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Isolation and Screening of Indigenous Entomopathogenic Fungi against Sucking Pests of Vegetables

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Abstract

Geographical variation and strain specificity can alter the host specificity of entomopathogenic fungi. Since entomopathogenic fungi is one of the safe and alternative tool for pest management and management of sucking pests in vegetables often demands repeated use of systemic insecticides, attempts were made to isolate indigenous strains of entomopathogenic fungi specific to sucking pests infesting vegetables grown in Kerala. Infected insects collected from various fields and soils collected from cropped, non cropped forests as well as sacred groves were used for isolation using insect bait method, soil dilution plating and Warcup method. The morphological characters were studied using Motic images and molecular characterization was done using tools like Polymerase Chain Reaction, ITS DNA amplification and sequencing. Among different isolates we could locate three entomopathogenic fungi belonging to Ascomycota coming under the genus Lecanicillium and its two closely related genera Eupenicillium and Simplicillium. Though all the three were infective to sucking pest, the isolate Ls Vs.1, identified as Lecanicillium saksenae was found to be the most promising one. Conidial suspension of L. saksenae at 6.98 X 10^7 spores ml^{-1} was found to cause 100 % mortality to Aphis craccivora within 24 hours. In this context we propose Lecanicillium saksenae (ITCC No: of Ls.Vs.1 -7714) collected from Vellayani soil as a potent entomopathogen effective to sucking **pests of vegetables.**

Keywords – entomopathogenic fungi; Lecanicillium saksenae; indigenous isolates; isolation; molecular characterisation

I. INTRODUCTION

Pest management in vegetable crops especially the sucking pests like aphids, mealy bugs, thrips etc. is mostly attained by repeated use of persistent insecticides that create problems of resistance, resurgence and residue. Entomopathogenic fungi (EPF) are alternative and attractive tool in pest management as they do not cause any hazardous effect on human health and environment. They are well suited for being developed as a contact biopesticide which can also act on sucking pests [1]. Moreover, their complex mode of action by breaching the cuticle, invasion of haemocoel and interfering with enzyme activity makes the chances of resistance development meagre .

Genus *Lecanicillium* is well known for its pathogenicity to sucking pests *viz*. aphids, mealy bugs and white flies. Though broad host range of EPF is an added advantage, strain specificity and geographical variation may alter their host specificity. Bischoff *et al*., (2009) pointed out that many isolates of EPF show geographical variations [2].

Hence we aimed to isolate the indigenous strains of fungi from soils of Kerala as well as infected cadavers collected from various fields, with a view to obtain geographically specific strains of entomopathogenic *Lecanicillium* and its closely related genera which are known to be potential in controlling sucking pests infesting vegetables commonly grown in our region.

II. MATERIALS AND METHODS

A. Isolation of indigenous strains of EPF

Entomopathogenic fungi occur naturally as infections in living hosts for a short period of time and during the remaining part of their life cycle they presumably lurk as dormant conidia in the soil mostly in the vicinity of the host cadaver. Hence attempts were made to isolate them from the soils as well as from infected insects**.**

B. From insect cadaver

Frequent monitoring was done in fields with heavy pest infestation. Insect specimens suspected to be infected by mycosis were brought to the laboratory and kept in moist chambers for development of symptoms or mortality. Upon death or mycosis the specimens were surface sterilized using one per cent sodium hypochlorite, followed by 70 per cent alcohol and three repeated changes of sterile distilled water. They were then inoculated on PDA slants and kept under incubation for one week under room temperature. On development of fungal mycelia they were transferred to fresh PDA slants for further growth. The pure cultures thus developed were stored under refrigeration for identification.

C. From soil samples

Soils from cropped, non cropped as well as forest area and sacred groves were collected for isolation of entomopathogenic fungi. Out of the 55 samples collected 30 were from cropped area, 20 from non cropped area, three from forest area and two from sacred groves. From each site, three spots were chosen and 500 g samples were drawn from each from a depth of 20 cm using an auger. A representative sample was arrived by pooling and quartering method. Samples were then homogenized, passed through 2 mm sieve and stored in plastic containers. These samples were used for detection of fungi within 48 h of collection. Two methods were adopted (i) insect bait method and (ii) serial dilution plate method.

D. Insect bait method

The bait insect used was the wax moth *Galleria mellonella*, reared in the laboratory using the method suggested by Meyling (2007) [3]. Fourth instar larvae (4 -5 week old) were selected from the culture and pre treated to avoid webbing. For this, they were immersed in hot water at 50ºC for 10 sec strained out and cooled under running tap water for 30 sec. They were then dried by placing on a tissue paper. After keeping in darkness for 3-5 h the larvae were transferred to plastic containers having 40g of soil sample @ 10 larvae/ bottle. The soil was moistened by sprinkling sterile water. The lid of the container was ventilated with pin holes. Second day, the containers were inverted to ensure uniform contact of the larvae with soil particles. This was repeated for 2-10 days and then observed at 24 h interval for symptom development. Suspected larvae with symptoms of mycosis were taken out and kept in a moist chamber for development of fungal growth, if any. Those which developed mycelial growth were surface sterilized in 0.1% sodium hypochlorite followed by 70% alcohol and three changes in sterile water. They were transferred to selective media using CTAB as the antibacterial and suppressing agent of fast growing contaminants.

E. Soil dilution plating

Attempt to isolate entomopathogenic fungi from soil samples were carried out using soil dilution plating method. 10 g of prepared sample was added to 90 ml of sterile water and mixed well for the release of propagules in the soil. 1 ml of the sample was spread on 2% plane agar plate with a rotary motion of the plate. Plates were then incubated at room temperature. As and when colonies appear they were picked up and placed on fresh PDA slants and brought under pure culture for further observations.

F. Soil Plating (Warcup method)

In this method five gram of soil sample was sprinkled in sterile petriplate. 15 ml molten agar incorporated with streptomycin @ 0.1 % was poured into it and dispersed by swirling motion. They were incubated for development of colonies. The emerging colonies were transferred to fresh PDA slants.

The fungal colonies isolated by the above methods were brought under pure culture for studying the morphological and molecular characters.

G. Identification of isolates

The culture characters were observed from the dorsal and ventral surfaces of the plates and the conidiophore and conidial characters were noted by preparing whole mount slides viewed under Motic Images, for genus level identification. To confirm its species identity we used molecular tools such as Polymerase Chain Reaction (PCR) and ITS DNA amplification and sequencing.

H. ITS DNA Amplification and Sequencing

Fungal cultures were grown in PDA at 28˚C for one week. Genomic DNA was isolated using Nucleospin ® Plant II Kit (Machrey – Nagel) following manufacturers specifications. The eluted DNA was stored at 4˚C. The quality of DNA was checked by agarose gel electrophoresis. The gels were visualized using UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). Complete ITS region was amplified using the primers ITS – 1F (forward: TCCGTAGGTGAACCTTGCGG) and ITS – 4R (reverse: TCCTCCGCTTATTGATATGC) [4]. PCR was carried out in a 20µl reaction mixture containing 1X Phire PCR buffer (contains 1.5mM $MgCl_2$), 0.2mM each dNTPs (dATP, ddGTP, dCTP, dTTP), 20ng DNA, 1U of PhireHotstart II DNA Polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers. Amplifications were carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using a 30 sec denaturation step at 98°C followed by 40 cycles consisting of denaturation at 98°C for 5 sec, primer annealing at 62°C for 10 sec and extension at 72°C for 15 sec with a final extension at 72°C for 60 sec. PCR products were checked by ethidiumbromide stained 1.2% agarose gel electrophoresis and purified by ExoSAP – IT treatment (GE HealthCare, UK).

PCR products were sequenced in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) following manufacturers protocol ; carrying out the reactions in ABI 3500 DNA Analyzer (Applied Biosystems, USA). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequence were carried out using Geneious Prov 5.1 [5]. The ITS sequences were compared with the sequences placed in Gen Bank Database (National Center for Biotechnology Information, NCBI) by using the BLAST (Basic Local Alignment Search Tool, NCBI).

III. RESULTS AND DISCUSSION

3.1 Isolation and identification of entomopathogens

Three entomopathogenic fungi from field collected insect specimens and soils of Vellayani area were isolated and identified morphologically as those belonging to the genera *Lecanicillium*, *Eupenicillium* and *Simplicillium*.

3.2 Morphological characters

The new isolate LsVs 1, *Lecanicillium* was obtained from Vellayani soils by dilution plate technique. It exhibited a concentric growth pattern, with vine red pigmentation on the reverse side of culture (Fig.1.A). On microscopic examination the conidiophores were found to be little differentiated from its vegetative hyphae. The phialides were attached to short side branches. Conidia were globular, hyaline and borne singly on apex (Fig.2). It measured 2.2 -4.4 *µm.* The second isolate EsVg 1, *Eupencillium* obtained by insect bait method had silvery white raised colonies with no pigmentation on media (Fig 1 B). Phialides were digitally arranged and 2-5 in number (Fig 3). Conidia are globose and smooth measuring 0.6 – 2.5 um. The white fly isolate SsVw 1, *Simplicillium* had white flat colonies with serrated border shrinking inward (Fig 1 C). There was no pigmentation on media. Conidia were smooth and globular measuring 0.9 – 2.5 um (Fig.4).

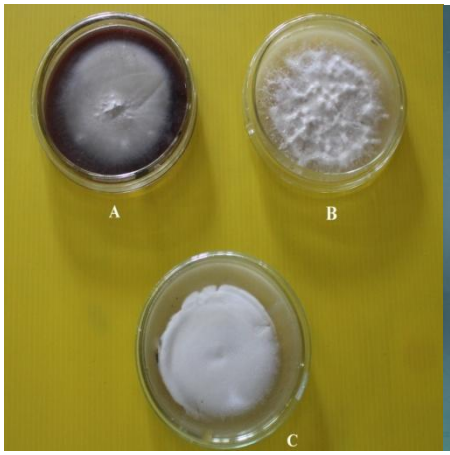

Figure 1. Macroscopic features of the indigenous isolates

Figure 2. Microscopy of L. saksenae

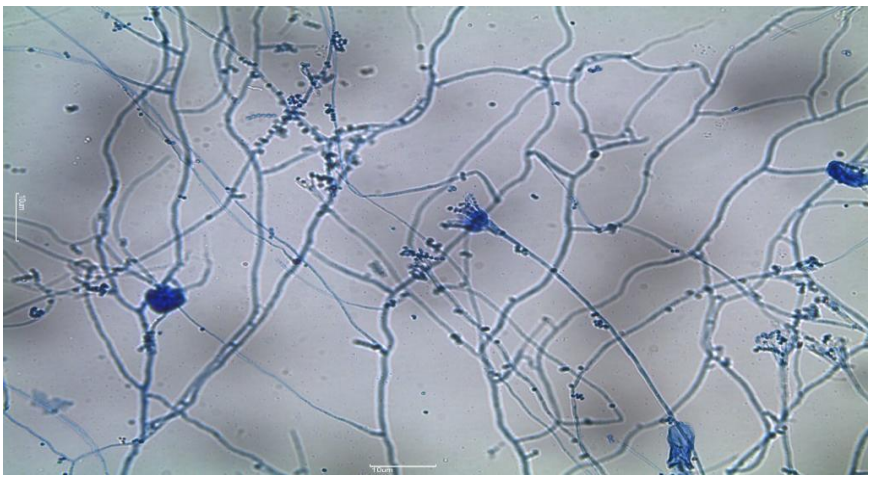

Figure 3. Microscopy of Eupenicillium shearii

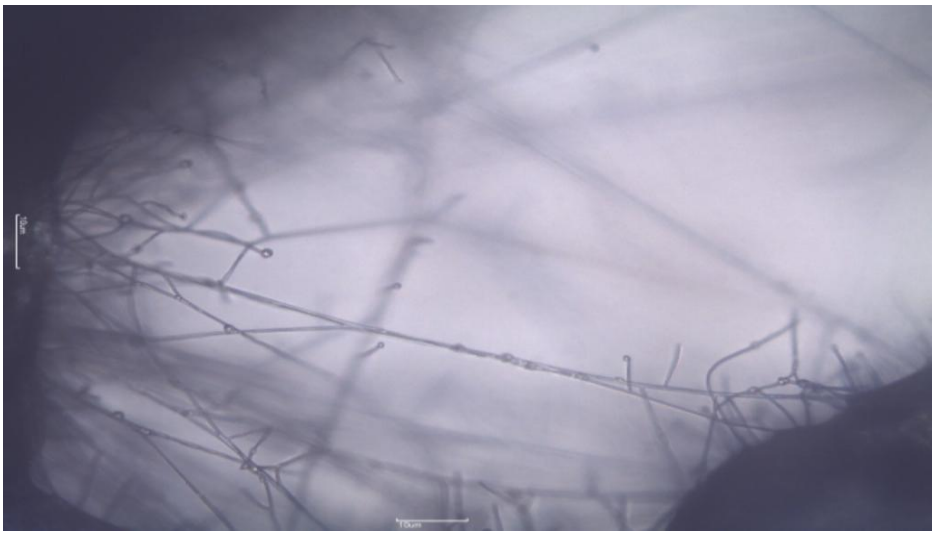

Figure 4. Microscopy of Simplicillium **sp.**

3.3 Molecular characters

PCR amplification of the isolated DNA with the primers mentioned above yielded 120 – 200 bp, 100 – 200 bp and 150 - 200 bp fragments for the isolates Ls Vs 1, Ss Vw 1 and EsVg 1 respectively when ran on agarose gel using a 2-log DNA ladder (NEB) as the molecular marker (Fig.5).

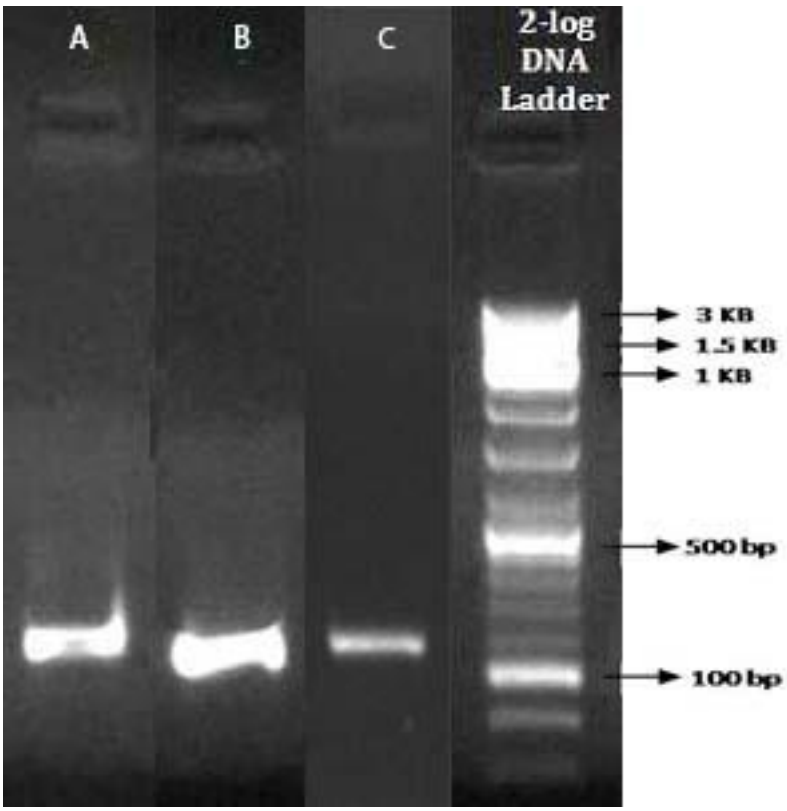

***Figure 5. Amplification products of 18S rRNA gene of the fungal isolates. DNA marker (number on the right are in kilobase). A, Lecanicillium saksenae; B, Eupenicillium shearii; C, Simplicillium* sp.**

Table 1. summarizes the results of 18S rRNA sequencing of the three fungal isolates. The amplified sequences were used as BLASTN query sequence against the NCBI database. A BLAST search for the ITS rRNA sequence of the three isolates Ls Vs 1, Ss Vw 1 and Es Vg 1 indicated that it was 100% similar to 12 isolates of *L. saksenae*, 99% similar to 2 isolates of *Simplicillium* sp and 99% similar to 6 isolates of *Eupenicillium shearii* respectively. Therefore three different species belonging to three different genera *Lecanicillium*, *Simplicillium* and *Eupenicillium* have been isolated all of which belong to the phylum Ascomycota.

Analysis of the ITS sequences confirmed the identity of new isolates as *L. saksenae*, *E. shearii* and *Simplicillium* sp. as per NCBI BLAST. ITS sequencing of LsVs 1, isolate showed hundred per cent homology to different *L. saksenae* isolates. Of the 13 sequences which showed 100 per cent similarity to the query sequence, 12 were from *L. saksenae*. With the second isolate EsVg 1, of the 10 sequences which showed 99% similarity to the query sequence, 6 were from *E. shearii* and with the third isolate SsVw1, of the 10 sequences which showed 99% similarity to the query sequence 2 were from *Simplicillium* sp. The results of BLASTN analysis is presented in Table 1.

Table 1. Genbank BLASTN match Acc.No:s of 18S rRNA sequence and percentage identity of the indigenous isolates of entompapthogenic fungi identified

IsolateNo:	Substratum	Site/host of Isolation	Identification of closest relative	BLAST Match Acc no:	Identity (%)
LsVs 1	Soil	Vellayani	*Lecanicillium saksenae*	KF472156.1 AB378520.1 AB360363.1 AB360362.1 AB360361.1 AB360360.1 AB360359.1 AB360358.1 AB360357.1 AB360352.1 AB360351.1 AB360350.1	100
EsVg 1	Insect	Galleria	*Eupenicillium shearii*	AF033420.1 AJ004893.1 AY232278.1 KF578127.1 JQ863221.1 GQ924907.1	99
SsVw 1	Insect	Whitefly	*Simplicillium* sp.	AB378534.1 EF641862.1	99

3.4 Pathogenicity and virulence of the isolates

Ability of the new isolates to cause infection to the common sucking pests found in the vegetable ecosystem of Kerala was assessed under laboratory conditions using field collected insects. The pathogenicity test revealed that *L. saksenae* is more efficient compared to *Simplicillium* sp. and *E. shearii*. It was found that *L. saksenae* had a broad spectrum action on sucking pests viz. aphids *Aphis craccivora*, *A. gossypii*, white fly *Bemisia tabaci*, jassid *Amrasca biguttula biguttula*, mealy bug *Coccidohysterix insolitus*, pod bug *Riptortus pedestris* as well as on phytophagous mite *Polyphagotarsonemus latus*. The time taken for mortality was only 24 h in some hosts where as it extended upto 48 h in some others (Table 2). *E. shearii* was infectious to *C. insolitus*, *A. craccivora* and *A. gossypii* only and the time taken for mortality extended upto 98 h. *Simplicillium* sp. was found to be infective to *Henosepilachna viginctioctopunctata*, *C. insolitus, and A. craccivora*. The death of

the treated insects occurred after 96 h. As *L. saksenae* was found to be a promising isolate for sucking pests, investigations on virulence was ascertained with this isolate alone.

Table 2. Pathogenecity of Indigenous isolates to different pests of vegetables

Crop	Insect species	*L. saksenae*		*E. shearii*		*Simplicillium* sp.	
		**I*	***T (h)*	**I*	***T (h)*	**I*	***T (h)*
Brinjal	Mealy bug *Coccidohysterix insolitus*	+ ve	24	+ve	96	+ve	48
	Jassid *Amrasca biguttula biguttula*	+ve	24	-ve	-	-ve	-
	Epilachna grub *Henosepilachna vignctioctopunctata*	-ve	-	+ve	96	-ve	
	Leaf roller *Antoba olivacea*	-ve	-	-ve	-	-ve	-
Cowpea	Aphid *Aphis craccivora*	+ve	24	+ve	96	+ve	48
	Pod Bugs *Riptortus pedestris*	+ve	48	-ve	-	-ve	-
	Green shield bug *Nezara virudula*	- ve	-	- ve	-	- ve	-
Bhindi	Aphids *A.gossypii*	+ ve	24	+ ve	96	-ve	-
	Whitefly *Bemisia tabacci*	+ ve	48	- ve	-	+ ve	96
	Red cotton bug *Dysdercus cingulatus*	- ve	-	- ve	-	- ve	-
Chilly	Mites *Polyphagotarsonaemus latus* Cut worm *Spodoptera litura*	+ ve	24	- ve	-	- ve	-

****Infectivity ** Time taken for mortality***

Virulence of *L. saksenae* was tested using the nymphs of *Aphis craccivora* as test insect. Dose dependent mortality worked out revealed 100 per cent mortality within 24 h of treatment with conidial suspension at a concentration of 6.9×10^7 spores ml $^{-1}$. The results are presented in Table 3.

Table 3. Dose dependent mortality of *L. saksenae* to cowpea aphid *A. craccivora*

Dose dependent mortality of *L.saksenae*	
Concentration (spores ml $^{-1}$)	**Mortality (%) 24 h**
6.981×10^7	100
6.981×10^6	73.3
6.981×10^5	50
6.981×10^4	23
6.981×10^3	3.3
6.981×10^2	0
Water (check)	0

All the three isolates were effective to sucking pests. However *E. shearii* and *Simplicillium* sp. were found to be weak insect pathogens. We have confirmed the species level identity by studying the ITS region. ITS region has been used widely as a phylogenetic marker [6, 7 and 8]. In

can be noticed in our study that isolate 1 LsVs1 has 100 percent homology with 12 *Lecanicillium* isolates isolated by [9] of which one was proposed as a new combination *L. saksenae* which was obtained from epiphytic and subterranean arthropods of east Kalimantan province of Indonesia. *L. saksenae* was never reported previously for its pathogenicity to sucking pests or any other crop pests in India or elsewhere until [10] reported it as a new entomopathogen effective to sucking pests of vegetables. Genus *Lecanicillium* is well known for its pathogenicity to scale insects, white fly [11]. Its high level pathogenicity to different species of aphids such as *Aphis gossypii*, *Macrosiphum euphorbiae, Brevicoryne brassicae* and *Myzus persicae* has been reported by several workers [12, 13 and 14] aphids, scales, mealy bugs and thrips but report on entomopathogenicity of the new isolate *L. saksenae* is for the first time in India and abroad. Pathogenicity of the genus to coreid bugs was not seen reported earlier. Conversely, in our study *L. saksenae* was found to be infective to cow pea pod bug as well.

The other two isolates *Eupencillium shearii* and *Simplicillium* sp. were found to be weak pathogens with narrow host range and delayed action. Sung *et al.*, (2014) has reported for the first time the entomopathogenicity of *S. lanosoniveum* on silk worms [15]. However the entomopathogenicity of *Eupenicillium shearii* or any other *Eupenicillium* sp. is not seen reported earlier. Basem *et al.*, (2012) isolated a marine species *Eupenicillium javanicum* from sediments of water of Gulf of Aqaba, Red sea [16].

1V. CONCLUSION

Considering the range of infectivity, pathogenicity and virulence factors we propose *L. saksenae* as a promising isolate with ample scope for development of a new biopesticide for sucking pests. It could yield cent per cent mortality of *A. craccivora* within 24 h of treatment.

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