MANAGEMENT OF MAJOR CHEWING PESTS, *Henosepilachna septima* (Dieke) AND *Diaphania indica* (Saund) INFESTING BITTER GOURD WITH BACTERIAL BIOAGENTS

by

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DECLARATION

I, hereby declare that this thesis entitled **"MANAGEMENT OF MAJOR CHEWING PESTS, *Henosepilachna septima* (Dieke) AND *Diaphania indica* (Saund) INFESTING BITTER GOURD WITH BACTERIAL BIOAGENTS"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

^{0}C	Degree Celsius
%	Per cent
cm	Centimetre
cl	Microlitre
DAT	Days after treatment
m^2	Metre square
et al.	And others
Fig.	Figure
KAU	Kerala Agricultural University
No.	Number
NS	Non significant
Sl.	Serial
sp or spp.	Species (Singular and Plural)
viz.	Namely
Kg	Kilogram
g	Gram
µg	Micro gram
nm	Nano meter
cfu	Colony forming units

M	Molar
mM	Milli molar
ng	Nano gram
rpm	Rotations per minute
ml	Millilitre
CD	Critical difference
SC	Suspension concentrate
EC	Emulsifiable concentrate
OD	Optical density
h	Hours
@	At the rate of
ha	Hectares

Introduction

1. INTRODUCTION

Momordica charantia (L.), commonly known as bitter gourd or bitter melon is an important cucurbitaceous vegetable crop grown all through the tropics. Bitter gourd fruits are high in nutrients such as beta-carotene, vitamin C, folic acid, magnesium, phosphorus and potassium (Wills *et al.*, 1984). Besides, it has immense medicinal properties owing to the presence of certain phytochemicals that possess antibiotic, antimutagenic, antioxidant, antiviral, antidiabetic and immune enhancing properties (Grover and Yadav, 2004). India is the largest producer of bitter gourd with a production of 9.4 lakh tons from 0.83 lakh ha (NHB, 2014) using approximately 350 tons of seeds (Dhillon *et al.*, 2016).

Bitter gourd is infested by a large number of pests that curtail the production and productivity of the crop substantially. Various chewing pests *viz.* red pumpkin beetle, *Aulacophora foveicollis* (Dejean), epilachna beetle, *Henosepilachna septima,* pumpkin caterpillar, *Diaphania indica* feed on the foliage causing considerable leaf area reduction affecting the photosynthetic efficiency of the crop (Rai *et al.,* 2014)

Epilachna beetle, *H. septima* and pumpkin caterpillar, *D. indica* are two major phytophagous foliage feeding insects infesting cucurbitaceous crops including bitter gourd in India. The adults and grubs of epilachna beetle chew the epidermal layers of leaves leading to skeletonisation, causing 35 -75 per cent leaf injury in infested crop (Srivastava and Katiyar, 1972). In addition, stem and fruit infestation are also reported in bitter gourd (Singh *et al.*, 2010).

The larvae of the lepidopteran pest *D. indica* feed on leaves of bitter gourd. Fruit infestation ranging from 3-14 per cent was also noticed, wherein the larvae scrape the epicarp in early instars and bore the base in later instars (Singh and Naik, 2006). Extensive feeding by these two pests on leaves causes defoliation, resulting in reduction in total photosynthetic area and considerable decline in fruit yield. Fruit infestation lowers the consumer preference leading to reduction in market value of produce.

To mitigate the crop loss by these pests, farmers often resort to frequent application of chemical pesticides in large quantities. Inspite of heavy insecticide application, the crop loss increases due to various reasons like development of resistance, pest resurgence and pest replacement besides negatively influencing environment and human health by way of leaving toxic residues. Hence there is a need to evolve eco friendly management strategies.

Microbial control utilizing beneficial micro organisms is an attractive option for the management of foliage feeding insects that helps to reduce the pesticide load on vegetables. Pathogenic microorganisms that cause diseases in insects are used for the biocontrol of many chewing pests including both epilachna beetle (Rajendran and Gopalan, 1999; Markandeya *et al.*, 2001) and pumpkin caterpillar (Neema *et al.*, 2010).

Besides entomopathogens, certain plant colonising bacteria that live on the nutrients obtained from plants are found to have pest control capability. These bacteria are called by different names depending on the sites they inhabit such as rhizosphere bacteria that survive within and surrounding the plant root zone, endophytic bacteria that are found residing the interior of plants without causing disease and phylloplane bacteria which are epiphytic surface dwellers of the host plant.

Phylloplane bacteria survive on the leaf surfaces colonizing on sites such as trichomes, stomata and epidermal cell wall junctions (Beattie and Lindow, 1995). Phytophagous epilachna beetles can be biologically controlled by chitinase secreting strain KPM-012A of *Alcaligenes paradoxus* (Castellani and Chalmers), isolated from tomato phylloplane which caused the degradation of peritrophic membrane in *Henosepilachna vigintioctopunctata* (Fabricus) (Otsu *et al.*, 2003). Phylloplane bacteria are also reported to reduce feeding and induce mortality of phytophagous insects (Otsu *et al.*, 2004). Aswathy (2015) isolated two entomopathogenic bacteria *Serratia marcescens* (Bizio) and *Pseudomonas aeruginosa* (Schroter) and a

phylloplane bacterium *Pseudomonas fluorescens* (Flugge) from brinjal ecosystem for the management of *H. vigintioctopunctata* infesting brinjal.

Being foliage feeders, both *H. septima* and *D. indica* are vulnerable to infection by entomopathogenic as well as phylloplane bacteria. The present study entitled "Management of major chewing pests, *Henosepilachna septima* (Dieke) and *Diaphania indica* (Saund) infesting bitter gourd with bacterial bioagents" was undertaken to evaluate and utilize entomopathogenic and phylloplane bacteria for the management of *H. septima* and *D. indica* with the following objectives.

- Isolation of bacteria from phylloplane of bitter gourd
- Pathogenicity testing of the isolated bacteria to larvae of *H. septima* and *D. indica*
- Characterisation of pathogenic phylloplane bacteria
- Evaluation of the efficacy of the bacterial bioagents against chewing pests of bitter gourd.

Review of Literature

2. REVIEW OF LITERATURE

Momordica charantia (L.), commonly known as bitter gourd or bitter melon is grown as a food crop all through the tropics and is one of the major vegetables in India. Both young shoots and immature fruits are commercially consumed as vegetables. Besides its high nutritional value as a rich source of vitamins and minerals (Wills *et al.*, 1984), it is also known to have pharmacological properties such as antihelminthic, purgative and antidiabetic action (Holm *et al.*, 1997). In India, bitter gourd is cultivated over an area of 0.83 lakh ha with an annual production of 9.4 lakh tons (NHB, 2014). In the state of Kerala, it is cultivated over an area of 2673 ha (FIB, 2017).

Insect pests are one among the major biotic constraints of vegetable production in India. Besides causing direct damage to crops, many of them also act as vectors for several viral diseases. Owing to insect attack, crop loss of 30-40 per cent have been reported in vegetable crops (Rai *et al.*, 2014).

Bitter gourd is heavily infested by a number of pests during different growth stages such as aphid, *Aphis gossypii* (Glover), red pumpkin beetle, *Aulacophora foveicollis*, epilachna beetle, *Henosepilachna septima*, pumpkin caterpillar, *Diaphania indica*, cucurbit fruit fly, *Bactrocera cucurbitae* (Coquillett), coreid bug, *Leptoglossus australis* (Guerin – Meneville) etc. (Nandakumar, 1999). Yield loss of 60-80 per cent was reported in bitter gourd due to the attack of insect pests (Shivalingaswamy *et al.*, 2002).

Insects with chewing mouth parts feeding on the foliage are identified as one of the yield limiting factors by way of defoliation leading to decline in photosynthetic efficiency as its vines and creepers provide enough hiding place for them (Carvalho *et al.*, 2014).

Foliage feeding insects at times also infest the fruits, lowering the consumer preference leading to reduction in market value of the produce (Arcaya *et al.*, 2004).

2.1 EPILACHNA BEETLE

Epilachna beetle (Coccinellidae; Coleoptera) is identified as notorious pest infesting plants belonging to Cucurbitaceae and Solanaceae families all over India as well as other countries. About 35-75 per cent leaf injury is reported by this pest in infested crops (Srivastava and Katiyar, 1972). *H. septima* infest cucurbitaceous crops (Anand *et al.*, 1988) and *H. vigintioctopunctata* is identified as a serious pest in solanaceous crops (Mall *et al.*, 1992).

Owing to the attack of *H. vigintioctopunctata*, substantial economic loss occurs in many crops, negatively affecting both quality and quantity of produce. Damage of 80 per cent was observed in potato due to scraping of leaves by grubs and adults of beetle (Rajagopal and Trivedi, 1989). The grubs and adults eat away the foliage, retarding the plant growth, leading to reduced fruit production thereby resulting in yield loss up to 60 per cent in brinjal (Mall *et al.*, 1992). Leaf infestation of more than 80 per cent was recorded in cowpea during summer season by (Haider and Srinivasan, 2011).

The phytophagous epilachna beetle feeding on bitter gourd in India was identified as *H. septima* (Anand *et al.*, 1988). *H. septima* has been found to be a destructive pest throughout the growth stages of bitter gourd resulting in yield loss (Sreekala and Ushakumari, 1999). Grubs and adults of *H. septima* feed on leaves, stem and fruits of bitter gourd causing great economic loss (Singh *et al.*, 2010). Epidermal scraping caused by grubs and adults result in skeletonisation of leaves in bitter gourd (Barma and Jha, 2013) and voracious feeding causes entire drying and death of the crop. Epilachna beetle infestation contributed an yield loss of more than 80 per cent in bottle gourd (Bhoumik and Saha, 2017).

2.2 PUMPKIN CATERPILLAR

Diaphania indica (Saund) (Pyralidae; Lepidoptera) popularly known as pumpkin caterpillar or cucumber moth is a widespread pest of cucurbitaceous crops (Morgan and Midmore, 2002; Arcaya *et al.*, 2004).

It is a foliage feeding pest in 13 cucurbitaceous plants in India out of which bitter gourd is one of the preferred hosts (Pandey, 1977). The newly hatched larvae feed on leaves where they cluster, fold and weave the leaves together. Upon high populations, the pest almost defoliates the plant. Fruit damage is also reported, where they feed on and puncture the surface of developing fruits mainly those near to the leaves in pointed gourd, in addition to 25 – 30 per cent foliage damage (Jhala *et al.*, 2005). Singh and Naik (2006) noticed fruit infestation of 3-14 per cent in bitter gourd by *D. indica* wherein the larvae scrape the epicarp in early instars and bore the base in later instars.

2.3 MICROBIAL MANAGEMENT OF INSECT PESTS

Development of ecofriendly management measures and their deployment in IPM has been a main objective for both academia and industry during the last decades (Ruiu *et al.* 2015).

Microbial control of insect pests utilizing beneficial microorganisms is an attractive alternative to chemical pesticides. The progressive discovery and use of various entomopathogenic microbial species including bacteria, virus, protozoa, fungi, microsporidia and nematodes has been exploited commercially (Vega and Kaya, 2012).

Pathogenic microorganisms are reported to cause diseases in large number of insect pests including epilachna beetle (Rajendran and Gopalan, 1999; Markandeya *et al.*, 2001) and pumpkin caterpillar (Neema *et al.*, 2010). Many entomopathogens are developed and registered as microbial pesticides by commercial companies and government agencies all over the world (EPA, 2013).

2.4 ENTOMOPATHOGENIC BACTERIA

Bacteria cause diseases in insects upon oral ingestion. They infect through the midgut epithelial cells resulting in death of the host by septicaemia and production of

toxins. Majority of entomopathogenic bacteria belong to Bacillaceae, Pseudomonadaceae and Enterobacteriacea, Streptococcaceae and Micrococcaceae families. Bacterial bioagents being selective, provides a better alternative to chemical pesticides (Tanada and Kaya, 1993). Microbial control witnessed a paradigm shift with the invention of potential entomopathogenic bacteria capable of achieving quick kill of the pests, like those species belonging to the genus *Bacillus* (Glare and O'Callaghan, 2000). Members of Bacillaceae particularly *Bacillus thuringiensis* have received considerable attention and occupy presently around 2 per cent of the total insecticidal market (Bravo *et al.*, 2011).

2.4.1 *Bacillus thuringiensis* Berliner

Bacillus thuringiensis (*Bt*) is a Gram positive, soil dwelling bacterium, known for its ability to produce crystalline inclusions upon sporulation (Cry toxins) which contains insecticidal proteins called δ endotoxin (El- Menofy *et al.*, 2014).

Endotoxins, secreted as water-soluble proteins, are proteolytically processed by midgut proteases which further binds to specific receptors in insect midgut epithelial cells, creating pores thus destroying the selective permeability of the membranes. The disturbances in osmotic equilibrium and lysis lead to insect paralysis, septicaemia and death (Bravo *et al.*, 2011).

Application of *Bt* has been reported against more than 40 species of insects (Burgus and Daoust, 1986). Insects susceptible to *Bt* have increased in number with the isolation of large number of sub species and strains (Hofte and Whitely, 1989). *Bt* has been reported as a microbial control agent against many chewing pests like cotton boll weevil, *Anthonomus grandis* (Boheman) (Monnerat *et al.*, 2012), gram pod borer, *Helicoverpa armigera* (Hubner), leaf caterpillar, *Spodoptera litura* (F.), red flour beetle, *Tribolium castaneum* (Herbst) (Kausarmalik, 2014), cabbage white butterfly, *Pieris brassicae* (L.) (Mohan *et al.*, 2014), bihar hairy caterpillar, *Spilarctia obliqua* (Walker) (Khan, 2015).

As per the results obtained from the study conducted by Markandeya *et al.* (2001) *H. vigintioctopunctata* fed with leaves dipped in 1 per cent *B. thuringiensis* formulation (Biotox) resulted in mortality of 39.03 per cent and 5.22 per cent in grubs and adults respectively. Besides, leaf area damage caused by grubs and adults was found low when compared with that in the control treatment.

Second instar larvae and adults of *H. vigintioctopunctata* were found susceptible to Ba9808 of *B. thuringiensis* which caused a mortality of 55 per cent and 26.7 per cent respectively. Ba9808 resulted in feeding inhibition, reduction of pupation percentage and reduction in egg production rate (Hong *et al.*, 2002). Ping *et al.* (2008) noted that the second instar larvae of *H. vigintioctomaculata* were vulnerable to strain WZ-9 of *B. thuringiensis*, which contributed 100 per ecnt mortality at 72 h with LC50 value of 2.95×10^{7} cell ml^{-1}.

Bt subsp *kurstaki* (*Btk*) is generally used against young lepidopteran larvae and includes different strains with significant commercial interest like HD-1, SA-11, SA-12, PB 54, ABTS-351 and EG2348 (Glare and O'Callaghan, 2000).

According to Wang *et al.* (1989), 10-50 per cent of *D. indica* died after consuming 0.05-2 per cent solution of *B. thuringiensis*. Field trial reports of the study revealed that, 0.1-0.2 per cent of *Bt* emulsion (Bt_1 and 8401), alone or in combination with pyrethrin or permethrin caused 62.5 per cent control of *D. indica* without significantly affecting its natural enemies (*Apanteles glomeratus* L.). Neema *et al.* (2010) reported native *B. thuringiensis* isolates KK7, KK8, KK9 and KY2 from western ghats pathogenic to *D. indica.*

2.4.2 *Pseudomonas*

A number of species of *Pseudomonas* are found associated with insects either as pathogens or as commensals in the digestive tract. Though *P. aeruginosa* is one of

the commonly isolated bacteria from insetcs, they seldom cause epizootics in field populations (Buchner, 1963).

P. fluorescens strains were found effective in killing or causing morphological defects in widely used laboratory insects (Pimenta *et al.*, 2003). *P. fluorescens* was reported to cause mortality of 70 per cent and 56 per cent in the larvae and adults of alder leaf beetle, *Agelastica alni* L. respectively within 7 days after treatment whereas, *P. chlororaphis* (Bergey) brought about 37 and 30 per cent mortality of larvae and adults respectively (Sezen *et al.*, 2004).

Sezen *et al.* (2007) determined the insecticidal effect of two species of *Pseudomonas* on *Melolontha melolontha* larvae, a serious pest of hastle nuts in Turkey. The insecticidal activity of isolates at 1.8×10^9 bacteria ml^{-1} dose, within 10 days on the larvae were 40 per cent and 50 per cent for the two species of *Pseudomonas*. According to Meca *et al.* (2009), *Pseudomonas* sp caused 70 per cent mortality of citrus leaf miner, *Phyllocnitis citrella* (Stainton) larvae at 72 h.

Entomopathogenic activity of different strains of *Pseudomonas* sp were tested against the 5^{th} instar larvae of migratory locust, *Locusta migratoria* (L.). The results obtained 1 week after treatment showed that, treated nymphs were sensitive to the bacteria with a mortality rate of 100 per cent and 98 per cent for *Pseudomonas* sp strain B3 (HF911369) and strain B4 (HF911366) respectively (Mohandkaci *et al.*, 2015).

Gopal *et al.* (2002) reported *P. alcaligens* (Monias) can cause septicaemia in rhinoceros beetle grubs under stress conditions. Of the 6627 grubs and 307 adults collected from various breeding sites of the pest, 5 per cent of the grubs and 22 per cent of the adults had natural viral infection caused by *Oryctes* virus, 3 per cent larvae died of *Metarhizium anisopliae* (Sorokin) mycosis and 20 per cent larvae died from bacterial septicaemia. *P. aeruginosa* isolated from dead grubs of epilachna beetle, *H. vigintioctopunctata* was reported to cause mortality of 73.01 per cent on these grubs (Aswathy, 2015).

2.4.3 *Serratia*

Serratia entomophila and *Serratia proteamacula* are used as effective biological pesticides against Newzeland grass grubs, *Costelytra zealandica* (White) (Hurst *et al.*, 2000).

Lauzon *et al.* (2003) stated that non-pigmenting strains of *S. marcescens* were pathogenic to apple maggot flies, *Rhagoletis pomonella* (Walsh) rendering rapid mortality within 24 hours after treatment. Reports prove that *S. marcescens* NMCC46 had potent mosquito larvicidal activity resulting from the red colour pigment prodigiosin causing 50 per cent mortality within the first 24 h of treatment (Patel *et al.*, 2011). *S. marcescens* isolated from dead grub of epilachna beetle, *H. vigintioctopunctata* from brinjal was found to cause 93.28 per cent mortality of the grub (Aswathy, 2015). Pu and Hou (2016) observed, *S. marcescens* caused a mortality of 56.75 per cent on the fourth instar larvae of red palm weevil, *Rhynchophorus ferrugineus* Oliver.

Serratia sp and *Pseudomonas* sp have been reported as pathogens of South American fruit fly, *Anastrepha frateraculus* (Wiedemann), Mediterraian fruit fly, *Ceratitis capitata* (Wiedemann) and the South American palm weevil, *R. palmarum* (L.) (Briceno, 2004). Meca *et al.* (2009) reported that *Serratia* sp had entomopathogenic effect on citrus leaf miner, *P. citrella* where it caused a per cent mortality of 80.4 per cent on the larvae between 48 and 72 h. Reports reveal significant reduction in feed consumption by lepidopteran insects (*H. armigera* and *S. litura*) fed on diet supplemented with *Serratia* sp. A maximum mortality of 94.3 per cent and 92.7 per cent was noted in *H. armigera* and *S. litura* respectively at 72 h after treatment (Chattopadhyay *et al.*, 2012).

2.4.4 Other Bacteria

Clostridium bifermentus (Weinburg and Seguin) was found pathogenic to mosquitoes and blackflies (Nicolas *et al.*, 1990). *Enterobacter* sp at high

concentrations was found to cause mortality in fruit flies, *Ceratitis capitata* and *Anastrepha fraterculus.* The principal symptoms of infection observed were septcaemia, inhibition of feeding, lack of motility and death at 24 to 72 h (Briceno, 2004). *Enterobacter aerogenes* was reported to cause mortality of 73 per cent in the larvae of *P. citrella* (Meca *et al.*, 2009). *Burkholderia* sp has recently been asserted to affect ovipositon and fecundity of the bean bug, *Riptortus pedestris* (Fabricius) (Kil *et al.*, 2014). Whole cell broth cultures of *B. rinojensis* (A396), rendered oral toxicity and contact effects against the beet armyworm *Spodoptera exigua* Hubner) (Kreylos *et al.*, 2013).

Ingestion of the pigment violecein (tryptophan derivative), synthesized by *Chromobacterium substugae* was found to cause toxic effects in different insects *viz.* Colorado potato beetle, *Lepinotarsa decemlineata* (Say), western corn root worm, *Diabrotica virgifera* (Le conte), southern corn root worm, *Diabrotica undecimpunctata* (L.), diamond back moth, *Plutella xylostella* (L.), sweet potato white fly, *Bemisia tabaci* (Gennadium) and southern green stink bug, *Nazara viridula* (L.) (Martin *et al.*, 2007).

Klebsiella pneumoniae (Trevisan) was isolated from dead grubs of red palm weevil, *R. ferrugineus* (Pu and Hou, 2016). *K. pneumoniae* and *S. marcescens* were also isolated from the gut region of red palm weevil by Josephrajkumar *et al.* (2017).

2.5 PLANT MICROBE INTERACTION MEDIATED BIOCONTROL

Biological control within broad ecological context includes plant microbe interactions also (Andrews, 1992). Many bacteria associated with plants are known to exert beneficial effects on the plants such as growth promotion (Glick, 1995), induced resistance to pathogens and pest control capability against insect herbivores (Bostock *et al.*, 2001). Bacteria that associate with plants in their natural environment are called by different names such as rhizosphere bacteria, endophytic bacteria and phylloplane bacteria depending on the sites they inhabit.

2.5.1 Rhizosphere Bacteria

Bacteria that survive in association with the rhizosphere are known as rhizosphere bacteria (Schroth and Hancock, 1982). About 2 - 5 per cent of rhizobacteria show a beneficial effect on plant growth and hence these bacteria are termed as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1989). PGPR enhance plant growth directly by facilitating nutrient uptake and indirectly by protecting the plant from pathogens and insect pests. Systemic resistance induced by these microbes enhance plant defense against pests and pathogens (vanLoon *et al.*, 1998).

Reports show PGPRs like *Pseudomonas, Bacillus* and *Serratia* are efficient root colonisers and protect plant from different crop pests (Tomczyk, 2006; Hanafi *et al.*, 2007; Siddiqui *et al.*, 2007).

2.5.1.1 Pseudomonas

Rhizospheric *P. maltophila* (Hugh) was found to affect the growth of corn ear worm, *H. zea* (Boddie) a major polyphagous pest of many agricultural crops contributing more than 60 per cent reduction in adult emergence of the pest (Bong and Sikorowski, 1991). *P. gladioli* (Zopf) was found to affect the relative growth rate, consumption rate and digestibility of feed by *H. armigera* in cotton (Qingwen *et al.*, 1998).

Plant growth promoting *P. fluorescens* strains Pf1, TDK 1 and PY 15 rendered notable reduction in the leaf folder damage in rice plants which is attributed to the enhanced activity of polyphenol oxidase, lipoxygenase, chitinase and proteinase inhibitors in PGPR treated plants. Besides this, increased natural enemy population in the PGPR treated plots was also noticed (Saravankumar *et al.*, 2007).

Melvin and Muthukumaran (2008) observed that tomato leaves treated with combined foliar application of defense inducer, jasmonic acid (JA) and *P. aeruginosa* recorded maximum larval mortality of *S. litura* under the pot culture condition. Combined treatment resulted in significant reduction in pupation rate,

adult emergence and adult longevity of the pest. The activity of proteinase inhibitor, polyphenol oxidase (PPO) and lipoxygenase molecules was promoted by JA treatment.

Maria *et al.* (2008) attributed the insecticidal property of two strains CHA0 and pf-5 of rhizospheric *P. fluorescens* to a large protein toxin termed Fit (*P. fluorescens* insecticidal toxin). Haemocoelic injection of even low doses of *P. fluorescens* CHA0 or Pf-5 was observed efficient in inducing mortal effects on larvae of the tobacco hornworm *Manduca sexta* (L.) and the greater wax moth *Galleria mellononella* (F.).

2.5.1.2 Bacillus

Zehnder *et al.* (1997) proved experimentally that PGPR treated cucumber plants caused significant reduction in feeding by dibroticine cucumber beetles due to reduction in cucurbitacin, which act as a phagostimulant for the beetle. *Bacillus pumilus* strain INR – 7 was effective against striped cucumber beetle, *Acalyma vittatum* (Barber) and the spotted beetle, *Diabrotica undecimpunctata howardi* as per their study.

A delay in population growth and population size of cotton aphids *Aphis gossypii,* on cucumber plants treated with PGPR (*Bacillus* spp.) was reported by Stout *et al.*, (2002). Hanafi *et al.* (2007) observed significant reduction in proliferation of *Bemisia tabaci* (Gennadius) on *B. subtilis* (Cohn) treated tomato plants. A significant increase in the mortality rate of cowpea aphid was reported in PGPR treated cowpea plants than the control where, *B. subtilis* was found the most efficient among the different PGPRs used (Kavitha, 2010).

2.5.1.3 Serratia

Serratia sp isolated from maize rhizosphere was found entomopathogenic on maize rootworms *Diabrotica virgifera virgifera* and the rootworm infestation subsequently increased the bacterial population (Prischmann *et al.*, 2008).

Rhizosperic *S. marcescens* was found effective against pod bug, *R. pedestris* in cowpea (Kavitha, 2010).

2.5.2 Endophytic Bacteria

Endophytic bacteria are those that inhabit the interior of plants, especially leaves, branches and stems, showing no apparent harm to the host they colonize (Sturz *et al.*, 2000). The presence of endophytic micro organisms in host plant can enhance plant vigour by way of rendering protection against insect pests and diseases, promoting growth and enhancing resistance in stress conditions (Azevedo *et al.*, 2000).

Thuler *et al.* (2006) reported that strain EN5 of endophytic *Alcaligenes piechaudii* (Kiredjian) reduced the incidence of *Plutella xylostella* by about 50 to 80 per cent. *B. subtilis* strains EPCO 102, EPCO 16 and *P. fluorescens* Pf1 reduced the aphid infestation in cotton (Rajendran *et al.*, 2011). Endophytic strains of *B. thuringiensis*, S1905 and S2122 caused 100 per cent and strain S2124 caused 58.33 per cent mortality in third instar caterpillars of diamond back moth, *Plutella xylostella* (Praca, 2012).

Fahey (1991) genetically modified xylem-inhabiting endophytic bacteria *Clavibacter xyli* subsp *cynodontis*, by the introduction of delta endotoxin gene of *B. thuringiensis,* and the recombinant provided resistance against lepidopteran and coleopteran pests. Gaofu *et al.* (2012), effectively modified an endophyte, *B. subtilis* WH2, isolated from rice seedlings by introducing *Pinellia ternata* agglutinin (PTA) gene with insecticidal properties and found it effective in controlling white backed plant hopper (WBPH) in rice.

2.5.3 Phylloplane Bacteria

Phylloplane is a natural habitat on leaf surface which supports growth of wide range of microorganisms. According to Beattie and Lindow (1995), phylloplane bacteria survive on the leaf surfaces colonizing on sites such as trichomes, stomata and epidermal cell wall junctions.

Andrews (1992) reported that microorganisms that stably colonize the surface of plant leaves act as potential biological agents to suppress foliar pathogens and insect defoliators. Mostly being commensalistic, some of the phylloplane bacteria can produce extracellular chitinase which in turn degrades the peritrophic membrane of chewing insects, thereby making them good biocontrol agents (Aggarwal *et al.*, 2015).

B. thuringiensis is indigenous to diverse environments such as soil, insect cadavers and leaves of plants (Smith and Couch, 1991). *Bt* has been reported as a phylloplane inhabitant in addition to entomopathogen and soil inhabitant (Meadows, 1993).

Otsu *et al.* (2003) proved scientifically that the phytophagous epilachna beetles can be biologically controlled by chitinase secreting strain KPM-012A of *Alcaligens paradoxus* isolated from tomato phylloplane, which caused the degradation of peritrophic membrane in *H. vigintioctopunctata*. Otsu *et al.* (2004) provided an experimental basis for the biological control of herbivorous insect pests using leaf inhabiting, entomopathogenic strain of *P. fluorescens*. The strain KPM-018P isolated from tomato leaves caused 70.5 $\pm$ 21.5 per cent mortality in larvae of *H. vigintioctopunctata*. This method was thus proved effective for decreasing the population of larvae and adults of the pest in the subsequent generation.

A study conducted by Maduell *et al.* (2002) reported that 60 per cent of total 256 isolates of *B. thuringiensis* isolated from phylloplane of *Piper* sp were toxic to *S. frugiperda* (Smith) and 40 per cent were toxic to southern house mosquito, *Culex quinquefasciatus* (Say). *B. thuringiensis* isolated by Gonzalez and Molla (2011) from the phylloplane of tomato plants could effectively control the tomato leaf miner, *Tuta absoluta* (Meyrick). Aswathy (2015) reported that *P. fluorescens* isolated from the phylloplane of brinjal, caused 63.25 per cent mortality of grubs of *H. vigintioctopunctata.*

Materials and Methods

3. MATERIALS AND METHODS

The study on the "Management of major chewing pests, *Henosepilachna septima* (Dieke) and *Diaphania indica* (Saund) infesting bitter gourd with bacterial bioagents" was carried out at the Department of Entomology, College of Agriculture, Vellayani during 2015 – 2017.

3.1 ISOLATION AND MAINTENANCE OF BACTERIAL CULTURES

3.1.1 Phylloplane Bacteria of Bitter gourd

Bacteria were isolated from phylloplane of bitter gourd by employing leaf impression method of Otsu *et al.* (2003). Completely developed leaves were randomly collected from bitter gourd plants. The upper and lower surfaces of the collected leaves were pressed and left for two-three minutes on separate M-9 minimal agar media plates with the composition of 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO4, 0.5 g NaCl, 1 g NH4Cl, 4 g glucose and 20 g agar in 1000 ml of water. The media was amended with colloidal chitin. Bacterial colonies developing on the media were streak purified in Nutrient agar (NA) media. Pure cultures were then transferred to NA slants and stored under refrigerated condition. Pure cultures were preserved as glycerol culture by mixing 750 µl bacterial suspension with 250 µl sterile glycerol and were stored at -80°C in a deep freezer until further use.

3.1.2 Other Bacteria

Besides phylloplane isolates, the following bacteria were also maintained in pure culture and used for the experiments.

Sl No.	Bacteria	Source	Media
1	*Serratia marcescens* (Hv3)	Entomopathogen isolated from *H. vigintioctopunctata*	Nutrient agar (NA)

2	*Pseudomonas fluorescens* (PNO26R)	Department of Agricultural Microbiology	Kings B (KB)
3	*Pseudomonas fluorescens* (psm13)	Brinjal phylloplane	Kings B (KB)

3.2 MAINTENANCE OF TEST INSECTS

3.2.1 Maintenance of *H. septima*

H. septima were reared on detached bitter gourd leaves in polypet jars with their mouth covered using muslin cloth from field collected adults. Adults obtained from the maintained culture were released at the rate of 10 per jar for egg laying. The egg masses laid on bitter gourd leaves were transferred daily to separate jars and the grubs emerged were maintained on fresh leaves. Second instar grubs obtained from the egg masses of same age were used for the experiments.

3.2.2 Maintenance of *D. indica*

Similarly, *D. indica* adults were reared on bitter gourd leaves in polypet jars with their mouth covered using muslin cloth. Adults obtained from the maintained culture were released at the rate of six per jar for egg laying. Ten per cent honey solution was provided as feed. Larvae soon after hatching were daily transferred to separate jars and were reared on bitter gourd leaves. Second instar larvae emerged from egg masses of same age were used for the experiment.

3.3 SCREENING OF BACTERIA FOR PATHOGENICITY

A preliminary screening of the thirteen phylloplane bacterial isolates, along with *S. marcescens* (Hv3), *P. fluorescens* (PN026R) and *P. fluorescens* (psm13) was done to test the pathogenicity to larvae of *H. septima* and *D. indica* by oral exposure.

3.3.1 Preparation of Bacterial Cell Suspensions

Bacterial isolates were cross streaked in NA media and incubated for 24 h. Cell suspension was prepared by adding 10 ml of sterile water. The Optical Density (OD) of the diluted suspension was read in a UV – Visible spectrophotometer and the same was adjusted with addition of distilled water to get an OD equivalent to 10^8 cfu ml^{-1} and was chosen for treatment.

Fresh leaves were dipped individually in each bacterial suspensions containing 10^8cfu ml^{-1} for five minutes and dried in room temperature. Larvae of the test insects were released separately to the treated leaves at the rate of 10 numbers per each treatment. Insects were observed daily for the development of symptoms and mortality.

Dead insects were surface sterilized with 0.1 per cent $HgCl_2$ for one minute and washed in sterile distilled water thrice and placed individually in NA medium for re - isolation of bacteria. The re - isolated bacteria were further provided to the test insects and mortality was observed thereby confirming Koch's postulates.

3.4 CHARACTERISATION OF SELECTED PHYLLOPLANE BACTERIA

3.4.1 Colony Morphology

Colony morphology of five bacteria (Isolate1, 3, 5, 7 and 12) found pathogenic to the test insects were studied by observing individual colonies on NA plates.

3.4.2 Gram Staining

Gram staining of the selected bacterial isolates was carried out by employing four steps *viz*. applying a primary stain (crystal violet) to a bacterial smear after heat fixing, followed by the addition of a mordant (Gram's Iodine), rapid decolorization with alcohol or acetone and finally, counterstaining with

safranin. All other stains except safranin were retained over the smear for one minute and washed off before the application of next stain. Safranin was kept for a period of 30 seconds and excess stain was removed by washing. The smear after staining was observed under oil immersion (100 X) objective.

3.5 LABORATORY SCREENING OF BACTERIA

3.5.1 Against *H. septima*

The phylloplane isolated bacteria pathogenic to *H. septima* (isolate 1, 5 and 7) along with *S. marcescens* (Hv3) and *P. fluorescens* (PNO26R) were screened against *H. septima* grubs with *B. thuringiensis* (0.25%) and flubendiamide 39.35 SC (0.004%) as checks.

3.5.2 Against *D. indica*

Similarly, screening against *D. indica* larvae was carried out using pathogenic phylloplane isolates (isolate 3, 5, 7 and 12) along with *S. marcescens* (Hv3) and *P. fluorescens* (PNO26R) with *B. thuringiensis* (0.25%) and flubendiamide 39.35 SC (0.004%) as checks.

3.5.3 Treatment of Bacterial Agents on Target Insects

Bitter gourd leaf discs of 3 cm diameter cut out with a sterile cork borer were dipped in bacterial suspension (10^8 cfu ml^{-1}) and dried by keeping in a Laminar Air Flow chamber. Second instar larvae of *H. septima* and *D. indica,* after starving for 12 h, were allowed to feed individually on the treated leaf discs kept in sterile glass vials. After ensuring complete feeding, ten insects each receiving same treatment were transferred to fresh leaves taken in sterile petri plates. Three replications were kept for each treatment each with ten insects. The insects fed on leaf disc dipped in sterile water were maintained as untreated control. The dead insects were removed periodically and noted for symptoms of infection.

3.5.4 Observations

Mortality caused by different treatments on the treated insects was observed regularly. Mortality of larvae and leaf area damage resulted were noted at one, three, five and seven days after treatment (DAT). Dead insects were transferred to moist tissue paper kept in petri plates and were observed for development of symptoms. Pathogenicity was confirmed by proving Koch's postulates. Per cent mortality was calculated using the following formula.

$$\text{Per cent mortality} = \frac{\text{Initial population} - \text{Final population}}{\text{Initial population}} \times 100$$

The total leaf area and leaf area damaged were determined at 1, 3, 5 and 7 DAT by sketching the leaf on a graph paper. The per cent leaf area damaged on each day was determined using the following formula.

$$\text{Per cent leaf area damaged} = \frac{\text{Total leaf area} - \text{Leaf area left undamaged}}{\text{Total leaf area}} \times 100$$

3.6 MOLECULAR CHARACTERISATION OF SELECTED PHYLLOPLANE ISOLATES

Molecular characterization of phylloplane bacteria (isolate 5 and 7) selected based on the laboratory screening was done by sequencing of DNA of 16S rRNA of these isolates with the help of microbial identification service at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram as detailed below.

3.6.1 Genomic DNA Isolation

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture was taken in a microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K were added and incubated at 56 °C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg ml^{-1}) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µl of 100 per cent ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

3.6.2 Agarose Gel Electrophoresis for DNA Quality and Quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% per cent sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8 per cent agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg ml^{-1} ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.3 PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg ml^{-1} BSA, 4 per cent DMSO, 5pM of forward and reverse primers and template DNA.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

16S rRNA

95 °C	-	5.00 min	
95 °C	-	30 sec	} 35 cycles
60 °C	-	40 sec	
72 °C	-	60 sec	
72 °C	-	7.00 min	
4 °C	-	∞	

3.6.4 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2 per cent agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.4.1 ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.6.4.2 Sequencing Using Big Dye Terminator

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.
The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

3.6.4.3 Post Sequencing PCR Clean up

1. Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.

2. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
3. 52 µl of master mix II was added to each reaction.
4. Contents were mixed by inverting and incubated at room temperature for 30 minutes
5. Spun at 14,000 rpm for 30 minutes
6. Decanted the supernatant and added 100 µl of 70 per cent ethanol
7. Spun at 14,000 rpm for 20 minutes.
8. Decanted the supernatant and repeated 70 per cent ethanol wash
9. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.6.5 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6.

The nucleotide sequence of 16S rDNA obtained was compared with the sequence available in the data base of National Centre for Biotechnology Information (NCBI) using the BLAST tool provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for homology and fixing the bacterial identity.

3.7 BIOCHEMICAL CHARACTERIZATION OF SELECTED PHYLLOPLANE BACTERIA

The identity of the phylloplane isolates (isolate 5 and 7) was further confirmed by carrying out biochemical analysis using Hi-Carbo (Part A, B and C), Hi- Bacillus, and Hi-Assorted kits (Himedia, Mumbai). Colour change observed on the biochemical amended media of the kit after spot inoculating culture suspension of isolate 5 and 7 @ 10^8 cfu ml^{-1} followed by incubation for 24 h

indicated the reaction with respect to different biochemicals/carbohydrates as positive or negative.

3.8 CHITINASE ACTIVITY

The chitinase activity of *S. marcescens* (pmc5) and *Klebsiella* sp was studied by both visual and quantitative methods by growing these bacteria in minimal media containing colloidal chitin as the sole carbon source.

3.8.1 Preparation of Colloidal Chitin

Ground crude chitin (40 g) was dissolved in 200 ml of cold concentrated Hydrochloric acid (HCl) by occasional stirring for 30 to 50 minutes. The resultant mixture obtained was then filtered through glass wool and was added drop wise into 2000 ml of ice cold water (5 to 10 °C) with continuous stirring using a magnetic stirrer. The creamy white gelationous precipitate thus formed was then separated by filtering through Whatman No. 1 filter paper. This was followed by repeatedly washing the precipitate by re suspending it in 1 litre of tap water. The process was repeated five to six times until the pH of the solution became neutral.

3.8.2 Detection of Chitinase Activity

The selected bacteria were tested for the production of chitinase by spot inoculation of the bacterial suspensions on minimal soft agar medium (1.5% agar) with 0.1% colloidal chitin as the sole carbon source following the plate assay method devised by Soumya *et al.* (2012). Inoculated plates were incubated for a period of seven days, thereafter, congo red solution (0.1%) was poured over the inoculated plates and the plates were observed for the development of clearance zone. The clear zone developed around the bacterial growth which indicates the chitinase activity was measured.

3.8.3 Quantification of Chitinase Activity

3.8.3.1 Enzyme Sample Preparation for Chitinase Assay

Chitin (0.3 g) was dissolved in 10 ml of distilled water and sterilized by autoclaving at 121°C for 20 minutes. A loopful of bacterial cells from an actively growing culture was inoculated into it and was incubated for 48 h at 28 °C (Avelizapa *et al.*, 1999; Avelizapa *et al.*, 2001). The same was centrifuged at 8000 rpm for ten minutes and the supernatant obtained was used as enzyme sample for further analysis.

3.8.3.2 Chitinase Assay Using Chitin-Azure

1 ml each of the enzyme sample and sodium phosphate buffer (0.2 M) was mixed with 5 mg of chitin azure. The mixture after incubation in a water bath at 50 °C for 3h, samples were centrifuged at 7000 rpm and absorbance was read at 560 nm. The quantity of enzyme that resulted an increase 0.01 in absorbance was equivalent to 1 unit of chitinase (Ramirez *et al.*, 2004).

3.9 ANTIBIOTIC RESISTANCE PATTERN OF SELECTED PHYLLOPLANE BACTERIA

Antibiotic resistance pattern of *Serratia marcescens* (pmc5) and *Klebsiella* sp was worked out by spot inoculating them in antibiotic amended plates at different concentrations.

The following antibiotics at the specified concentration were tested.

Ampicillin, neomycin and streptocycline at 50, 75 and 100 ppm, tetracycline at 10, 20 and 50 ppm, spectinomycin at 25, 50 and 100 ppm and rifampicin at 25, 50 and 75 ppm.

3.10 MANAGEMENT OF CHEWING PESTS OF BITTER GOURD WITH BACTERIAL BIOAGENTS (POT CULTURE EXPERIMENT)

A pot culture experiment was conducted to evaluate the efficacy of foliar application of the following bacterial bioagents against *H. septima* and *D. indica.*

Design : CRD

Replication : 3

Treatments

T_1 : *Serratia marcescens* @ 10^8 cfu ml^{-1}

T_2 : *Pseudomonas fluorescens* (PN026R) @ 10^8 cfu ml^{-1}

T_3 : *Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}

T_4 : *Klebsiella* sp @ 10^8 cfu ml^{-1}

T_5 : *Bacillus thuringiensis* @ 0.25%

T_6 : Flubendiamide 39.35 SC (0.004%)

T_7 : Quinalphos 25 EC (0.05%)

T_8 : Untreated control

Seeds of bitter gourd (variety: Preethi) were sown in grow bags filled with potting mixture containing sand, soil and cow dung in the ratio of 1:2:1. The crop was grown following the KAU package of practice recommendations (2016).

A consistent population of *H. septima* and *D. indica* were maintained in bitter gourd raised for application of treatments, avoiding plant protection measures. Pre-treatment count of the insects was noted from each plant. Cell suspension of bacterial agents at 10^8 cfu ml^{-1} was prepared as per the method described in 3.3.1 and was sprayed on to the plants covering the entire plant at flowering stage. Flubendiamide 39.35 SC (0.004 %) and quinalphos 25 EC (0.05%) were sprayed as chemical checks and application of sterile water served as untreated control.

3.10.1 Observations

3.10.1.1 Mortality and Leaf Area Damage

Mortality and leaf area damage caused by the pests on plants after treatment with bacterial bioagents at 1, 3, 5 and 7 DAT were noted as described in 3.5.4.

3.10.1.2 Population of H. septima and D. indica

Post treatment count of both the pests from plants sprayed with different treatments was noted to assess the population of the target insect pests after treatment. Number of pests per plant was counted at time intervals of 1, 3, 5 and 7 DAT and mortality was worked out.

3.10.1.3 Other Pests and Natural Enemies

Pests other than *H. septima* and *D. indica*, and natural enemies were also monitored during the study. Observations on the number of non target pests and natural enemies in treated plants were recorded on per plant basis from each of the treated plant.

3.11 STATISTICAL ANALYSIS

Data of each experiment were analyzed with the help of suitable analytical methods. Data on mortality, leaf area damage and mean population were analyzed by one way analysis of variance after square root transformation.

Results

4. RESULTS

4.1 ISOLATION OF PHYLLOPLANE BACTERIA FROM BITTER GOURD

The bacterial colonies obtained from the phylloplane of bitter gourd upon leaf impression on M-9 minimal agar media were streak purified and brought to pure culture. The thirteen bacterial isolates thus obtained were numbered serially as isolate 1 to isolate 13 in the order of their isolation.

4.2 PRELIMINARY SCREENING OF BACTERIA FOR PATHOGENICITY TO *H. septima* AND *D. indica* LARVAE

Mortality exhibited by different bacterial agents on larvae of *H. septima* and *D. indica* at 5 DAT is presented in Table 1 and 2 respectively.

Upon preliminary screening, out of the thirteen bacteria isolated, only three *viz*. isolate 1, 5 and 7 were found pathogenic to *H. septima*. 13.33 per cent of grubs treated with isolate 1 died 5 DAT. 83.33 per cent and 46.66 per cent mortality were observed in case of isolate 5 and 7 respectively at 5 DAT. Four isolates *viz*. isolate 3, 5, 7 and 12 were found pathogenic to the larvae of *D. indica* with 23.33 per cent, 93.33 per cent, 53.33 per cent and 26.66 per cent mortality respectively at 5 DAT.

Pathogenicity of phylloplane isolates (isolate 1, 5 and 7) against *H. septima* and (isolate 3, 5, 7 and 12) *D. indica* were further confirmed by proving the koch's postulates. Among the entomopathogenic bacteria tested, *S. marcescens* (Hv3) and *P. fluorescens* (PNO26R) were also found to infect both *H. septima* nd *D. indica*.

Mortality of 90.00 per cent and 96.66 per cent in *H. septima* grubs and *D. indica* larvae respectively were noticed in case of *S. marcescens* (Hv3) and 30.00 per cent and 20.00 per cent in case of *P. fluorescens* (PNO26R) at 5 DAT. *P. fluorescens* (psm13) failed to infect the larvae of both the insects. Pathogenicity of

Table 1. Screening of entomopathogenic and phylloplane bacteria against *H. septima* grubs

Isolates	Source	Per cent mortality*	Koch's postulates
		5 DAT	
Serratia marcescens (Hv3)	Entomopathogen isolated from *H. vigintioctopunctata*	90 (9.48)[a]	+
Pseudomonas fluorescens (PN026R)	Department of Agricultural Microbiology	30 (5.42)[c]	+
Pseudomonas fluorescens (psm13)	Brinjal phylloplane	0 (0.707)[e]	-
Isolate 1	Bitter gourd phylloplane	13.33 (3.60)[d]	+
Isolate 2	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 3	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 4	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 5	Bitter gourd phylloplane	83.33 (9.12)[a]	+
Isolate 6	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 7	Bitter gourd phylloplane	46.66 (6.82)[b]	+
Isolate 8	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 9	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 10	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 11	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 12	Bitter gourd phylloplane	0 (0.707)[e]	-
Isolate 13	Bitter gourd phylloplane	0 (0.707)[e]	-
CD (0.05)		1.149	

*Mean of three replications (Values in the parentheses are square root transformed)

Table 2. Screening of entomopathogenic and phylloplane bacteria against *D. indica* larvae

Isolates	Source	Per cent mortality*	Koch's postulates
		5 DAT	
Serratia marcescens (Hv3)	Entomopathogen isolated from*H.vigintioctopunctata*	96.66 (9.85)[a]	+
Pseudomonas fluorescens (PNO26R)	Department of Agricultural Microbiology	20 (3.87))[c]	+
Pseudomonas fluorescens (psm 13)	Brinjal phylloplane	0 (0.707)[c]	-
Isolate 1	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 2	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 3	Bitter gourd phylloplane	23.33 (4.71)[bc]	+
Isolate 4	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 5	Bitter gourd phylloplane	93.33 (9.68)[a]	+
Isolate 6	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 7	Bitter gourd phylloplane	53.33 (7.33)[ab]	+
Isolate 8	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 9	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 10	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 11	Bitter gourd phylloplane	0 (0.707)[c]	-
Isolate 12	Bitter gourd phylloplane	26.66 (5.04)[bc]	+
Isolate 13	Bitter gourd phylloplane	0 (0.707)[c]	-
CD (0.05)		2.691	

*Mean of three replications (Values in the parentheses are square root transformed)

the isolates was confirmed by proving koch's postulates. The phylloplane isolates 1, 3, 5, 7 and 12 along with *S. marcescens* (Hv3), *P. fluorescens* (PNO26R) were selected for further evaluation based on the results obtained from the preliminary screening.

4.2.1 Disease Symptoms in Larvae Treated with Phylloplane Bacteria

4.2.1.1 In H. septima

a. Isolate 1

The first symptom appeared 24 h after treatment as light brownish discolouration in the abdominal region of the treated grubs. Shrinking of grubs was observed from 3 DAT. Per cent mortality of 13.33 was observed at 5 DAT (Plate 1a).

b. Isolate 5

On treatment with isolate 5, pinkish red discolouration of grubs was noticed at 24 h after treatment with cessation of feeding. Later the grubs turned dark brown to black in colour. Mortality began 24 h after treatment. 83.33 per cent of the larvae were killed 5 DAT (Plate 1b).

c. Isolate 7

The grubs after treatment with isolate 7 were active at 24 h after treatment. Grubs turned light brown from yellow at 3 DAT along with swelling of body. 46.66 per cent mortality was observed 5 DAT (Plate 1c).

4.2.1.2 In D. indica

a. Isolate 3

Larvae, fed with leaves treated with isolate 3, exhibited slight swelling with light brownish discolouration beginning from head region 24 h after treatment. Mortality of 23.33 per cent was noticed 5 DAT (Plate 2a).

Plate 1a: Isolate 1

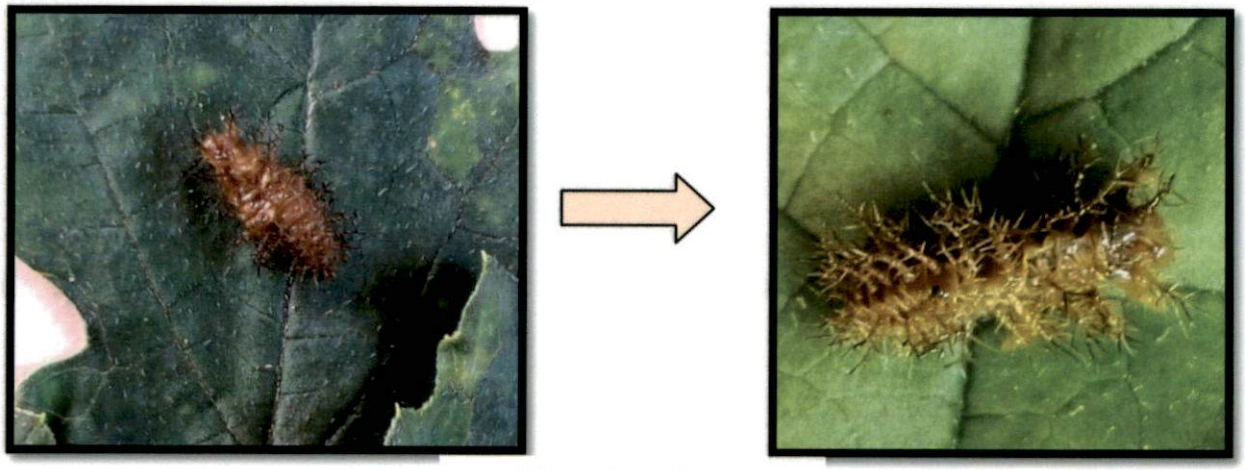

Plate 1b: Isolate 5

Plate 1c: Isolate 7

Plate 1: Disease symptoms in *H. septima* grubs treated with phylloplane bacteria

b. Isolate 5

Larvae upon treatment with isolate 5 exhibited reddish discolouration beginning from head region 24 h after treatment, which proceeded backwards. Mortality of 93.33 per cent was obtained 5 DAT. Dead larvae turned entirely black. Oozing of body fluid having putrified smell was also noticed (Plate 2b).

c. Isolate 7

Larvae treated with isolate 7 were with light brownish discolouration 24 h after treatment. Swelling of whole body was observed 3 DAT, along which dark brown to black discolouration. Mortality at 5 DAT was 53.33 per cent (Plate 2c).

d. Isolate 12

On treatment with isolate 12, the larvae were observed with light brownish discolouration 3 DAT. Oozing of body fluid was noticed 5 DAT with a mortality of 26.66 per cent (Plate 2d).

4.3 CHARACTERISATION OF SELECTED BACTERIA

4.3.1 Colony Morphology

Following colony characters were observed for the phylloplane bacteria grown on NA plates.

a. Isolate 1

Colonies were bright yellow, crinkled with serrated edges. Growth was slow in NA plates (Plate 3a).

b. Isolate 3

Bacterial isolate grew rapidly in nutrient agar medium with yellow colouration. The colonies were flat circular with concentric rings (Plate 3b).

Plate 2a: Isolate 3

Plate 2b: Isolate 5

Plate 2c: Isolate 7

Plate 2d: Isolate 12

Plate 2: Disease symptoms in *D. indica* larvae treated with phylloplane bacteria

c. Isolate 5

The isolate produced dark red, circular colonies with thick slightly raised centre and lobate periphery. Colonies were smooth and shiny and were fast growing in nutrient agar medium (Plate 3c).

d. Isolate 7

Large circular creamish yellow colonies with thick raised granular centre and smooth periphery was noticed (Plate 3d).

e. Isolate 12

Colonies were small and flat and exhibited spreading nature in nutrient agar medium. Cream colour was observed initially, which later turned to light yellow (Plate 3e).

4.3.2 Gram Staining

Isolate 1, 5 and 7 were Gram negative. Isolate 3 and 12 were found to be Gram positive.

4.4 LABORATORY SCREENING OF BACTERIAL ISOLATES AGAINST CHEWING PESTS

4.4.1 *H. septima*

4.4.1.1 Mortality

Mortality of grubs of *H. septima* treated with different bacteria is shown in Table 3.

S. marcescens (Hv3) exhibited the highest per cent mortality at 1, 3, 5 and 7 DAT which was found to be on par with isolate 5 and significantly superior over all other treatments.

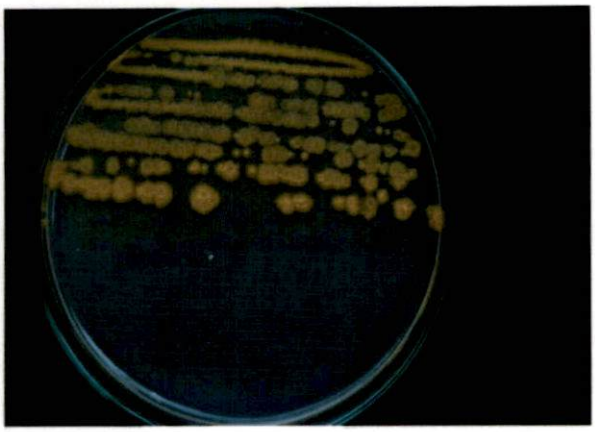

Plate 3a: Isolate 1

Plate 3b: Isolate 3

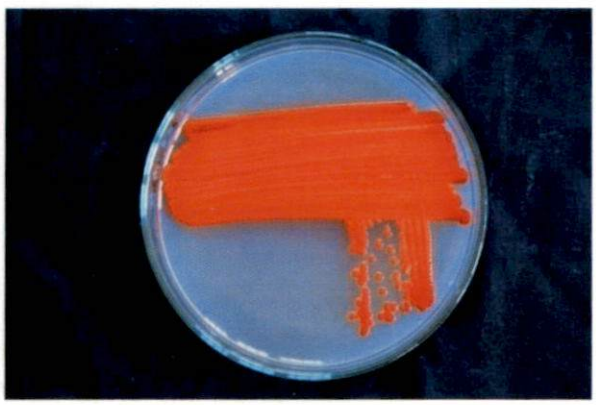

Plate 3c: Isolate 5

Plate 3d: Isolate 7

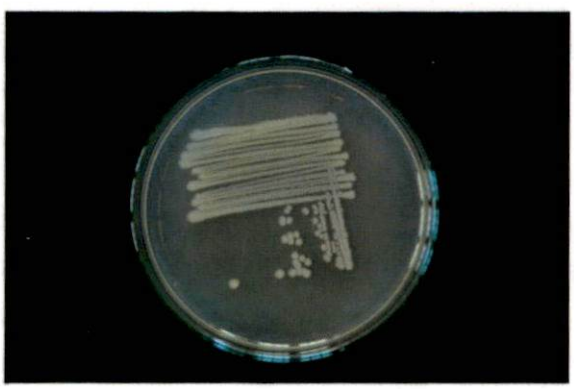

Plate 3e: Isolate 12

Plate 3: Colony morphology of phylloplane bacteria

At 1 DAT, 83.33 per cent and 76.67 per cent mortality were observed for *S. marcescens* (Hv3) and isolate 5 respectively. Isolate 7 which exhibited a mortality of 36.67 per cent was the next best treatment. Isolate 1 and *P. fluorescens* (PNO26R) caused only 10 and 6.67 per cent mortality respectively. No mortality was observed at 1 DAT in the chemical check (flubendiamide) and commercial biocontrol agent (*B. thuringiensis*).

At 3 DAT, mortality in treatment with *S. marcescens* (Hv3) increased to 90.00 per cent which was on par with isolate 5 with 80.00 per cent mortality. This was followed by isolate 7 which caused 43.33 per cent mortality. *P. fluorescens* and isolate 1 produced low mortality of 16.67 per cent and 13.33 per cent respectively. *Bt* did not produce any mortality at 3 DAT also.

The same trend was observed at 5 and 7 DAT with respect to *S. marcescens* (Hv3), isolate 5 and isolate 7 with mortality of 90.00, 83.33 and 50 per cent respectively at 7 DAT. An increase in mortality (16.67 to 30 per cent) was observed in grubs treated with *P. fluorescens* (PNO26R) from 3 DAT to 5 DAT.

4.4.1.2 Leaf Area Damage

Leaf area damage caused by grubs treated with different bacteria are shown in Table 4.

Per cent leaf area damage was found to be the least in treatment with *S. marcescens* (Hv3) at all intervals of observation which was followed by isolate 5.

At 1 DAT, grubs treated with *S. marcescens* (Hv3) and isolate 5 produced significantly low leaf area damage of 36.20 per cent and 38.15 per cent respectively, in comparison to the rest of the treatments. *P. fluorescens* (PNO26R) caused 40.15 per cent damage. Isolate 7 and isolate 1 treatments resulted in 53.14 and 56.12 per cent damage respectively. Grubs treated with flubendiamide

Tabe 3. Mortality of *H. septima* grubs treated with bacterial bioagents

Treatments		Per cent mortality*			
		1 DAT	3 DAT	5 DAT	7 DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	83.33 (9.16)[a]	90.00 (9.47)[a]	90.00 (9.50)[a]	90.00 (9.50)[a]
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	6.70 (2.4)[c]	16.67 (4.03)[cd]	30.00 (5.47)[c]	36.67 (6.08)[b]
T_3	Isolate 1 @ 10^8 cfu ml^{-1}	10.00 (3.24)[c]	13.33 (3.56)[d]	13.33 (3.66)[d]	16.67 (4.09)[c]
T_4	Isolate 5 @ 10^8 cfu ml^{-1}	76.67 (8.78)[a]	80.00 (8.94)[a]	83.33 (9.15)[a]	83.33 (9.15)[a]
T_5	Isolate 7 @ 10^8 cfu ml^{-1}	36.67 (6.08)[b]	43.33 (6.57)[b]	46.67 (6.86)[b]	50.00 (7.09)[b]
T_6	*Bacillus thuringiensis* @ 0.25%	0 (0.909)[d]	0 (0.909)[e]	3.33 (1.56)[e]	6.67 (2.4)[d]
T_7	Flubendiamide 39.35 SC,0.004%	0 (0.909)[d]	26.67 (5.09)[c]	33.33 (5.80)[bc]	36.67 (6.06)[b]
T_8	Untreated control	0 (0.909)[d]	0 (0.909)[e]	0 (0.909)[f]	0 (0.701)[d]
	CD (0.05)	1.312	1.214	1.376	1.432

*Mean of three replications comprising ten grubs each
(Values in the parentheses are square root transformed)

(59.91%) and *Bt* (60.48%) did not significantly reduce the leaf area damage over untreated check (61.47%).

The leaf area damage caused by grubs treated with *S. marcescens* (Hv3) and isolate 5 decreased to 10.5 and 13.7 per cent respectively at 3 DAT which were statistically significant over other treatments. Decrease in leaf area damage was also observed in case of isolate 7 (36.64 %). The leaf area damage at 3 DAT increased in all the other treatments *viz. P. fluorescens* (PNO26R) causing 45.77 per cent damage, isolate 1 (73.77%), flubendiamide (74.06%), *Bt* (75.88%) and untreated control (82.21%).

Significantly low per cent leaf area damage was observed in treatment with *S. marcescens* (Hv3) (7.93 %) at 5 DAT followed by isolate 5 (14.68 %) as against 100 per cent in untreated control. Leaf area damage increased in all other treatments except chemical check where slight decrease was observed.

At 7 DAT, 11.26 per cent and 18.89 per cent damage in the leaf area were caused by the grubs in treatments with *S. marcescens* (Hv3) and isolate 5 respectively which were statistically superior to the other treatments. Leaf area damage of 47.53 per cent and 68.62 per cent were recorded in treatments with isolate 7 and *P. fluorescens* (PNO26R) as against 100 per cent in the untreated control. There was no significant difference in the leaf area damage in the grubs treated with flubendiamide, isolate 1 and *Bt* which were on par with untreated control.

4.4.2 *D. indica*

4.4.2.1 Mortality

Mortality of grubs of *D. indica* treated with different bacteria is shown in Table 5.

Table 4. Leaf area damage caused by *H. septima* grubs treated with bacterial bioagents

Treatments		Per cent leaf area damage*			
		1 DAT	3 DAT	5 DAT	7 DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	36.20 $(6.02)^{d}$	10.50 $(3.21)^{d}$	7.93 $(2.56)^{e}$	11.26 $(3.00)^{c}$
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	40.15 $(6.34)^{c}$	45.77 $(6.77)^{b}$	59.94 $(7.78)^{bc}$	68.62 $(8.31)^{b}$
T_3	Isolate 1 @ 10^8 cfu ml^{-1}	56.12 $(7.49)^{b}$	73.77 $(8.58)^{a}$	79.42 $(8.93)^{ab}$	85.6 $(9.28)^{ab}$
T_4	Isolate 5 @ 10^8 cfu ml^{-1}	38.15 $(6.18)^{cd}$	13.70 $(3.69)^{d}$	14.68 $(3.90)^{d}$	18.89 $(4.36)^{c}$
T_5	Isolate 7 @ 10^8 cfu ml^{-1}	53.14 $(7.28)^{b}$	36.64 $(6.05)^{c}$	43.48 $(6.63)^{c}$	47.53 $(6.93)^{b}$
T_6	*Bacillus thuringiensis* @ 0.25%	60.48 $(7.78)^{a}$	75.88 $(8.70)^{a}$	91.42 $(9.59)^{a}$	92.82 $(9.65)^{ab}$
T_7	Flubendiamide 39.35 SC,0.004%	59.91 $(7.74)^{a}$	74.06 $(8.61)^{a}$	69.86 $(8.37)^{b}$	70.35 $(8.41)^{ab}$
T_8	Untreated control	61.47 $(7.83)^{a}$	82.21 $(9.07)^{a}$	100.00 $(10.03)^{a}$	100.00 $(10.03)^{a}$
	CD (0.05)	0.213	0.454	1.187	1.512

*Mean of three replications comprising ten grubs each
(Values in the parentheses are square root transformed)

S. marcescens (Hv3) along with isolate 5 were found to be superior over all other bacterial agents and were on par with each other at all intervals of observation.

At 1 DAT, isolate 5 and *S. marcescens* (Hv3) resulted in 86.67 per cent and 73.33 per cent mortality of larvae of *D. indica* which were significantly superior over other treatments and on par with chemical check (90 %). Isolate 7 caused 43.33 per cent mortality on the larvae. Per cent mortality of 13.33 and 6.67 were noticed in larvae treated with isolate 12 and isolate 3 respectively. *P. fluorescens* (PNO26R) and *Bt* did not show any mortality at 1 DAT.

Mortality caused by *S. marcescens* (Hv3) and isolate 5 increased to 93.33 per cent 3 DAT which was on par with the chemical check (100 %). Isolate 7 exhibited 50.00 per cent mortality which was on par with *Bt* which exhibited a rapid rise in the per cent mortality to 56.67 per cent at 3 DAT. Per cent mortality of 23.33 and 20.00 per cent were caused by isolate 12 and isolate 3 respectively. *P. fluorescens* (PNO26R) exhibited only 6.67 per cent mortality.

At 5 and 7 DAT, treatments with *S. marcescens* (Hv3) and isolate 5 were found to be significantly superior and were on par with both chemical check and *B. thuringiensis* treatment. 96.67 and 93.33 per cent mortality were observed at 7 DAT for *S. marcescens* (Hv3) and isolate 5 respectively. At 7 DAT, Isolate 7 caused 56.67 per cent mortality. Treatments with isolate 12 (26.67 and 36.67%), isolate 3 (23.33 and 33.33%) and *P. fluorescens* (PNO26R) (20 and 23.33 %) were on par with each other at 5 and 7 DAT.

4.4.2.2 Leaf Area Damage

Leaf area damage caused by larvae at different intervals after treatment with bacteria is shown in Table 6.

Table 5. Mortality of *D. indica* larvae treated with bacterial bioagents

Treatments		Per cent mortality*			
		1 DAT	3 DAT	5 DAT	7 DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	73.33 $(8.57)^{ab}$	93.33 $(9.67)^{ab}$	96.67 $(9.85)^{a}$	96.67 $(9.85)^{a}$
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8cfu ml^{-1}	0 $(0.909)^{d}$	6.67 $(1.99)^{d}$	20 $(3.86)^{de}$	23.33 $(4.72)^{c}$
T_3	Isolate 3 @ 10^8 cfu ml^{-1}	6.67 $(4.77)^{bc}$	20 $(3.86)^{cd}$	23.33 $(4.72)^{de}$	33.33 $(5.80)^{c}$
T_4	Isolate 5 @ 10^8 cfu ml^{-1}	86.67 $(9.687)^{a}$	93.33 $(9.69)^{ab}$	93.33 $(9.69)^{ab}$	93.33 $(9.69)^{a}$
T_5	Isolate 7 @ 10^8 cfu ml^{-1}	43.33 $(6.61)^{b}$	50.00 $(7.08)^{bc}$	53.33 $(7.33)^{bc}$	56.67 $(7.53)^{b}$
T_6	Isolate 12 @ 10^8 cfu ml^{-1}	13.33 $(3.16)^{c}$	23.33 $(4.72)^{c}$	26.67 $(5.04)^{cd}$	36.67 $(6.08)^{bc}$
T_7	*Bacillus thuringiensis* @ 0.25%	0 $(0.909)^{d}$	56.67 $(7.55)^{b}$	83.33 $(9.15)^{ab}$	86.67 $(9.34)^{a}$
T_8	Flubendiamide 39.35 SC,0.004%	90.00 $(9.50)^{a}$	100.00 $(10.03)^{a}$	100.00 $(10.03)^{a}$	100.00 $(10.03)^{a}$
T_9	Untreated control	0 $(0.909)^{d}$	3.33 $(1.55)^{d}$	6.67 $(2.4)^{e}$	6.67 $(2.4)^{d}$
	CD (0.05)	2.489	2.672	2.441	1.486

*Mean of three replications comprising ten larvae each
(Values in the parentheses are square root transformed)

Least leaf area damage was detected when larvae were treated with *S. marcescens* (Hv3) and isolate 5 and was found to be superior over all other bacterial treatments at all intervals of observation.

At 1 DAT, a leaf area damage of 25.62 and 27.62 per cent were caused by larvae treated with *S. marcescens* (Hv3) and isolate 5. *S. marcescens* (Hv3) treatment was found to be significantly superior over other treatments except application of flubendiamide which caused only 7.66 per cent damage. Per cent damage of 30.05 was caused by isolate 12 treated larvae. Larvae treated with isolate 7, isolate 3 and *Bt* resulted in 31.5, 32.48 and 33.00 per cent leaf area damage respectively which were on par with each other. Treatment with *P. fluorescens* (PNO26R) was found inferior to all other treatments with 36.03 per cent damage.

At 3 DAT, a rapid reduction was noticed in the per cent leaf area damaged by larvae treated with isolate 5 (6.65%) as against 47.75 per cent damage in the untreated control, and was found statistically superior over the other bacterial agents. *S. marcescens* (Hv3) treated larvae caused 12.87 per cent damage. Comparatively high leaf area damage was observed in all other treatments.

S. marcescens (Hv3) and isolate 5 treated larvae were found statistically superior over other bacterial agents and on par with the chemical check, exhibiting only 2.71 and 5.23 per cent damage respectively as against 77.75 per cent damage in the untreated control at 5 DAT. Larvae treated with isolate 7 caused 35.14 per cent damage which was on par with that of *Bt* (28.90%). More than 50.00 per cent damage was caused by isolate 12, isolate 3 and *P. fluorescens* (PNO26R) treated larvae.

At 7 DAT, per cent leaf area damage of only 4.51 and 8.31 per cent were caused by larvae treated with *S. marcescens* (Hv3) and isolate 5 treated larvae respectively as against 100 per cent damage in the untreated check, and were on par with the chemical check. Leaf area damage resulted by isolate 7 treated larvae

was more (59.34%) and the treatment was inferior to *Bt* (16.19%). Damage resulted by larvae treated with *P. fluorescens* (PNO26R), isolate 12 and isolate 3 were high *viz*. 67.10, 74.57 and 77.24 per cent respectively.

Based on the results obtained from laboratory experiment, isolate 5 and 7 were found to be superior among the pathogenic phylloplane isolates tested, both in terms of high mortality and low leaf area damage. These phylloplane bacteria along with *S. marcescens* (Hv3) and *P. fluorescens* (PNO26R) found effective against chewing pests were selected for further evaluation.

4.5 MOLECULAR CHARACTERISATION OF SUPERIOR PHYLLOPLANE ISOLATES

16s rDNA sequence of internal transcribed region of superior phylloplane isolates (isolate 5 and 7) amplified using CAGGCCTAACACATGCAAGTC as forward primer and GGGCGGWGTGTACAAGGC as reverse primer are presented in Table 7. Blast details of the most matching sequence homology in NCBI data base with respect to isolate 5 and 7 are presented in Table 8.

Isolate 5 and 7 exhibited 99 per cent and 100 per cent sequence identity with *Serratia marcescens* Db11, complete genome and *Klebsiella* sp T-3-1 16S ribosomal RNA gene respectively.

4.6 BIOCHEMICAL CHARACTERISATION OF THE SELECTED ISOLATES

Results obtained from biochemical characterisation of the superior phylloplane isolates are presented in Table 9.

Isolate 5 exhibited positive reaction to the utilisation of carbohydrates, glucose, arabinose, galactose, dextrose, fructose, xylose, maltose, melibiose, glycerol and mannitol and negative reaction to the utilization of adonitol, lactose, malonate, sucrose, mannitol, rhamnose, trehalose, raffinose, inulin, dulcitol, inositol and erythritol. It showed positive reaction with respect to the enzymes,

Table 6. Leaf area damage caused by *D. indica* larvae treated with bacterial bioagents

Treatments		Per cent Leaf area damage*			
		1 DAT	3 DAT	5 DAT	7 DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	25.62 $(5.07)^f$	12.87 $(3.63)^d$	2.71 $(1.46)^e$	4.51 $(1.72)^d$
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	36.03 $(6.00)^b$	40.12 $(6.37)^{ab}$	57.77 $(7.62)^{ab}$	67.1 $(8.20)^{ab}$
T_3	Isolate 3 @ 10^8 cfu ml^{-1}	32.48 $(5.70)^c$	39.71 $(6.33)^b$	58.29 $(7.64)^{ab}$	77.24 $(8.79)^{ab}$
T_4	Isolate 5 @ 10^8 cfu ml^{-1}	27.62 $(5.25)^e$	6.65 $(2.64)^e$	5.23 $(2.16)^e$	8.31 $(2.64)^{cd}$
T_5	Isolate 7 @ 10^8 cfu ml^{-1}	31.5 $(5.61)^{cd}$	27.44 $(5.28)^c$	35.14 $(5.96)^{cd}$	59.34 $(7.22)^b$
T_6	Isolate 12 @ 10^8 cfu ml^{-1}	30.05 $(5.48)^d$	35.33 $(5.96)^b$	53.00 $(7.29)^{bc}$	74.57 $(8.64)^{ab}$
T_7	*Bacillus thuringiensis* @ 0.25%	33.00 $(5.74)^c$	37.2 $(6.14)^b$	28.90 $(5.42)^d$	16.19 $(4.05)^c$
T_8	Flubendiamide 39.35 SC,0.004%	7.66 $(2.77)^g$	0.68 $(1.05)^f$	0 $(0.909)^e$	0 $(0.909)^d$
T_9	Untreated control	38.49 $(6.20)^a$	47.75 $(6.95)^a$	77.75 $(8.44)^a$	100.00 $(10.00)^a$
	CD (0.05)	0.168	0.577	1.431	1.920

*Mean of three replications comprising ten larvae each
(Values in the parentheses are square root transformed)

Table 7. DNA sequence of 16S rRNA of superior phylloplane bacteria

Isolates	Sequence
5	TGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGG ATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGG GGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGG TGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAG GCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACG TTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC ACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACT GCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGT AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCC TGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA TACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGG CGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAA GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG TTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTT CCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTC GTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA TCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTA CACACGTGCTACAATGGCGTATACAAAGAGAAGCGACCTCGCGAGAGCAAGCG GACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGA AGTCGGAATCGCTAGTAATCGTAGATCAGA
7	TCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGA GGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAA AGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTA GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGAT GACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGA AGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAAGGTTA ATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA AAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTG GGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCC AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCG GCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCC CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACG GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG CATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACA GAACTTGCCAGAGATGGCTTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCAT GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCA ACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAG TGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACC AGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGA GCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGAC TCCATGAAGTCGGAATCGCTAGTAATCGTAGATC

Table 8. Blast search details of the DNA of rRNA sequences of superior phylloplane isolates in NCBI data base

Isolates	Description	Max score	Total score	Query cover	E value	Identity	Accession No.
5	*Serratia marcescens* subsp *marcescens* Db11, complete genome	2324	16169	100%	0.0	99%	NZ_HG326223.1
7	*Klebsiella* sp T-3-1 16S ribosomal RNA gene, partial sequence	2344	2344	100%	0.0	100%	FJ577968.1

citrate, nitrate and negative reaction to arginine, Voges Proskeur, catalase, lysine, ornithine, urease, phenyl alanine, α-methyl- D glucoside and no H_2S production.

Isolate 7 exhibited positive reaction to the utilisation of carbohydrates, glucose, arabinose, malonate, trehalose, raffinose, galactose, dextrose, fructose, xylose, maltose, melibiose, glycerol, inulin, dulcitol, inositol and mannitol and negative to the utilization of adonitol, lactose, sucrose, mannitol, rhamnose and erythritol. With respect to enzymes, isolate 7 showed positive reaction to citrate, urease, nitrate and negative to lysine, ornithine, phenyl alanine, arginine, Voges Proskeur, catalase, α-methyl- D glucoside and no H_2S production.

4.7 CHITINASE ACTIVITY

Upon application of congo red on bacterial growth on media containing colloidal chitin as the sole carbon source, development of clear transparent zone was detected around the colony in both isolate 5 (*S. marcescens* pmc5) and isolate 7 (*Klebsiella* sp). Development of halo with diameter 1.40 cm and 0.8 cm was noticed in *S. marcescens* (pmc5) (Plate 4a) and *Klebsiella* sp (Plate 4b) respectively. This indicate the digestion of colloidal chitin in the media by chitinase enzyme produced by these bacteria.

Chitinase enzyme activity of 1.24 units and 1.07 units were detected in *S. marcescens* (pmc5) and *Klebsiella* sp respectively in assay using chitin- azure as substrate (Table 10).

4.8 ANTIBIOTIC RESISTANCE PATTERN

Growth exhibited by *S. marcescens* (pmc5) and *Klebsiella* sp in antibiotic amended plates is shown in Table 11.

S. marcescens (pmc5) when grown individually in different antibiotic amended NA media, exhibited growth in Ampicillin 50, 75 and 100 ppm,

Table 9. Biochemical characterisation of selected phylloplane bacteria

Sl. No.	**Carbon source utilisation**	*S. marcescens* (pmc5) (Isolate 5)	*Klebsiella* sp (Isolate 7)
1	Dextrose	+	+
2	Xylose	+	+
3	Maltose	+	+
4	Melibiose	+	+
5	Glucose	+	+
6	Adonitol	-	-
7	Arabinose	+	+
8	Lactose	-	-
9	Inulin	-	+
10	Dulcitol	-	+
11	Inositol	-	+
12	Mannitol	+	+
13	Erythritol	-	-
14	Malonate	-	+
15	Glycerol	+	+
16	Sucrose	-	-
17	Mannitol	-	-
18	Rhamnose	-	-
19	Trehalose	-	+
20	Raffinose	-	+
21	Galactose	+	+
22	Fructose	+	+
	Biochemical reaction		
1	Citrate	+	+
2	Ornithine	-	-
3	Urease	-	+
4	Lysine	-	-
5	Phenyl alanine	-	-
6	Nitrate	+	+
7	Arginine	-	-
8	Voges Proskeur	-	-
9	Catalase	-	-
10	H_2S production	-	-
11	α-methyl – D glucoside	-	-

Table 10. Chitinase activity of selected phylloplane bacteria.

Sl. No.	Bacteria	Clear halo diameter (cm)	Chitinase activity (units)
1	*Serratia marcescens* (pmc5)	1.4	1.24
2	*Klebsiella* sp	0.80	1.07

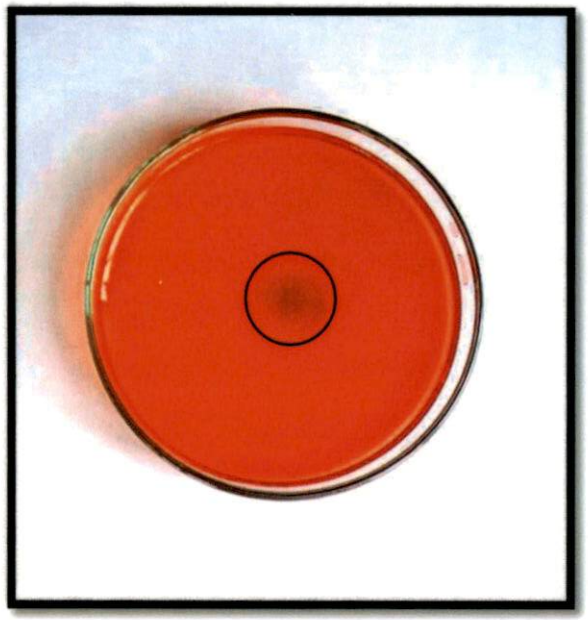

Plate 4a: *Serratia marcescens* (pmc 5)

Plate 4b: *Klebsiella* sp

Plate 4: Chitinase activity of superior phylloplane bacteria

Tetracycline 10, 20 and 50 ppm, Spectinomycin 25, 50 and 100 ppm, Streptocycline 50, 75 and 100 ppm and Rifampicin 25, 50 and 75 ppm.

Similarly, *Klebsiella* sp showed growth in Ampicillin 50, 75 and 100 ppm, Tetracycline 10, 20 and 50 ppm, Neomycin 50, 75 and 100 ppm, Spectinomycin 25, 50 and 100 ppm, Streptocycline 50, 75 and 100 ppm and Rifampicin 25, 50 and 75 ppm.

Based on this combined antibiotic resistance, pattern worked out for the isolates are shown below.

S. marcescens (pmc5) exhibited growth in NA plates amended with combination of Ampicillin^{100}, Tetracycline^{50}, Spectinomycin^{100}, Streptocycline^{100}, Rifampicin^{75}.

Klebsiella sp showed growth in NA plates amended with combination of Ampicillin^{100}, Tetracycline^{50}, Neomycin^{100}, Spectinomycin^{100}, Streptocycline^{100}, Rifampicin^{75}.

4.9 MANAGEMENT OF CHEWING PESTS OF BITTER GOURD WITH BACTERIAL BIOAGENTS (POT CULTURE EXPERIMENT)

Data on efficacy of foliar application of bacterial bioagents in managing the major chewing pests, *H. septima* and *D. indica* of bitter gourd raised in pots is presented below.

4.9.1 Mortality of *H. septima*

Mortality of *H. septima* grubs on plants treated with bacterial bioagents at different intervals is presented in Table 12.

At 1 DAT, the phylloplane isolated *S. marcescens* (pmc5) treated plants recorded 38.33 per cent mortality of *H. septima* grubs which was on par with quinalphos (0.05%) treatment. *S. marcescens* (Hv3) with 23.41 per cent mortality

Table 11. Antibiotic resistance of selected phylloplane bacteria

Sl. No.	Antibiotic	Concentration (ppm)	*S. marcescens* (pmc5) (Isolate 5)	*Klebsiella* sp (Isolate 7)
1	Ampicillin	50	+	+
		75	+	+
		100	+	+
2	Tetracycline	10	+	+
		20	+	+
		50	+	+
3	Neomycin	50	-	+
		75	-	+
		100	-	+
4	Spectinomycin	25	+	+
		50	+	+
		100	+	+
5	Streptocycline	50	+	+
		75	+	+
		100	+	+
6	Rifampicin	25	+	+
		50	+	+
		75	+	+

+ bacterial growth observed
- no bacterial growth

was on par with *S. marcescens* (pmc5). However, the chemical check, flubendiamide 39.35 SC (0.004%), *P. fluorescens* (PNO26R) and *Bt* were found less effective with mortality of 6.67, 5.56 and 0 per cent respectively.

At 3 DAT also *S. marcescens* (pmc5) produced maximum mortality (55 %) which was on par with *S. marcescens* (Hv3) with 37.9 per cent mortality. The mortality in plants treated with *Klebsiella* sp increased to 26.49 per cent and was on par with *S. marcescens* (Hv3). Flubendiamide (12.22%) and *P. fluorescens* (PNO26R) (10.32%) exhibited low mortality. *Bt* did not show any mortality at 3 DAT also.

At 5 and 7 DAT, *S. marcescens* (pmc5) recorded the highest mortality (65.83 %) among the bacterial bioagents and was equally effective as quinalphos 0.05 %. *S. marcescens* (Hv3) with 42.06 per cent mortality at 5 and 7 DAT was statistically on par with *S. marcescens* (pmc5) treatment. Mortality of grubs in plants treated with *Klebsiella* sp and *P. fluorescens* (PNO26R) were 33.09 and 15.87 per cent respectively at 5 DAT which increased to 37.86 and 21.43 per cent respectively at 7 DAT. *Bt* did not exhibit any mortality on the grubs at any of the intervals of observation.

4.9.2 Leaf Area Damage by *H. septima*

Leaf area damage caused by *H. septima* grubs on plants after treatment with bacterial agents at different intervals of observation is shown in Table 13.

At all intervals after treatments, the chemical check (quinalphos 0.05 %) was found to significantly reduce the leaf area damage by *H. septima* than the other treatments.

At the first day after foliar application of bacterial bioagents, leaf area damage of 22.69 per cent was noticed on *S. marcescens* (pmc5) treated plants. The plants treated with quinalphos 0.05 % exhibited significantly low leaf area damage (7.87 %). *S. marcescens* (Hv3) treatment resulted in 28.21 per cent

Table 12. Mortality of *H. septima* grubs on plants treated with bacterial bioagents (Pot culture)

Treatments		Per cent mortality *			
		1 DAT	3 DAT	5 DAT	7 DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	23.41 $(4.87)^{bc}$	37.90 $(6.18)^{b}$	42.06 $(6.50)^{b}$	42.06 $(6.50)^{b}$
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	5.56 $(1.85)^{d}$	10.32 $(2.86)^{bc}$	15.87 $(4.05)^{c}$	21.43 $(4.61)^{bc}$
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	38.33 $(6.17)^{ab}$	55.00 $(7.38)^{ab}$	65.83 $(8.14)^{ab}$	65.83 $(8.14)^{ab}$
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	15 $(3.42)^{bc}$	26.49 $(5.08)^{b}$	33.09 $(5.56)^{bc}$	37.86 $(6.07)^{bc}$
T_5	*Bacillus thuringiensis* @ 0.25%	0 $(0.909)^{d}$	0 $(0.909)^{d}$	0 $(0.909)^{d}$	0 $(0.909)^{d}$
T_6	Flubendiamide 39.35 SC @ 0.004%	6.67 $(1.99)^{cd}$	12.22 $(3.12)^{bc}$	18.89 $(4.39)^{bc}$	18.89 $(4.39)^{c}$
T_7	Quinalphos 25 EC @ 0.05%	77.78 $(8.85)^{a}$	100.00 $(10.03)^{a}$	100.00 $(10.03)^{a}$	100 .00 $(10.03)^{a}$
T_8	Untreated control	0 $(0.909)^{d}$	5.56 $(1.85)^{cd}$	5.56 $(1.85)^{d}$	5.56 $(1.85)^{d}$
	CD (0.05)	2.788	2.823	2.144	2.061

*Mean of three replications
(Values in the parentheses are square root transformed)

damage which was on par with *S. marcescens* (pmc5) and *Bt* (24.84%). High leaf area damage of 34.72 per cent, comparable to untreated check (33.49 %) was noticed in plants treated with *Klebsiella* sp (34.72%).

At 3 DAT, *S. marcescens* (pmc5) treated plants exhibited 20.49 per cent damage on leaves which was statistically on par with that of *S. marcescens* (Hv3) (27.17 %). *S. marcescens* (pmc5) treatment resulted in significantly low damage than the other bacterial bioagents except *S. marcescens* (Hv3). The other bacterial agents as well as flubendiamide treatment did not produce any significant result than the control.

At 5 DAT, *S. marcescens* (pmc5) and *S. marcescens* (Hv3) treated plants were observed with per cent leaf area damage of only 18.77 and 23.34 per cent respectively as against 60.18 per cent in untreated control and these treatments were statistically superior over all other treatments except quinalphos 0.05 % (0 %). Increase in leaf area damage was observed in the rest of the treatments which did not differ significantly from untreated control.

At 7 DAT, *S. marcescens* (pmc5) (16.26 % leaf area damage) produced significantly superior results than all other treatments other than quinalphos 0.05 %. *S. marcescens* (Hv3) treated plants exhibited 36.16 per cent damage which was superior than control. All the other treatments did not significantly reduce the leaf area damage than untreated control.

4.9.3 Mean Population of *H. septima*

Mean population of *H. septima* grubs per plant at different intervals after treatment with different bacterial bioagents was recorded to observe the population change, the results of which are presented in Table 14.

The population of *H. septima* grubs were found to be homogenous in all the plants upon analysis of the pre treatment count. Plants treated with quinalphos

Table 13. Leaf area damage caused by *H. septima* grubs on plants treated with bacterial bioagents (Pot culture)

Treatments		Per cent leaf area damage*			
		1 DAT	3 DAT	5 DAT	7 DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	28.21 (5.31)^{ab}	27.17 (5.20)^{bc}	23.34 (4.82)^{b}	36.16 (6.01)^{b}
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	29.89 (5.46)^{ab}	32.05 (5.65)^{ab}	40.66 (6.38)^{a}	44.44 (6.65)^{ab}
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	22.69 (4.75)^{b}	20.49 (4.41)^{c}	18.77 (4.18)^{b}	16.26 (3.94)^{c}
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	34.72 (5.87)^{a}	38.67 (6.18)^{ab}	47.22 (6.79)^{a}	54.34 (7.17)^{ab}
T_5	*Bacillus thuringiensis* @ 0.25%	24.84 (4.96)^{b}	36.71 (6.02)^{ab}	57.48 (7.55)^{a}	64.32 (7.98)^{a}
T_6	Flubendiamide 39.35 SC @ 0.004%	28.67 (5.32)^{ab}	34.47 (5.86)^{ab}	41.29 (6.42)^{a}	44.80 (6.69)^{ab}
T_7	Quinalphos 25 EC @ 0.05%	7.87 (2.79)^{c}	0.11 (0.32)^{d}	0 (0.909)^{c}	0 (0.909)^{d}
T_8	Untreated control	33.49 (5.79)^{a}	45.98 (6.78)^{a}	60.18 (7.75)^{a}	68.28 (8.26)^{a}
	CD (0.05)	0.787	1.162	1.451	1.714

*Mean of three replications
(Values in the parentheses are square root transformed)

0.05 % could bring about significant reduction in population of *H. septima* than the other treatments at all intervals after treatment.

A post treatment count of 11.84 grubs plant^{-1} observed on *S. marcescens* (pmc5) treated plants was the lowest among the bacterial bioagents which was statistically on par with quinalphos 0.05 % at 1 DAT. The rest of the treatments failed to significantly reduce the population than untreated control.

At 3 DAT, no grubs were observed in plants treated with quinalphos (0.05 %). *S. marcescens* (pmc5) which recorded 8.61 grubs plant^{-1} was observed to be the next best treatment after quinalphos, and was statistically on par with *S. marcescens* (Hv3) which recorded 14.00 grubs plant^{-1}. These were followed by *Bt*, flubendiamide 0.004 %, *Klebsiella* sp and *P. fluorescens* (PNO26R) with 15.07, 15.07, 16.15 and 18.30 grubs plant^{-1} respectively which did not differ significantly from untreated control (17.23 grubs plant^{-1}).

At 5 and 7 DAT, plants treated with *S. marcescens* (pmc5) exhibited significantly low population of *H. septima* (6.46 grubs plant^{-1}) than all other treatments except quinalphos 0.05 %. The population did not differ significantly from untreated control in rest of the treatments.

4.9.4 Mortality of *D. indica*

Mortality of *D. indica* larvae on plants after treatment with bacterial agents at different intervals of observation is presented in Table 15.

At 1 DAT, the phylloplane isolated *S. marcescens* (pmc5) with 28.89 per cent mortality was found to be superior after both the chemical checks in causing mortality of the larvae. *S. marcescens* (Hv3) treatment caused a per cent mortality of 10.32 per cent. *Klebsiella* sp, *Bt*, and *P. fluorescens* (PNO26R) did not cause mortality on the larvae.

Table 14. Mean population of *H. septima* grubs on bitter gourd after treatment with bacterial bioagents (Pot culture)

Treatments		Mean population*			
		1DAT	3DAT	5DAT	7DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	17.23^{ab}	14.00^{ab}	12.92^{a}	12.92^{a}
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	19.38^{a}	18.30^{a}	17.23^{a}	16.15^{a}
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	11.84^{bc}	8.61^{b}	6.46^{b}	6.46^{b}
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	18.30^{ab}	16.15^{a}	15.08^{a}	14.00^{a}
T_5	*Bacillus thuringiensis* @ 0.25%	15.08^{ab}	15.07^{a}	15.08^{a}	15.07^{a}
T_6	Flubendiamide 39.35 SC @ 0.004%	16.15^{ab}	15.07^{a}	14.00^{a}	14.00^{a}
T_7	Quinalphos 25 EC @ 0.05%	5.38^{c}	0^{c}	0^{c}	0^{c}
T_8	Untreated control	18.30^{ab}	17.23^{a}	17.23^{a}	17.23^{a}
	CD (0.05)	6.545	5.588	5.813	5.711

*Mean of three replications

At 3 DAT, treatments with chemical checks, flubendiamide and quinalphos which recorded 100 per cent and 95.24 per cent mortality respectively were observed as the most superior treatments. Among the bacterial agents, *S. marcescens* (pmc5) with 58.90 per cent mortality was found statistically on par with *S. marcescens* (Hv3) with 31.75 per cent mortality and superior than rest of the treatments. The other bacterial agents tested, though superior than untreated control, were statistically on par with each other with respect to mortality.

Mortality of 100 per cent was observed at 5 and 7 DAT in both the chemical checks. At 5 DAT, *S. marcescens* (pmc5) recorded the high mortality of 58.90 per cent among the bacterial bioagents and was on par with *S. marcescens* (Hv3) with 49.01 per cent mortality. *Klebsiella* sp resulted in 22.26 per cent mortality, on par with *Bt* (32.22 %). All the bacterial agents were found to be superior than the untreated control.

At 7 DAT, *S. marcescens* (pmc5) treated plants exhibited 87.78 per cent mortality of the larvae, which was statistically on par to both the chemical checks each with 100 per cent mortality. *S. marcescens* (Hv3) treatment with 57.14 per cent mortality of larvae was on par with *S. marcescens* (pmc5).

4.9.5 Leaf Area Damage by *D. indica*

Leaf area damage caused by *D. indica* larvae on plants treated with bacterial agents at different intervals of observation is shown in Table 16.

Application of chemical pesticides was found to significantly reduce the leaf area damage by *D. indica* at 1, 3, 5 and 7 DAT.

1 DAT, plants treated with the phylloplane isolated *Klebsiella* sp and *S. marcescens* (pmc5) treated larvae were observed with 20.39 and 23.19 per cent damage respectively, which were the best after the chemical checks, flubendiamide (1.81 %) and quinalphos (8.70 %). Rest of the treatments did not produce significant reduction in leaf area damage than the untreated control.

Table 15. Mortality of *D. indica* larvae on plants treated with bacterial bioagents (Pot culture)

Treatments		Per cent mortality *			
		1DAT	3DAT	5DAT	7DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	10.32 $(2.89)^c$	31.75 $(5.67)^{bc}$	49.01 $(6.98)^{bc}$	57.14 $(7.56)^b$
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	0 $(0.909)^d$	12.22 $(3.12)^d$	15.87 $(4.05)^d$	16.98 $(4.17)^{de}$
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	28.89 $(5.39)^b$	58.90 $(7.70)^b$	58.90 $(7.70)^b$	87.78 $(9.38)^{ab}$
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	0 $(0.909)^d$	13.33 $(3.25)^d$	22.26 $(4.61)^d$	25.56 $(5.02)^{cd}$
T_5	*Bacillus thuringiensis* @ 0.25%	0 $(0.909)^d$	18.89 $(4.39)^{cd}$	32.22 $(5.63)^{cd}$	37.78 $(6.18)^{bc}$
T_6	Flubendiamide 39.35 SC @ 0.004%	93.33 $(9.67)^a$	100.00 $(10.03)^a$	100.00 $(10.03)^a$	100.00 $(10.03)^a$
T_7	Quinalphos 25 EC @ 0.05%	65.08 $(8.07)^a$	95.24 $(9.77)^{ab}$	100.00 $(10.03)^a$	100.00 $(10.03)^a$
T_8	Untreated control	0 $(0.909)^d$	0 $(0.909)^e$	6.67 $(1.99)^e$	11.43 $(3.07)^e$
	CD (0.05)	2.053	2.278	2.341	1.878

*Mean of three replications
(Values in the parentheses are square root transformed)

At 3 DAT, among the bacterial bioagents, *S. marcescens* (pmc5) recorded the least leaf area damage of 21.29 per cent and was on par with *S. marcescens* (Hv3) with 24.53 per cent damage. In plants treated with *Klebsiella* sp, 26.15 per cent leaf area damage was observed making it on par with *S. marcescens* (Hv3). These three bacterial treatments were found to be significantly superior than untreated check (34.81 %) whereas *Bt* and *P. fluorescens* (PNO26R) treatments did not show superiority over control.

At 5 and 7 DAT, plants treated with *S. marcescens* (pmc5) with leaf area damage of 13.55 and 6.99 was found to be significantly superior than rest of the bacterial bioagents in reducing the damage. At 5 DAT, *S. marcescens* (Hv3), *Bt*, *P. fluorescens* (PNO26R) and *Klebsiella* sp treatments with 21.62, 25.13, 27.81 and 28.04 per cent leaf area damage respectively were found superior than control (48.07 %). However, *Klebsiella* sp and *P. fluorescens* (PNO26R) failed to differ significantly from control at 7 DAT.

4.9.6 Mean Population

The population fluctuation of *D. inidca* larvae on plants treated with bacterial agents is presented in Table 17.

Since the pre count of the larvae did not differ significantly, the population is considered homogenous at the beginning of the experiment.

Significant population reduction than rest of the treatments was observed in both chemical checks at all intervals after treatment folowed by *S. marcescens* (pmc5) where the per plant population of *D. indica* larvae was found to decrease gradually from 12.48 to 2.08 from 1 to 7 DAT.

At 1 DAT, *Klebsiella* sp, *Bt*, and *P. fluorescens* (PNO26R) treatments recorded with mean populations of 16.64, 16.64 and 18.72 larvae plant^{-1} did not differ significantly from that of untreated control.

Table 16. Leaf area damage caused by *D. indica* larvae on plants treated with bacterial bioagents (Pot culture)

Treatments		Per cent leaf area damage*			
		1DAT	3DAT	5DAT	7DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	23.52 $(4.84)^{ab}$	24.53 $(4.99)^{cd}$	21.62 $(4.68)^{b}$	30.8 $(5.99)^{b}$
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	24.64 $(4.96)^{ab}$	30.31 $(5.54)^{ab}$	27.81 $(5.29)^{b}$	41.28 $(6.44)^{ab}$
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	23.19 $(4.82)^{b}$	21.29 $(4.67)^{d}$	13.55 $(3.73)^{c}$	6.99 $(2.45)^{c}$
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	20.39 $(4.51)^{b}$	26.15 $(5.16)^{bcd}$	28.04 $(5.32)^{b}$	40.93 $(6.41)^{ab}$
T_5	*Bacillus thuringiensis* @ 0.25%	23.61 $(4.85)^{ab}$	29.39 $(5.47)^{abc}$	25.13 $(5.06)^{b}$	35.71 $(5.98)^{b}$
T_6	Flubendiamide 39.35 SC @ 0.004%	1.81 $(1.35)^{d}$	0.03 $(0.73)^{f}$	0 $(0.701)^{d}$	0 $(0.701)^{d}$
T_7	Quinalphos 25 EC @ 0.05%	8.70 $(2.94)^{c}$	2.08 $(1.60)^{e}$	0.02 $(0.72)^{d}$	0 $(0.701)^{d}$
T_8	Untreated control	28.25 $(5.30)^{a}$	34.81 $(5.93)^{a}$	48.07 $(6.95)^{a}$	63.48 $(7.95)^{a}$
	CD (0.05)	0.473	0.489	0.862	1.684

*Mean of three replications
(Values in the parentheses are square root transformed)

3 DAT, population in plants treated with *Bt* and *S. marcescens* (Hv3) got reduced to 13.52 per plant making it significantly superior than untreated check.

At 5 DAT, the population of *D. indica* in plants treated with *S. marcescens* (pmc5) was only 2.08 per plant making it on par with application of chemical insecticides. However, at 7 DAT, the population did not show further reduction. At 7 DAT, *S. marcescens* (pmc5) and *S. marcescens* (Hv3) treatments with mean population of 2.08 and 8.32 larvae plant^{-1} were found to be on par and superior over control in reducing the population.

4.9.7 Mean Population of Other Pests in Bitter gourd After Treatment

Mean population of other pests of bitter gourd recorded 5 DAT is presented in Table 18.

Though the attack of *Aphis gossypii* and *Bactrocera cucurbitae* were observed, their population did not differ significantly among the treatments.

4.9.8 Mean Population of Natural Enemies in Bitter gourd After Treatment

There was no significant difference in the mean population of natural enemies of pests of bitter gourd among the different treatments (Table 19).

4.9.9 Yield of Bitter Gourd Treated with Different Phylloplane and Pathogenic bacteria

Yield per plant of bitter gourd treated with different bacterial bioagents is presented in Table 20.

Though the plants treated with phylloplane isolates, *S. marcescens* (pmc5) and *Klebsiella* sp recorded the higher yields (1.86 and 1.80 kg plant^{-1} respectively), there was no statistical significance with respect to yield per plant.

Table 17. Mean population of *D. indica* larvae on bitter gourd after treatment with bacterial bioagents (Pot culture)

Treatments		Mean population*			
		1DAT	3DAT	5DAT	7DAT
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	17.68^a	13.52^b	9.36^c	8.32^d
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	18.72^a	16.64^{ab}	15.6^{ab}	15.6^{ab}
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	12.48^b	7.28^c	2.08^d	2.08^d
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	16.64^a	14.56^{ab}	12.48^{bc}	12.48^{bc}
T_5	*Bacillus thuringiensis* @ 0.25%	16.64^a	13.52^b	11.44^{bc}	10.4^{cd}
T_6	Flubendiamide 39.35 SC @ 0.004%	1.04^d	0^d	0^d	0^e
T_7	Quinalphos 25 EC @ 0.05%	6.24^c	1.04^d	0^d	0^e
T_8	Untreated control	18.72^a	18.72^a	17.68^a	16.64^a
	CD (0.05)	3.48	4.67	4.67	3.98

*Mean of three replications

Table 18. Mean population / infestation of other pests in bitter gourd at 5 DAT with bacterial bioagents

Treatments		Mean population per plant of *Aphis gossypii**	*Bactrocera cucurbitae* infested fruits per plant*
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	29.33	38.27
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	36.33	35.71
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	26.77	43.48
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	0	37.00
T_5	*Bacillus thuringiensis* @ 0.25%	28.00	41.47
T_6	Flubendiamide 39.35 SC,0.004%	0	33.63
T_7	Quinalphos 25 EC, 0.05%	24.33	35.16
T_8	Untreated control	32.00	39.88
		NS	NS

* Mean of three replications

Table 19. Mean population of natural enemies in bitter gourd at 5 DAT with bacterial bioagents

Treatments		Predators		Parasitoids
		Spider	Praying mantid	*Apanteles flavipes*
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	0.33	0	1.33
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	0	0.66	1.33
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	0	0	1.00
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	0.67	1.00	0.33
T_5	*Bacillus thuringiensis* @ 0.25%	0	0	2
T_6	Flubendiamide 39.35 SC @ 0.004%	0	0.33	0
T_7	Quinalphos 25 EC @ 0.05%	0.33	0.33	1.00
T_8	Untreated control	1.00	0.67	1.67
		NS	NS	NS

* Mean of three replications

Table 20. Yield of bitter gourd treated with bacterial bioagents

	Treatments	Yield (Kg Plant^{-1})
T_1	*Serratia marcescens* (Hv3) @ 10^8 cfu ml^{-1}	1.47
T_2	*Pseudomonas fluorescens* (PNO26R) @ 10^8 cfu ml^{-1}	0.36
T_3	*Serratia marcescens* (pmc5) @ 10^8 cfu ml^{-1}	1.86
T_4	*Klebsiella* sp @ 10^8 cfu ml^{-1}	1.80
T_5	*Bacillus thuringiensis* @ 0.25%	0.45
T_6	Flubendiamide 39.35 SC, 0.004%	0.41
T_7	Quinalphos 25 EC, 0.05%	0.95
T_8	Untreated control	0.36
		NS

* Mean of three replications

Discussion

5. DISCUSSION

Epilachna beetle, *Henosepilachna septima* (Dieke) has been identified as a destructive pest of bitter gourd where the grubs and adults feed on leaves throughout the growth stages of the crop (Sreekala and Ushakumari, 1999). The pumpkin caterpillar or cucumber moth, *Diaphania indica* (Saund) is another widespread foliage feeding pest of bitter gourd (Morgan and Midmore, 2002; Arcaya *et al.*, 2004). These chewing pests defoliate the plants reducing the photosynthetic efficiency leading to substantial yield loss. In addition, they also infest the fruits at times reducing its quality and market value (Singh and Naik, 2006). Considering the negative impacts due to the over reliance on insecticides by farmers to manage these pests, microbial management is assumed as an eco friendly alternative.

Epilachna beetle and *D. indica* were reported to be susceptible to certain pathogenic micro organisms (Markandeya *et al.*, 2001; Neema *et al.*, 2010). Being chewing insects, larvae of both *H. septima* and *D. indica* consume the phylloplane microorganisms that stably colonize both internal and exposed sites of plant along with the foliage. Phylloplane bacteria survive on the leaf surfaces colonizing on sites such as trichomes, stomata and epidermal cell wall junctions (Beattie and Lindow, 1995). Andrews (1992) reported that phylloplane microorganisms have immense potential to act as biological agents to suppress foliar pathogens and insect defoliators. Some of them produce extracellular chitinase which in turn degrades the peritrophic membrane of chewing insects, thereby making them good biocontrol agents (Aggarwal *et al.*, 2015). Hence, attempts were made in the present study to isolate and characterize phylloplane bacteria from bitter gourd and to utilize them along with other entomopathogens, in the biocontrol of major chewing pests, *H. septima* and *D. indica*.

Bacteria were isolated from bitter gourd phylloplane by taking leaf impression of both surfaces of bitter gourd leaves in M-9 minimal agar media containing colloidal chitin as the sole carbon source. The bacteria that showed growth in this medium were thus confirmed to have the ability to degrade chitin.

Thirteen bacteria were isolated and serially numbered from1 to 13 in the order of isolation. Upon preliminary screening by oral exposure, out of these thirteen isolates, three isolates (isolate 1, 5 and 7) were found pathogenic to *H. septima* grubs and four (isolate 3, 5, 7 and 12) to *D. indica* larvae. Pathogenicity of these five phylloplane isolates were further confirmed by proving Koch's postulates. The larvae of *H. septima* and *D. indica* upon bacterial infection produced symptoms such as discolouration, feeding cessation and swelling of body before death followed by appearance of bacterial ooze along with putrified smell. These symptoms are typical of bacterial infection (Tanada and Kaya, 1993).

The pathogenic phylloplane isolates were characterised by studying the colony morphology and Gram staining. Colonies of isolate 1 were observed as slow growing in NA plates. All other isolates, isolate 3, 5, 7 and 12 were found to be fast growing. White to creamish yellow colour was exhibited by 1, 3, 7 and 12 whereas isolate 5 was observed as dark red smooth and raised colonies. Red coloured colonies are observed in many *Serratia marcescens* isolates due to the production of pigment prodigiosin (Patel *et al.*, 2011). Upon Gram staining, isolate 1, 5 and 7 were noticed as Gram negative and isolate 3 and 12 were found to be Gram positive.

Laboratory evaluation of bacterial isolates against chewing pests was done employing the infective phylloplane isolates (isolate 1, 5 and 7 against *H. septima* and isolate 3, 5, 7 and 12 against *D. indica*) along with entomopathogen, *S. marcescens* (Hv3), isolated from *H. vigintioctopuncatata* (Aswathy, 2015). Commercial bacterial biocontrol agents, *P. fluorescens* (PNO26R) (Anith *et al.*, 2003) and *B. thuringiensis* were also included as treatments besides flubendiamide 0.004 % as chemical check.

S. marcescens (Hv3) and phylloplane isolate 5 treatments caused more than 80 per cent mortality of *H. septima* 3 DAT onwards, an effect significantly superior than rest of the treatments including the chemical check. The same bacteria produced more than 90 per cent mortality in *D. indica* also at 3 DAT making it as effective as flubendiamide 0.004 % as well as *B. thuringiensis*. *S. marcescens* was reported to

multiply rapidly inside insect hemocoel bringing about death of the insect within 3 DAT (Tanada and Kaya, 1993). In the present study, 83.33 and 73.33 per cent mortality were obtained in *S. marcescens* (Hv3) treated larvae of *H. septima* and *D. indica* respectively, within 24 h of treatment. Reports show the entomopathogenic activity of *S. marcescens* against corn ear worm, *H. zea* (Boddie), tobacco bud worm, *Heliothis virescens* (F.), cotton boll weevil, *Anthonomus grandis* and pecan weevil, *Curculio caryae* (Horn) (Sikorowski and Lawrence, 1998). *S. marcescens* isolated from dead grubs of epilachna beetle, *H. vigintioctopunctata* was found to cause mortality of 93.28 per cent (Aswathy, 2015). In the present study, the same bacteria was found to be effective against another epilachna beetle, *H. septima* and *D. indica*. Both *S. marcescens* (Hv3) and the red coloured isolate 5 could bring about death of more than 70 per cent of the larvae 24 h after treatment. Mosquito larvicidal activity by *S. marcescens* resulting from the red colour pigment prodigiosin causing 50 per cent mortality within the first 24 h of treatment was reported by Patel *et al.* (2011). The production of prodigiosin pigment by *S. marcescens* might be the probable reason for inducing mortality in insects.

After *S. marcescens* (Hv3) and isolate 5, the isolate 7 produced the next best result with 50 and 56.67 per cent mortality in *H. septima* and *D. indica* respectively at 7 DAT. *P. fluorescens* (PNO26R), though slow in action could result in mortality of 36.67 and 23.33 per cent in *H. septima* and *D. indica* repectively at 7 DAT. *P. fluorescens* strains were reported to cause mortality and morphological defects in laboratory insects (Pimenta *et al.*, 2003). Reports reveal that *P. fluorescens* resulted in mortality of 70 and 56 per cent in the larvae and adults of alder leaf beetle, *Agelastica alni* L. respectively within 7 days after treatment (Sezen *et al.*, 2004).

B. thuringiensis though ineffective against *H. septima*, was found equally effective as *S. marcescens* (Hv3) and isolate 5 against *D. indica*. *Bt* subsp *kurstaki* (*Btk*) is generally used against young lepidopteran larvae and includes different strains with significant commercial interest like HD-1, SA-11, SA-12, PB 54, ABTS-351 and EG2348 (Glare and O'Callaghan, 2000). *Bt* has been reported as a microbial control agent against many lepidopterans like Gram pod borer, *H. armigera,* leaf

caterpillar, *S. litura*, (Kausarmalik, 2014), cabbage white butterfly *Pieris brassicae* (Mohan *et al.*, 2014) and bihar hairy caterpillar, *Spilarctia obliqua* (Khan, 2015).

Leaf area damage resulted by larvae of *H. septima* and *D. indica* after treatment with each of the bacterial agents was recorded in the study since foliar protection is the main objective of pest management. Foliar damage was assessed to check the efficacy of bacterial agents in reducing damage since there are reports showing phylloplane isolated bacteria, *Alcaligenes paradoxus* KPM-012A could suppress leaf feeding and oviposition by *Epilachna vigintioctopunctata* (Otsu *et al.*, 2003). In the present study, treatment with *S. marcescens* (Hv3) recorded significantly low leaf area damage (7.93 %) by *H. septima* at 5 DAT followed by isolate 5 (14.68 %) as against 100 per cent in untreated control. Isolate 7 also reduced the leaf area damage considerably over control. *S. marcescens* (Hv3) and isolate 5 treatments were found statistically superior over other bacterial agents in reducing leaf area damage by *D. indica* and on par with the chemical check, exhibiting only 2.71 and 5.23 per cent damage respectively as against 77.75 per cent damage in the untreated control at 5 DAT. Larvae treated with isolate 7 caused 35.14 per cent damage which was on par with that of *Bt* (28.90%). This reduction in leaf area damage was found to be proportional to mortality. So leaf area damage reduction in the present study is attributed to the high mortality of larvae rather than feeding suppression. Significant reduction in feed consumption by lepidopteran insects, *H. armigera* and *S. litura* on treatment with *Serratia* sp was reported, where mortality of 94.3 per cent and 92.7 per cent were noticed in *H. armigera* and *S. litura* respectively at 72 h after treatment (Chattopadhyay *et al.*, 2012). Isolate 5 and 7 observed superior among the pathogenic phylloplane isolates, both in terms of high mortality and low leaf area damage, were selected for further evaluation.

Inorder to determine the identity, the internal transcribed regions of DNA of 16s rRNA of isolate 5 and 7 were amplified using CAGGCCTAACACATGCAAGTC as forward primer and GGGCGGWGTGTACAAGGC as reverse primer in PCR (Josephrajkumar *et al.* 2017). The 1.5 KB fragment obtained was sequenced. Blast search of amplified DNA

sequence in NCBI data base revealed the identity of isolate 5 and 7 as *Serraia marcescens* and *Klebsiella* sp respectively with 99 per cent and 100 per cent homology. The identity of the bacteria were further confirmed by biochemical analysis in which isolate 5 exhibited negative reaction with respect to urease, malonate and raffinose as carbon source, characteristic to *S. marcescens* whereas isolate 7 exhibited urease, melibiose and glucose positive reaction (Holt *et al.*, 1994). Tanada and Kaya (1993) reported more than 70 species of insects belonging to Lepidoptera, Orthoptera, Coleoptera, Diptera and Hymenoptera were susceptible to infestation by *Serratia* sp. Pu and Hou (2016) isolated *Klebsiella pneumoniae* from dead grubs of red palm weevil, *R. ferrugineus*. *K. pneumoniae* and *S. marcescens* were also isolated from the gut region of red palm weevil by Josephrajkumar *et al.* (2017).

Bacterial pathogens incapable of infesting insects through cuticle often initiate infection through midgut epithelial cells (Pigott and Ellar, 2007). The midgut epithelial cells of chewing insects are protected from pathogens by the presence of chitinous peritrophic membrane (Chapman, 2013). Chitinase activity of the bacterial isolates was studied both visually and quantitatively since it act as an indicator of pathogenicity in chewing insects due to its ability to degrade the chitinous peritrophic membrane. Chitinase activity detected by inoculating bacterial isolates in media containing colloidal chitin as the sole carbon source showed that isolate 5 (*S. marcescens* pmc5) and 7 (*Klebsiella* sp) produced clear halo around the growth with diameter of 1.4 cm and 0.8 cm respectively which was visualized by pouring congo red. Digestion of chitin results in clear transparent zone formation (Soumya *et al.*, 2012). Since chitinase could bring about mortality by way of degrading chitinous peritrophic membrane, its efficacy in chewing pest management has been linked with the amount of chitinase produced. *S. marcescens* pmc5 and *Klebsiella* sp recorded chitinase activity of 1.24 units and 1.07 units respectively, when quantified using chitin-azure by the method devised by Ramirez *et al.* (2004). Gohel *et al.* (2006) stated that bacterial isolates such as *B. circulans* (Jordan), *P. maltophila* and *S. marcescens* released high levels of extracellular chitinolytic enzymes. *S. marcescens*

strain SEN was reported to possess good chitinase activity, where it could bring about 70.8 per cent mortality in larvae of *S. litura* (Aggarwal, 2015). Chitinase activity of 1.4 units was reported in *S. marcescens* (Hv3) by Aswathy (2015). Chitinase producing microorganisms were found to be pathogenic to insects. Potential of chitinolytic microbes as biocontrol agent has been demonstrated against beet army worm, *S. exigua* (Hubner) (Liu *et al.* 2002) and *R. ferrugineus* (Al-Kaabi *et al*. 2006).

Antibiotic resistance pattern of *S. marcescens* (pmc5) and *Klebsiella* sp was worked out and results indicated multiple antibiotic resistance in both isolates. Based on this combined antibiotic resistance, *S. marcescens* (pmc5) was recorded with resistance pattern as Ampicillin^{100}, Tetracycline^{50}, Spectinomycin^{100}, Streptocycline^{100} and Rifampicin^{75} and that of *Klebsiella* sp as Ampicillin^{100}, Tetracycline^{50}, Neomycin^{100}, Spectinomycin^{100}, Streptocycline^{100} and Rifampicin^{75}. Since *S. marcescens* (pmc5) and *Klebsiella* sp were found to have specific antibiotic resistance pattern, it offers the future prospect of screening them based on the resistance pattern for phylloplane and environmental persitance.

Efficacy of foliar application of superior bacterial bioagents obtained in laboratory screening was studied under field conditions in bitter gourd raised in grow bags. The treatments included phylloplane bacteria *S. marcescens* (pmc5) and *Klebsiella* sp along with *S. marcescens* (Hv3), *P. fluorescens* (PN026R), *B. thuringiensis* and two chemical checks. The phylloplane isolate, *S. marcescens* (pmc5) exhibited maximum mortality of *H. septima* at 5 and 7 DAT (65.83 %), an effect statistically similar to treatment with quinalphos 0.05 %. *S. marcescens* (Hv3) with more than 40 per cent mortality at 5 DAT was equally effective as *S. marcescens* (pmc5) treatment.

S. marcescens (pmc5) and *S. marcescens* (Hv3) also exhibited substantial reduction of leaf area damage by *H. septima* over untreated control than the other bacterial bioagents (Fig. 1), though inferior to quinalphos 0.05 %. Plants treated with *S. marcescens* (pmc5) and *S. marcescens* (Hv3) were observed with 68.78 and 61.30

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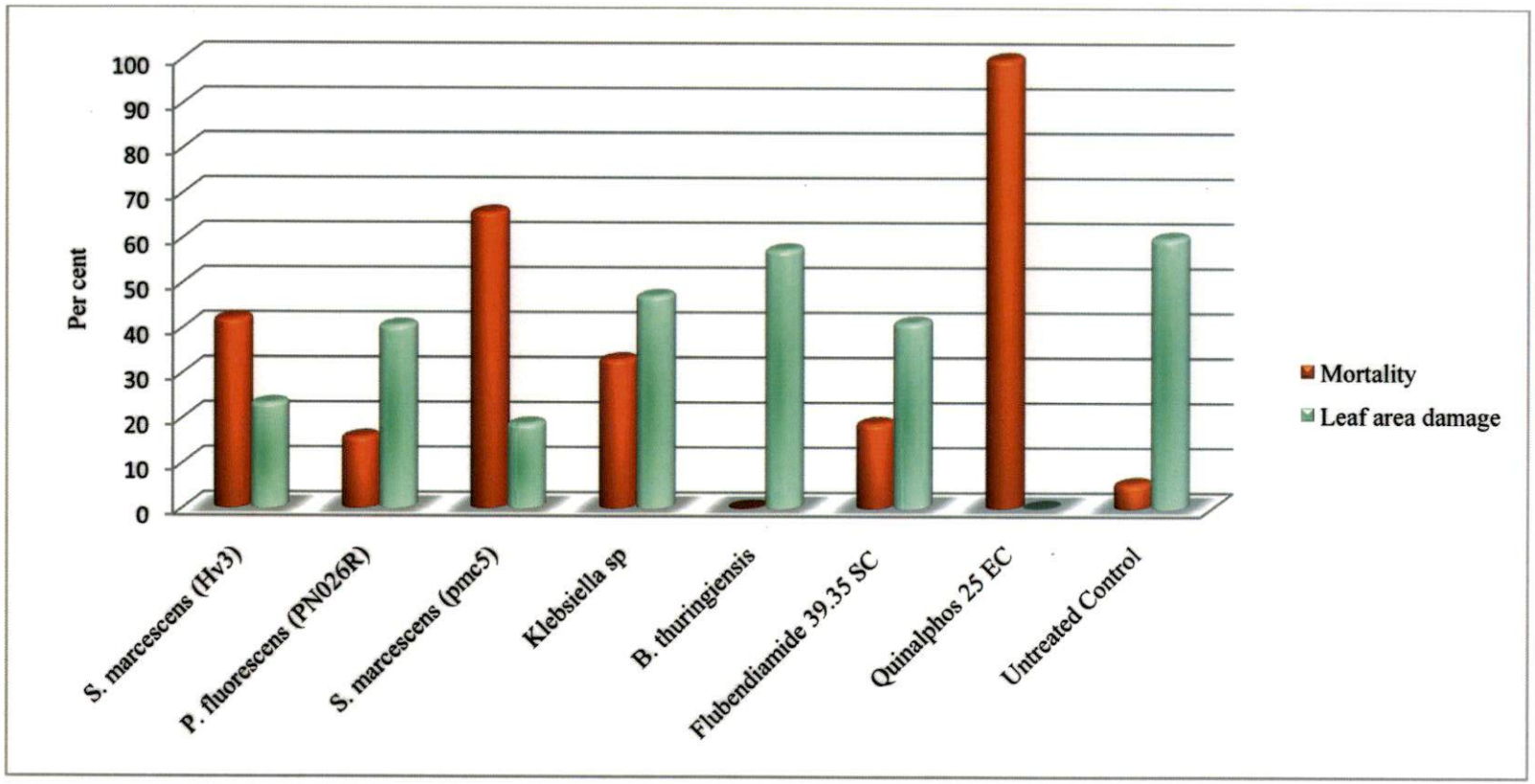

Fig.1. Relation between mortality of *H. septima* grubs and leaf area damaged

per cent reduction in leaf area damaged over untreated control by *H. septima* at 5 DAT, (Fig. 2). The reason might be the high mortality of larvae in the plants treated with these bacteria. The superiority of *S. marcescens* (pmc5) in bringing significant population reduction at all intervals, 1 DAT is evident from the fig. 3. More than 60 per cent reduction in population of *H. septima* over untreated control was recorded in plants treated with *S. marcescens* (pmc5) at 5 DAT.

S. marcescens (pmc5) treatment was also found effective against the lepidopteran *D. indica* producing 58.90 per cent mortality at 3 and 5 DAT which increased to 87.78 per cent at 7 DAT making it as effective as chemical pesticide. Reduction in leaf area damage with respect to increase in mortality was observed at all intervals (Fig. 4). More than 60 per cent reduction in population of *D. indica* (Fig. 6) and leaf area damage by it (Fig. 5) was observed in plants treated with *S. marcescens* (pmc5). *S. marcescens* (Hv3) found equally effective to *S. marcescens* (pmc5) with respect to mortality, with more than 40 per cent reduction in population and leaf area damage over untreated control.

Post population of other pests of bitter gourd and natural enemies of bitter gourd pests was recorded. However, there was no statistical significance in the population with respect to any of the treatments.

Bacteria that colonize the surface of plant leaves are potential biological vectors for the production of molecules suppressive to insect defoliators (Andrews, 1992). In this point of view, Otsu *et al.* (2003) isolated phylloplane bacteria, *Alcaligenes paradoxus* for the first time from tomato leaves, where the chitinase secreting strain (KPM-012A) of the bacteria could degrade the peritrophic membrane of phytophagous epilachna beetle, *H. vigintioctopunctata*. Otsu *et al.* (2004) provided an experimental basis for the biological control of herbivorous insect pests using leaf inhabiting, entomopathogenic strain of *P. fluorescens*. The strain KPM-018P isolated from tomato leaves caused 70.5 $\pm$ 21.5 per cent mortality in larvae of *H. vigintioctopunctata*. This method was thus proved effective for decreasing the population of larvae and adult insect pests in the subsequent generation. A study

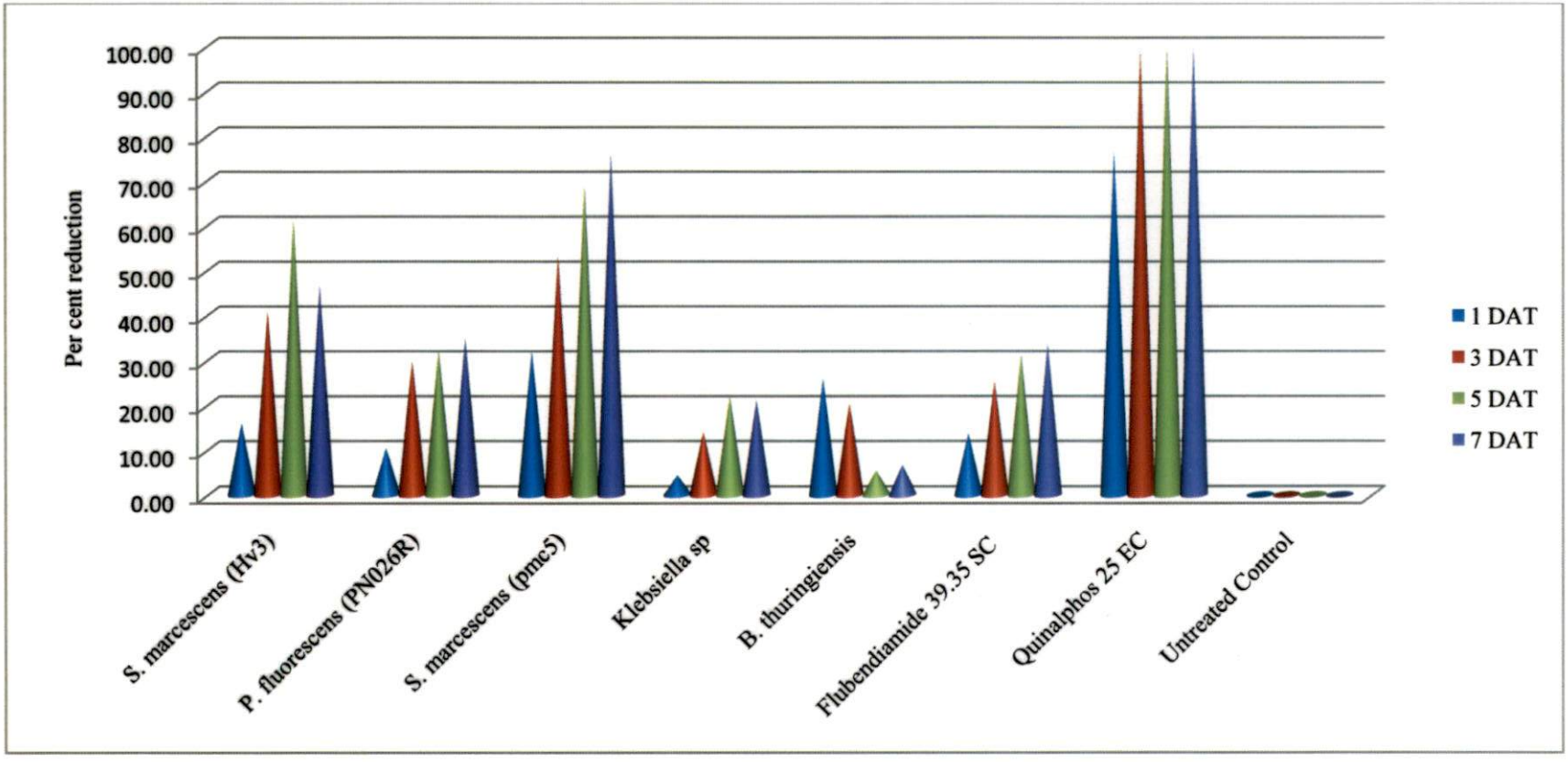

Fig. 2. Per cent reduction over control in leaf area damaged by *H. septima* grubs on plants treated with bacterial bioagents

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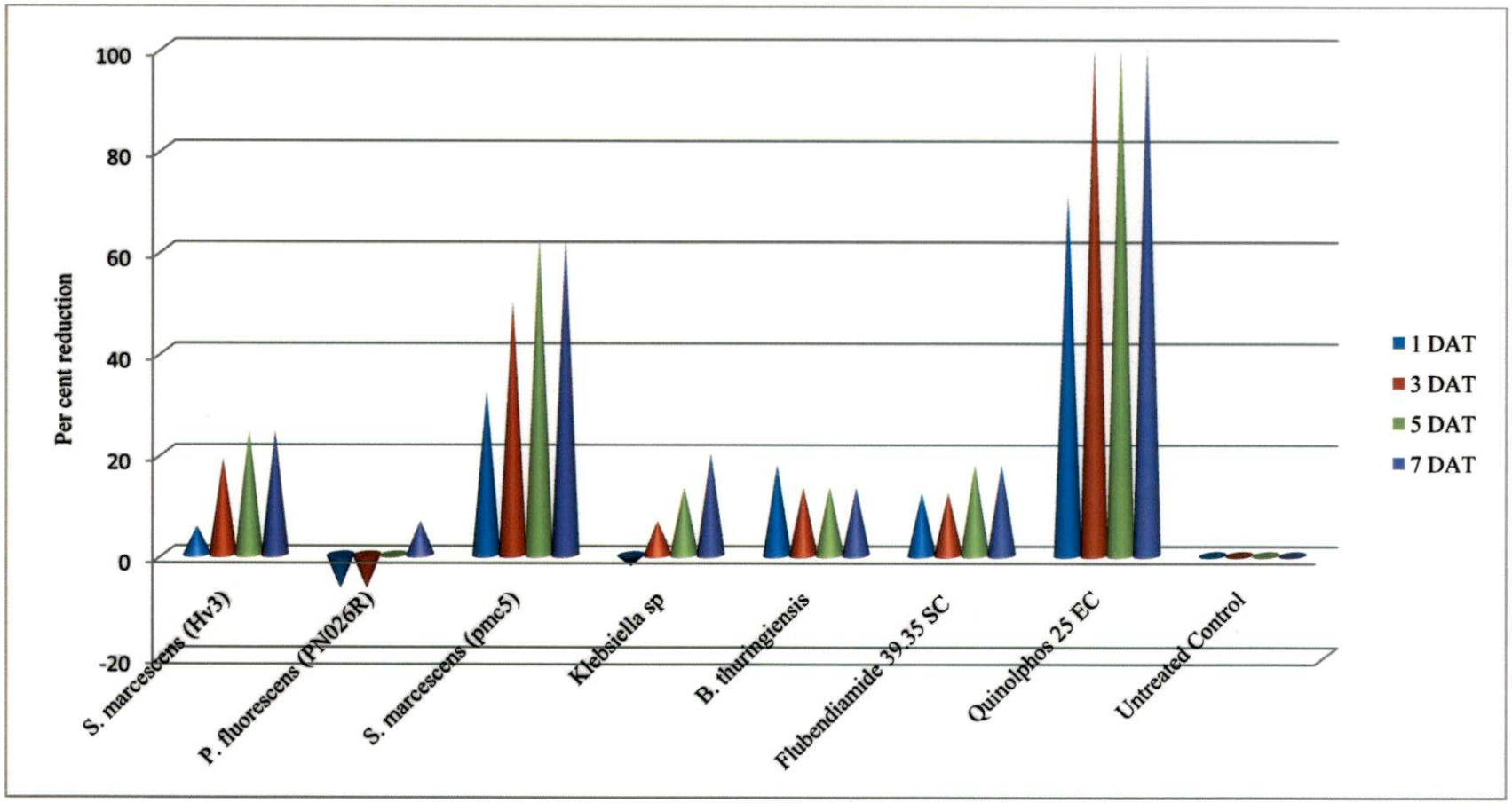

Fig. 3. Per cent reduction over control of *H. septima* grub population on plants treated with bacterial bioagents

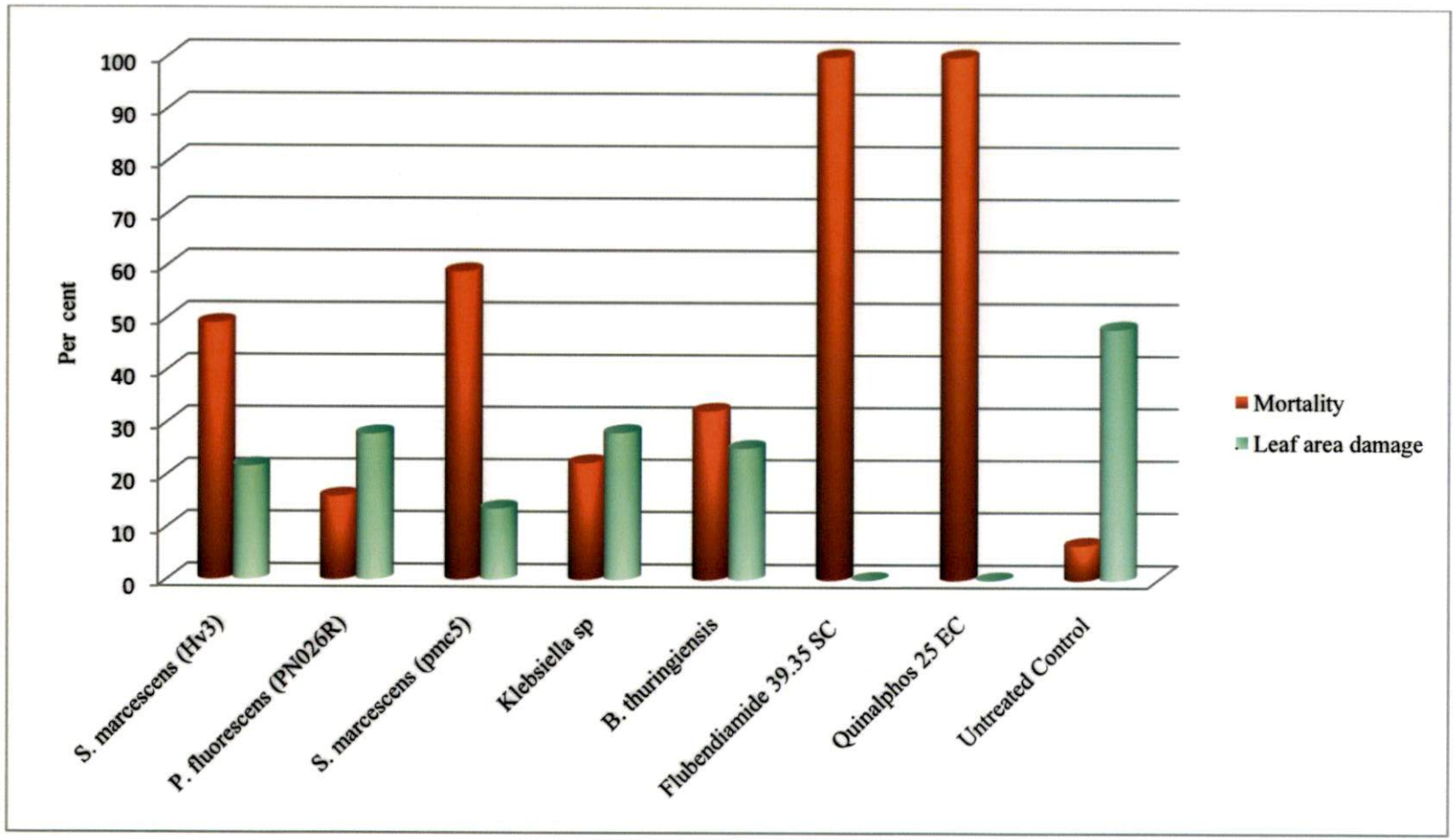

Fig. 4. Relation between mortality of *D. indica* larvae and leaf area damaged

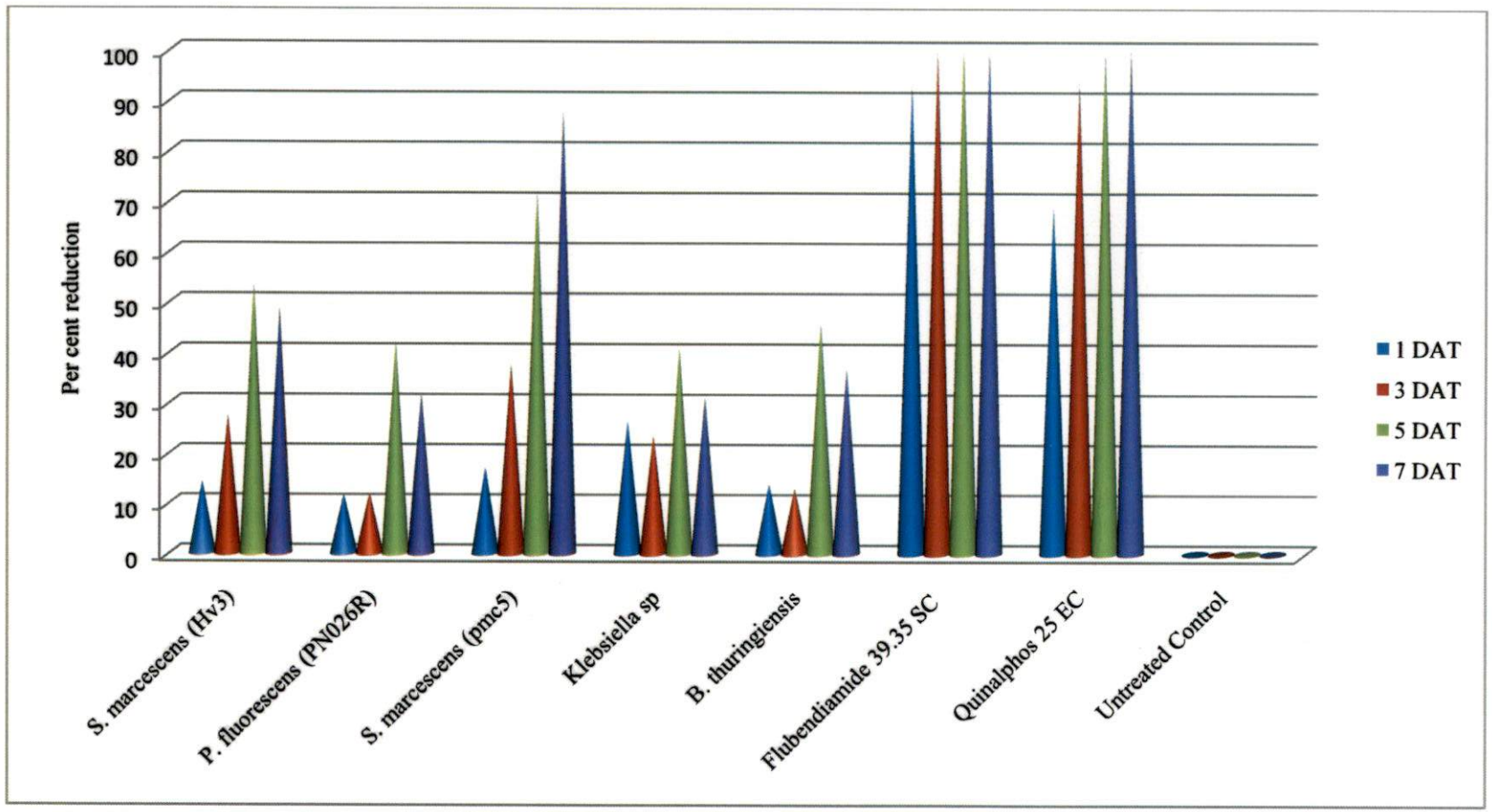

Fig. 5. Per cent reduction over control in leaf area damaged by *D. indica* larvae on plants treated with bacterial bioagents

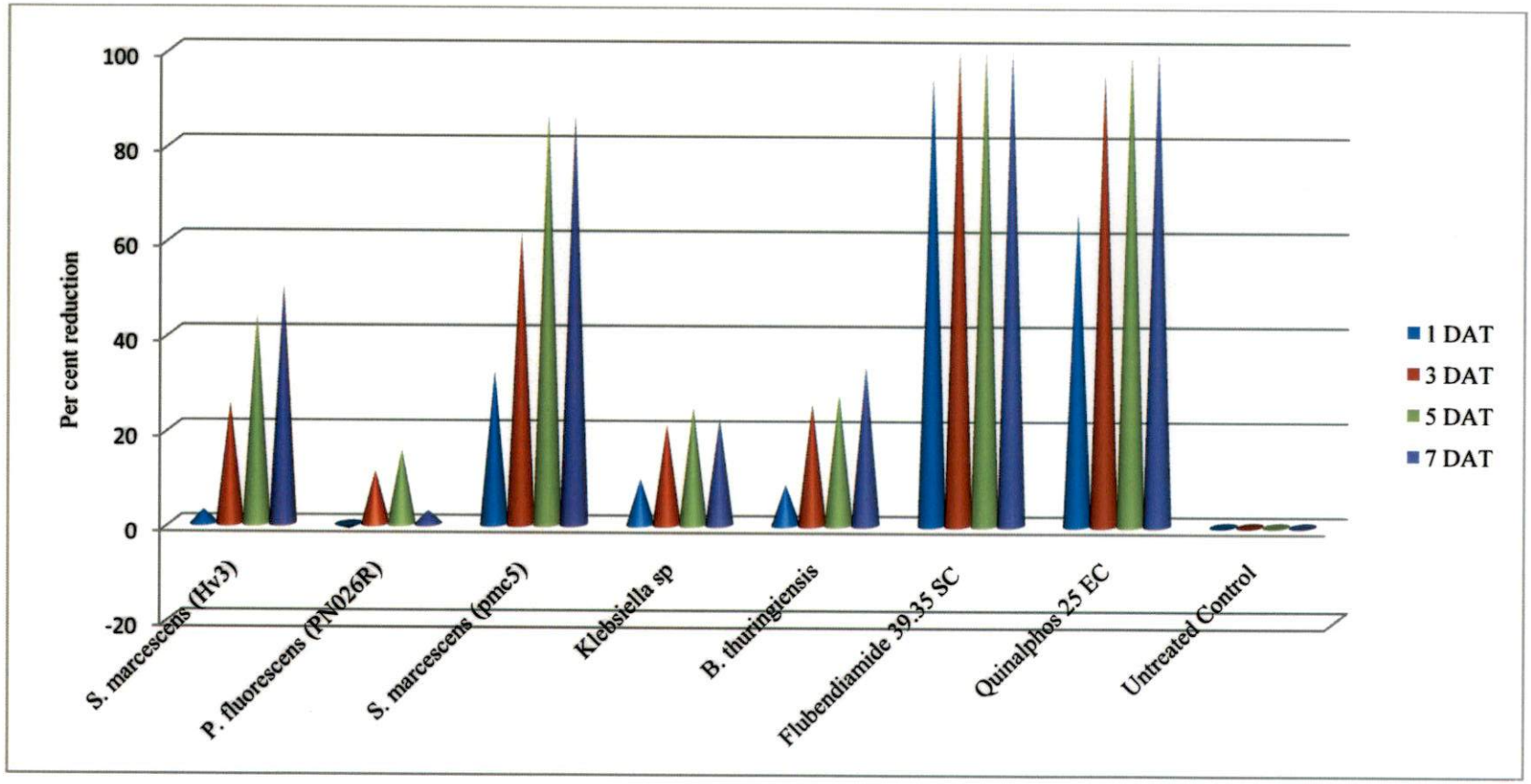

Fig. 6. Per cent reduction over control of *D. indica* larvae population on plants treated with bacterial bioagents

conducted by Maduell *et al.* (2002) reported 60 per cent of total 256 isolates of *B. thuringiensis* isolated from phylloplane of *Piper* sp, toxic to *S. fugiperda* and 40 per cent were toxic to southern house mosquito, *Culex quinquefasciatus. B. thuringiensis* isolated by Gonzalez and Molla (2011) from the phylloplane of tomato plants could effectively control the tomato leaf miner, *Tuta absoluta.* Aswathy (2015) reported that *P. fluorescens* isolated from the phylloplane of brinjal, caused 63.25 per cent mortality of *H. vigintioctopunctata.*

The role of phylloplane bacteria in the biocontrol of insect pests is obvious from the above citations. In the present study, the phylloplane isolated *S. marcescens* (pmc5) was found effective against chewing insects, *H. septima* and *D. indica* followed by entomopathogen *S. marcescens* (Hv3) with respect to three parameters *viz.* mortality, leaf area damage reduction and population reduction. However, since *S. marcescens* and *Klebsiella* sp are recognized as opportunistic pathogens capable of causing infections in animals and human beings in addition to insects, their pest control use in live form is limited. Since the metabolites like chitinase and prodigiosin produced by *S. marcescens* have pest control role, identification, characterization and biosafety evaluation of secondary metabolites may yield products having biocontrol potential.

Summary

6. SUMMARY

Over the years, insect attack has been found as one among the major biotic constraints of vegetable production across the world. *Momordica charantia* (L.), popularly known as bitter gourd is one of the prominent vegetables grown in India. The crop is heavily infested by foliage feeding chewing pests, *Henosepilachna septima* (Dieke) and *Diaphania indica* (Saund). Farmers often resort to frequent application of chemical pesticides to manage these pests. But insecticide resistance has been reported by these pests. Besides, considering the negative effects on environment, an alternate strategy to manage these pests is needed. There arise the choice of employing microbial agents to manage insect pests. In this context, the study entitled "Management of major chewing pests, *Henosepilachna septima* (Dieke) and *Diaphania indica* (Saund) infesting bitter gourd with bacterial bioagents" was conducted during the period 2015-2017, in the Department of Agricultural Entomology at College of Agriculture, Vellayani. The objective was to manage the chewing pests using phylloplane and pathogenic bacteria.

The salient findings of the present study are described below.

Thirteen bacteria were isolated from the phylloplane of bitter gourd by taking leaf impression on M-9 minimal agar media. Upon preliminary screening for pathogenicity, three isolates (isolate 1, 5 and 7) were found to be pathogenic to *H. septima* and four (isolate 3, 5, 7 and 12) to *D. indica*. Symptoms similar to that of bacterial infection were exhibited by larvae of *H. septima* and *D. indica* following treatment with pathogenic bacteria.

Laboratory evaluation of pathogenic phylloplane isolates was done by oral exposure adopting leaf disc method, along with other entomopathogenic bacteria *viz*, *Serratia marcescens* (Hv3) obtained from *H. vigintioctopunctata*, *Pseudomonas fluorescens* (PN026R) and *Bacillus thuringiensis* var. *kurstaki*. Treatments with *S. marcescens* (Hv3) and isolate 5 were found to be significantly

superior in causing mortality to *H. septima* grubs at 1, 3, 5 and 7 DAT. Mortality of 90 per cent and 83.33 per cent were observed for *S. marcescens* (Hv3) and isolate 5 respectively at 5 DAT. Leaf area damage was also found to be significantly low in treatments with *S. marcescens* (Hv3) and isolate 5 (7.93 per cent and 14.68 per cent respectively) than control (100 per cent) at 5 DAT.

S. marcescens (Hv3) and isolate 5 were found to be effective against *D. indica* also, causing 96.67 per cent and 93.33 per cent mortality at 5 DAT, which were equally effective as chemical insecticide (Flubendiamide 39.35 SC, 0.004%) and commercial microbial pesticide, *B. thuringiensis*. Larvae treated with *S. marcescens* (Hv3) and isolate 5 caused leaf area damage of 2.71 per cent and 5.23 per cent only as against 77.75 per cent in untreated control.

Molecular characterization of superior phylloplane bacteria (isolate 5 and 7) selected based on the laboratory screening was done by sequencing of internal transcribed region of 16S rDNA of these isolates with the help of microbial identification service at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. Isolate 5 and 7 were identified as *Serratia marcescens* and *Klebsiella* sp as it exhibited 99 and 100 per cent sequence homology with them.

The identity of the bacteria were further confirmed by biochemical analysis in which isolate 5 exhibited negative reaction with respect to urease, malonate and raffinose characteristic to *S. marcescens,* and isolate 7 exhibited urease, melibiose and glucose positive reaction, characteristic to *Klebsiella* sp.

The production of chitinase enzyme, required for breching the peritrophic membrane of chewing insects was detected for *Serratia marcescens* (pmc5) and *Klebsiella* sp, as evidenced by clear zone formation in media containing colloidal chitin as the sole carbon source. Chitinase activity of 1.24 and 1.07 units were recorded in *S. marcescens* (pmc5) and *Klebsiella* sp respectively.

S. marcescens (pmc5) showed multiple antibiotic resistance of Ampicillin^{100}, Tetracycline^{50}, Spectinomycin^{100}, Streptocyclin^{100} and Rifampicin^{75}

whereas *Klebsiella* sp exhibited Ampicillin^{100}, Tetracycline^{50}, Neomycin^{100}, Spectinomycin^{100}, Streptocyclin^{100}, Rifampicin^{75} resistance.

Field evaluation of efficacy of foliar application of selected bacteria @ 10^8 cfu ml^{-1} against chewing insects in bitter gourd was done in pot culture. The phylloplane isolate *S. marcescens* (pmc5) produced significantly high mortality in both *H. septima* (65.83 per cent) and *D. indica* larvae (87.78 per cent) at 7 DAT, which was on par with chemical treatment quinalphos 0.05 per cent.

Treatments with *S. marcescens* (Hv3) obtained from *H. vigintioctopunctata* which produced high mortality of 42.06 per cent in *H. septima* and 49.01 per cent and 57.14 per cent in *D. indica* at 5 and 7 DAT was the next best treatment. In case of *H. septima* grubs, *Klebsiella* sp which exhibited 33.09 per cent morality was found following these treatments whereas in *D. indica* larvae, *Klebsiella* sp was found inferior to *Bt* with 22.26 and 32.22 per cent respectively.

At 5 DAT, plants treated with *S. marcescens* (pmc5) showed significantly low population of *H. septima* grubs (6.46 grubs $plant^{-1}$) and *D. indica* (2.08 larvae $plant^{-1}$) than control (17.23 grubs $plant^{-1}$ and 17.68 larvae $plant^{-1}$). The next best treatment was *S. marcescens* (Hv3) with population of 12.92 grubs $plant^{-1}$ for *H. septima* and 9.36 larvae $plant^{-1}$ for *D. indica*, respectvely.

Plants treated with *S. marcescens* (pmc5) showed 68.78 per cent and 71.91 per cent leaf area damage reduction over untreated control by *H. septima* and *D. indica* respectively at 5 DAT. *S. marcescens* (Hv3) treatment caused 61.30 per cent and 53.33 per cent reduction of leaf area damage by *H. septima* and *D. indica* respectively over untreated control at 5 DAT.

With respect to all the three parameters such as high per cent mortality, low post treatment population and high per cent reduction of leaf area damage, *S. marcescens* (pmc5) was found to be superior over all other bacterial agents,

against the larvae of *H. septima* and *D. indica*. This was followed by *S. marcescens* (Hv3).

Thus, *S. marcescens* (pmc5), isolated from phylloplane of bitter gourd @ 10^8 cfu ml^{-1} is found effective against chewing pests of bitter gourd. However, since the opportunistic human pathogenic nature of *S. marcescens* limits its direct use in the field, identification of insect toxic secondary metabolites produced by it have to be investigated to develop biocontrol products.

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Management of major chewing pests, *Henosepilachna septima* (Dieke) and *Diaphania indica* (Saund) infesting bitter gourd with bacterial bioagents

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ABSTRACT

The study entitled "Management of major chewing pests, *Henosepilachna septima* (Dieke) and *Diaphania indica* (Saund) infesting bitter gourd with bacterial bioagents" was conducted during the period 2015-2017, in the Department of Agricultural Entomology at College of Agriculture, Vellayani. The objective was to manage the chewing pests using phylloplane and pathogenic bacteria.

Thirteen bacteria were isolated from the phylloplane of bitter gourd by taking leaf impression on M-9 minimal agar media. Upon preliminary screening for pathogenicity, three isolates (isolate 1, 5 and 7) were found to be pathogenic to *H. septima* and four (isolate 3, 5, 7 and 12) to *D. indica*.

Laboratory evaluation of pathogenic phylloplane isolates was done by oral exposure adopting leaf disc method, along with other entomopathogenic bacteria *viz*, *Serratia marcescens* (Hv3) obtained from *H. vigintioctopunctata*, *Pseudomonas fluorescens* (PN026R) and *Bacillus thuringiensis* var. *kurstaki*. Treatments with *S. marcescens* (Hv3) and isolate 5 were found to be significantly superior in causing mortality to *H. septima* grubs at 1, 3, 5 and 7 DAT. Mortality of 90 per cent and 83.33 per cent were observed for *S. marcescens* (Hv3) and isolate 5 respectively at 5 DAT. Leaf area damage was also found to be significantly low in treatments with *S. marcescens* (Hv3) and isolate 5 (7.93 per cent and 14.68 per cent respectively) than control (100 per cent) at 5 DAT.

S. marcescens (Hv3) and isolate 5 were found to be effective against *D. indica* also, causing 96.67 per cent and 93.33 per cent mortality at 5 DAT, which was equally effective as chemical insecticide (Flubendiamide 39.35 SC, 0.004%) and commercial microbial pesticide, *B. thuringiensis*. Larvae treated with *S. marcescens* (Hv3) and isolate 5 caused leaf area damage of 2.71 per cent and 5.23 per cent only as against 77.75 per cent in control.

Internal transcribed regions of DNA of 16S rRNA of isolate 5 and 7 were amplified using CAGGCCTAACACATGCAAGTC as forward primer and GGGCGGWGTGTACAAGGC as reverse primer in PCR. Blast search of amplified DNA in NCBI data base revealed the identity of isolate 5 and 7 as *Serratia marcescens* and *Klebsiella* sp respectively with 99 per cent and 100 per cent homology. The identity of the bacteria were further confirmed by biochemical analysis in which isolate 5 exhibited negative reaction with respect to urease, malonate and raffinose characteristic to *S. marcescens,* and isolate 7 exhibited urease, melibiose and glucose positive reaction, characteristic to *Klebsiellla* sp.

Chitinase activity of 1.24 and 1.07 units were recorded in *S. marcescens* (pmc5) and *Klebsiellla* sp respectively using chitin azure method, indicating the potential of these phylloplane bacteria in breaching the peritrophic membrane of chewing insects.

A pot culture experiment was carried out to evaluate the efficacy of foliar application of selected bacteria @ 10^8 cfu ml^{-1} against chewing insects in bitter gourd. The phylloplane isolate *S. marcescens* (pmc5) produced significantly high mortality in both *H. septima* (65.83 per cent) and *D. indica* larvae (87.78 per cent) at 7 DAT, which was on par with chemical treatment quinolphos 0.05%. Treatments with *S. marcescens* (Hv3) obtained from *H. vigintioctopunctata* which produced high mortality of 42.06 per cent in *H. septima* and 49.01 per cent and 57.14 per cent in *D. indica* at 5 and 7 DAT was the next best treatment.

At 5 DAT plants treated with *S. marcescens* (pmc5) showed significantly low population of *H. septima* grubs (6.46 grubs $plant^{-1}$) and *D. indica* (2.08 larvae $plant^{-1}$) than control (17.23 grubs $plant^{-1}$ and 17.68 larvae $plant^{-1}$).

Plants treated with *S. marcescens* (pmc5) showed 68.78 per cent and 71.91 per cent leaf area damage reduction over untreated control by *H. septima* and *D. indica* respectively at 5 DAT. *S. marcescens* (Hv3) treatment caused 61.30 per

cent and 53.53 per cent reduction of leaf area damage by *H. septima* and *D. indica* respectively over untreated control at 5 DAT. Thus, *S. marcescens* (pmc5), isolated from phylloplane of bitter gourd is found effective against chewing pests of bitter gourd. Since the field use in live form of *S. marcescens* is limited due to its opportunistic animal pathogenic nature, investigations have to be undertaken on insect toxic secondary metabolites produced by it to yield biocontrol products.

Appendices

APPENDIX-I

COMPOSITION OF MEDIA USED

1. Nutrient Agar

NaCl	-	5 g
Peptone	-	5 g
Beef extract	-	3 g
Agar	-	20 g
Distilled water	-	1000 ml

Peptone, NaCl and beef extract were dissolved in 500 ml of distilled water and the volume was made up to 1000 ml. Agar powder (20 g) was added to this mixture and sterilized by autoclaving at 121 ^{0}C for 15 minutes.

2. King's Medium B

Peptone	-	20 g
K_2HPO_4	-	1.5 g
$MgSO_4$	-	1.5 g
Glycerol	-	10 ml
Agar	-	20 g
Distilled water	-	1000 ml

Peptone, K_2HPO_4 and $MgSO_4$ were dissolved in distilled water, followed by addition of glycerol. Agar was added to this mixture and sterilized by autoclaving at 121 ^{0}C for 15 minutes.

APPENDIX- II

COMPOSITION OF STAIN USED

1. CRYSTAL VIOLET

One volume saturated alcohol solution of crystal violet in four volumes of one per cent aqueous ammonium oxalate.

2. GRAM'S IODINE

Iodine crystals	-	1 g
Potassium iodide	-	2 g
Distilled water	-	300 ml

3. SAFRANIN

Ten ml saturated solution of safranin dissolved in 100 ml of distilled water

4. MALACHITE GREEN

Malachite green	-	5 g
Distilled water	-	100 ml