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Bioefficacy and Characterisation of bitter gourd phylloplane bacteria against chewing pests

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ABSTRACT
*Phylloplane bacteria, which are found colonising plant leaf surfaces are found to play significant role as efficient biocontrol agents against crop pests. So far, the phylloplane of bitter gourd (*Momordica charantia L.*) has not been reported to have the presence of bacterial bioagents. Thirteen bacteria were isolated from bitter gourd phylloplane by adopting leaf impression method and were screened for pathogenicity against the larvae of chewing pests, Henosepilachna septima (Dieke) and Diaphania indica (Saund). Out of the thirteen bacteria, two isolates found causing high per cent mortality in the larvae were identified as Serratia marcescens and Klebsiella sp on the basis of biochemical and molecular characterisation.*
Key words: *phylloplane, Henosepilachna septima , Diaphania indica, Serratia marcescens , Klebsiella*

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INTRODUCTION

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 [19], it is also known to have pharmacological properties such as antihelminthic, purgative and antidiabetic action [8]. Insect pests are one among the major biotic constraints of vegetable production in India. Bitter gourd is heavily infested by a number of pests during different growth stages. 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 [17]. The pumpkin caterpillar or cucumber moth, *Diaphania indica* (Saund) is another widespread foliage feeding pest of bitter gourd [12, 3].

Phylloplane bacteria survive on the leaf surfaces colonizing on sites such as trichomes, stomata and epidermal cellwall junctions [5]. Andrews [2] reported that phylloplane microorganisms have immense potential to act as potential 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 [1].

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. Some of the phylloplane bacteria are capable of producing diseases in insects and hence are potential biocontrol agents. Inorder to employ biocontrol potential of the bacteria, they need to get isolated, screened and identified as effective agents against the pests. Attempts were made in the present study to isolate and characterize phylloplane bacteria from bitter gourd and to utilize them along with other entomopathogens, for the biocontrol of major chewing pests *viz. H. septima* and *D. indica*.

MATERIAL AND METHODS

Isolation of Phylloplane bacteria

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_2PO_4, 0.5 g NaCl, 1 g

NH_4Cl, 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.

Maintenance of insect cultures

H. septima and *D. indica* 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 eggs laid on bitter gourd leaves were transferred daily to separate jars and the larvae emerged were maintained on fresh leaves. Second instar larvae obtained from the eggs of same age were used for the experiments.

Screening of bacteria

The pathogenicity of phylloplane bacterial isolates obtained, along with *S. marcescens* Hv3 from *H. vigintioctopunctata* [4] and two strains of *P. fluorescens*, PN026R and psm13 and *Bacillus thuringiensis* was tested.

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. The pathogenic bacteria were re-isolated from cadaver and Koch's postulates was proved.

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.

Bacteria found infective to *H. septima* (isolate 1, 5 and 7) and *D. indica* (isolate 3, 5, 7 and 12) in preliminary testing were screened against respective test 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 petriplates. 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.

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.

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

Bacterial identification

Promising phylloplane bacteria (isolate 5 and 7) obtained were identified using 16S rDNA sequencing. 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 and sequenced. Identity was established using the blast tool of NCBI data base.

RESULTS AND DISCUSSION

Phylloplane bacteria have been isolated and proved to be efficient against many pests. *Bacillus thuringiensis* has been reported as a phylloplane inhabitant in addition to entomopathogen and soil inhabitant [11]. Otsu *et al*. [13] 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*. [14] provided an experimental basis for the biological control of herbivorous insect pests using leaf inhabiting, entomopathogenic strain of *Pseudomonas fluorescens*. The strain KPM-018P isolated from

tomato leaves caused 70.5 ± 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. Recently, *P. fluorescens* was isolated from the phylloplane of brinjal, where it caused 63.25 per cent mortality of grubs of *H. vigintioctopunctata* [4].

Table 1: Morphological characteristics of the pathogenic phylloplane bacteria

Isolates	Colony morphology	Colour	Gram reaction
Isolate 1	Crinkled with serrated edges	Bright yellow	Gram negative
Isolate 3	Flat circular with concentric rings	Yellow	Gram positive
Isolate 5	Circular, raised smooth and shiny	Dark red	Gram negative
Isolate 7	Large circular with granular centre and lobate periphery	Creamish yellow	Gram negative
Isolate 12	Small, flat, spreading	Light yellow	Gram positive

Table 2: 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)

Table 3: 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)

Plate 1a: Isolate 1

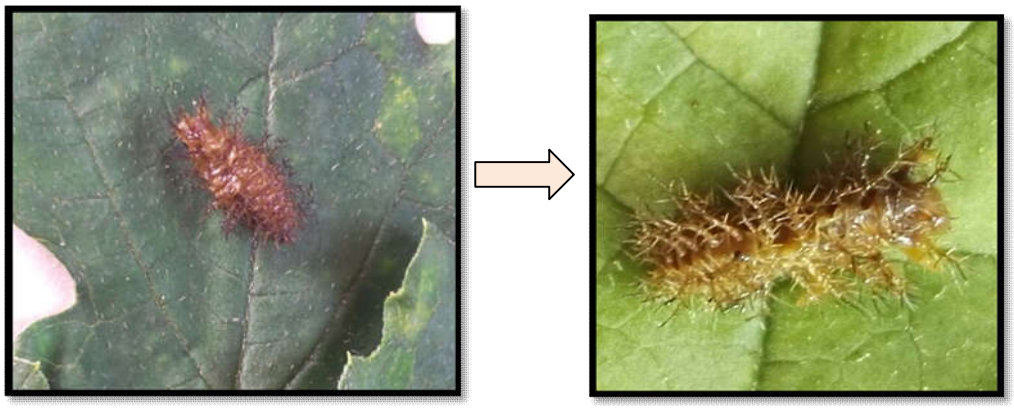

Plate 1b: Isolate 5

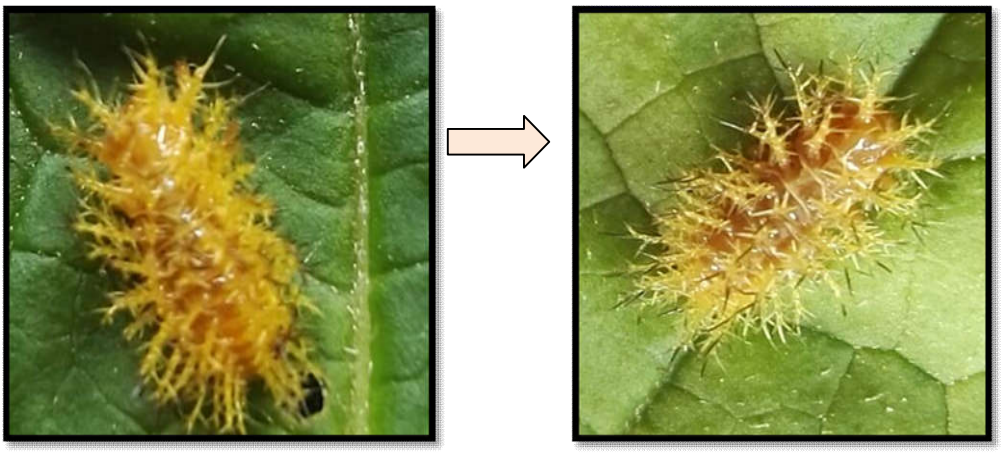

Plate 1c: Isolate 7

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

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

A study conducted by Maduell *et al*. [10] 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 [7] from the phylloplane of tomato plants could effectively control the tomato leaf miner, *Tuta absoluta* (Meyrick).

Phylloplane bacteria can be isolated from leaf surfaces by adopting leaf impression technique devised by Otsu *et al*. [13]. 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. 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. Treated larvae exhibited different disease symptoms such as shrinking, swelling, putrification, oozing of body fluid etc (Plate 1 and 2). Colony morphology and gram reaction of the pathogenic phylloplane isolates are described in Table 1.

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 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.

Isolate 5 and isolate 7 were obtained superior among the thirteen isolates in terms of causing high per cent mortality in larvae of *H. septima* and *D. indica*. Isolate 5 and 7 resulted in 83.33 and 46.67 per cent mortality respectively in larvae of *H. septima* whereas 93.33 and 53.33 per cent mortality was caused against larvae of *D. indica* by these isolates (Table 2 and 3). These superior isolates were identified by sequencing of 16s rDNA of these isolates. Blast search details of isolate 5 and isolate 7 in NCBI data base revealed their identity as *Serratia marcescens* and *Klebsiella* sp with 99 and 100 per cent homology.

S. *marcescens* was reported to multiply rapidly inside insect hemocoel bringing about death of the insect within 3 DAT [18]. Significant reduction in feed consumption by lepidopteran insects (*Helicoverpa armigera*, Hubner and *Spodoptera litura*, (Fab.) 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 [6]. *S*. *marcescens* isolated from dead grubs of epilachna beetle, *H. vigintioctopunctata* was found entomopathogenic to it causing mortality of 93.28 per cent [4].

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*. [15]. The production of prodigiosin pigment by *S*. *marcescens* might be the probable reason for inducing mortality in insects.

Klebsiella pneumoniae was isolated from dead grubs of red palm weevil, *Rhynchophorus ferrugineus* Oliver [16]. *Klebsiella pneumoniae* along with *Serratia marcescens* were isolated from the gut region of red palm weevil in the study carried out by Josephrajkumar *et al*. [9].

CONCLUSION AND FUTURE PROSPECTS

The study reports the first evidence of the presence of phylloplane bacteria in bitter gourd with biocontrol potency. The phylloplane isolated *Serratia marcesecns* and *Klebsiella* sp were found pathogenic to larvae of both *H. septima* and *D. indica*. From the study, it is clear that phylloplane bacteria can be effectively employed in pest management, thereby reducing the negative impacts of chemical insecticides. 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.

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