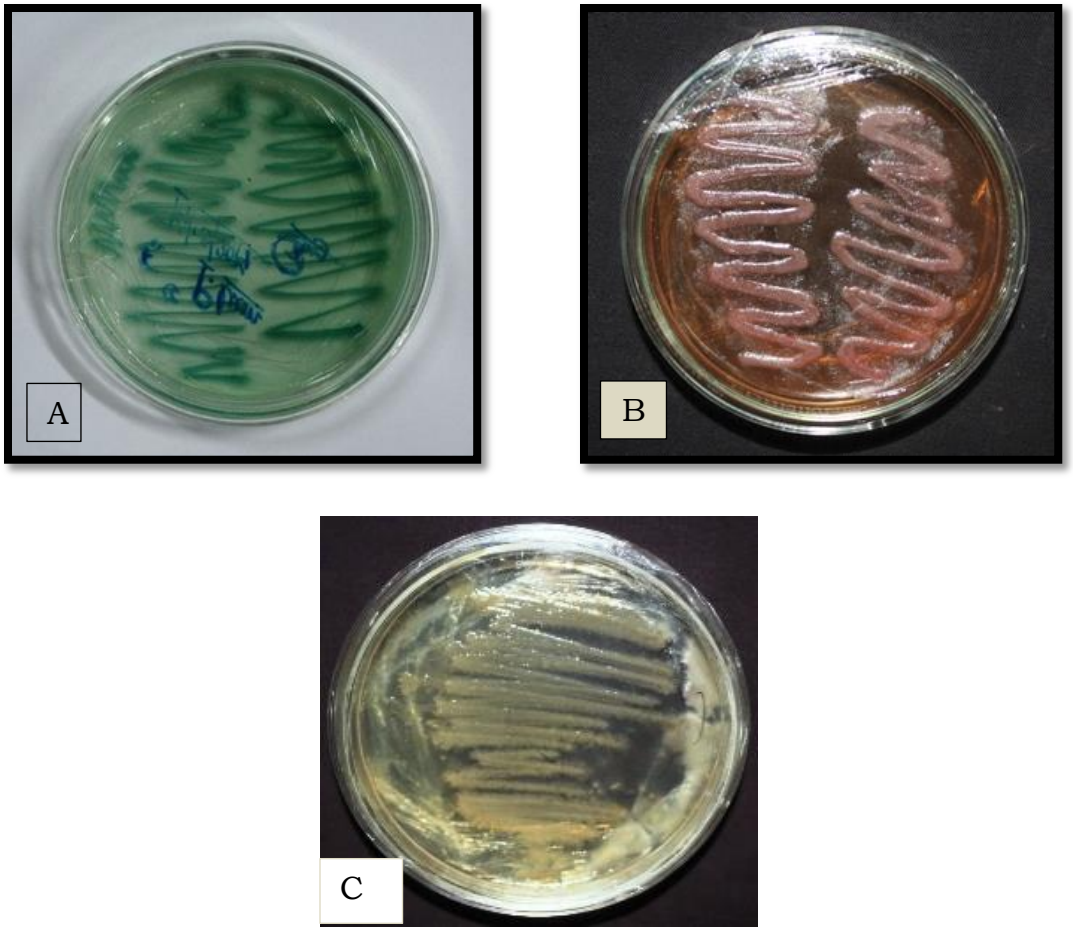


Plate 10: Growth of *Photorhabdus luminescens* on different media A: NBTA, B: Mac Conkey agar and C: Nutrient agar medium

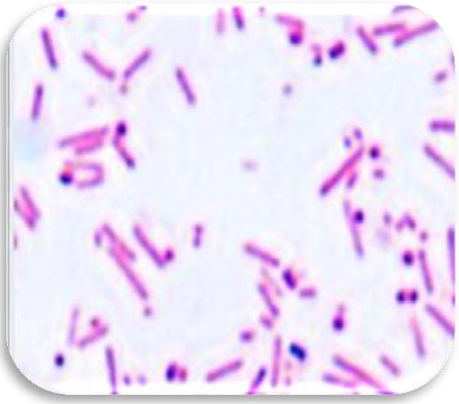


Plate 11: *Photorhabdus luminescens* (100X magnification)

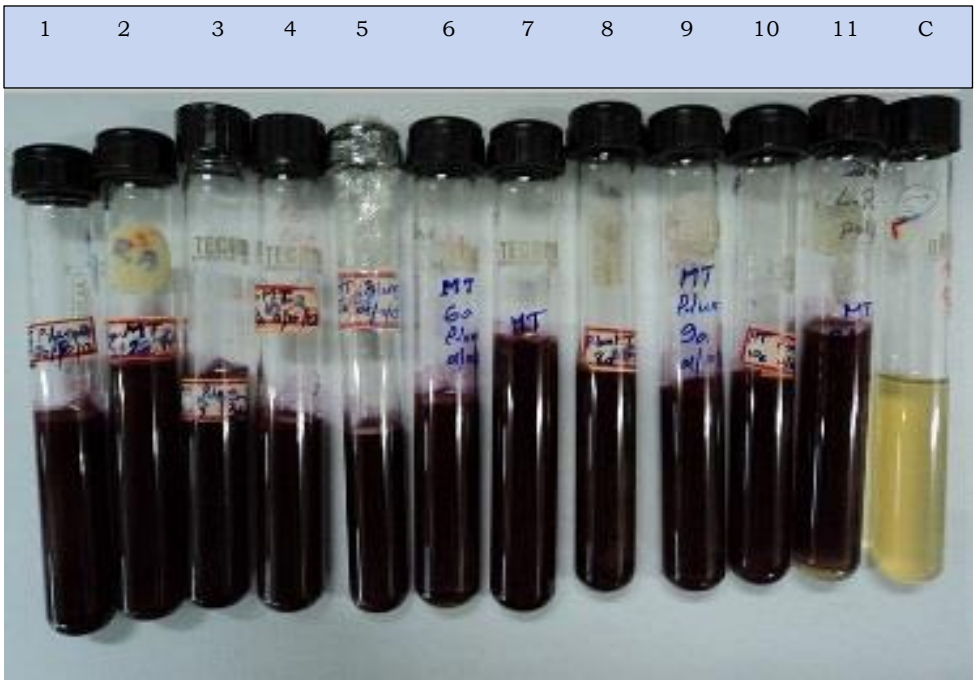


Plate 12: Motility test.

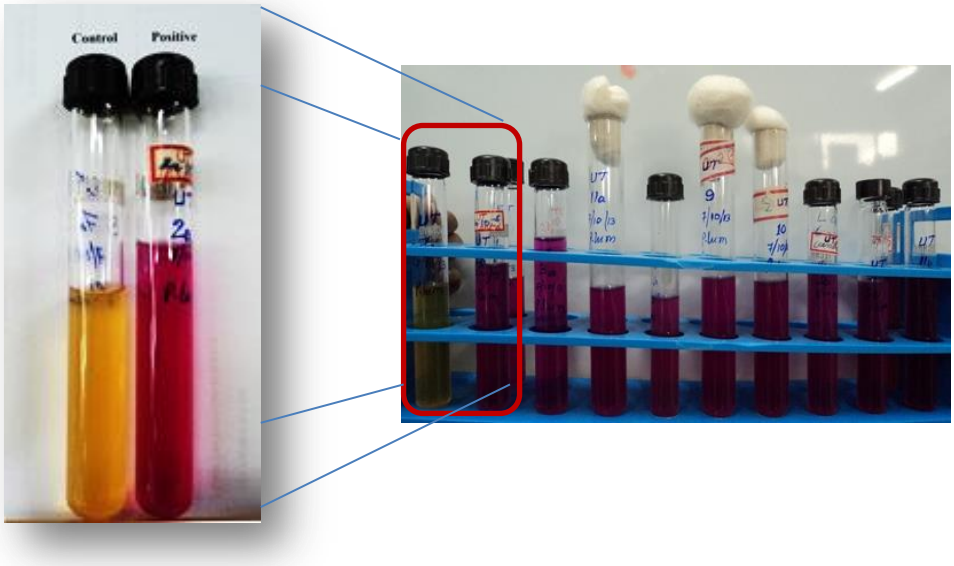


Plate 13: Urease test

1) Strain 1, 2) Strain 2, 3) Strain 3, 4) Strain 4, 5) Strain 5, 6) Strain 6, 7) Strain 7, 8) Strain 8, 9) Strain 9, 10) Strain 10, 11) Strain 11, C) Control.

Table 6: Morphological characters of *Photorhabdus luminescens* isolates

Sl. No.	Isolates	Colony morphology on NBTA			Cell shape
		Colour	Shape	Nature	
1	**Strain 1**	Blue	Round	N.S	Rod
2	**Strain 2**	Blue	Round	N.S.	Rod
3	**Strain 3**	Blue	Irregular	S	Rod
4	**Strain 4**	Blue	Round	N.S.	Rod
5	**Strain 5**	Blue	Round	N.S.	Rod
6	**Strain 6**	Blue	Round	N.S.	Rod
7	**Strain 7**	Blue	Round	N.S.	Rod
8	**Strain 8**	Blue	Irregular	N.S.	Rod
9	**Strain 9**	Blue	Round	N.S.	Rod
10	**Strain 10**	Blue	Round	N.S.	Rod
11	**Strain 11**	Blue	Irregular	S.	Rod

S – Spreading, NS – Non Spreading

Table 7: Biochemical and physiological characters of *Photorhabdus luminescens* isolates

Sl. No.	Isolates	Gram reaction	Lactose fermentation	Motility test	Gelatin liquefaction	Urease test
1	**Strain 1**	-	+	+	+	+
2	**Strain 2**	-	+	+	+	+
3	**Strain 3**	-	+	+	+	+
4	**Strain 4**	-	+	+	+	+
5	**Strain 5**	-	+	+	+	+
6	**Strain 6**	-	+	+	+	+
7	**Strain 7**	-	+	+	+	+
8	**Strain 8**	-	+	+	+	+
9	**Strain 9**		+	+	+	+
10	**Strain 10**	-	+	+	+	+
11	**Strain 11**	-	+	+	+	+

+ = positive - = Negative

4.2.1.3.4 Lactose fermentation test (Acid gas production)

All bacterial isolates were found positive for lactose lactose fermentation test indicated by the change in colour of the medium to yellow and appearance of gas bubbles in the Durham's tube (Table 7).

4.3 Insect bio-control activity of *Photorhabdus luminescens*

Insect bioassay of the *P. luminescens* strains were carried out using *Galleria mellonella* (Greater Wax Moth) and *Plutella xylostella* i.e. Diamond Back Moth (DBM) (Plate 8 and Plate 9 respectively). Mortality response was recorded at every 8 h. after treatment. Percent mortality responses of *G. mellonella* as well as *P. xylostella* at 24h. and 40 h. after treatment to different dosages of all 11 isolates of *P. luminescens* strains were compared (Table 8, Table 9, Table 10 and Table 11). Probit analysis for the mortality response was done at 24 h. after the treatment as at this time it is showing at least fifty per cent death in the highest concentration dose.

The LD_{50} for the 11 symbiotic bacterial isolates on *G. mellonella* larvae treated with bacterial cell concentrations 1×10^3 (T 1), 1×10^4 (T 2), 1×10^5 (T 3), 1×10^6 (T4), 1×10^7 (T5) cells per ml and sterile distilled water as control. Fiducial limit and chi^2 values are presented in Table 12 and for *P. xylostella* in Table 13. Among all the 11 isolates strain 10 shown lowest LD_{50} values for both the insect pests under the present study i.e. 0.2403×10^6 for *G. mellonella* and 0.2541×10^6 for *P. xylostella*.

The source of variation in susceptibility of *P. xylostella* as well as *G. mellonella* to 11 isolates of *P. luminescens* bacteria was determined by F test and found that the pathogens varied significantly ($P<0.0001$) in their virulence in both the methods. The mean per cent mortality induced by the 11 isolates of *P. luminescens* for varied dose was tested using Duncan's Multiple Range Test (DMRT) followed by F test and found to be significantly different ($P<0.05$) for the 11 isolates of *P. luminescens* (Table 14 and Table 15). The mean per cent mortality obtained was significantly different ($P<0.05$) for each concentration with in the strains. Strain 10 caused highest mortality 63.33% against *P. xylostella* at highest dose (T_5) while other 10 strains caused only around 56.96% mortality. Strain 10 also caused highest mortality 60% against *G. mellonella* while other 10 strains caused around 53.66% mortality.

Table 8: Percentage mortality of *G. mellonella* larvae to different dosage of *P. luminescens* 24 h. after treatment

Treatment	Percent mortality										
	Strain 1	**Strain 2**	**Strain 3**	**Strain 4**	**Strain 5**	**Strain 6**	**Strain 7**	**Strain 8**	**Strain 9**	**Strain 10**	**Strain 11**
Control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
T 1	26.667	26.667	26.667	23.333	26.667	30.000	26.667	26.667	23.333	23.333	26.667
T 2	36.667	36.667	36.667	30.000	36.667	36.667	33.333	33.333	33.333	43.333	26.667
T 3	40.000	43.333	40.000	36.667	36.667	43.333	43.333	40.000	43.333	50.000	43.333
T 4	46.667	46.667	46.667	43.333	46.667	46.667	46.667	50.000	46.667	53.333	46.667
T 5	56.667	60.000	60.000	56.667	56.667	60.000	53.333	56.667	56.667	63.333	53.333

Control 0.00, T1 (1×10^3), T2 (1×10^4), T3 (1×10^5), T4 (1×10^6), T5 (1×10^7) cells/ml.

Table 9: Percentage mortality of *G. mellonella* larvae to different dosage of *P. luminescens* 40 h. after treatment

Treatment	Percent mortality										
	Strain 1	**Strain 2**	**Strain 3**	**Strain 4**	**Strain 5**	**Strain 6**	**Strain 7**	**Strain 8**	**Strain 9**	**Strain 10**	**Strain 11**
Control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
T 1	66.667	70.000	66.667	66.667	66.667	70.000	66.667	66.667	63.333	63.333	66.667
T 2	76.667	76.667	76.667	70.000	76.667	76.667	73.333	73.333	73.333	83.333	70.000
T 3	83.333	83.333	80.000	76.667	76.667	83.333	86.667	80.000	86.667	90.000	86.667
T 4	86.667	86.667	86.667	83.333	86.667	86.667	86.667	93.333	86.667	90.000	90.000
T 5	96.667	96.667	96.667	96.667	96.667	100.000	96.667	96.667	96.667	100.000	96.667

Control 0.00, T1 (1×10^3), T2 (1×10^4), T3 (1×10^5), T4 (1×10^6), T5 (1×10^7) cells/ml.

Table 10: Percentage mortality of *P. xyllostella* larvae to different dosage of *P. luminescens* 24 h. after treatment

Treatment	Percent mortality										
	Strain 1	**Strain 2**	**Strain 3**	**Strain 4**	**Strain 5**	**Strain 6**	**Strain 7**	**Strain 8**	**Strain 9**	**Strain 10**	**Strain 11**
Control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
T 1	20.000	23.333	23.333	26.667	26.667	26.667	26.667	23.333	23.333	33.333	26.667
T 2	30.000	33.333	33.333	33.333	40.000	36.667	33.333	33.333	26.667	40.000	33.333
T 3	36.670	40.000	43.333	36.667	43.333	43.333	40.000	43.333	36.667	50.000	43.333
T 4	43.370	46.667	46.667	46.667	46.667	46.667	43.333	46.667	46.667	53.333	43.333
T 5	56.670	53.333	53.333	53.333	53.333	53.333	56.667	56.667	50.000	60.000	50.000

Control 0.00, T1 (1×10^3), T2 (1×10^4), T3 (1×10^5), T4 (1×10^6), T5 (1×10^7) cells/ml.

Table 11: Percentage mortality of *P. xyllostella* larvae to different dosage of *P. luminescens* 40 h. after treatment

Treatment	Percent mortality										
	Strain 1	**Strain 2**	**Strain 3**	**Strain 4**	**Strain 5**	**Strain 6**	**Strain 7**	**Strain 8**	**Strain 9**	**Strain 10**	**Strain 11**
Control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
T 1	60.000	73.333	63.333	73.333	66.667	66.667	66.667	63.333	63.333	73.333	66.667
T 2	70.000	80.000	73.333	73.333	80.000	76.667	73.333	73.333	66.667	83.333	76.667
T 3	76.667	83.333	83.333	86.667	83.333	83.333	80.000	83.333	76.667	86.667	83.333
T 4	83.333	86.667	86.667	93.333	90.000	86.667	83.333	90.000	86.667	93.333	86.667
T 5	96.667	93.333	93.333	93.333	93.333	93.333	96.667	96.667	96.667	100.000	96.667

Control 0.00, T1 (1×10^3), T2 (1×10^4), T3 (1×10^5), T4 (1×10^6), T5 (1×10^7) cells/ml.

Table 12: Probit analysis of dosage mortality response of *Photorhabdus luminescens* to the *G. mellonella* larvae at 24 h. after treatment

Sl. No.	Isolate	Chi^2 (d.f.= 13)	Regression equation (Y=bx+a)	LD_{50} (cells/ml)	Fiducial limit (cells/ml)
1.	**Strain 1**	1.329	0 .183x-1.143	1.715×10^{6}	130698.234- 2.934×10^{11}
2.	**Strain 2**	2.263	0.200x-1.193	0.9125×10^{6}	85822.866- 1.192×10^{9}
3.	**Strain 3**	2.847	0.201x-1.214	1.110×10^{6}	105554.268- 2.135×10^{9}
4.	**Strain 4**	1.358	0.216x-1.398	3.027×10^{6}	303679.689- 9.788×10^{9}
5.	**Strain 5**	1.811	0. 184x-1.164	2.120×10^{6}	160391.665- 6.735×10^{11}
6.	**Strain 6**	1.070	0.181x-1.080	0.9028×10^{6}	65367.667- 2.573×10^{10}
7.	**Strain 7**	1.584	0.175x-1.120	2.456×10^{6}	163654.171- 4.659×10^{13}
8.	**Strain 8**	1.743	0.201x-1.235	1.353×10^{6}	129186.945- 3.674×10^{9}
9.	**Strain 9**	1.706	0.212x-1.308	1.461×10^{6}	155090.890- 1.531×10^{9}
10.	**Strain 10**	3.137	0.234x-1.261	0.2403×10^{6}	26638.032- 6099402.385
11.	**Strain 11**	2.126	0.195x-1.256	2.766×10^{6}	230577.303- 1.394×10^{11}

Table 13: Probit analysis of dosage mortality response of *Photorhabdus luminescens* to the *P. xylostella* larvae at 24 h. after treatment

Sl. No.	Isolate	Chi^2	Regression equation (Y=bx+a)	LD_{50} (cells/ml)	Fiducial limit (cells/ml)
1.	**Strain 1**	1.974	0.237x-1.526	2.757×10^{6}	332672.543- 1.393×10^{9}
2.	**Strain 2**	1.313	0.195x-1.259	2.748×10^{6}	230704.926- 1.305×10^{11}
3.	**Strain 3**	1.783	0.195x-1.237	2.252×10^{6}	192489.889- 6.898×10^{10}
4.	**Strain 4**	1.554	0.176x-1.161	3.815×10^{6}	243524.662- 3.414×10^{14}
5.	**Strain 5**	1.588	0.156x-0.988	2.119×10^{6}	104750.629- 2.086×10^{24}
6.	**Strain 6**	1.631	0.166x-1.053	2.291×10^{6}	133164.414- 3.960×10^{16}
7.	**Strain 7**	2.249	0.185x-1.186	2.614×10^{6}	195386.174- 1.434×10^{12}
8.	**Strain 8**	1.706	0.212x-1.308	1.461×10^{6}	155090.890- 1.531×10^{9}
9.	**Strain 9**	2.293	0.200x-1.352	5.972×10^{6}	452372.041- 1.207×10^{12}
10.	**Strain 10**	3.150	0.170x-0.921	0.2541×10^{6}	7261.631- 8.719×10^{8}
11.	**Strain 11**	2.282	0.150x-1.025	7.076×10^{6}	271304.759- 3.690×10^{79}

Table 14: Mean per cent mortality of *G. mellonella* caused by different strains of *P. luminescens* at different concentrations of cell suspension (cells ml^{-1}).

Strain	Mean per cent mortality after 24 h. of treatment				
	T1	**T2**	**T3**	**T4**	**T5**
S1	26.667 (30.983)^{ji}	36.667 (37.209)^{fhig}	40.000 (39.215)^{fheg}	46.667 (43.059)^{fdec}	56.667 (48.826)^{bac}
S2	26.667 (30.983)^{ji}	36.667 (37.209)^{fhig}	43.333 (41.137)^{fdeg}	46.667 (43.059)^{fdec}	60.000 (50.832)^{ba}
S3	26.667 (30.983)^{ji}	36.667 (37.209)^{fhig}	40.000 (39.131)^{fheg}	46.667 (43.059)^{fdec}	60.000 (50.832)^{ba}
S4	23.333 (28.768)^{j}	30.000 (33.197)^{jhi}	36.667 (37.209)^{fhig}	43.333 (41.137)^{fdeg}	56.667 (48.826)^{bac}
S5	26.667 (30.983)^{ji}	36.667 (37.209)^{fhig}	36.667 (37.209)^{fhig}	46.667 (43.059)^{fdec}	56.667 (48.826)^{bac}
S6	30.000 (33.197)^{jhi}	36.667 (37.209)^{fhig}	43.333 (41.137)^{fdeg}	46.667 (43.059)^{fdec}	60.000 (50.748)^{ba}
S7	26.667 (30.983)^{ji}	33.333 (35.203)^{jhig}	43.333 (41.137)^{fdeg}	46.667 (43.059)^{fdec}	53.333 (46.903)^{bdac}
S8	26.667 (30.983)^{ji}	33.333 (35.203)^{jhig}	40.000 (39.215)^{fheg}	50.000 (44.981)^{bdec}	56.667 (48.826)^{bac}
S9	23.333 (28.768)^{j}	33.333 (35.203)^{jhig}	43.333 (41.137)^{fdeg}	46.667 (43.059)^{fdec}	56.667 (48.826)^{bac}
S10	23.333 (28.768)^{j}	43.333 (41.137)^{fdeg}	50.000 (44.981)^{bdec}	53.333 (46.903)^{bdac}	63.333 (52.754)^{a}
S11	26.667 (30.983)^{ji}	26.667 (30.983)^{ji}	43.333 (41.137)^{fdeg}	46.667 (43.059)^{fdec}	53.333 (46.903)^{bdac}

Control 0.00, T1 (1×10^{3}), T2 (1×10^{4}), T3 (1×10^{5}), T4 (1×10^{6}), T5 (1×10^{7}) cells/ml.

Table 15: Mean per cent mortality of DBM caused by different strains of *P. luminescens* at different concentrations of cell suspension (cells ml^{-1}).

Strain	Mean percent mortality after 24 h. of treatment				
	T1	**T2**	**T3**	**T4**	**T5**
S1	20.000 (26.554)^{j}	30.000 (32.989)^{jhig}	36.667 (37.210)^{fheg}	43.333 (41.138)^{fdec}	56.667 (48.826)^{ba}
S2	23.333 (28.769)^{ji}	33.333 (35.204)^{fhig}	40.000 (39.216)^{fdeg}	46.667 (43.060)^{bdec}	53.333 (46.904)^{bac}
S3	23.333 (28.769)^{ji}	33.333 (35.204)^{fhig}	43.333 (41.138)^{fdec}	46.667 (43.060)^{bdec}	53.333 (46.904)^{bac}
S4	26.667 (30.983)^{jhi}	33.333 (35.204)^{fhig}	36.667 (37.210)^{fheg}	46.667 (43.060)^{bdec}	53.333 (46.904)^{bac}
S5	26.667 (30.983)^{jhi}	40.000 (39.216)^{fdeg}	43.333 (41.138)^{fdec}	46.667 (43.060)^{bdec}	53.333 (46.904)^{bac}
S6	26.667 (30.983)^{jhi}	36.667 (37.210)^{fheg}	43.333 (41.138)^{fdec}	46.667 (43.060)^{bdec}	53.333 (46.904)^{bac}
S7	26.667 (30.983)^{jhi}	33.333 (35.204)^{fhig}	40.000 (39.132)^{fdeg}	43.333 (41.138)^{fdec}	56.667 (48.826)^{ba}
S8	23.333 (28.769)^{ji}	33.333 (35.204)^{fhig}	43.333 (41.138)^{fdec}	46.667 (43.06)^{bdec}	56.667 (48.826)^{ba}
S9	23.333 (28.769)^{ji}	26.667 (30.983)^{jhi}	36.667 (37.210)^{fheg}	46.667 (43.060)^{bdec}	50.000 (44.982)^{bdac}
S10	33.333 (35.204)^{fhig}	40.000 (39.132)^{fdeg}	50.000 (44.982)^{bdac}	53.333 (46.904)^{bac}	60.000 (50.832)^{a}
S11	26.667 (30.983)^{jhi}	33.333 (35.204)^{fhig}	43.333 (41.132)^{fdec}	43.333 (41.138)^{fdec}	50.000 (44.982)^{bdac}

Control 0.00, T1 (1×10^{3}), T2 (1×10^{4}), T3 (1×10^{5}), T4 (1×10^{6}), T5 (1×10^{7}) cells/ml.

4.4 Standardization of protocol for RAPD analysis

4.4.1 Amplification conditions

In order to obtain high amplification rate and reproducible banding pattern PCR amplification conditions were optimized based on the protocol outlined by Abbas *et al.* (1996) with minor modifications. Different durations for hot start, denaturation, and primer annealing and primer extension were tried. The PCR reaction was evaluated for 30, 35 and 40 cycles using Taq Buffer A from GeNei^{TM}. The optimum conditions for each cycle of PCR were developed for high amplification levels. The PCR optimum conditions consisted of the following steps.

Initial strand separation or start at 95°C for five minute followed by, 40 cycles

i. Denaturation at 95°C for 5 minutes.
ii. Primer annealing at 35.5°C for 1 minute.
iii. Primer extension at 72°C for 2 minutes.
iv. Final extension at 72°C for 8 minutes.
v. Final hold at 4°C for ∞.

4.4.3 Reaction parameters

It is important to optimize the concentration of PCR mixture, in order to produce informative and reproducible RAPD fingerprints. Hence different concentrations of template DNA (10-15ng, 20-30ng, 30-50ng) were tried by keeping other PCR components and conditions same. A concentration of 30-50ng of template was found to be optimum for obtaining intense and clear banding pattern in symbiotic bacterial isolates. In all these cases, 2 µl of 20 pico moles of primer and 0.5 µl of 3 unit/µl per reaction were used. However, fluctuation in the concentration of template DNA did affect the amplification, with lower concentration of template DNA (10-15 ng) causing either reduced or no amplification of smaller fragments (Table 16).

Table 16: Optimum concentration and conditions for PCR amplification of random primers

Variable	Conditions/ Concentration	
	Evaluated	**Optimum**
PCR amplification		
Hot start (95°C)	2 min, 3 min, 5 min.	5 min
Denaturation (94°C)	30 sec, 1 min, 2 min.	1 min
Primer annealing (37°C)	1min, 1.5 min, 2.0 min, 3min	1 min
Extension (72°C)	1 min, 1.5 min, 2.0 min, 3 min	2 min
Number of cycles	35, 40, 45 cycles	40
RAPD Protocol		
Template DNA	10-15ng, 20-30ng, 30-50ng	30-50ng

4.5 RAPD analysis of *Photorhabdus luminescens* isolated from agro climatic zone 06 of Karnataka

The symbiotic bacterial cultures from agro climatic zone 06 of Karnataka which showed positive for physiological and biochemical tests as *P. luminescens* were tested with random primer and subjected to diversity analysis. 11 random primers screened, four random primers showed sharp intense banding pattern with good amplification were selected for the analysis.

4.5.1 RAPD characterization

A total of 33 bands produced from selected four primers were used for genetic diversity estimation among eleven isolates of *P. luminescens* bacteria. For the purpose of illustration, the RAPD fingerprints generated for eleven isolates using four primers are presented in Plates 14, 15, 16 and 17.

Number of bands scored for each primer varied from 7 to 11 with an average of 8.25 bands per primer. Out of 33 amplification bands, 3 bands (9.09%) were monomorphic, 6 bands (18.18%) were unique and 21 bands (63.64%) were shared polymorphic, which were informative in revealing the relationship among the bacterial isolates.

4.5.2 Cluster analysis of 11 isolates of *P. luminescens* bacteria

Cluster analysis based on 33 RAPD bands revealed that the 11 isolates of *P. luminescens* formed one major cluster in the dendogram (Fig. 1). This is subdivided into two sub cluster at similarity coefficient 0.25. In which strain 3 found distinct on similarity coefficient 1.00 at sub cluster one. Sub cluster two is further subdivided into two sub-sub cluster at similarity coefficient 0.70. Strain 11 is found distinct under the sub-sub cluster one. Sub-sub cluster two is again divided into two groups at similarity coefficient 0.90, in which strain 5 alone represent first group while second group is further divided into two groups at similarity coefficient 0.97. Under these two groups first group is represented by strain 1 and strain 2, while second group is represented by strain 7, strain 8, strain 9, and strain 10.

4.5.3 Principal component analysis (PCA)

To visualize the genetic relatedness among the 11 strains of *P. luminescens* isolated in detail principal component analysis (PCA) was done for 33 bands generated by 10 decamer random primers. The description of the data was done using three dimensions and the same is presented in Fig 2.

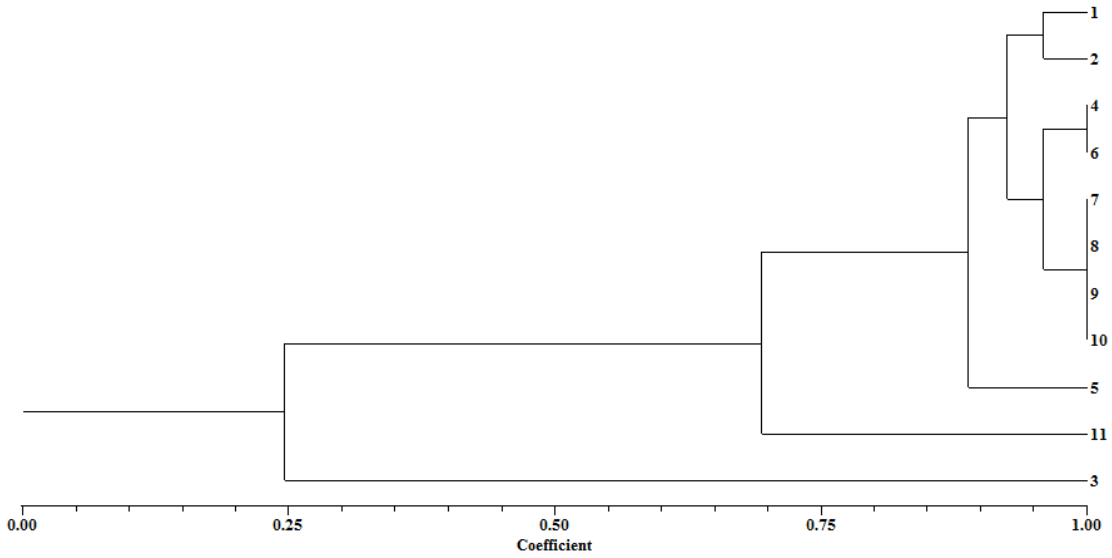


Fig. 1: Dendrogram based on RAPD profile of 11 *Photorhabdus luminescens* bacterial isolates

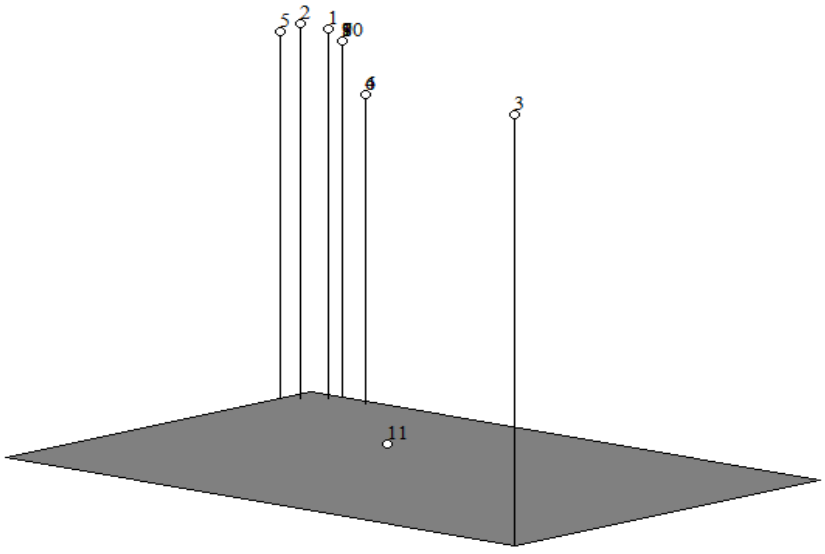


Fig. 2 Principal Component Analysis

(1) Strain 1, (2) Strain 2, (3) Strain 3, (4) Strain 4, (5) Strain 5, (6) Strain 6, (7) Strain 7, (8) Strain 8, (9) Strain 9, (10) Strain 10, (11) Strain 11

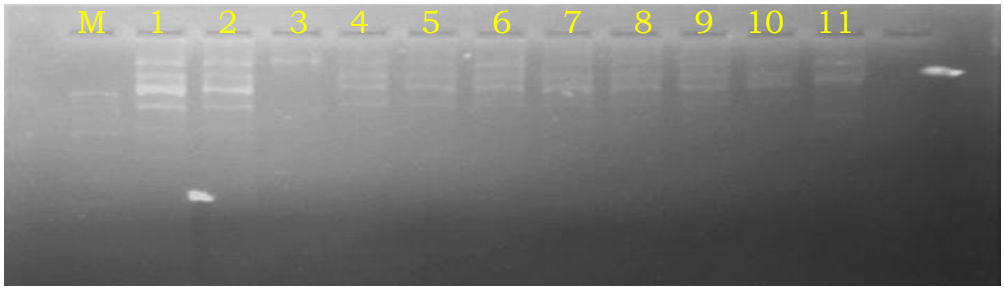


Plate 14: RAPD gel profile of symbiotic bacterial isolates generated using 10-mer random primer OPA 17.

Lane: M: marker, 1) Strain 1, 2) Strain 2, 3) Strain 3, 4) Strain 4, 5) Strain 5, 6) Strain 6, 7) Strain 7, 8) Strain 8, 9) Strain 9, 10) Strain 10, 11) Strain 11.

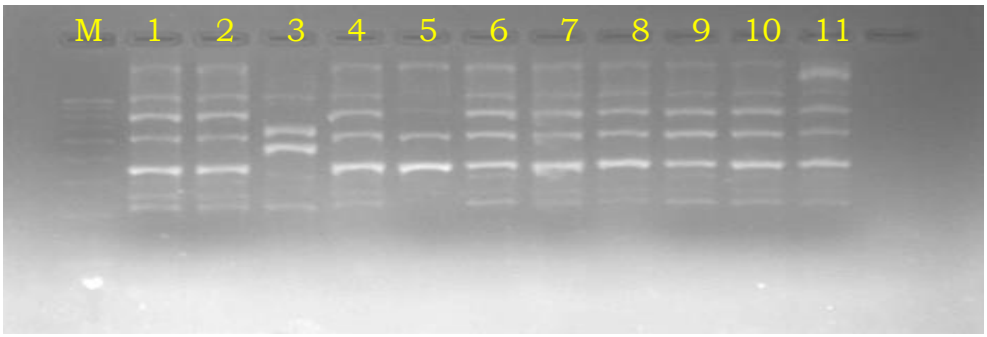


Plate 15: RAPD gel profile of symbiotic bacterial isolates generated using 10-mer random primer OPA 18.

Lane: M: marker, 1) Strain 1, 2) Strain 2, 3) Strain 3, 4) Strain 4, 5) Strain 5, 6) Strain 6, 7) Strain 7, 8) Strain 8, 9) Strain 9, 10) Strain 10, 11) Strain 11.

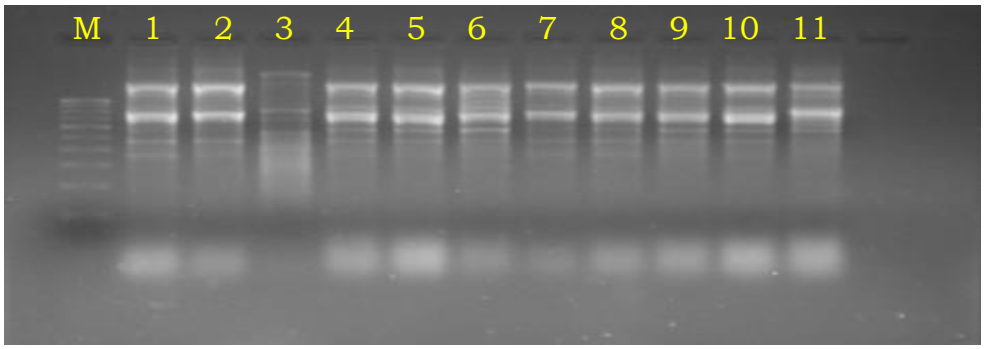


Plate 16: RAPD gel profile of symbiotic bacterial isolates generated using 10-mer random primer OPA 3

Lane: M: marker, 1) Strain 1, 2) Strain 2, 3) Strain 3, 4) Strain 4, 5) Strain 5, 6) Strain 6, 7) Strain 7, 8) Strain 8, 9) Strain 9, 10) Strain 10, 11) Strain 11.

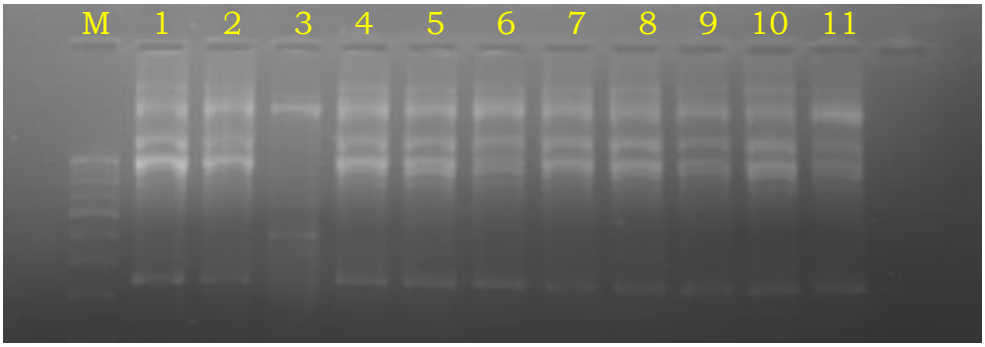


Plate 17: RAPD gel profile of symbiotic bacterial isolates generated using 10-mer random primer OPA 13.

Lane: M: marker, 1) Strain 1, 2) Strain 2, 3) Strain 3, 4) Strain 4, 5) Strain 5, 6) Strain 6, 7) Strain 7, 8) Strain 8, 9) Strain 9, 10) Strain 10, 11) Strain 11.

Table 17: Jaccard's similarity matrix of 11 isolates of *P. luminescens* strains based on RAPD analysis

	Strain1	**Strain2**	**Strain3**	**Strain4**	**Strain5**	**Strain6**	**Strain7**	**Strain8**	**Strain9**	**Strain10**	**Strain11**
Strain1	1.00										
Strain2	0.95	1.00									
Strain3	0.23	0.20	1.00								
Strain4	0.92	0.88	0.27	1.00							
Strain5	0.87	0.83	0.17	0.87	1.00						
Strain6	0.92	0.88	0.27	1.00	0.87	1.00					
Strain7	0.95	0.91	0.24	0.95	0.91	0.95	1.00				
Strain8	0.95	0.91	0.24	0.95	0.91	0.95	1.00	1.00			
Strain9	0.95	0.91	0.24	0.95	0.91	0.95	1.00	1.00	1.00		
Strain10	0.95	0.91	0.24	0.95	0.91	0.95	1.00	1.00	1.00	1.00	
Strain11	0.67	0.64	0.33	0.74	0.62	0.74	0.70	0.70	0.70	0.70	1.00

V. DISCUSSION

Entomopathogenic nematodes could be used in integrated pest management approaches to bring together all available insect pests control options. The bacterium *Photorhabdus luminescens* symbiotically lives within entomopathogenic nematodes and shows mutualistic relation with entomophagous nematodes. *Photorhabdus luminescens* secretes high-molecular-weight toxin complexes following its release into the insect hemocoel upon nematode invasion. The toxin complex has both oral and injectable activities against a wide range of insects (Blackburn *et al.,* 1998).

The biocontrol activity of *Photorhabdus luminescens* determines the ability of its complex toxin to kill specific insect pests. The morbidity rate of the insect pests goes increasing while steady increase in the concentration of the *Photorhabdus luminescens* which shows its potency to control the insect pests and it has been proved economically very beneficial in the integrated pest management of the crops.

The characterization of the different strains of *Photorhabdus luminescens* gives an idea about the genetic similarity and dissimilarity between the strains isolated from the soils of different locations. Further diversity analysis is especially imperative in evolutionary relationship of the strains.

A symbiotic bacterium is one of the best alternates to biological control of insect pests unlike biological pesticides for instance *Bacillus thuringiensis* because the widespread use of the Bt toxin leads to resistance among insect pests. The genome of the *Photorhabdus luminescens* with its many insect toxins provides some ways. Sometimes it is good to have toxins from two organisms, because if one develops resistance then another will be useful to control the insect pests (Kumar *et al*., 2011).

The results of the experiments conducted for the present study are discussed below under the following headings:

5.1 Isolation and identification of *Photorhabdus luminescens.*

5.2 Bio-control activity of *Photorhabdus luminescens* against insect pest *G. mellonella* and *P. xylostella*.

5.3 Molecular diversity analysis of *Photorhabdus luminescens* isolates using RAPD marker.

5.1 Isolation and identification of *Photorhabdus luminescens*

P. luminescens was isolated by streaking the haemolymph of the dead larva of *Galleria* on nutrient agar and NBTA plates. Similar kinds of results were reported by Kumar *et al.* (2014). Identification was done based on gram staining, colony morphology formed in different growth media and different types of biochemical tests discussed below.

Microscopic observations of symbiotic bacterial isolates were further examined for their Gram's reaction and shape. All the isolates were found Gram negative and rod shaped long to medium rod shaped, gram negative, motile, bioluminescent bacterium. (Rajagopal and Bhatnagar, 2002; Martin *et al.*, 2004; Kumar *et al.*, 2014).

On NBTA agar, the bacteria formed greenish colonies (Rajagopal and Bhatnagar 2002) and on MacConkey agar all the symbiotic bacterial isolates absorbed neutral red and formed bright-pink to red-colored, convex, opaque colonies (Boemare and Akhurst, 1988; Saux *et al.*, 1999). On nutrient agar media all symbiotic bacterial isolates formed characteristic buff or cream coloured colonies. Similar kind of report of *P. luminescens* colony morphology (Nagesh *et al*, 2002) was smooth, small, circular, and glistering, convex with entire margin and white with shiny in colour.

These bacteria were further identified based on biochemical characters like Gelatin liquefaction, motility test, Urease test, Lactose fermentation test as given in the results.

All isolates were found positive to gelatin liquefaction as indicated by the production of clear zone after flooding of *P. luminescens* spotted gelatin agar plates with 12 % $HgCl_2$ solution for 20 minutes (Marokházi *et al.*, 2004). Kumar *et al.*, (2014) reported positive for motility test for all isolates indicated by the appearance of dispersed red colour growth in the motility test medium. All isolates were found positive for urease test as indicated by the change in colour from yellow to red (An and Grewal 2010).

All bacterial isolates were found positive for lactose fermentation test indicated by the change in colour of the medium to yellow and appearance of gas bubbles in the Durham's tube (Kumar *et al.*, 2011).

5.2 Bio-control activity of *Photorhabdus luminescens* against insect pests

Highest mortality of *G. mellonella* was recorded by strain 10 with a dose of 1×10^7 cells/ml ($T_{5)}$, 63.33% and 100.00% at 24 h. and 40 h. after treatment respectively (Fig.3, Fig. 4). Highest mortality of *P. xylostella* was recorded by strain 10 at dose of 1×10^7 cells/ml (T_5) (60.00% and 100.00% at 24 h. and 40 h. after treatment respectively) (Fig.5 and Fig. 6).

Among all the 11 isolates strain 10 shown lowest LD_{50} values for both the insect pests under the present study i.e. 0.2403×10^6 for *G. mellonella* and 0.2541×10^6 for *P. xylostella* (Fig. 7 to 12).

Abdel-Razek (2003) reported relationship between *Xenorhabdus nematophilus* and *Photorhabdus luminescens* against diamondback moth, *Plutella xylostella* pupae and showed pathogenic capability of *P. luminescens* over that of *X. nematophilus*. He reported 60 and 40% mortality with LD_{50} values of 5×10^5 and 5.5×10^5 cells/ml respectively.

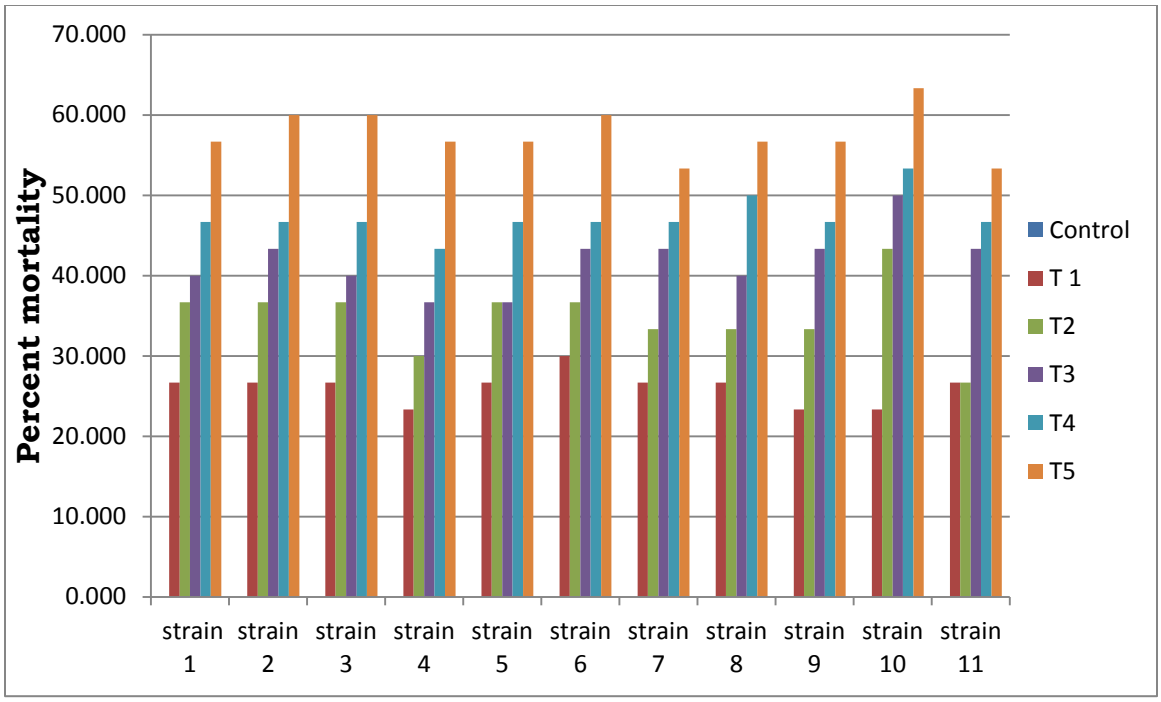


Fig. 3: Percentage mortality of *G. mellonella* larvae to different dosage of *P. luminescens* at 24 h. after treatment

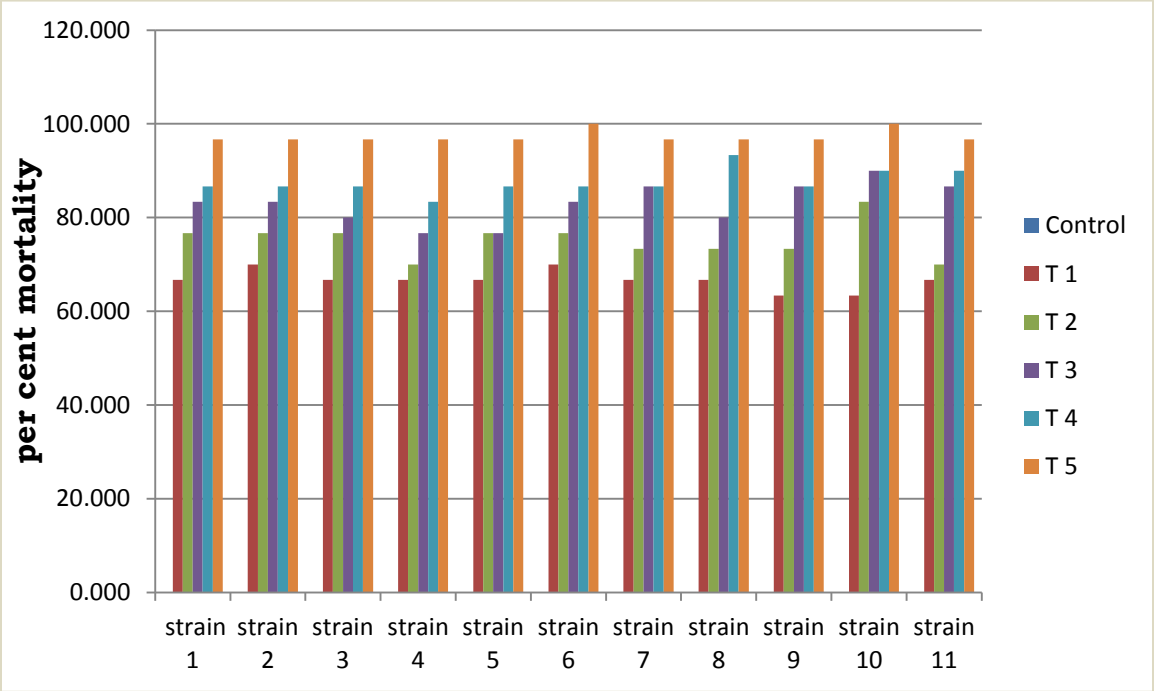


Fig. 4: Percentage mortality of *G. mellonella* to different dosage of *P. luminescens* at 40 h. after treatment

Control 0.00, T1 (1×10^3), T2 (1×10^4), T3 (1×10^5), T4 (1×10^6), T5 (1×10^7) cells/ml.

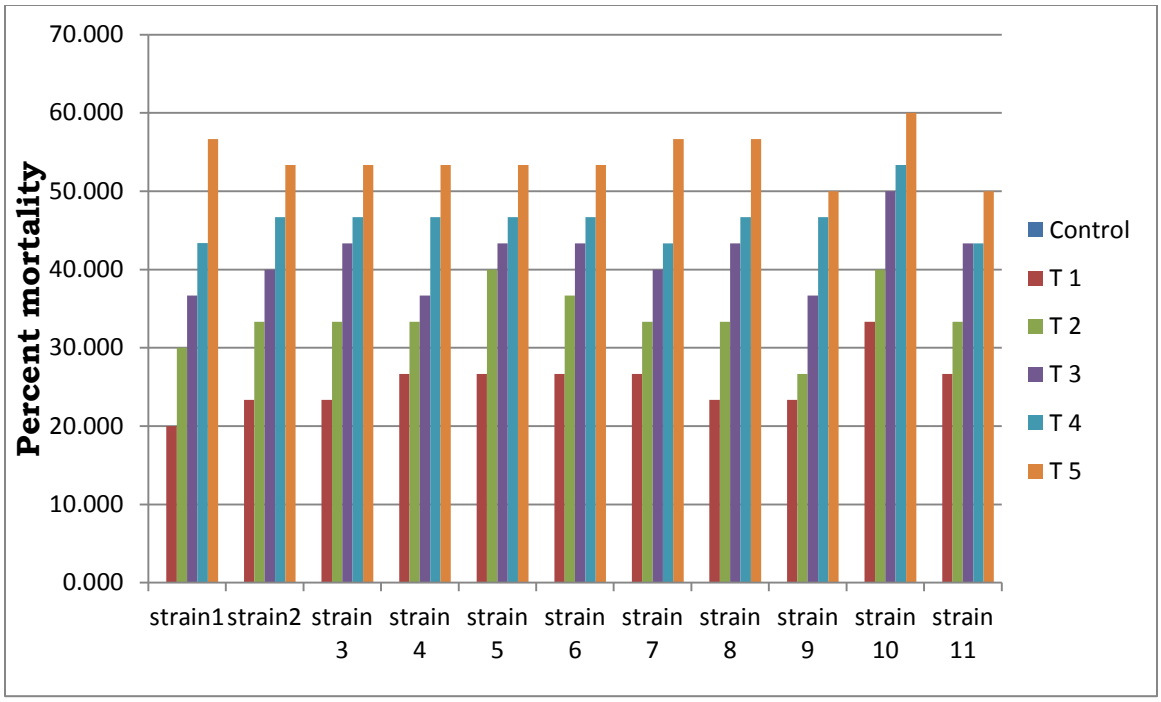


Fig. 5: Percentage mortality of *P. xylostella* larvae to different dosage of *P. luminescens* at 24 h. after treatment

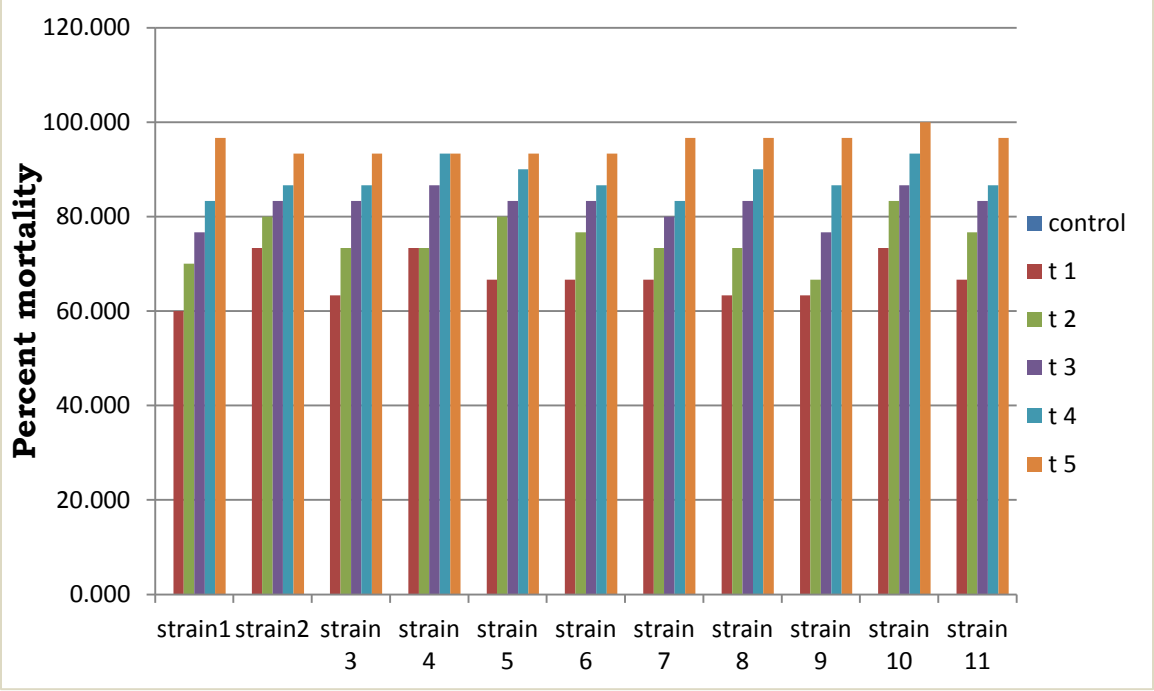


Fig. 6: Percentage mortality of *P. xylostella* larvae to different dosage of *P. luminescens* at 40 h. after treatment

Control 0.00, T1 (1×10^3), T2 (1×10^4), T3 (1×10^5), T4 (1×10^6), T5 (1×10^7) cells/ml.

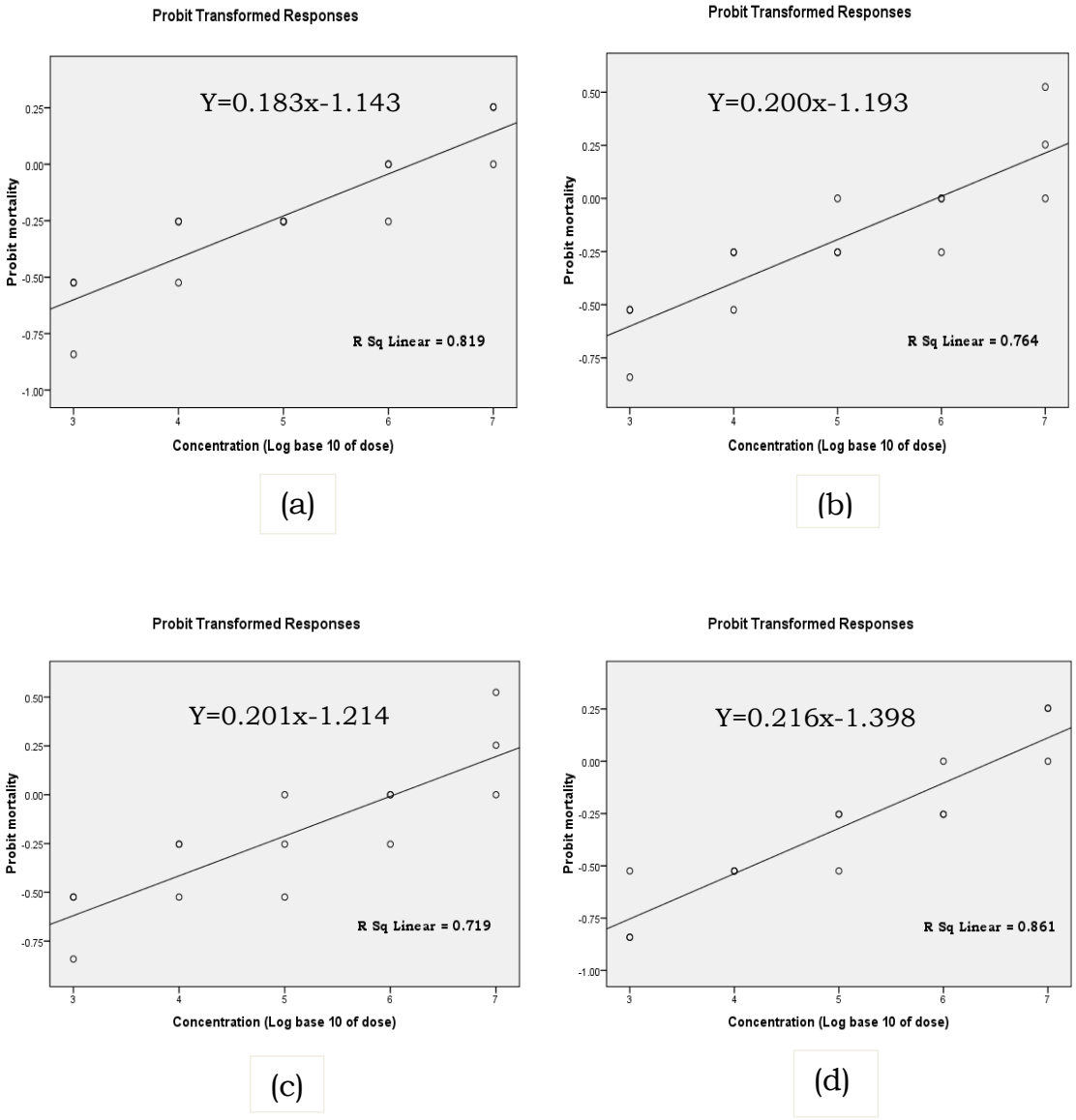


Fig. 7: Concentration mortality regression line for larvae of *G. mellonella* treated with different doses of *P. luminescens* bacterial isolates Strain 1 (a), Strain 2 (b), Strain 3 (c) and Strain 4 (d).

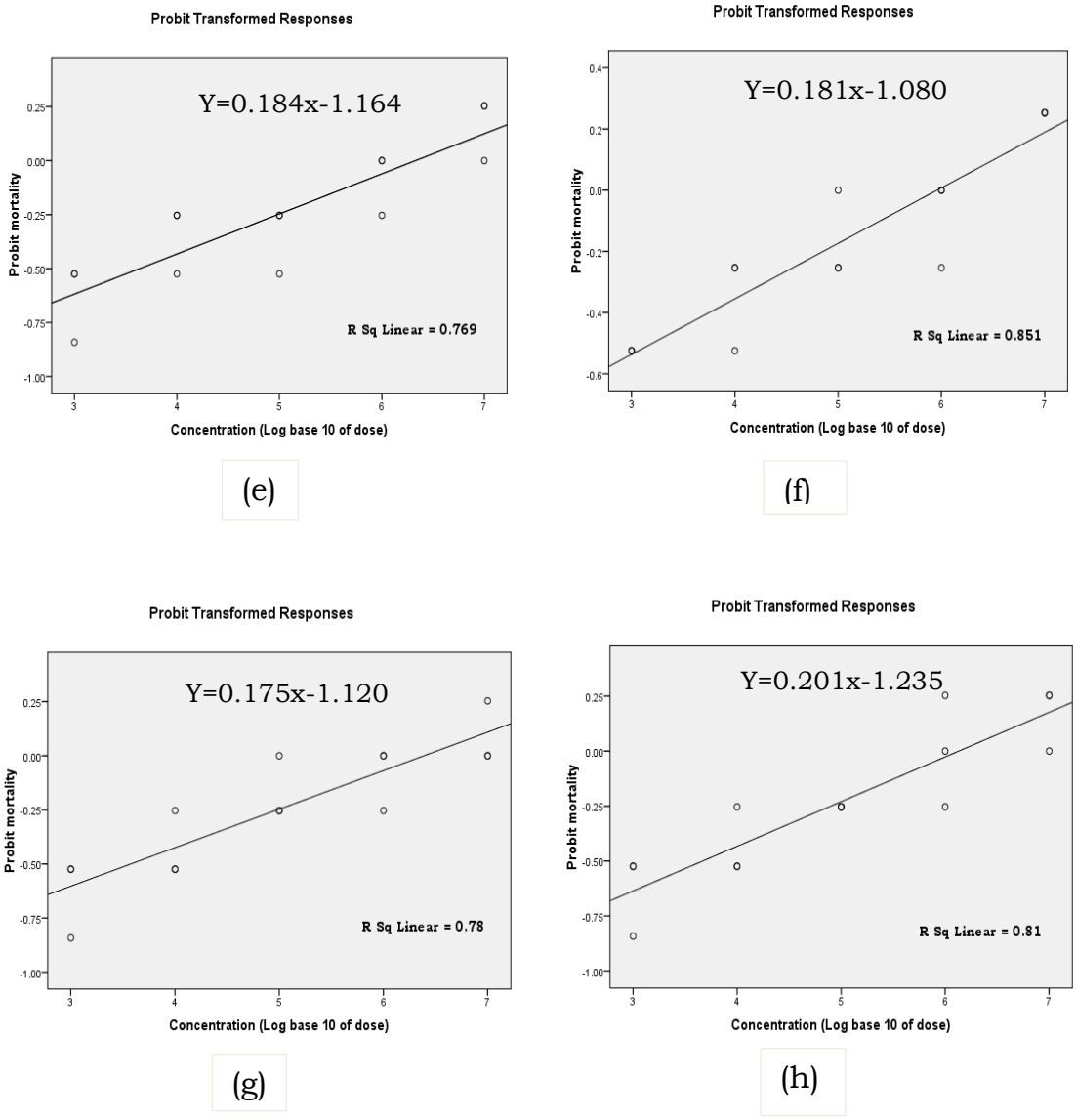


Fig. 8: Concentration mortality regression line for larvae of *G. mellonella* treated with different doses of *P. luminescens* bacterial isolates Strain 5 (e), Strain 6 (f), Strain 7 (g) and Strain 8 (h).

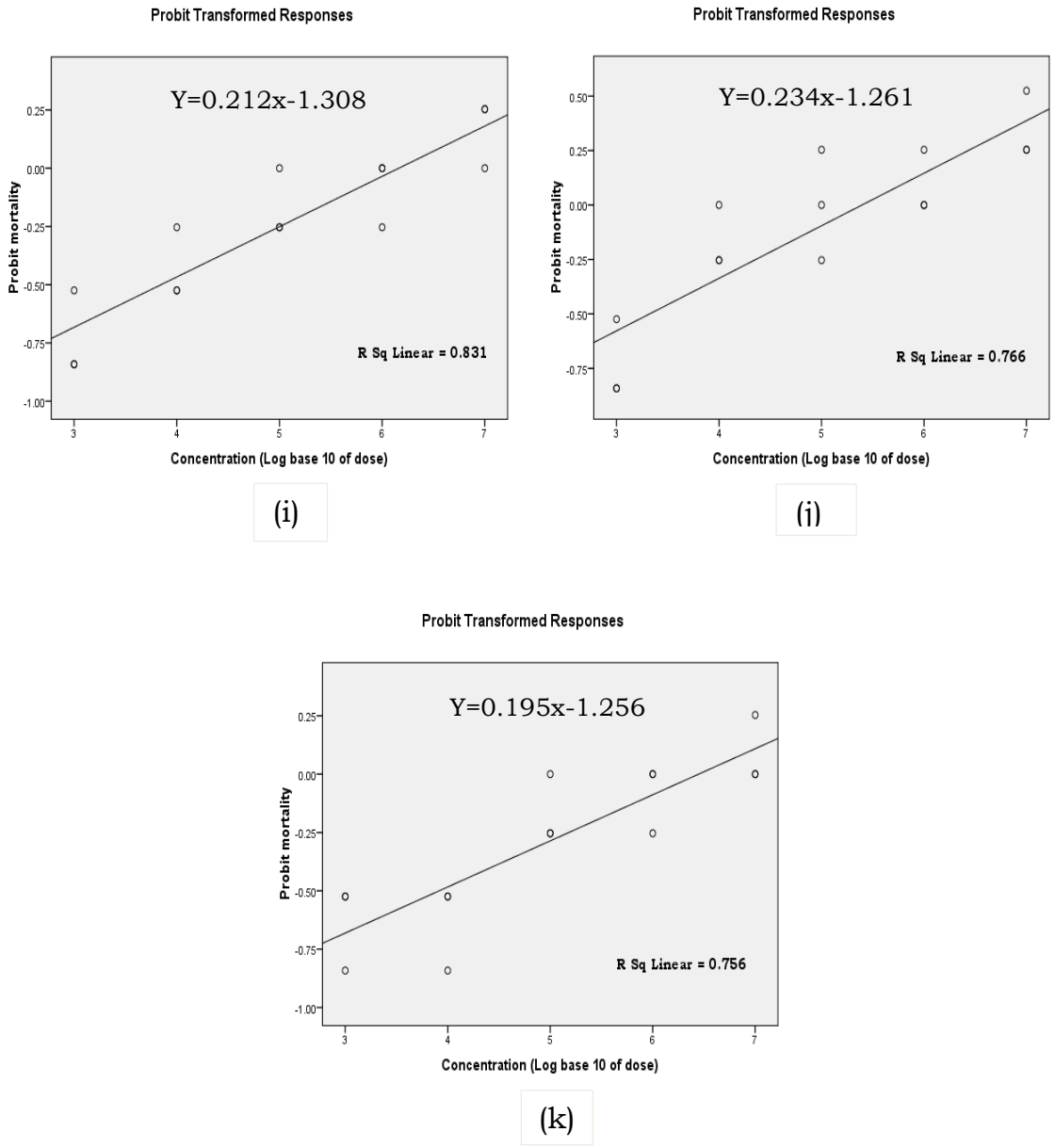


Fig. 9: Concentration mortality regression line for larvae of *G. mellonella* treated with different doses of *P. luminescens* bacterial isolates Strain 9 (i), Strain 10 (j) and Strain 11 (k)

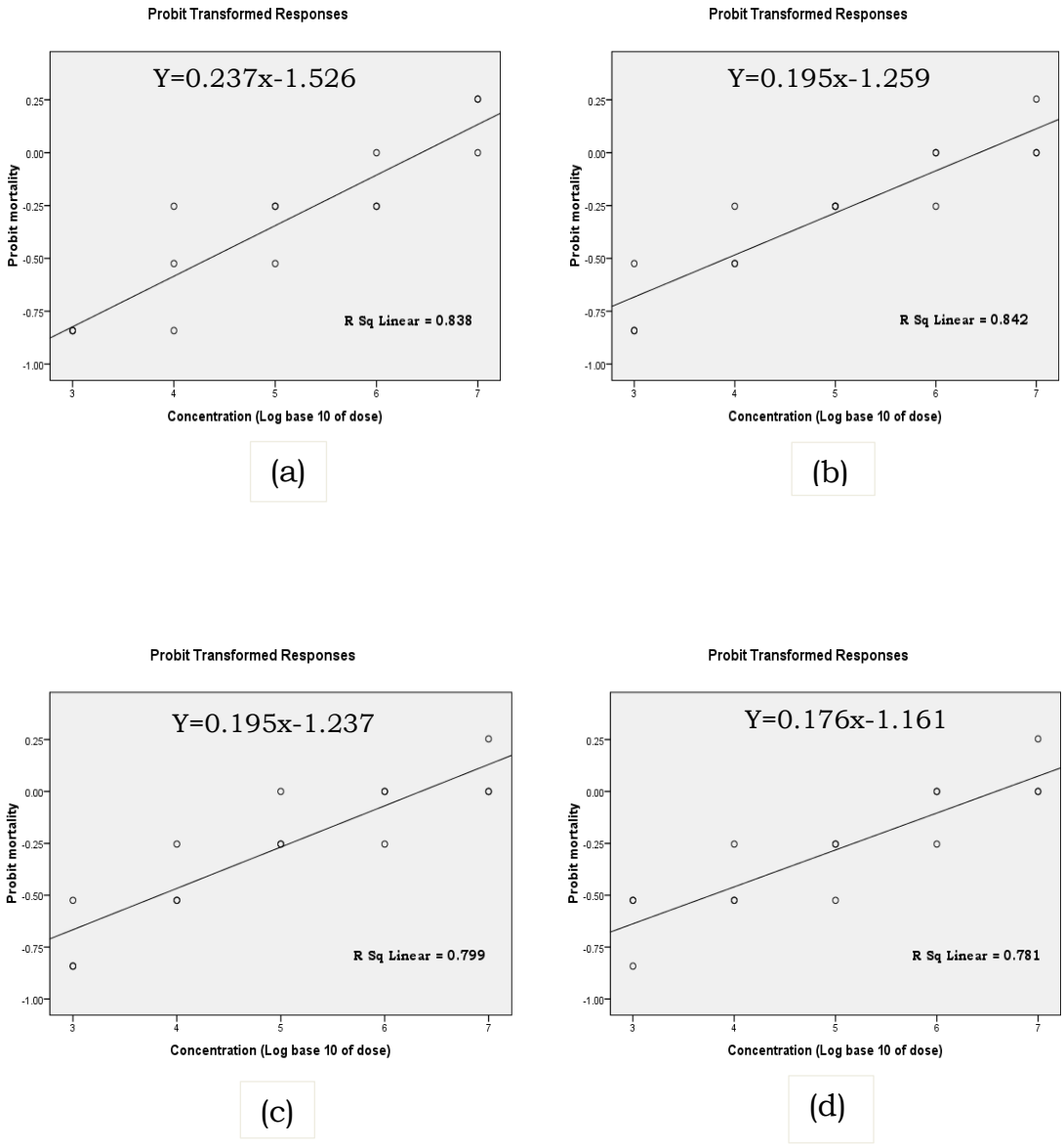


Fig. 10: Concentration mortality regression line for larvae of *P. xylostella* treated with different doses of *P. luminescens* bacterial isolates Strain 1 (a), Strain 2 (b), Strain 3 (c) and Strain 4 (d).

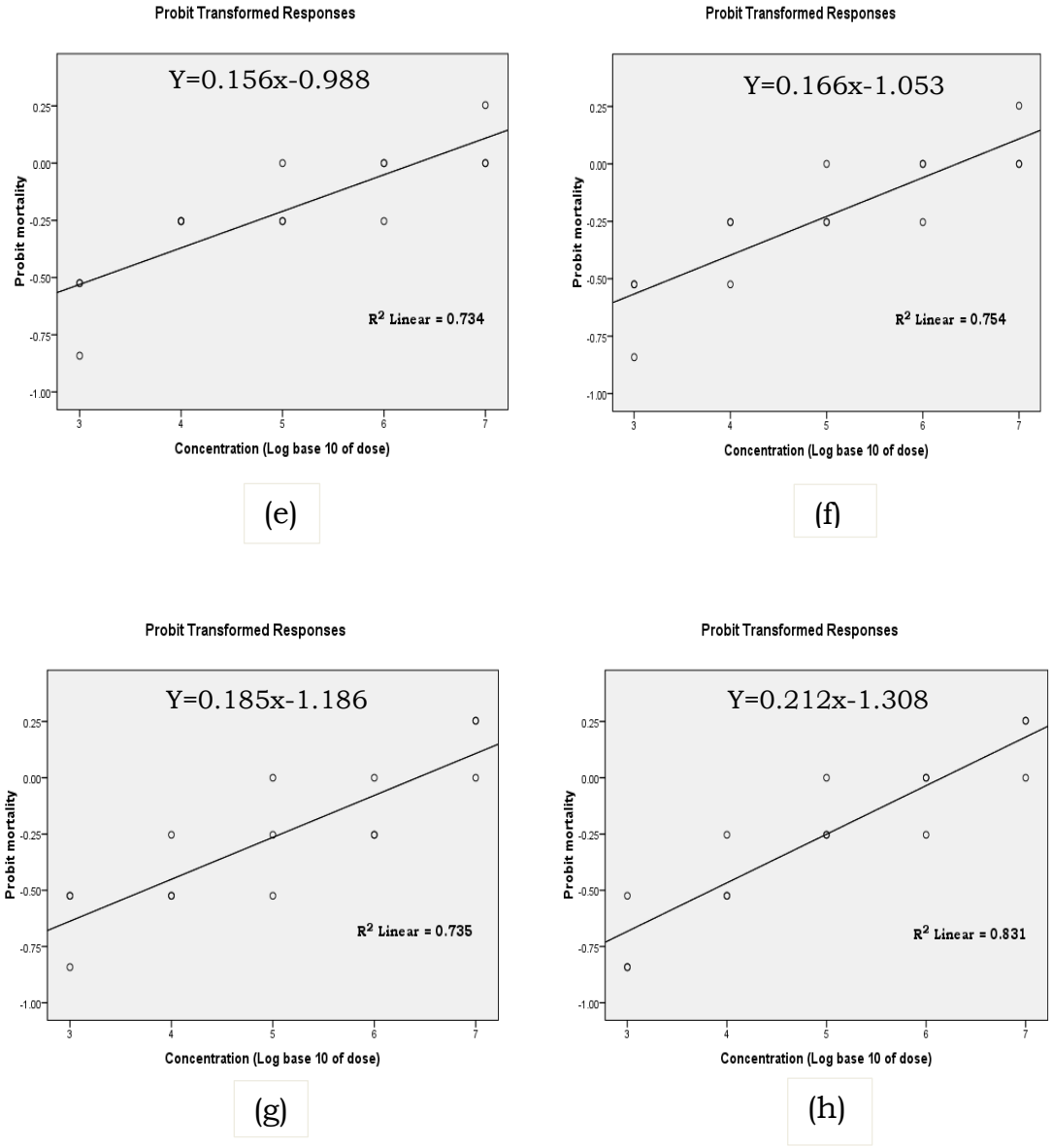


Fig. 11: Concentration mortality regression line for larvae of *P. xylostella* treated with different doses of *P. luminescens* bacterial isolates Strain 5 (e), Strain 6 (f), Strain 7 (g) and Strain 8 (h).

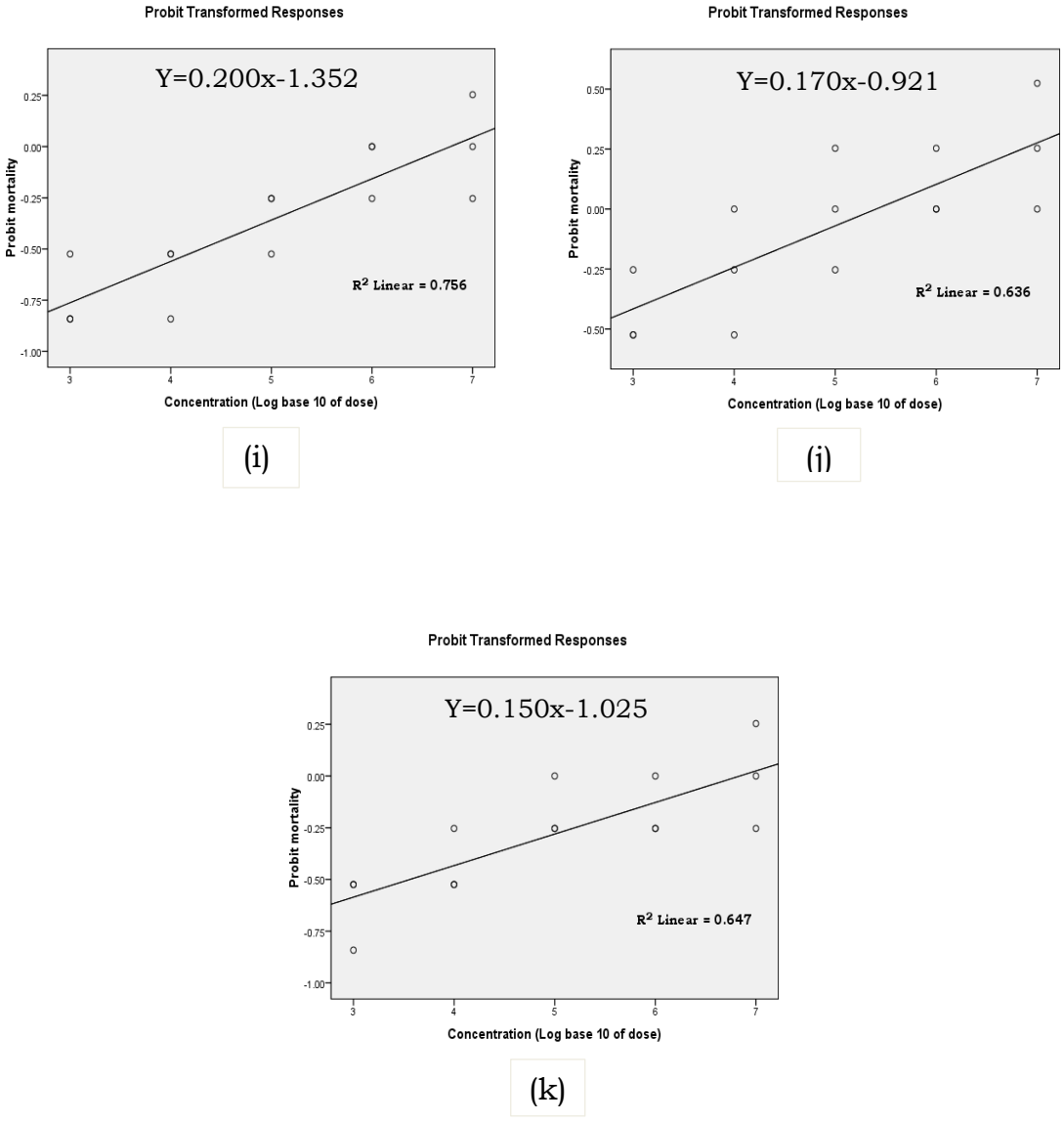


Fig. 12: Concentration mortality regression line for larvae of *P. xylostella* treated with different doses of *P. luminescens* bacterial isolates Strain 9 (i), Strain 10 (j) and Strain 11 (k).

Ansari *et al.* (2003) reported pathogenicity study of the symbiotic bacteria *Photorhabdus luminescens*. Three microliters of a bacterial suspension (containing 0, 25, 250, 2500, or 25,000 cells) were injected into the hemocoel of third instar of *H. philanthus* and last instar *Galleria mellonella*. *P. luminescens* killed 100% of the larvae of both species after 72 h.

Shahina *et al.* (2011) reported similar pathogenicity of *P. luminescens*. *P. luminescens* suspension in broth applied at different rates caused 95 and 98% mortality of *G. mellonella* and *Macrotermes spp*., respectively and the LD_{50} value of bacterial doses was 190.4, 27.4 bacterial cell/ml respectively.

5.3 Molecular diversity analysis of *Photorhabdus luminescens* isolates using RAPD marker

Cluster analysis based on 33 RAPD bands revealed that the 11 isolates of *P. luminescens* formed one major cluster in the dendogram (Fig. 11). This is subdivided into two sub cluster at similarity coefficient 0.25, in which strain 3 found distinct on similarity coefficient 1.00 at sub cluster one. Sub cluster two is further subdivided into two sub-sub cluster at similarity coefficient 0.70. Strain 11 is found distinct under the sub-sub cluster one. Sub-sub cluster two is again divided into two groups at similarity coefficient 0.90, in which strain 5 alone represent first group while second group is further divided into two groups at similarity coefficient 0.97. Under these two groups first group is represented by strain 1 and strain 2, while second group is represented by strain 7, strain 8, strain 9, and strain 10.

Random amplified polymorphic DNA (RAPD) was used to differentiate a total of 38 strains of entomopathogenic fungi isolated from 20 geographic regions of Taiwan and mainland China. Fungal isolates were obtained from 15 insect species. Banding patterns were generated from 3 selected primers (OPM 12, 18, and 20). Isolates were grouped into 10 clusters according to similarity, following cluster analysis using Jeffrey's coefficients. Three distinct genotypes were observed among the 38 isolates tested. On the basis of RAPD patterns, 2 genera and 1 species were recognized, namely Beauveria, *Metarhizium anisopliae* var. *anisopliae*, and *Nomuraea*. Nomuraea exhibited a more conservative banding pattern than each of the other genera. RAPD markers may be useful as identification biomarkers of specific bio control strains in a limited geographical area (Kao *et al.,* 2002).

Gajbhiye *et al.* (2007) reported evaluation of genetic diversity of *Bacillus* isolates using Randomly Amplified Polymorphic DNA (RAPD) molecular marker. They used 20 isolates for diversity study with 20 random primers. Out of 20 primers 18 were found polymorphic. They analysed RAPD gel profiles with the help of NTSYSpc programme and dendogram was generated. They found five major clusters at 61% similarity level.

VI. SUMMARY

Photorhabdus is entomopathogenic bacteria that are symbiotically associated with entomopathogenic nematode of the family Heterorhabditidae. This bacterium is found to be effective biocontrol agent against many insect pests.

The present study was carried out to isolate and identify symbiotic bacteria *Photorhabdus luminescens* associated with entomopathogenic nematodes from agro climatic zone 06 of Karnataka, bio-control activity of *Photorhabdus luminescens* against insect crop pest Diamondback moth (*Plutella xylostella*) and honey bee pest and model bio control testing insect greater wax moth (*Galleria mellonella*) and molecular diversity analysis of *Photorhabdus luminescens* isolates using RAPD marker was carried out in Department of Plant Biotechnology, University of Agricultural Sciences, GKVK campus, Bangalore with the objectives:

1. Isolation and identification of *Photorhabdus luminescens* from soils collected from V C farm, Mandya (agro climatic Zone-6 of Karnataka).
2. Bio-control activity of *Photorhabdus luminescens* against insect pest *G. mellonella* and *P. xylostella*.
3. Molecular diversity analysis of *Photorhabdus luminescens* isolates using RAPD marker.

Keeping in view of the above objectives, collection of EPNs was done by placing *G. mellonella* baited traps in soils of different crop fields of V C farm, Mandya (agro climatic zone 06 of Karnataka). The traps were harvested at 4^{th}, 7^{th} and 12^{th} day after placing the traps in the soils collected. The total percentage mortality of *G. mellonella* larvae to EPNs was recorded as follows. maize field 6%, ragi field 8%, upland rice 14%, cowpea field 6%, non-cultivated land 4%, sugarcane 2%. A total of twenty nematode cultures were harvested.

Eleven strains of symbiotic bacteria were isolated from the nematodes collected. The bacterial strains were identified as *P. luminescens* through a number of microbiological and biochemical tests such as colony morphology in different growth medium like NBTA, MacConkey, and Nutrient Agar. Gram-staining shows negative and microscopic observation shows rod shaped flagellated motile bacteria. Biochemical tests such as Gelatin liquefaction test, Urease test, Motility test and Lactose fermentation test confirmed identity of symbiotic bacteria as *P. luminescens*.

Bio control activity of insect pest was carried out using *P. xylostella* and *G. mellonella* to different concentrations of *P. luminescens* viz. 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 cells per mililitre. The mean per cent mortality induced by the 11 isolates of *P. luminescens* for varied dose was tested using Duncan's Multiple Range Test (DMRT) followed by F test and found to be significantly different ($P<0.05$) for the 11 isolates of *P. luminescens.* Probit analysis using software SPSS 17.0 showed varied median lethal dose among 11 isolates. Strain 10 showed least LD_{50} value of 0.2403×10^6 and 0.2541×10^6 against *G. mellonella* and *P. xylostella* respectively. Strain 4 showed highest value for

LD_{50} of 3.027×10^6 cells per ml against *G. mellonella.* Strain 11 showed highest value for LD_{50} of 7.076×10^6 cells per ml against *P. xylostella.* These findings suggest toxic activity vary among different isolates of *P. luminescens*. Toxic activity also varies against different insect pests.

Molecular diversity of 11 isolates *Photorhabdus luminescens* using RAPD marker showed all the strains formed a major cluster and strain 3 was found distinct at similarity coefficient 1.00 in sub cluster one. Strain 11 also showed distinctness in sub cluster two. All other strains showed relativeness in the dendogram. The less variability among strains in this study is probably due to sampling from sites close to each other with same type of agro climatic condition. No correlation has been found between strain distinctness and killing ability. However, strain variation was observed between the strains collected in soils from different crop fields and uncultivated land.

Future line of work

- Isolation and identification of *P. luminescens* bacteria from diverse area of the country with high insect bio control activity.
- Biochemical and molecular characterization of the toxin genes.
- Development of quick and mass culture procedure *in vitro* for *P. luminescens*.
- Evaluation of symbiotic bacterial culture against more number of insect pests of diverse habitat.
- How insect pathogenicity varies whether due to difference in immune response in different insect pest or simply feeding and foraging behavior difference.
- Evaluation of toxic activity in green house as well as field condition of effective strains.
- Developing strategy for incorporation of insect bio control activity of *P. luminescens* into integrated pest management system.

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APPENDIX- I

Composition of different media:

NBTA medium

Nutrient agar	37 g
Bromothymol blue	25 mg
1% Tetrazolium chloride	4 ml
Distilled water	1000 ml

MacConkey agar medium

Peptic digest animal tissue	20 g
Agar	15 g
Lactose	10 g
Sodium chloride	5 g
Bile salt	1.5 g
Neutral red	0.05 g
Crystal violet	0.001 g
pH	7.2
Distilled water	1000 ml

Nutrient agar (NA)

Peptone	5 g
Beef extract	3 g
Sodium chloride	5 g
Glucose	5 g
Agar-agar	17 g
Distilled water	1000 ml

Gelatin Agar Medium

Peptone	5 g
Beef extract	3 g
Gelatin	30 g
Agar	15 g
Distilled water	1000 ml

Lactose fermentation broth medium

Peptone	10 g
Sodium chloride	5 g
Meat extract	3 g
Andrade's indicator	10 ml

Distilled water	1000 ml
Adjust pH to	7.1

(Andrade's indicator: add 0.5 gm of Acid fuchsin to 16 ml of 1N NaoH and makeup the volume to 100 ml by adding distilled water).

Urease test medium

Peptone	1 g
Glucose	1 g
Sodium chloride	5 g
Monobasic potassium phosphate (KH_2PO_4)	2 g
Phenol red	0.012 g
Adjust pH to	7.0

Add 100ml of 20% urea (separately sterilized)
Adjust final volume to 1000ml

Motility test medium

Beef extract	3 g
Peptone	10 g
Nacl	5 g
Agar	4 g
Distilled water	1000 ml
Adjust pH to	7.4
TTC	0.5 gm (Add after autoclaving)

Gram staining

i) Prepare smear on a clean dry slide

ii) Air dry and heat fix the smear

iii) Stain with crystal violet for 30 seconds.

iv) Rinse with water.

v) Add Grams iodine and leave it for 30 seconds.

vi) Rinse with water.

vii) Decolourise with 95 percent alcohol. (Dip the slide in jars containing alcohol for 10-20 seconds). Rinse with water.

viii) Counter stain with safronin for 20 –30 seconds, air dry observe under oil immersion Gram –ve (red cells) Gram + ve (violet cells).

APPENDIX- II

Preparation of DNA Extraction Reagents

i) Cetyl-Trimethyl Ammonium Bromide (CTAB) / NaCl solution:

4.1 g of NaCl was dissolved in 80 ml of water to which 10 g of CTAB was added slowly, in order to increase the rate of dissolving it was heated to 65°C. Finally the volume was made up to 100 ml by adding sterile distilled water.

ii) NaCl (5M)

The stock solution of NaCl was prepared by dissolving 29.2 g salt in 80 ml water. Volume was adjusted to 100 ml with water and later it was sterilized by autoclaving and stored at room temperature.

iii) Tris base (1M, pH 8.0)

The stock solution of Tris base was prepared by dissolving 11 g of Tris base in 80 ml of water then the pH was adjusted to 8.0 with concentrated HCl. It was sterilized by autoclaving and stored at room temperature.

iv) EDTA (0.5M)

EDTA of 18.61 g was added in 80 ml water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaoH pellets and then it was sterilized by autoclaving and stored at room temperature.

v) Chloroform: Isoamyl alcohol (24:1 v/v)

Chloroform of 96 ml was mixed with 4 ml of isoamyl alcohol and stored under refrigerator conditions.

vi) Phenol: chloroform: Isoamyl alcohol (25:24:1 v/v)

Equilibrated phenol of 50 ml was mixed with 48 ml of chloroform and 2 ml of isoamyl alcohol and stored at 4°C.

vii) TE (Tris EDTA pH 8.0) buffer

Tris base (IM, pH 8.0) of 1 ml was mixed with 0.2 ml of EDTA (0.5 M, pH 8.0) and finally the volume was adjusted to 100ml with sterile water and finally this solution was sterilized by autoclaving.

viii) RNAase

RNAase of 10 mg was dissolved in 1 ml of Tris EDTA buffer (pH 7.8) and it was heated to near boiling point. This was finally stored under freezing temperature.

ix) TBE (5X, pH 8.0)

Tris base of 27 g and 13.75 g of boric acid were dissolved in 200 ml of sterile water to which 10 ml of EDTA (0.5 M) was added and finally volume was adjusted to 500 ml by adding sterile water.

x) Ethidium Bromide (100mg /ml)

Ethidium bromide of 1 g was added to 100 ml of sterile water and was stirred using magnetic stirrer until the dissolution of dye. Later the container was wrapped in aluminum foil and stored at room temperature.

xi) 70 Per cent ethanol

Absolute alcohol of 70 ml was mixed with 30 ml of distilled water and it was stored at room temperature.