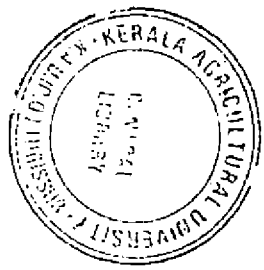
PATHOGENICITY AND BIOCHEMICAL PROPERTIES OF ENTOMOPATHOGENIC FUNGUS *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno



by

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DECLARATION

I hereby declare that this thesis entitled **"Pathogenicity and biochemical properties of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno"** 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 of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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Jasmy. Y

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LIST OF ABBREVIATIONS AND SYMBOLS USED

@	At the rate of
%	Per cent
µg	Micro gram
µL	Microlitre
µm	Micro meter
A	Absorbance value
CD	Critical difference
Cfu	Colony forming units
cm	Centimeter
DAI	Days after inoculation
DAT	Days after treatment
EPF	Entomopathogenic fungi
et al.	And other co workers
Fig.	Figure
g	Gram
g-1	Per gram
h	Hours
HAT	Hours after treatment
i.e.	that is
Kg	Kilo gram
Kg^{-1}	Per Kilogram
L	Litre
l^{-1}	Per litre
M	Molarity
mg	Milli gram

mg^{-1}	Per milligram
Min	Minutes
mL	Millilitre
mL^{-1}	Per millilitre
N	Total number
ng	nanogram
nm	Nano meter
No.	Number
NS	Non Significant
°C	Degree Celsius
pH	Negative logarithm of hydrogen ions
ppm	Parts per million
Rf	Retension factor
rpm	Revolution per minute
Rs.	Rupees
sp. or spp	Species (Singular and plural)
U	Unit
viz.	Namely

Introduction

I. INTRODUCTION

The reorientation of agriculture towards ecologically sustainable practices has motivated the researchers to search for biological alternatives for pest control. An economic increment of 20 per cent has been directed to explore biotechnological products of microbial origin, such as biofertilizers, biopesticides and microbial enzymes used for crop protection (Tergerdy and Szakács, 1998). With the worldwide consumer perception that the chemical usage in crop protection needs to be reduced considerably, there had been an increasing interest in the exploitation of microbial pathogens for pest management. It is therefore the need of the hour to develop biological control strategies, especially for the growing organic market. Presently biopesticides occupy 1.3 per cent of the world's total pesticide market of which, 90 per cent of them are used as insecticides (Bailey *et al.*, 2010). According to the reports of CPL Business Consultants (2010), entomopathogenic fungi occupies second place among the microbials used in pest management.

Pest management using entomopathogenic fungi (EPF) is an exciting and rapidly developing research area, which serves as a potent alternate tool where chemical pesticides are banned or being phased out or in cases where pests have attained resistance to insecticides. The degree of specificity, non hazardous nature and unique mode of action render them as a safe and sustainable tool in Good Agricultural Practices (GAP).

EPF like *Beauveria, Metarhizium, Lecanicillium, Hirsutella, Nomuraea* and *Isaria* are gaining importance in pest management due to the availability of simpler, easier and cheaper mass production techniques. Among them, the genus *Lecanicillium* is known for its pathogenicity to insects, mites as well as nematodes infesting various crop plants and some of them have been developed as

commercial biopesticides. (Hall and Papierok, 1982; Derakhshan *et al.*, 2008). Some species of the genus antagonistic to plant pathogenic fungi (Kim *et al.*, 2007).

Despite the effectiveness of EPF in controlling the target pests, they are reported to be slow in action and sensitive to the environment. Attempts to overcome such problems encompass the identification of indigenous and geographically distinct isolates that are highly pathogenic and environmentally stable and *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno is a new indigenous isolate (ITCC No. LsVs 1-7714), from the cultivated soils of Vellayani, Kerala, morphologically and molecularly characterized by Rani *et al.* (2014). Preliminary investigations conducted at College of Agriculture, Vellayani revealed that this isolate was infective to sucking pests in general and multiplies well under the normal temperature range prevailing in Kerala unlike the National Bureau of Agricultural Insect Resources (NBAIR) isolate of *Lecanicillium lecanii* (Zimmermann) Zare and Gams which requires a lower temperature range. Apart from its adaptability *L. saksenae* was found to be more effective in speed of kill.

The uplift of an indigenous isolate as an effective and safe bioagent needs basic information on its host range, pathogenicity, cross infectivity to crop plants and safety to non target organisms. In addition to the innate traits like pathogenicity and virulence, the success of an entomopathogen largely depends on its amenability to mass production. Standardization of cost effective and enriched substrates that supports growth and sporulation is an inevitable step in its development. Elucidation of its biochemical properties which are the key determinants of pathogenicity is also required, to improve its efficacy. Considering these factors the present investigation was undertaken to generate information on the following aspects of *L. saksenae*

- Information on its pathogenicity to insects and mites infesting crop plants
- Its potential use against plant parasitic nematodes

- Pathogenicity to crop plants on which it is to be applied
- Safety to non target organisms such as predators, parasitoids, pollinators and other beneficial insects
- Cost effective enriched substrates for mass production
- Cuticle degrading enzymes secreted by the fungus
- Toxins produced by the fungus and its bioefficacy

Review of Literature

2. REVIEW OF LITERATURE

Biological control using entomopathogenic fungi (EPF) is an exciting and rapidly advancing research area that plays an important role in safer pest management. They are unique in their mode of action unlike the other entomopathogens such as bacteria and viruses. The mode of entry through the cuticle make them suitable for management of sucking pests as well. Sustainability, comparatively broader host range, safety to other invertebrates and amenability to easy and cheaper mass production are some other attributes of these bioagents.

Out of the 700 known species of fungi, nearly 90 genera are pathogenic to insects (Khachatourians and Sohail, 2008). They mainly belong to the orders Entomophthorales and Hypocreales (formerly called Hyphomycetes) which includes *Entomophthora* sp., *Septobasidium* sp., *Neozygites* sp., *Aspergillus* sp., *Beauveria* sp., *Fusarium* sp.., *Hirsutella* sp., *Metarhizium* sp., *Nomuraea* sp., *Paecilomyces* sp., *Verticillium* sp., *Erynia* sp., *Furia* sp., *Pandora* sp., *Zoophthora* sp., etc. Of the various entomopathogens, the most important ones that have been commercially exploited are *Metarhizum anisopliae* (Metsch.) Sorokin, *Beauveria bassiana* (Balsam) Vullemin, *Lecanicillium lecanii* (Zimmermann) Zare and Gams, *Hirsutella thompsonii* (Fisher) and *Isaria fumosorosea* (Wize) Brown and Smith (formerly called *Paecilomyces fumosoroseus*) (Perez *et al.*, 2014).

The genus *Verticillium* comprising a wide variety of species with diverse host range including arthropods, nematodes, plants and fungi has been redefined using rDNA sequencing, placing all the insect pathogenic members to a new genus, *Lecanicillium* (Zare and Gams, 2001). *L. lecanii* is one such species which was formerly designated as *V. lecanii*. Other species of *Lecanicillium* pathogenic to insects are *Lecanicillium antillanum* (Britt.) Standl., *Lecanicillium attenuatum* Zare and Gams, *Lecanicillium evansii* Zare and Gams, *Lecanicillium fungicola* (Preuss) Zare and Gams, *Lecanicillium kalimantanense* Kurihara and Sukarno,

Lecanicillium longisporum (Petch) Zare and Gams *Lecanicillium muscarium* (Petch) Zare and Gams and *Lecanicillium psalliotae* (Treschew) Zare and Gams.

The widely popular member *L. lecanii,* the white halo fungus, is known for its pathogenicity to sucking pests such as aphids, scales, mealy bugs, whiteflies etc. (Ekbom, 1979; Kanagaratnam *et al.,*1982., Cuthbertson *et al.*, 2005., Faria and Wraight, 2007). It was also found pathogenic to Orthoptera, Coleoptera and Lepidoptera (Banu, 2013).

L. saksenae was first described as a new species of *Verticillium* by Kushwaha (1980). He reported it as a keratin degrading fungus, isolated from soils of Madhya Pradesh. Sukarno *et al.* (2009), described the species as *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno which was from epiphytic spiders and subterranean arthropods of east Kalimantan province of Indonesia. Its efficiency as a biodegrader of pesticides was later reported by Pinto *et al.* (2012). A new isolate (ITCC Acession No: Ls.Vs.1-7714) from the soils of Vellayani, Kerala identified and characterized as *L. saksenae* was found to be promising against sucking pests of vegetables (Rani *et al.*, 2014, 2015), which was the first report on its entomopathogenicity. It had 100 per cent r DNA sequence homology with *Lecanicillium* isolated by Sukarno *et al.* in 2009 from Indonesia.

2.1 PATHOGENICITY OF *Lecanicillium* TO INSECT PESTS, MITES AND NEMATODES

Pathogenicity is a qualitative trait of a pathogen to cause disease and it is determined by a myriad of biological, physiological and environmental factors. The host spectra vary widely with genus, species or even isolates. The genus *Lecanicillium* is an entompathogenic group having a wide range of hosts, extending from arthropod pests and nematodes to plant pathogenic fungi. It is one of the most wide spread and vital *Hyphomycetes* occurring frequently in the order

Homoptera, and occasionally in other orders such as Diptera, Hymenoptera, Lepidoptera and Acarina under all climatic regimes.

2.1.1 Insect Pests

2.1.1.1 Hemiptera

2.1.1.1.1. Aphids

L. lecanii is a species which regularly cause mortality of aphids under natural conditions. Harper and Huang (1986),who studied the role of *L. lecanii* in pest control showed that it reduced the population of *Aphis pisum* (Harris) by 60 to 90 per cent, *Myzus dirhodum* (Walker) by 32 to 85 per cent and *Mysus persicae* (Sulzer) by 50-100 per cent within eight days. Sahayaraj and Namashivayam (2000) while studying the bioefficacy of *L. lecanii* against the aphid *Aphis craccivora* (Koch) on groundnut reported that when the concentration of the fungus was increased from 10^6 to 10^{10}spores mL^{-1}, the mortality also increased from 10 to 63.7 per cent on the second day after treatment (DAT). Kim (2007) reported that when conidia of *L. attenuatum* at a spore concentration of 10^4 and 10^8 conidia mL^{-1}, was applied to the first instar of the cotton aphid, *Aphis gossypii* Glover, life span was significantly reduced to 10.8 and 8.4 days from 12 days. Roditakisa *et al.* (2008) studied the reproduction phase of green peach aphid *M. persicae* treated with *L. longisporum* KV71 under laboratory conditions and found that the reproduction phase was significantly reduced by two days. Vu *et al.* (2007), observed that among the several fungi tested for the control of aphids, *L. lecanii* was the most potent one resulting in 100 per cent mortality to both *M. persicae* and *A. gossypii* within five and two DAT respectively. Parmar *et al.* (2008) reported that *L. lecanii* showed 77.16 per cent mortality of the mustard aphid *Lipaphis erysimi* (Kaltenbach) 10 DAT at a spore concentration of 10^5 spore mL^{-1}. Ujjan and Shahzad (2012) studied the effect of various strains of EPF *viz., L. lecanii* (PDRL922), *Paecilomyces lilacinus* (Thom.) Samson (PDRL812), *B. bassiana* (PDRL1187) and *M. anisopliae* (PDRL526)

against *L. erysimi* and found that *L. lecanii* strain PDRL922 at 10^9spores mL^{-1} was more effective since it produced 100 per cent mortality of the aphid after three days of inoculation.

Increase in spore concentration of *L. lecanii* from 10^3 to 10^8spores mL^{-1} was reported to increase the mortality rate of *A. craccivora* from 3.33 to 100 per cent by Saranya *et al.* (2010). Suresh *et al.* (2012) reported that among different concentrationsof *L. lecanii viz.*,10^7, 10^8 and 10^9 spores mL^{-1}, the concentration of 10^8spores mL^{-1} showed higher percent mortality of 73.99 and 57.73 of adult and nymphs respectively of *A. craccivora,* whereas in the field condition 10^9 spores mL^{-1} resulted in higher mortality of 71.62 per cent. While studying the pathogenicity of *L. longisporum* strain LRC 190, Nazemi *et al.* (2014) reported that a concentration of 10^8 spores mL^{-1} caused 90 per cent mortality of adult aphid *Cinara pini*, over seven days, while the same level of control was achieved in nymphs with 10^7 spores mL^{-1}. Kim and Roberts (2015) reported that *L. attenuatum* treated against different developmental stages of *A. gossypii* caused100 per cent mortality of third instar and adults onthe fourth day, but with first instar, this was achieved only on the seventh day. Rani *et al.* (2015) reported that the new and indigenous species *L. saksenae* from Vellayani, Kerala was effective at 10^7 spores mL^{-1} causing cent per cent mortality of *A. craccivora* and *A. gossypii* within 24 HAT.

2.1.1.1.2. Mealy bugs

Kulkarni and Mote (2003) evaluated the efficacy of *L. lecanii* against pomegranate mealybug *Phencoccus solenopsis* (Tinsley) and reported that the formulation of the fungus at two to six g L^{-1} of water was effective causing more than eighty per cent mortality after seven days (Banu *et al.*,2009). A native isolate of *L. lecanii* obtained from Coimbatore was found to be more effective than *M. anisopliae* and *B. bassiana* in managing the mealy bug *Paracoccus marginatus* Williams and Granara de Willinkand *P. solenops is* infesting cotton in India (Banu *et al.*, 2010). A comparative study

conducted by Kulkarni and Patil (2013) using three wettable powder formulations of *L. lecanii*, *M. anisopliae* and *B. bassiana* @ 1.15 per cent (10^5 spores g^{-1}) revealed that *L. lecanii* was the best treatment in managing custard apple mealy bug *Maconellicocus hirsutus* Green, *Planococcus citri* Risso and *Ferrisia virgata* Cockerell. Rani *et al.* (2015) reported that *L. saksenae* was effective against the brinjal mealy bug *Coccidohysterix insolitus* Green when it was sprayed @ 10^7 spores mL^{-1} causing 100 per cent mortality within 24 HAT.

2.1.1.1.3. Whiteflies

The experiment carried out by Kanagaratnam *et al.* (1982) revealed the effectiveness of fortnightly or monthly sprays of *L. lecanii* at 10^7 spores mL^{-1} in managing a heavy infestation of cucumber whitefly *Trialeurodes vaporariorum* Westwood in glass houses. They reported 85 to 95 per cent mortality within four DAT. *L. lecanii* was reported as an effective biological control agent against cucumber whitefly *T. vaporariorum* by Kim *et al.* (2002). They found that lower concentrations (10^3 to 10^7conidia mL^{-1}) of the fungus exhibited relatively low mortality of adult whitefly while higher concentrations (10^8 to 10^9conidia mL^{-1}) exhibited 100 per cent mortality within seven DAT. Wang *et al.* (2004) used spore suspensions of six strains of *L. lecanii* at concentrations ranging from 10^2 to 10^7 conidia $mL^{-1}$for the treatment of whiteflies *Bemisia tabaci* Gennadius in sweet potato and found that the cumulative mortality ranged from 10.4 to 94.7 per cent and that the mortality increased with increase in concentration of the conidial suspension. *L. muscarium* (10^8 spores mL^{-1}) resulted in 90 per cent mortality of *B. tabaci* in sweet potato fields (Cuthbertson *et al.*, 2005). Raheem *et al.* (2009) studied the pathogencity of two isolates of EPF such as *L. lecanii* and *B. bassiana* under three concentrations (10^5, 10^6 and 10^7 conidia mL^{-1}) and they found that both *L. lecanii* and *B. bassiana* induced death from fourth DAT and that 100 percent of mortality occurred after the seventh day. The spore concentration of 10^7 conidia mL^{-1} in both *L. lecanii* and *B. bassiana* was highly toxic to the adult of

B. tabaci compared the other two concentrations. Under field conditions the concentration (10^7) was found to be the best against whitefly and *L. lecanii* was slightly superior to *B. bassiana*. Park and Kim (2010) identified *Lecanicillium* isolates, Btab01 and 4078 as most effective against *B. tabaci*. Bioassay studies using these isolates proved their effectiveness against egg, larval and adult stages causing 71 to 77 per cent, 75 to 84 per cent and 93 to 96 per cent mortality, respectively. The indigenous isolate *L. saksenae* was found to be pathogenic to *B. tabaci* in tomato when treated at a concentration of 10^7 spores mL^{-1} and resulted in 100 per cent mortality within 48h as reported by Rani *et al.* (2015).

2.1.1.1.4. Scale insects

In 1861, Nivter identified *L. lecanii* parasitizing a soft scale insect, *Lecanium coffeae* Walker (now named as *Saissetia coffeae*) in Ceylon. In India Easwaramoorthy and Jayaraj (1978) reported for the first time that *L. lecanii* was highly effective to coffee green scale, *Coccus viridis* Green when sprayed at a spore concentration of 10^6 spores mL^{-1}. They could attain 73.10 per cent mortality under field conditions after two sprayings. In an attempt to manage sucking pests in vegetables using the entomopathogens *B. bassiana*, *M. anisopliae*, *L. lecanii* and *P. fumosoroseus,* Rabindra and Ramanujam (2007) observed that *L. lecanii* at a concentration of 10^6spores mL^{-1} was effective in managing the green scale *C. viridis* causing 73.10 per cent mortality within a week. Liu *et al.* (2014) studied the pathogenicity of *L. lecanii, L. fungicola* and *Fusarium incarnatum - equiseti* against Japanese pine bast scale, *Matsucoccus matsumurae* Kuwana at 10^7 spores mL^{-1} and found *L. lecanii* as the most virulent species causing 100 per cent mortality eight DAT.

2.1.1.1.5. Jassids

Ghelani *et al.* (2014) tested the field efficacy of various insecticides against major sucking pests of cotton and found that among the biopesticides tested, *L. lecanii* @ 2.5 kg ha^{-1} resulted in more than fifty per cent mortality

whereas *B. bassiana* resulted 47 per cent mortality after 15 days of application. Nithya (2015) while studying pathogenicity of *L. lecanii* against sucking pests of cowpea found that it was infective to the jassids, *A. biguttula biguttula* at 10^7 conidia $mL^{-1}$where the mortality was recorded from the third day onwards. Rani *et al.* (2015) in their attempt to manage sucking pests in vegetables found that *L. saksenae* is effective in controlling *A. bigutulla bigutulla* @ 10^7 spores mL^{-1}. They reported 100 per cent mortality of the jassids within 24 HAT.

2.1.1.1.6. Bugs

Reiview of literature points out that *Lecancillium* is more often pathogenic to homopterans and nonpathogenic to hetreopterans. Rani *et al.* (2014), reported that *L. sakaenae* at 10^8 spores mL^{-1} is infective to cowpea pod bug *Riptortus pedestris* F. resulting in 100 per cent mortality at 48 HAT.

2.1.1.2. Lepidoptera

L. lecanii was found to be less infectious to most of the lepidopterans. Draganova and Markova (2006) identified different isolates of EPF - four *B. bassiana,* two *M. anisopliae* and one *L. lecanii* and evaluated them against the larvae of *Ephestia kuhniella* Zeller. After eight DAT, the isolate 383Bb of *B. bassiana* caused the highest lethal effect to larvae with a cumulative mortality of 87.78 per cent ± 13.18, followed by isolates 399Bb and 382Bb of *B. bassiana* 60.00 per cent ± 8.14 and 58.89 per cent ± 8.75, respectively where as the isolate 32V1 of *L. lecanii* showed less lethal effect in comparison with the other isolates. In a laboratory evaluation carried out by Paciulyte *et al.* (2010), the conidial suspensions of the two fungi *L. psalliotae* and *Fusarium solani* (Mart.) Sacc. at 10^5 to 10^8 conidia mL^{-1} were tested against Pine defoliator, *Bupalus piniaria* L. larvae. It was found that only the highest concentration of *F. solani* induced mortality of fourth instar larvae, from 29.6 to 30.7 per cent after ten days of spraying and the others were ineffective in managing the pests. Mathur*et al.* (2012) evaluated *L. lecanii* and *B. bassiana* against the brinjal shoot

and fruit borer, *Leucinodes orbonalis* Guenee. and found that both the fungi were not much effective, resulting only 37.7 and 38.7 per cent mortality. Asi *et al.* (2013) studied the potential of EPF such as *I. fumosorosea, L. lecanii, M. anisopliae* and *B. bassiana* for the control of *Spodoptera litura* F. and found that the highest mortality was caused by *B. bassiana* followed by *I. fumosorosea, M. anisopliae* and *L. lecanii.* The time taken for fifty per cent mortality of third instar larvae was 187 h in *B. bassiana*, 192 h in *I. fumosorosea* at concentration of 10^8 conidia mL^{-1} and in the case of *L. lecanii* it took 240 h at a concentration of 10^7conidia mL^{-1}.

2.1.1.3. Coleoptera

There are a few reports on *Lecanicillium* being effective to coleopteran pests. Santharam *et al.* (1978) while evaluating the efficacy of *L. lecanii* at a concentration of 10^7 spores mL^{-1} against epilachna beetle *Henosepilachna vignitiopunctata* F. found that both the larvae and pupae were more susceptible to fungal suspension than the adults. Ghatak and Mondal (2008) while studying the effect of various biopesticides on the management of *H. vignitiopunctata* found that there was a reduction upto 60.99 per cent when treated with talc formulation of *L. lecanii* at a concentration of 20g L^{-1}.

2.1.2. Mites

L. lecanii infects a wide range of sucking pests including mites. Chandler *et al.* (2005) assessed 40 isolates of EPF from six genera, *viz., Lecanicillium, Hirsutella, Metarhizium, Beauveria, Tolypocladium and Paecilomyces* against *Tetranychus urticae L.* with a spore suspension of 10^7 spores mL^{-1} and reported that only three isolates, *Lecanicillium* 450.99, *Metarhizium* 442.99, and *Hirsutella* 457.99 were pathogenic causing 43, 38 and 35.7 per cent mortality respectively at six DAT. In a laboratory experiment conducted by Sreenivas *et al.* (2005) it was revealed that *M. anisopliae* (10^8cfu mL^{-1}), *B. bassiana* (10^8cfu mL^{-1}) and *L. lecanii* (10^8 cfu mL^{-1}) were effective against red spider mite,

Tetranychus neocaledonicus Zacher. Amjad *et al.* (2012) studied the effect of various strains of *M. anisopliae, P. fumosoroseus* (Pf n32) and *L. lecanii* on two spotted spider mite, *T. urticae.* Their results indicated that Pf n32 (10^8 conidia mL^{-1}) resulted in 50 per cent mortality within four days, *L. lecanii* V17 (10^8 conidia mL^{-1}) within five days and *M. anisopliae* within eight days. Rani *et al.* (2015) observed that *L. saksenae* is effective in managing chilly mite *Polyphagotarsonemus latus* Banks causing 100 per cent mortality within 24 h.

2.1.3 Egg Parasitism on Root Knot Nematode

The parasitism of *L. lecanii* on cysts or eggs of plant parasitic nematode such as sugar beet nematode *Heterodera schachtii* Schmidt and root knot nematode *Meloidogyne incognita* (Kofoid) Chit. Were reported by several workers. Hanssler (1990) while studying the parasitism of *L. lecanii* on the egg mass of *H. schachtii* found that the cyst wall was penetrated by the fungus after 60 h of inoculation during which the fungus secretes some specific enzymes into the culture medium which enable the fungus to degrade constituents of the cyst as well as the eggshell. Meyer and Meyer (1995) studied the effects of mutant strains of *L. lecanii* against soyabean cyst nematode, *Heterodera glycines* Ichinohein green house and revealed that under laboratory and green house conditions, the fungus parasitises the eggs of *H. glycines* and thus reduce the nematode populations. *L. psalliotae, L. antillanum,* and other *Lecanicillium* species were reported to be parasitic on the eggs of the root knot nematode *M. incognita* by Gan *et al.* (2007). Nguyen *et al.* (2007) while studying the egg parasitism of *M. incognita* by *L. antillanum* revealed most of the eggs were parasitized by the fungus and the hyphae grew fully inside the eggs, two days after treatment. There was 90 per cent parasitism after three DAI.

2.2 BIOSAFETY OF *Lecanicillium*

2.2.1. Cross Infectivity to Crop Plants

Lecanicillium being closely related to *Verticillium,* a genera consisting of plant pathogens, chances of them being pathogenic to crop plants need to be ruled out. Cuthbertson *et al.* (2005) found that the application of *L. muscarium* at 10^7 spores mL^{-1} could control, *B .tabaci* in tomato and verbena without causing any damage to the plants. Gurulingappa *et al.* (2010) tested *B. bassiana, L. lecanii* and *Aspergillus parasiticus* Spear at10^8 spores $mL^{-1}$and confirmed their safety to crop plants such as cotton (*Gossypium hirsutum* L.), wheat (*Triticum aestivum* L.), bean (*Phaseolus vulgaris* L.), corn (*Zea mays* L.), tomato (*Lycopersicum esculentum* L.), and pumpkin (*Cucurbita maxima* L.), through leaf and soil inoculation methods.

2.2.2. Safety to Non Target Organisms

Any intervention in the ecosystem using a living component needs assessment of the risk factors involved in its adoption. Safety of entomopathogens to non target organisms is to be ascertained before integrating them into the pest management package.

2.2.2.1 Predators

L. lecanii was found to be safe to aphid predators *Cycloneda sanguinea* L., *Oxyptamus gastrostacus* and *Zelus* sp. when applied at 10^7 spores mL^{-1} (Rondon *et al.*, 1982). Its safety to predatory mite *Phytoseiulus persimilis* Athias-Henrio, when applied at the rate of 10^7 spores mL^{-1} was reported by Koike *et al.* (2005). Reddy *et al* (2013) reported that *L. lecani. B. bassiana* and *M. anisopliae* @ 10^8 spores mL^{-1}were safe to the predatory mirid bug *Cyrtorhinus lividipennis* Reuter and the spiders of rice ecosystem. There was no significant variation in the number of mirid bugs and spiders before and after spraying

2.2.2.2 Parasitoids

Kim *et al.* (2005) reported that *L. lecanii* at 10^8 spores mL^{-1} was nonpathogenic to the aphid parasitoid *Aphidius colemani* Viereck. Malarvannan *et al.* (2010) conducted biosafety studies of *L. lecanii* to the egg parasitoids *Trichogramma chilonis* Ishii and *Trichogramma japonicum* Ashmead and found that even at the highest concentration of 10^7 spores mL^{-1} *T. chilonis* and *T. japonicum* parasitized normally on their host insect *Corcyra cephalonicam* Stainton.

2.2.2.3 Pollinators

EPF are known to be safer to pollinators. Though the safety of *Metarhizium* and *Beauveria* has been validated earlier, the literature pertaining to the safety aspects of *Lecanicillium* to the insect pollinators are found to be lacking.

2.2.2.4 Productive Insects

Soni and Takur (2011) on studying the biosafety of EPF such as *M. anisopliae* (10^8 spores mL^{-1}) *B. bassiana* (10^8 spores mL^{-1}) and *L. lecanii* (10^7 spores mL^{-1}) to the honey bee *Apis mellifera* L. found that the mortality recorded till the 20^{th} day did not vary in the treated and non treated larvae indicating that they are safe to the bees.

2.4 MASS PRODUCTION OF ENTOMOPATHOGENIC FUNGI

Amenability to mass production is the most important criteria for an entomopathogen for its development as a biocontrol agent. Fungi belonging to the genus *Metrahizium, Beauveria, Lecanicilium, Hirsutella* and *Isaria* (*Paecelomyces*) are being commercialized due to their easiness in massproduction. The criteria for selection of an ideal substrate are its availability, cost effectiveness and nutrient parameters that supports maximum sporulation

without mycelial inhibition. Substitution of synthetic media with naturally available substrates makes mass production cost effective.

2.4.1. Natural Solid Substrates

Feng *et al.* (2000) assessed the spore yield of *L. lecanii* on solid substrates such as cooked rice, rice bran, rice husk and their mixtures and concluded that both cooked rice and rice bran gave higher spore production of 10^9 spores g^{-1} of the substrate. Similarly, Lakshmi (2001) also ascertained the suitability of cooked rice and rice bran for mass production of *L. lecanii,* where they reported an yield of 10^9 spores $g^{-1}$of the substrate. Nirmala *et al.* (2006) while assessing the substrates for mass production of four isolates of *Lecanicillium* observed that spore production in biphasic system (PDB and rice) ranges from 0.23 to 1.75 x 10^9 spores mL^{-1} after ten days of incubation (DAI). Derakshan *et al.* (2008) while evaluating different media for the production of *L. lecanii* recorded the superiority of rice grains compared to wheat, maize, sorghum and ragi in terms of spore count as well as number of colony forming units. They could obtain an yield of 10^9 spores mL^{-1} after 14 DAI. The number of viable colonies was reported as 10^9 cfu mL^{-1}. Sahayaraj and Namasivayam (2008) while evaluating various agricultural products and by products for mass production of *L. lecanii*, *B. bassiana* and *P. fumosoroseus* observed that for the mass production of *L .lecanii*, sorghum recorded the maximum yield of spores compared to vegetable wastes, seeds, rice husk and saw dust . The spore yield obtained was 10^9spores mL^{-1} for sorghum and 10^8 spores mL^{-1} for vegetable wastes, seeds, rice husk and saw dust respectively. Prasad and Pal (2014), while working out the economics of mass production of *L. lecanii, B. bassiana* and *M. anisopliae* grown on nine industrial agricultural products found that the cost involved in the mass production of *L. lecanii* was low when farm yard manure was used as substrate. On comparison of the spore yield it was on par (1.85 X 10^8spores mL^{-1}) with that of the synthetic media, SDB (1.80 X 10^8 spores mL^{-1}).

2.4.2 Enriched Solid Substrates

Liu *et al.*(1990) on studying the application of EPF such as *B. bassiana* and *Isaria javanicus* (Friedrichs and Bally) Samson for the control of *Leucaena* psyllid, *Heteropsylla cubana* Crawford found that on mass production of the fungi in chitin enriched rice bran increased the sporulation of both the fungi to 10^9 spores mL^{-1}. Puzari *et al.* (1997) reported that rice husk supplemented with two per cent dextrose resulted higher sporulation in *M. anisopliae* (10^{10} spores mL^{-1}). Palma-Guerrero *et al.* (2010) studied the effect of chitosan supplementation in sporulation of *L. psalliotae, B. bassiana, M. anisopliae, P.lilacinus* and *Pochonia chlamydosporia* (Goddard) Zare and Gams and found that the addition of two per cent chitosan to corn meal agar (CMA) profoundly increased conidiation from five to sixty per cent. Reyez – Hernandez *et al.* (2014) reported that among the 13 media tested cameron media (SDA + chitin) @ 10g L^{-1} resulted in higher conidiation of *L. lecanii* isolates, ATCC26854 and V3. The spore yield recorded by them was 10^9 spores mL^{-1} in isolate ATCC26854 and 10^8 sporesmL^{-1} in isolate V3.

2.4.3 Natural Liquid Substrates

L. lecanii was observed to give a spore yield of 10^9 spores mL^{-1} after nine days of incubation when it was multiplied in Sabouraud maltose agar yeast (SMAY) medium. (Feng *et al.*, 2000) while its yield was 10^8 spores mL^{-1} when mass produced in Molasses yeast broth (Lakshmi, 2001). Among the various liquid substrates tested for mass production of *L. lecanii viz.,* Molasses yeast broth (MYB), Potato dextrose broth (PDB), Potato carrot broth (PCB), Jaggery yeast broth (JYB), Sucrose yeast broth (PYB) and Potato sucrose broth (PSB) it was Molasses yeast broth (MYB) that was superior in spore yield (Derakshan *et al.*, 2008). The spore yield obtained were 2.28 X 10^9 spores mL^{-1} for MYB, 2.28 X 10^9 spores mL^{-1}, 2.12 X 10^9 spores mL^{-1} for PCB, 2 X 10^9 spores mL^{-1} for PSB, 1.2 X 10^9 spores mL^{-1} for JYB and 1.2 X 10^9 spores mL^{-1} for PDB and the number of viable colonies reported were 2.10 X 10^9 cfu mL^{-1} for MYB, 1.89 X 10^9 cfu

mL^{-1} for PCB, 1.87 X 10^9 cfu mL^{-1} for PSB, 1.5 X 10^9 cfu mL^{-1} for JYB and 1.2 X 10^9 cfu mL^{-1} for PDB. Sahayaraj and Namasivayam (2008)tested various liquid media such as coconut water, rice and wheat washed water and rice cooked waterfor mass production of *L. lecanii* , *B. bassiana* and *P. fumosoroseus.* The results showed that coconut water supported maximum sporulation of 1.21 X 10^9 spores mL^{-1} for *B. bassiana* and 1.01 X 10^9 spores mL^{-1} for *P. fumosoroseus* followed by 5.27 X 10^8 spores mL^{-1} for *L. lecanii*

2.4.4 Enriched Liquid Substrates

Rachappa *et al.* (2005) on evaluating the suitability of various liquid media on mass production of *M. anisopliae* revealed that coconut water and rice gruel fortified with yeast one per cent supported the growth and conidial production rather than molasses fortified with yeast one per cent. The spore yield recorded was 8.32 X 10^8 spores mL^{-1} and 4.2 X 10^8 spores mL^{-1}respectively. Srikanth and Santhalakshmi (2012) on studying the effects of various media additives on the production of *B. brongniartii* found that the addition of calcium chloride (0.5 to 3 per cent) + molassess, chitin (0.1 to 0.6 per cent) + molasses and lactic acid (0.5 to 3 per cent) + molasses increased the sporulation of the fungus to 10^{10}spores mL^{-1} in all the tested media. Nithya (2015) reported that the spore yield of *L. lecanii* could be enhanced from 10^7 to 10^8 spores mL^{-1} by enriching SDB with five per cent chitin or five per cent chitosan or five per cent yeast. The number of viable colonies recorded were 10^5 cfu mL^{-1} for all the tested media such as SDB, SDB + chitin, SDB + chitosan and SDB + yeast. She also observed that spores harvested from enriched media resulted in better mortality of *A. craccivora.* The mortality recorded was 100 per cent for five per cent enriched SDB + chitin, SDB + chitosan and SDB + yeast, where as non enriched SDB resulted only 59 per cent at 72 HAT.

2.5 METABOLITES OF ENTOMOPATHOGENIC FUNGI

Fungi secrete an array of compounds with biological activity against other organisms. These are mostly products of secondary metabolism. Some metabolites serve as antibiotics that protect them from antagonistic organisms, some prevent saprophytes and some others are important pathogenicity determinants.

2.5.1 Cuticle Degrading Enzymes

Production of a range of cuticle degrading enzymes such as lipases, proteases and chitinases is an important event in the interaction of EPF and host. Enzymes which break down the insect cuticle are important virulence determining factor of the EPF. Recently, these enzymes have been employed directly as insect control agents, thus opening the new potential use of EPF.

EPF produces a number of extracellular enzymes which plays an important role in the cuticle penetration of insect host cuticle *viz*., proteases, chitinases, and lipases to penetrate the barriers and expedite infection (St. Leger *et al*., 1986). While studying the cuticle degrading enzymes produced by *M. anisopliae*, *B. bassiana* and *N. rileyi* and their effect on pathogenicity to *Helicoverpa armigera* Hubner, found that *M. anisopliae* had high levels of chitinase (1.05 U mL^{-1}), protease (0.89 U mL^{-1}) and lipase (0.29 U mL^{-1}) and thus exhibited more than 70 per cent mortality. *B. bassiana* secreted the enzymes to the tune of 0.33 U mL^{-1}chitinase, 0.08 U mL^{-1} protease and 0.02 U mL^{-1} lipase. The mortality recorded was70 per cent. They observed a decline in mortality with decrease in enzyme levels. *N. rileyi* did not exhibit detectable chitinase levels even upto 120 h whereas it showed protease activity (0.27 U mL^{-1}) and lipase activity (0.22 U mL^{-1}). The mortality recorded on the test insect was less than 50 per cent.

Kang *et al.*(1999) detected an increase in the chitinolytic activity of *M. anisopliae* (8.66 U mL^{-1}) when colloidal chitin (two per cent) was the sole carbon source compared to Yeast extract peptone dextrose medium – YEPD (2 per cent yeast extract, one per cent peptone, and two per cent dextrose). Chitinase activity recorded in YEPD medium was 1.25 UmL^{-1}. Cardenas *et al.* (2001) reported that the clear zones around the colonies on agar plates using tributyrin as a substrate indicate production of lipase. According to Nahar *et al.* (2004) *M. anisopliae* secretes the enzymes protease, lipase, and two chitin-metabolizing enzymes, *viz.*, chitin deacetylase and chitosanase when grown in 0.7 per cent colloidal chitin containing Potato dextrose agar (PDA) medium. They recorded its chitinase activity as 0.01-0.0398 U mL^{-1}, protease activity as 0.01 – 0.02 U mL^{-1} and lipase activity as 0.312 and 0.015 U mL^{-1}. Dias *et al.* (2008) while working on the production of cuticle-degrading proteases produced by *B. bassiana* reported that the protease index on mineral agar medium amended with gelatin was 3.56 and 3.25 and in mineral agar medium with casein it was 1.87 and 2.2 respectively at pH 6.8 and 8.5. Kaur and Padmaja, (2009) studied the mode of action of various enzymes produced by *B. bassiana* and tested its virulence on *H. armigera.* They found that the cuticle degrading enzymes such as chitinase, protease and lipase helped in weakening the cuticular barrier of the insect. Besides this, enzymes penetrate into the intestine and cause damage to the peritrophic membrane as a result of which they stop feeding and finally die.

When two strains of *L. lecanii* (V3.4504 and No.V3.4505) were grown for eightdays continuously on the culture medium with cuticle material of the scale insect *Rhodococcus sariuoni* Borchsenius, it was observed that protease activity of the strain No. V3.4504 increased continuously in the first six days with a maximum value (33.94 ±1.61) U g^{-1}, and then decreased (Guoliang *et al.*, 2009). Meanwhile, its chitinase activity maintained which was at a lower level at the earlier stage, began to rise from fifth day reaching the maximum value of 7.28 ±1.36 U g^{-1}. A similar trend was observed in the protease and chitinase activities of the strain no. V3.4505. It indicated that, protease of the fungus play an

important role in the early phase to decompose protein in the cuticle of the scale infested by *L. lecanii.*

Protease and chitinase activity of *L. lecanii* cultured on the cuticlular substrate of the three scale insect species revealed that the protease activity of *L. lecanii* cultured on *Dorcus koreanus* Jang and Kawai and *R. sariuoni* was similar, their peak value emerging at the sixth day with 34.58 ± 1.01 U g^{-1} and 33.94±1.61 Ug^{-1} respectively (Xie *et al.*, 2010) They also reported a gradual increase in chitinase secrtetion during the first six days, followed by a rapid reduction. The protease activity on *Cybister japonicas* Sharp was obviously lower during the first six days and reached the peak of 34.30± 0.48 U g^{-1} on the seventh day.

Bai *et al.* (2012) observed that nine isolates of *M. anisopliae* showed chitinolytic activity ranging from 0.525 to 1.560 U mL^{-1}, protease activity from 0.020 to 0.114 U $mL^{-1}$and lipase activity from 0.153 to 0.500 U mL^{-1}.

2.5.2 TOXINS

EPF synthesize different metabolites and depsipeptides which act as toxins, playing an important role in the infection process resulting in a series of symptoms in insects such as convulsions, lack of coordination, behaviour alteration, feeding ceassation, paralysis and death.

The first systematic study of toxin production by fungal entomopathogens *in vitro* was conducted on *M. anisopliae* and led to the discovery of two novel insecticidal substances destruxin A and destruxin B. The LD50 of destruxin A and B when injected into silkworm larvae was 0.015± 0.030 g^{-1}, 24 h post-injection, but these compounds were 10 to 30 times less active in *Galleria* larvae (Kodaira,1961). Kodaira, (1962) isolated destruxin A and B from the active syrup extracted from the culture filtrate and the fungal mats of *Oospora destructor* (Metch.) Delac. It showed strong toxicity to silkworms and mice by injection, but the toxicity to the potato lady beetles when fed with poisoned leaves was not so

strong as in case of the injection. Later Suzuki *et al.* (1970) isolated three compounds, desmethyl destruxin B, D and C, from *M. anisopliae* culture filtrates.

Lecanicillium produces mycotoxins such as beauvericin, and bassianolide (Suzuki *et al.*, 1977; Kanaoka *et al.*, 1978). Ferron (1981) opined that an insect dies due to mechanical pressure exerted by excess fungal growth as well as by the action of mycotoxins. Some *Lecanicillium* species are known to produce toxic metabolites *invitro*, which may be implicated in the ability of the fungus to overcome its host (Claydon and Grove, 1982). They also reported that *Lecanicillium* produces dipicolinic acid. Yeh *et al.* (1996) could detect several mycotoxins like, beauvericin, beauverolides and bassianolide from cultures of *B. bassiana, L. lecanii,* and *Paecilomyces* spp and Destruxins A, B, C, D, E, F from cultures of *M. anisopliae.* He opined that these toxins act like poisons in insects. Samuels (1998) purified a toxic cyclodepsipeptide and Destruxin A from the culture filtrates of *M. anisopliae* grown in four day old Czapek's liquid medium using Reverse-Phase HPLC, and assayed for its effects on the heart beat of larval *Manduca sexta* (L.). He found that it accelerated the heart beat leading to the death. Soman *et al* (2001) reported that vertilecanin A1, decenedioic acid and 10 - hydroxyl - 8- decenedioic acid are the toxins secreted by *Lecanicillium.*

The major secondary metabolite produced by *B. brongniartii* was oosporein as reported by Strasser (2000). They reported that the fungus yielded toxin 270 mg L^{-1}, four days after incubation. Both *in vitro* and *in vivo* studies of oosporein revealed that it posed no risk to man and animals. Bandani *et al.*(2000) reported that EPF such as *Tolypocladium cylindrosporum* Gams, *Tolypocladium niveum* (Rostr) Bissett and *Tolypocladium parasiticum* Barron produced the toxin efrapeptin (116, 80 and 2 mg L^{-1} respectively) at 14 DAI. It was found to be toxic to the final instar larvae of *Galleria mellonella* L. and *M. sexta.* The LD 50 value of the efrepeptin toxin to the final instar larvae of *G. mellonella* and *M. sexta* were 30 and 47 ng respectively.

Assaf *et al.* (2005), isolated dipicolinic acid from *P. fumosoroseus* using Thin layer chromatography (TLC) performed using 0.051 M cerium ammonium sulphate which produced an yellow spot which was similar to the spot developed by the dipicolinic acid standard. The toxin was detected to the extent of 0.04 per cent.

2.5.3 Bioefficacy of Toxins

Rani (2001) isolated the mycotoxin, fusaric acid from seven day old culture filtrate of EPF *Fusarium pallidoroseum* (Cooke) Sacc in Czapek's liquid medium. Bioefficacy studies conducted revealed that the toxin at 500 and 1000 ppm caused 46 and 100 per cent mortality of *A. craccivora* respectively at the end of 72 HAT. Wang *et al.*, (2007) studied the toxicity of crude toxin extracts of two isolates of *L. lecanii* against sweet potato whitefly, *B. tabaci.* They noted that nymphs were the most susceptible stages followed by adults. The crude toxin of the isolate V 2850 @ 111 mg L^{-1} resulted in fifty per cent mortality. In the case of isolate V 3450, 50 per cent mortality was caused by178 mg L^{-1} at 48 HAT.

Materials and Methods

3. MATERIALS AND METHODS

The present research work entitled 'Pathogenicity and biochemical properties of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno.' was carried out at the Biocontrol Laboratory for Crop Pest Management, Department of Entomology, College of Agriculture, Vellayani during 2014-2016 .

3.1. PATHOGENICITY OF *L. saksenae* TO INSECT PESTS AND MITES

Laboratory experiments were carried out to assess the pathogenicity of *L. saksenae* to different groups of insect pests and mites.

Insect pests

Hemiptera

a) Cow pea aphid (*Aphis craccivora* Koch)
b) Brinjal mealy bug (*Coccidohysterix insolitus* Green)
c) White fly (*Bemisia tabaci* Gennadius)
d) Jassid (*Amrasca biguttula biguttula* Ishida)
e) Scale insect (*Lecanium* sp.)
f) Rice bug (*Leptocorisa acuta* Thunberg)
g) Cowpea pod bug *(Riptortus pedestris* F.)
h) Leaf footed bug (*Leptoglossus phyllopus* L.)
i) Green shield bug (*Nezara viridula* L.)
j) Red cotton bug (*Dysdercus cingulatus F.*)

Lepidoptera

a) Bhindi leaf roller (*Sylepta derogata* F.)
b) Cut worm (*Spodoptera litura* F.)
c) Brinjal fruit and shoot borer (*Leucinodes orbonalis* Guenee)
d) Cucumber moth (*Diaphania indica* Saunders)

Coleoptera

a) Ash weevil (*Myllocerus* sp.)

b) Brinjal epilachna beetle (*Henosepilachna vignitiopunctata* F.)

c) Banana pseudostem weevil (*Odoiporus longicollis* Oliver)

d) Banana rhizome weevil (*Cosmopolites sordidus* Germ.)

Mites

a) Red spider mite (*Tetranychus* sp.)

b) Chilli mite (*Polyphagotarsonemus latus* Banks)

3.1.1. Maintanence of fungal culture

L. saksenae isolate ITCC No: of Ls.Vs.1 -7714, maintained at Biocontrol Laboratory for crop pest management, Department of Entomology, College of Agriculture, Vellayani was utilized for the study. The fungus was sub cultured and maintained in Sabouraud Dextrose Agar (SDA). Fourteen day old cultures stored under refrigeration were used for carrying out various experiments. The virulence of the isolate was maintained by passing the fungus periodically through the brinjal mealy bug *C. insolitus*, and reisolating it.

3.1.2. Maintanence of test organisms

Stock culture of insects and mites were maintained separately by raising the respective host plants raised in pots of 45 cm diameter. The seeds of host plants were obtained from Department of Olericulture, College of Agriculture, Vellayani. They were maintained healthy by irrigation and manuring. Sequential planting was done to maintain fresh cultures of test organisms. All the test organisms were reared in the laboratory except the scale insect and mites.

3.1.2.1. Hemiptera

3.1.2.1.1. A. craccivora

Aphid colonies were located in field and the gravid females were collected from them and released into potted cowpea plants described in para 3.1.2. using a fine camel hair brush. The newly emerged young ones were transferred to new plants to begin a new culture.

3.1.2.1.2. C. insolitus

The field collected colonies of mealy bugs were released into potted brinjal plants (3.1.2) for carrying out pathogenicity studies. The newly emerged nymphs were transferred to new plants to begin a fresh culture which provided sufficient adults and nymphs needed for the experiment.

3.1.2.1.3. B. tabaci

Nymphs and adults collected from field were released into potted bhindi plants described in para 3.1.2. The first instar nymphs were transferred to new plants to maintain a fresh culture which could provide the required number of adults and nymphs. They were collected using inverted test tubes and used for pathogenicity studies.

3.1.2.1.4. A. biguttula biguttula

Fieldcollected nymphs and adults were released into potted bhindiplants described as in para 3.1.2, were kept in a rearing cage made measuring 164 x 100 x 118 cm^3.Freshly emerged nymphs were transferred to new plants to maintain the hopper culture. From this the adults and nymphs needed for the experiment were collected using inverted glass tubes.

3.1.2.1.5. L. acuta

Egg masses were collected from field and surface sterilized using 0.1 per cent sodium hypochlorite solution for two Min. and washed in sterile water. They were kept for emergence in a rearing jar of 17 x 10 cm^2, covered with muslin cloth. After one week, the emerged first instar wingless nymphs were transferred to potted paddy seedlings raised in pots mentioned in para 3.1.2. They were then placed inside the rearing cage mentioned above.

3.1.2.1.6. R. pedestris

Rearing was intiated with surface sterilized egg mass (para 3.1.2.1.5.). The newly emerged nymphs were transferred to new rearing jars provided with fresh bits of cowpea pod replaced on alternate days. The mouth of the jar was covered with a muslin cloth.

3.1.2.1.7. L. phyllopus

Stock culture was maintained as mentioned in para 3.1.2. The food source provided was tender bitter gourd fruits replaced every three days.

3.1.2.1.8. N. viridula

The culture was initiated using surface sterilized eggs collected from brinjal field, maintained in a rearing jar the mouth of which was closed with a muslin cloth. The food source was fresh brinjal leaves kept afresh by providing moistened cotton at the cut end.

3.1.2.1.9. D. cingulatus

D. cingulatus culture was initiated using surface sterilized eggs collected from bhindi field and maintained as in para3.1.2.1.8. Tender bhindi fruits kept afresh with the help of moistened cottonwere provided as food.

3.1.2.1.10. Lecanium sp.

Detached twigs of red gram infested with *Lecanium* sp. were kept in laboratory under observation for four days to rule out any infection. The healthy colonies were confined in fresh twigs which were kept turgid with the help of moistened cotton wool wound at the tip. The culture was maintained in in rearing jars (17 x 10 cm^2) covered with muslin cloth.

The cultures were kept under constant observation to remove natural enemies if any from the colonies. Healthy and uniform aged adults and nymphs were selected from the stock culture for pathogenicity studies. In the case of homopterans two day old nymphs and in the case of heteropterans seven day old nymphs were selected as test organisms

3.1.2.2. Lepidoptera

3.1.2.2.1. S. derogata

Larvae of *S. derogate* were collected from bhindi fields and released in rearing jars (15 x 20 cm) provided with fresh bhindi leaves. The mouth of the jar was covered with muslin cloth. It was kept under observation to rule out any infection. Pupae were transferred into another jar for adult emergence. Newly emerged adults were fed with 10 per cent honey solution in the form of soaked cotton kept on inner walls of the jar. Egg masses were transferred as and when they were laid by cutting off the leaf portion bearing them, to a new jar. Upon emergence the first instar larvae were transferred to another jar provided with tender leaves of bhindi.

3.1.2.2.2. S. litura

Egg masses collected from tomato plants were surface sterilized (para 3.1.2.1.5) and kept for emergence in a rearing jar covered with muslin cloth. The

newly emerged larvae were transferred to another jar along with the leaf and were fed with fresh castor leaves. Four day old caterpillars of uniform age were collected for pathogenicity studies and the rest were allowed to grow by providing them with fresh leaves taken in a rearing trough of 15 x 20 cm. Care was taken to minimize cannibalism by providing fresh leaves every day. All the rearing trough, muslin cloth etc. were kept hygienic to minimize viral infection. When the caterpillars were full grown and showed signs of pupation they were transferred to another trough provided with soil for enabling pupation. The adults on emergence were fed with 10 per cent honey solution.

3.1.2.2.3. L. orbonalis

Infested fruits collected from field were brought to the laboratory and kept for pupation outside feeding tunnels. The newly emerged adults were transferred to another jar for continuing their life cycle and were fed by transferring as in para3.1.2.2.2.

3.1.2.2.4. D. indica

Larvae of *D. indica* collected from field were released into glass jars provided with bitter gourd leaves which were kept afresh with the help of moistened cotton attached to the detached end of petiole. The rearing jars were cleaned every day and fresh feed was provided on alternate days. The mouth of the jars were covered using muslin cloth. On pupation, the cocoons were transferred into another glass jar for adult emergence. The newly emerged adults were fed as mentioned in para 3.1.2.2.2.and were kept for oviposition. On hatching, the first instar larvae were transferred to new jars to continue rearing.

Healthy and uniform aged moths and larvae were utilized for pathogenicity studies.

3.1.2.3 Coleoptera

3.1.2.3.1. Myllocerus sp.

Adult weevils were collected from the field and reared using fresh brinjal leaves kept afresh by keeping the twig immersed in a small vial containing water soaked cotton. The mouth of the rearing jar was covered with muslin cloth. The culture was kept under observation for two days to rule out any infection. Fresh twigs were provided on every four days. The eggs laid on each day were transferred into separate rearing troughs provided with fresh and tender leaves. The full grown grubs were transferred to rearing trays provided with sterile soil for pupation. The emerging adults were maintained as explained earlier to continue its lifecycle.

3.1.2.3.2. H. vignitiopunctata

Brinjal leaves carrying egg masses of epilachna were collected from the field and brought to the laboratory. They were kept for emergence in 9 cm Petridishes. Upon emergence they were transferred to rearing troughs provided with fresh twigs as mentioned in para 3.1.2.2. Twigs were replaced as and when needed till the grubs reached the pupation. The emerging beetles were transferred to another rearing trough to continue their life cycle.

3.1.2.3.3. O. longicollis

Infested pseudostem along with the grubs were brought to the laboratory and cut into 10 cm long pieces. Each bit carrying the grub was kept in separate rearing troughs mentioned in para 3.1.2.2. On deterioration of the pseudostem bits the grubs were transferred to new bits till they attained pupation. The emerging adults were transferred to rearing jars of 15 x 20 cm, the mouth of which was covered with muslin cloth. The jars were provided with 10 cm long outer sheath of pseudostem to enable egg laying. On hatching, the eggs were transferred to pseudostem bits and rearing was continued as described above.

3.1.2.3.4. C. sordidus

Infested rhizomes containing the grubs of *C. sordidus* were brought to the laboratory and cut into pieces of convenient size. Each bit carrying the grub was placed inside the rearing trough, the mouth of which was closed with muslin cloth. The rhizomes were replaced as and when they decay, until pupation. The emerging adults were transferred to new rearing trough with fresh rhizomes to continue the lifecycle.

Healthy and uniform aged beetles and grubs were utilized for pathogenicity test.

3.1.2.4 MITES

3.1.2.4.1 Tetranychus sp.

Tetranychus colonies collected from castor plants were brought to the laboratory and observed under microscope for any symptoms of infection. The colonies along with the leaves were kept under observation for 48 h in rearing troughs. The leaf bits were kept afresh by dipping the leaf petiole in small vials containing water.

3.1.2.4.2 Polyphagotarsonemus latus

P. latus colonies collected from chilli plants were utilized for pathogenicity studies afterruling out natural infection asdescribed in para 3.1.5.

After 48 h the mite colonies were observed under microscope to rule out any infection and the healthy colonies were used for pathogenicity studies.

3.1.3 Preparation of Spore Suspension

Spore suspension of *L. saksenae* was prepared from 14 day old culture grown in Sabouraud dextrose broth (SDB) by shaking and filtering through a double layered muslin cloth.

3.1.2. Pathogenicity Tests

To conduct pathogenicity test on various test organisms mentioned in para 3.1.2, the fungal spore suspension was standardized to a concentration of 10^7spores mL^{-1} and used.

3.1.4.1. Hemiptera

Detached leaf method (Yokomi and Gottwald, 1988) was used to conduct the pathogenicity of *L. saksenae* against *A. craccivora, A. biguttula biguttula, C. insolitus, Tetranychus* sp. and *P. latus*. Tender cowpea leaves detached along with petiole was kept turgid by covering the petiole with moist cotton. These were then placed in upside down position in a petridish of 9 cm diameter lined with filter paper moistened to saturation level. *A. craccivora* were transferred to these petridishes by tapping the leaves gently while *A. biguttula biguttula, C. insolitus* and *Tetranychus* sp. and *P. latus* were transferred using a soft fine moistened brush. Spore suspension was applied topically on the nymphs and adults using an atomizer.

B. tabaci collected using inverted test tube were transferred to small glass jars (10.5 x 6 cm^2) enabling free movement. Chilli leaves treated with spore suspension were provided inside the jar instead of topical application to cause minimum damage to the wings.

Bugs *viz., R. pedestris , L. phyllopus, N. viridula, D. cingulatus* and *L. acuta* were transferred into rearing jars of 17 x 10 cm^2 with their respective

food source mentioned in para 3.1.2.1. They were sprayed with spore suspension and then covered with a muslin cloth for proper aeration.

In the case of aphids, mealy bugs, white flies, jassids, scales and mites 50 insects served as one replication and other test organisms *viz.*, rice bug, pod bug, green shield bug, ash weevil, pseudostem weevil and rhizome weevil 20 insects were used as one replication. All the treatments were replicated thrice. Test organisms sprayed with sterile water served as control. Dead insects / mites were transferred to a moist chamber (Petridishes lined with a moistened filter paper) and observed for fungal mycelial growth if any. Mortality was recorded at 24 h interval for a period of 120 h. Percentage mortality was corrected using Abbot's formula (Abbot, 1925). The fungus was re-isolated from the cadaver and examined under microscope for mycelial and spore characteristics to confirm the pathogenicity.

3.1.4.2. Lepidoptera

The fungus was tested for its pathogenicity to caterpillars of test insects, *S. derogata*, *S. litura*, and *D. indica*. Freshly emerged adults and caterpillars of uniform age were selected for the experiment. The test insects were taken in a rearing jar and a fine spray of the spore suspension was sprayed using an atomizer. For each species three replications were maintained with ten caterpillars per replication and six adults per replication. Test insects sprayed with sterile water alone served as control. The treated adults were fed with 10 per cent honey solution after wiping out the excess moisture from the jar. The treated caterpillars were transferred to sterile Petridishes lined with filter paper and provided with fresh food as mentioned in para 3.1.2.2. They were kept under observation for symptoms of mycosis or mortality during the experimental period.

In the case of *L. orbonalis* larvae, spore suspension was sprayed over the caterpillars of uniform age taken in a sterile Petridish. After an hour, they were transferred to separate rearing troughs provided with tender brinjal fruit. Five

larvae were released into each fruit. After 48 h, one set of fruits were carefully cut open to observe the symptom of infection or mortality. Another set of fruits were split open after one week. For each set, five replications were maintained and another set of five fruits were maintained for untreated caterpillar. Upon pupation they were observed for normal adult emergence. Adults treated with spore suspension were maintained and observed as in the case of other lepidopterans mentioned above.

3.1.4.3. Coleoptera

Adults and grubs of *Myllocerus* sp. and *H. vignitiopunctata*, were treated with spore suspension as mentioned in para 3.1.4.2. Three replications were maintained separately for adults and grubs with 10 insects per replication.

In the case of *O. longicollis* and *C. sordidus* the uniform aged grubs collected from the culture was sprayed with the spore suspension. After an hour they were allowed to bore into pseudostem and rhizome as mentioned in para 3.1.2.3 3 and 3.1.2.3 4 respectively. Observations were recorded as mentioned in the case of *L. orbonalis* larvae. For each set, five replications were maintained and another set of five pseudostem bits and rhizome bitswere maintained for untreated grubs. Upon pupation they were observed for normal adult emergence.

Adults treated with spore suspension were maintained in rearing jars provided with outer sheath of pseudostem in the case of *O. longicollis* and rhizome bits for *C. sordidus*. They were observed for symptom development and mortality at 24 h interval.

For each species, three replications were maintained with six insects per replication. The insects treated with sterile water served as control. The treated insects except larval stage of *L. orbonalis, O. longicollis* and *C. sordidus* were observed for symptoms of infection and mortality at 24 h interval until hundred per cent mortality was noted.

3.1.5 Determination of Effective Dose

Effective dose of *L. saksenae* was determined for those test organisms which were found susceptible. For this, six different spore concentrations ranging from 10^3 to 10^8 spores ml^{-1}were prepared by serial dilution method. Spore count was eneumerated using a Neubauer haemocytometer. Test organisms were treated using the procedure adopted for pathogenicity test (para 3.1). Each treatment was replicated thrice with 50 insects per replication except for rice bug and pod bug where the number was 20 per replication. Mortality was recorded at 24 h interval till hundred per cent mortality occurred in any one of the treatment.

The dose that recorded more than 90 per cent mortality within three days was selected as the effective dose.

3.2 PARASITISM ON ROOT KNOT NEMATODE

Parasitism of *L. saksenae* to the eggs of root knot nematode *Meloidogyne incognita* (Kofoid and White) chit.was tested by extracting the eggs from samples of infested tomato plants. The plants showing symptoms of nematode attack were carefully uprooted along with the soil and the shoot portion was detached using a sharp razor blade. The root systems along with the galls were washed in running tap water to remove the soil. The roots were then cut into two to three pieces and rinsed thoroughly in tap water. The egg masses were separated using a forceps. They were then surface sterilized using 0.1 per cent sodium hypochlorite solution for one minute, followed by three sequential cleaning with sterile water. The sterilized eggs were then placed on agar plates. The eggs were then inoculated with mycelial mat of the fungus cut out from a culture plate. They were incubated for seven days and then examined for fungal growth under a stereo microscope after staining with lacto phenol. These were then placed on water taken in a sterile petridish, to observe normal emergence of nematodes.

3.3 CROSS INFECTIVITY TO CROP PLANTS

The vegetable crops in which *Verticillium* infection was reported *viz.*, cowpea, bhindi, brinjal and tomato were selected for the study. The seedlings from portray were transplanted to 45 cm diameter pots for 1:1:1 sand : soil: cowdung potting mixture. Soil and leaf inoculation methods were followed to test the infectivity. One month old plants were selected for the study.

3.3.1 Leaf Inoculation Method

The terminal three leaves were marked and pin pricks were made on them using a sterile disposable needle. Small cotton balls soaked in spore suspension (10^7spores mL^{-1}) was spreaded on the marked area. The treated leaves were kept undisturbed by covering with transparent polythene covers. Five plants were inoculated from each species. Plants smeared with sterile water were kept as control. The plants were kept under observation for a period of two months for occurrence of disease symptoms.

3.3.2 Soil Inoculation Method

Another set of potted plants were selected to carry out the soil inoculation method. To the one month old potted plants 100 ml of spore suspension at 10^7 spores mL^{-1} was drenched uniformly around the plant. Plants drenched with sterile water served as control. Five plants were treated from each species and kept under observation for a period of two months for observation on disease symptoms if any.

3.4 SAFETY TO NON TARGET ORGANISMS

3.4.1 Predators

3.4.1.1 Coccinellids

The common coccinellid predators *Chilomenes sexmaculata* F.and *Coccinella septumpunctata* L.seen in the colonies of sucking pests*viz.*, aphids, mealy bugs, white flies, scales and mites were selected as the test insects. Both the adults and grubs were tested for their susceptibility to *L. saksenae.*

3.4.1.1.1. Rearing of Coccinellids

The coccinellid eggs collected from vegetable ecosystem were kept in Petridishes for emergence. The grubs on emergence were fed with aphid colonies for two days. On the third day, they were transferred to a rearing trough, provided with aphids colonized on cowpea twigs which were kept afresh as described in para 3.1.4. A set of one week old grubs were separated for pathogenicity studies and the rest were fed continuously in the same trough till they pupated. The adult beetles on emergence were fed with 10 per cent honey solution and were utilized for testing the infectivity.

3.4.1.1.2. Infectivity Test

Three sterile Petridishes of 9cm diameter, lined with filter paper at the bottom were used for the experiment. Into each petridish ten grubs were transferred carefully from the stock culture, using a fine camel hair brush. Spore suspension at 10^7 spores ml^{-1} was applied topically using an atomizer. After an hour the treated insects were provided with aphids which were replenished daily after changing the filter paper. The grubs were observed for disease symptoms or mortality till they completed their life cycle.

The adult insects collected from the stock culture were tested for infectivity as described in the case of grubs except for the food source which was 10 per cent honey solution. Observations were recorded on symptoms of mycosis and mortality.

3.4.1.2 Syrphids

The syrphid predators selected for the study were *Ischiodon scutellare* F. and *Xanthogramma scutellare* Thorell.

3.4.1.2.1. Rearing of Syrphids

The pupae collected from aphid colonies were kept for emergence in the laboratory. Adults of the two species were maintained separately in rearing troughs (15 x 20 cm) provided with small cotton balls soaked in dilute honey kept on the walls of the trough. Simultaneously, fresh cowpea twigs having established aphid colonies were kept afresh in a small vial with water soaked cotton plug, to enable oviposition. The colonies were examined for eggs daily with a magnifying glass. The maggots were transferred to Petridishes lined with filter paper and fresh twigs of cowpea bearing aphid colonies. A set of maggots of same age were selected for the study. The emerging maggots were fed with aphids till they pupated. The newly emerged adults were fed as in the case of coccinellid beetles and utilized for the study.

3.4.1.2.b Infectivity Test

The procedure followed for coccinellids was followed in the case of syrphids. Symptom of mycosis and mortality were recorded.

3.4.1.3 Spiders

Infectivity test on predatory spiders were carried out using field collected population of *Tetragnatha maxillosa* Thorell. and *Oxyopus* sp. collected from rice

and vegetable ecosystem respectively were tested for their susceptibility to *L. saksenae*. They were brought to the laboratory and fed with aphid colonies kept in rearing troughs as described in para 3.4.1.1.1. After ruling out any natural infection, they were treated with *L. saksenae* spore suspension using the dose and method mentioned in the case of other test organisms. Fifteen spiders of each species were maintained separately for the test and kept under observation for symptom development and mortality.

3.4.2. Parasitoids

3.4.2.1 Bracon brevicornis F. *and Goniozus nephantidis* Muesebeck

Adults of *B. brevicornis* and *G. nephantidis* obtained from parasite breeding station, Department of Agriculture at Parottukonam, Thiruvananthapuram were made use of for the study.

Adults of both the species were taken seperately in 10 cm long glass vials provided with a thin plastic strip coated with sugar solution. The walls of the glass vial were given a fine spray of spore suspension, prior to release of the parasitoids. Each treatment was replicated thrice with ten insects per replication. They were kept under observation for 48 h for any behavioural changes or fungal development. Upon death, the treated insects were surface sterilized and placed on PDA slants to for mycelial growth if any. Treatment with sterile water served as control.

3.4.2.2. Trichogramma spp

The egg cards of *Trichogramma japonicum* Ashmead and *Trichogramma chilonis* Ishii were procured from the State Biocontrol Laboratory, Department of Agriculture, Kerala. Three bits were randomly detached from three different cards of each species. The trichobits of different cards were placed on the fungal cultures of *L. saksenae* grown in Petridishes for one hour, in such a way that the eggs were in contact with fungal mat. They were then transferred to

Petridishes, each Petridishes bearing three bits of the same card. The bits were observed for emergence of adults and the number of adults emerged in each case was noted. The percentage emergence was worked out using the formula.

$$\frac{\text{Number of adults emerged in treatment X 100}}{\text{Number of adults emerged in control}}$$

Upon death of the treated insects, they were surface sterilized and placed on PDA slants to observe mycelial growth, if any. Treatment with sterile water served as control.

3.4.3 Pollinators

3.4.3.1 Bees

Infectivity test on carpenter bee, *Xylocopa* sp. was carried out using field collected population. Adult bees were collected from brinjal field and tested for their susceptibility to *L. saksenae*. In the laboratory they were fed with 10 per cent honey solution kept in rearing troughs as described in para 3.4.1.1.1. After ruling out any natural infection, they were treated with the spore suspension using the dose and method mentioned in the case of other test organisms. Observations on symptoms of mycosis and mortality were recorded till all the bees were dead at least in one replication. The treatment was replicated thrice with 10 bees per replication.

3.4.3.2 Wasp

Infectivity test on wasp *Vespula* sp. was carried out using field collected population. Susceptibility to *L. saksenae* was tested as described in the case of bees. Observations on symptoms of mycosis and mortality were recorded till all the wasps were dead at least in one replication. Ten wasps were maintained separately for the test.

3.4.4 Productive Insects

3.4.4.1 Apis cerana indica F.

Infectivity test on honey bees was carried out in the Indian bee *A. cerana indica* collected from apiaries. The experiment was laid out and susceptibility was assessed as described above.

3.4.4.1 Apis mellifera L.

Infectivity test on honey bees was carried out in the Italian bee *A. mellifera* collected from apiaries. The experiment was laid out and observations were recorded as described in para 3.4.3.1.

3.4.4.2 Tetragonula iridipennis Smith

Infectivity test on stingless bees, *T. iridipennis* was carried out using bees collected from apiaries. The experiment was laid out and susceptibility assessed based on observations similar to that mentioned in para 3.4.3.1.

3.5 IDENTIFICATION OF COST EFFECTIVE CHITIN ENRICHED SUBSTRATES FOR MASS PRODUCTION

3.5.1 Solid Substrates

The natural substrates such as rice bran and wheat bran and both ammended with the natural biopolymers *viz.*, chitin and chitosan at five per cent were tested for the suitability for mass production with respect to mycelial growth and sporulation. The concentration of chitin and chitosan was fixed based on the findings of Nithya (2015). The virulence of the spores produced in each substrate was evaluated by determining the mortality caused in *C. insolitus*

3.5.1.1 Preparation of the Substrates

Thirty gram each of the substrates (wheat and rice bran) were weighed and made just wet by adding water. They were then transferred to 250 mL conical flasks, the mouth of which was closed with cotton plugs. The cotton plugs were then wrapped with paper and fastened using rubber band. Similarly thirty gram wheat bran plus 1.5 g crude chitin, thirty gram wheat plus 1.5 g crude chitosan, thirty gram rice bran plus 1.5 g crude chitin, thirty gram wheat bran plus 1.5 g crude chitosan were the other substrates tested. Chitin and chitosan were obtained from Matsyafed, Neendakara, Kollam.

The media were then sterilized in an autoclave at 1.06 Kg cm^{-2} and 121°C, 40 Min., cooled and inoculated with 5 mL spore suspension of *L. saksenae* aseptically in a laminar air flow and incubated at room temperature (28^0 C) for 14 days. Fungus cultured in SDB served as control.

3.5.2 Liquid Substrates

The liquid media tested for its sustainability for mass multiplication were rice water, coconut water and their amendmends with 0.3 per cent chitin and chitosan. The concentration was fixed based on the finding of Shanmugaiah *et al.* (2004).

3.5.2.1 Preparation of liquid media

100 mL of each media was poured into 250 mL conical flasks. Another set of these media to which chitin and chitosan were added separately in the colloidal form served as the other treatments. (Sterilization was carried out as described in para 3.5.1.1)

3.5.2.2 Preparation of Colloidal Chitin

Crude chitin (40 g) was slowly added into 250 mL of cold 0.25 N HCl with vigorous stirring and kept overnight at 4 ° C in a refrigerator. The mixture was filtered through glass wool into 2 L ice cold water with rapid stirring using a magnetic stirrer. The gelatinous white material formed beneath was separated by filtration through a Whatmann No. 1 filter paper. The chitin pellet was washed repeatedly with tap water until the pH became neutral (Roberts and Selitrennikoff, 1988).

3.5.2.3 Preparation of Colloidal Chitosan

The method described by Fenton *et al.* (1981) was followed for the preparation of colloidal chitosan. 10 g powdered chitosan was first dissolved in 0.2 M HCl and brought to pH 5.5 by adding 0.2 N NaOH. The white gelatinous material formed was separated by filtration through a Whatmann No. 1 filter paper and dried.

3.5.2.4 Estimation of Spore Count

100 mL of sterile distilled water was added to each of the solid substrate and stirred vigorously for one to two Min. The mixture was then squeezed and filtered through a double layered muslin cloth, into another conical flask using a sterile glass funnel. The spore count of the filtrate was estimated using 10 µL culture filtrate taken in a Haemocytometer. In the case of liquid substrates the suspension of the culture was shaken well and strained through a double layered muslin cloth and the spore count of the suspension was eneumerated as in the case of solid media. Spore count was estimated at 14, 21, and 28 DAI.

3.5.2.5. Estimation of Cfu

Colony forming units (cfu) were estimated by dilution plate method (Aneja, 1996 and 2003). The filtrate was serially diluted to get concentrations of

10^{-2}, 10^{-4} and 10^{-6}. From the serially diluted filtrate, one mLwas poured on each sterile Petri dish. To this 15 mL molten SDA with rose bengal media was added and gently rotated for uniform spreading of the spore suspension. The plates were then incubated at room temperature. Three replications were maintained for each treatment. Cfu was estimated at 14, 21 and 28 days, using the formula

$$\text{Cfu} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of sample (mL)}}$$

3.5.5. Determination of Mortality

For this study, the spores collected from 14, 21 and 28 day old cultures from different growth media were used. They were applied topically using an atomizer on the nymphs of *C. insolitus* collected from the laboratory culture mentioned in para 3.1.2.1.2. The procedure adopted was same as that mentioned in pathogenicity studies. Thirty insects were maintained in each replication. The treated insects were kept under observation to record the mortality at 24 h interval on the number of insects died and was continued till 100 per cent mortality was observed in any one of the treatments. Insects sprayed with sterile water served as control. For each treatment three replications were maintained

3.5.6 Cost Factor of the Substrates

The cost effectiveness of the substrates used for mass production was worked out by comparing the cost per kilogram of each of the substrates used for, with that of the standard synthetic medium SDB.

3.6. DETECTION AND QUANTIFICATION OF ENZYMES

3.6.1 Detection

3.6.1.1 Preparation of Culture Filtrate

L. saksenae was pure cultured in Sabouraud Dextrose Agar Yeast (SDAY). The plates were incubated for 14 days at room temperature. Spore suspension was prepared by pouring 10 mL of sterile water into the plates and swirling it uniformly for one to two Min. The concentration of spore suspension was adjusted to 10^7spores mL^{-1}. This suspension was used as the inoculant for enzyme assay.

3.6.1.2 Qualitative Assay

To study the effect of chitin and chitosan amended media on enzyme activity, the media listed below were used with SDB as the standard check.

1. SDB + colloidal chitin (0.3 per cent)
2. SDB + colloidal chitosan (0.3 per cent)
3. Peptone + colloidal chitin 2 per cent (sole carbon source)
4. Peptone + colloidal chitosan 2 per cent (sole carbon source)
5. SDB (standard check)

The media were sterilized as described in Para 3.5.1.1. 100 mL of media taken in 250 mL conical flasks were inoculated with 5 mL of inoculum and incubated at room temperature for seven days. The culture was filtered through Whatman No.1 filter paper to remove mycelia. The filtrate was further passed through Millipore (0.22 μm) for complete removal of mycelial spores. The culture filtrates obtained from each of the above media were utilized for enzyme assay.

3.6.1.3.1 Chitinase Activity

Presence of chitinase was measured by adopting plate assay method suggested by De Boer *et al.* (2004). Plates were prepared using chitin yeast extract agar (CYEA).

Composition of CYEA

1. Colloidal chitin - 20 g
2. Yeast extract - 0.5 g
3. Agar - 20 g
4. Congo red - 0.1 g
5. Water - 1000 mL

The media were sterilized as described in para 3.5.1.1 and cooled to 45°C. They were then plated in petridishes of 9cm diameter. On solidification a central well of 5 mm diameter was cut using a cork borer. 25 µL each of the culture filtrate prepared in different media mentioned in para 3.5.1 was added to the well and incubated for two days. Each plate served as a replicate with three replications per treatment. Plate without chitin served as control.

After two days of incubation, the zone of clearance around the well (halo diameter) was measured. Enzyme index was calculated using the formula

$$\text{Enzyme index} = \frac{\text{Diameter of halo in mm}}{\text{Diameter of well in mm}}$$

3.6.1.4. Protease Activity

Presence of protease was assessed by measuring the clear zone produced by degradation of milk protein in a pH indicator medium suggested by St. Leger *et al.* (1999) with skimmed milk as substrate.

Composition of pH indicator medium

1. Yeast extract - 0.1g
2. Agar agar - 20g
3. Bromocresol purple - 0.1g
4. Skimmed milk - 10 g
5. Water - 1000 mL

The pH was adjusted to 5.2 by adding 0.2 N NaoH. The media was sterilized and plated as described in para 3.6.1.1. The culture filtrates (25 µL each) mentioned in para 3.6.1.1 were added separately into the central well of each plate, as mentioned in para 3.6.2.1. The plates were incubated for two days. Each plate served as a replicate and three replications were maintained for each treatment. Plates without skimmed milk served as control. After two days of incubation, the enzyme index was worked out in detail as in para 3.6.2.1.

3.6.1.4 Lipase Activity

Presence of lipase was measured by adopting plate assay method suggested by Bai *et al.* (2012). Lipase index in solid medium was measured by the clearing zone produced by degradation of lipids in tributyrin agar.

Composition of medium

1. Peptone - 5 g
2. Yeast extract - 3 g
3. Agar - 20 g
4. Methyl red - 1g
5. Tributyrin - 10 mL
6. Water - 1000 mL

Tributryin was sterilized by heating it to 100 °C in a hot water bath. It was cooled to 80 °C before adding to the other constituents of the medium. The ingredients were mixed thoroughly to emulsify the tributyrin completely. The sterilized medium (para 3.5.1.1) was cooled and plated in 9 cm petridishes and lipase production was assessed by working out the enzyme index as detailed in para 3.6.1.2.1. Three replications were maintained for each treatment and each plate served as the replicate.

3.6.2 Quantification of Enzymes

3.6.2.1 ChitinaseProduction

The production of chitinase was assayed using the method followed by Vladimir *et al.* (2002). For this five reaction mixtures were prepared, each consisting of a mixture of 0.2 mL colloidal chitin, 0.3 mL of 1M sodium acetate buffer and 1 mL of culture filtrate taken in 1.5 ml eppendorf tubes. They were incubated at 40 °C for six h and then centrifuged at 12,225 g for 5 Min. at 6°C. The supernatant was collected and an aliquot of 0.75 mL supernatant taken in a 1.5 mL eppendorf tube was mixed with 1 mL of one per cent dinitrosalicylic acid (DNSA). To this mixture, 0.1 mL each of 0.7 M NaOH and 10M NaOH was added.Five such reaction mixtures were prepared using five different culture filtrates obtained from media as mentioned in para 3.5.1. They were then heated to 100 °C using water bath for five Min. and then cooled. The absorbance of reaction mixture at 582 nm (A^{582}) was measured using a spectrophotometer (SSL 117, Scanning Mini Spec). Each tube served as a replicate and three replications were maintained for each treatment. A calibration curve was plotted using the standard curve of N-acetyl D-glucosamine (NAGA) developed by Sigma Aldrich (2016). This was made use of to determine the concentration of reducing sugar which is an indication of chitinolytic activity. The concentration of reducing sugar present in different culture filtrates obtained in $\mu g\ mL^{-1}$ from the curve was

then converted to μ mole mL^{-1}so that the values can be substituted in the equation provided for unit conversion.(NAGA-U).

$$\text{Units } mL^{-1} \text{ enzyme} = \frac{(\mu \text{ mole NAG released}) (\text{Initial volume of assay})}{(6)\ (1.5)\ (1)}$$

6 = Conversion factor for converting 6 hours to 1 hour as per the Unit definition

1.5 = Volume (mL) of supernatant used in colorimetric determination

1= Volume (mL) of enzyme solution used

Chitinolytic activity is expressed in NAGA units with 1 NAGA unit (NAGA-U) defined as 1 1μ mole of NAGA released under the described culture conditions.

3.6.2.2 Protease Production

Protease production was assayed using the method suggested by Hossain *et al.* (2006). The reaction mixture was prepared with the following constituents.

Caesin	- 3 mL
Citrate phosphate buffer 0.1 M	- 3 mL
Crude culture filtrate	- 0.3 mL

The pH of the reaction mixture was adjusted to 7.0 and incubated at 40°C for 1 h. The reaction was stopped by the addition of 5 mL of Trichloro acetic acid (TCA, 20 per cent (w/v). The absorbance of the solution was measured at 650 nm in a spectrophotometer. Three replications were maintained with one tube per replication. The amount of amino acids released was calculated from a standard curve plotted against known concentrations of tyrosine. The quantity of tyrosine liberated (μg mL^{-1}) was estimated using the Standard Curve provided by Monreal and Reese (1969). One unit of enzyme is defined as the amount of enzyme that

released 1μg of tyrosine mL^{-1} of substrate.The following equation was used for unit conversion.

$$\text{Units } mL^{-1} \text{ enzyme} = \frac{\mu g \text{ tyrosine released x Total volume (mL) of assay}}{(1)\ (60)\ (1.5)}$$

1 = Volume (mL) of enzyme solution used

60 = Time of assay (Min.) as per the unit definition

1.5 = Volume (mL) used in colorimetric determination

3.6.2.3. Lipase Production

Lipase productionwas measured using the method described by Kamimura *et al.* (1999). A titrimetric assay was carried out using the following reaction mixture.

The reaction mixture consisted of 5 mL emulsion containing 25 per cent olive oil and 75 per cent gum arabic and 2 mL 10 mM phosphate buffer (pH 7). One mL of crude culture filtrate was added to the reaction mixture and was incubated at 37 °C for 30 Min. The reaction was stopped by the addition of 15 mL acetone-ethanol (1:1) mixture. To this methyl red indicator (0.01g) was added and titrated with 0.05 N NaOH. Olive oil in the reaction mixture acts as the substrate for the reaction. End point of the reaction was determined by the change in colour of the mixture from red to yellow. Each flask served as a replicate and three replications were maintained per treatment.

One unit of lipase is defined as the amount of enzyme that released 1μ mole of fatty acids per Min. under these conditions (Bai *et al.* 2012).

$$\text{Units mL}^{-1} = \frac{\text{Volume of NaOH consumed (mL) x Normality of NaOH (N) x 1000}}{\text{Time of incubation (Min.) x Volume of enzyme solution used (mL)}}$$

3.6.5 Effect of Crude Enzymes on Virulence of the Fungus

To study the effect of crude enzymes present in the culture media amended with chitin and chitosan, the five culture filtrates of *L. saksenae* obtained as in para 3.6.1.1. were made use of. They were applied topically using an atomizer on the nymphs collected from the laboratory culture mentioned in para 3.1.2.1.2. The procedure adopted was same as that mentioned in pathogenicity studies. The treated insects were kept under observation to record the mortality at 24 h interval. Insects sprayed with sterile water served as control. For each treatment three replications were maintained.

3.6.6 Correlation of Cuticle Degrading Enzymes with Virulence

To find out the correlation between enzymes and virulence correlation coefficient was worked out at 48 h and 96 h respectively.

3.7. TOXINS

3.7.1 Maintenance of Fungal Culture

The *L. saksenae* culture maintained as in para 3.1.1.was utilized for the toxin study. The fungus was grown in Sabouraud Dextrose Yeast Agar (SDYA) plated on 9 cm petridish kept under incubation for 14 days at 27°C. The aerial conidia were harvested using 0.05 percent Tween 80 and were used as the inoculum.

3.7.2. Isolation of Toxin

The fungus was cultured in 500 mL conical flasks containing 150 mL of medium standardized by Fargues *et al.* (1992). The composition of the media was as follows

1. Glucose - 30 g
2. Yeast extract - 3 g
3. Potassium dihydrogen phosphate - 0.39 g
4. Sodium Dihydrogen Phosphate Dodecahydrate - 1.42 g
5. Magnesium sulfate heptahydrate - 0.60 g
6. Ammonium nitrate - 0.70 g
7. Potassium chloride - 1.00 g

Before sterilization the pH of the medium was adjusted to 5.6 using 0.2 N HCl. The media after inoculation was incubated for six days at 27 ^{o}C and 150 rpm in a cooling incubator shaker (ROSI - 1, ROTEK BOD).

Six day old culture was centrifuged at 10,000 rpm at 4 ^{o}C for 10 Min to remove mycelia and spores. The supernatant was then filtered through a 0.1 μm millipore membrane. The filtrate, 150 mL was divided into 3 aliquots of 50 mL each and each of them separated repeatedly with equal volume of chloroform using a separating funnel. The extraction was carried out twice allowing 15 Min. for each extraction. All the chloroform extracts were combined and evaporated on a hot water bath. The extracts were then concentrated using freeze vaccum drier. The dried extracts were reconstituted to 5 mL with chloroform.

The fractions in the crude toxin were separated using a chromatographic column of 55 cm length and 2 cm diameter, using a low pH phosphate buffer (pH 1.8) as eluent. The fractions were collected separately in 50 mL beakers.

These fractions were subjected to Thin Layer Chromatography (TLC) on silica gel coated plates with 1 - butanol : water : acetic acid in the ratio 12 : 5 : 3 by volume. The plate was dried in a hot plate and sprayed with 0.051 M cerium ammonium sulphate solution to detect the colour. It was illuminated under UV light (254 nm) for better visibility and the Rf value was noted. A dipicolinic acid standard was usedfor detecting its presence, or for the presence of any related non-polar compounds as one of the main toxin produced by genus *Lecanicillium* was dipicolinic acid, as per literature.

3.7.3 Effect of Crude Toxin on Mortality of *C. insolitus*

Effect of crude toxin on virulence of the fungus was assessed by applying the crude toxin at varying concentrations of 1000 ppm, 500 ppm, 100 ppm and 10 ppm on *C. insolitus* topically. For this, stock solution of 1000 ppm of crude toxin was prepared by dissolving 100 mg of crude toxin in 100 mL of chloroform. From this stock solution, 500, 100 and 10 ppm were prepared by serial dilution technique. One mL each of the concentrations were sprayed on *C. insolitus* nymphs and observations were recorded on mortality at 24 h interval. Each replication contained fifty nymphs. The fungal spore suspension at a concentration of 10^7 spore mL^{-1} was kept as control. In order to rule out the effect of chloroform which was used as the solvent, one ppm chloroform was also used as one of the treatments.

3.7.4 Quantification of Dipicolinic Acid

The dipicolinic acid fraction present in the crude toxin was quantified using High Performance Liquid chromatography (HPTLC) analysis.

3.7.4.1 Preparation of Standard Solution

A stock solution of standard (100 µg mL^{-1}) was prepared by transferring one mg of standard dipicolinic acid (Merck, India) weighed into a 200 mL volumetric flask and made upto 10 mL with water.

3.7.4.2 Preparation of Sample Solution

Accurately weighed 200 mg of dried extract of crude toxin and dissolved it in 1mL of HPLC water (Merck, India).

3.7.4.3 Detection of Dipicolinic Acid Using HPTLC

HPTLC was performed on a precoated HPTLC plates (Silica gel 60 F254, 10 cm × 10 cm with aluminium baking (Merck, Mumbai, India). Different concentrations of the standard as well as the samples were applied as eight millimetre bands two millimetre apart by using CAMAG HPTLC (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 µL syringe (Hamilton, USA). The plate after spotting was kept in a CAMAG (10 cm × 10 cm) twin trough chamber previously saturated with the developing solvent (butanol: water : acetic acid 12: 5: 3 (v/v/v) for 20 Min. with a developing distance of 80 mM from the lower edge of plate. The plates were then dried in a stream of cold air and then scanned at 270 nm with a CAMAG TLC Scanner equipped with deuterium lamp by means of Wincats software. The Rf values of peaks were noted separately. The data were matched with the standard dipicolinic acid and compared with the inbuilt CAMAG drug to identify the dipicolinic acid fraction in the crude toxin and a chromatogram was developed.

Results

4. RESULTS

4.1. PATHOGENICITY OF *Lecanicillium saksenae* (KUSHWAHA) KURIHARA AND SUKARNO

L. saksenae was found to be pathogenic to the homopteran pests *viz.*, the black pea aphid, *Aphis craccivora* Koch, the brinjal mealy bug *Coccidohysterix insolitus* Green, the jassid *Amrasca biguttula biguttula* Ishida, the white fly *Bemisia tabaci* Gennadius and the brown soft scale *Lecanium* sp. It was also found to be infective to the heteropteran pests *viz.*, the rice bug *Leptocorisa acuta* Thunberg, the pod bug *Riptortus pedestris* F., the red spider mite *Tetranychus* sp. and the chilly mite *Polyphagotarsonemus latus* Banks. The other heteropterans screened for their susceptibility namely the leaf footed bug *Leptoglossus phyllopus* L., the green shield bug *Nezara viridula* L., and red cotton bug *Dysdercus cingulatus* F., did not take any infection. Neither the lepidopteran nor the coleopteran pests tested were found to be susceptible to the fungus. Treatment with the spore suspension at a concentration of 10^7 spores mL^{-1} manifested the symptoms of mycosis in the vulnerable insects and mites as detailed below.

4.1.1 Disease Symptoms

4.1.1.1. A. craccivora

Both the nymphs and adultsof *A. craccivora* were found to be normal as in the case of untreated insects, but gradually they turned inactive and pale. After 24 hours of treatment (HAT) 85.66 per cent of the nymphs and 75.66 per cent of the adults were found dead. By the end of 48 HAT, the mortality was 100 and 90.66 per cent respectively in the case of nymphs and adults. 100 per cent mortality of adults was recorded at 72 HAT. The body was covered with fluffy mycelia two days after death (Plate 1A).

A) *Aphis craccivora*

B) *Coccidohysterix insolitus*

C) *Bemisia tabaci*

D) *Amrasca bigutulla bigutulla*

Plate 1. Symptoms of mycosis by *Lecanicillium saksenae*

4.1.1.2. C. insolitus

The treated nymphs and adults shed their mealy out growth and turned naked and brownish, within 24 HAT. Ninety per cent of the nymphs and 85.66 per cent of the adults were found dead within a day. By the end of 48 HAT, the mortality was 100 and 95.33 per cent respectively in the case of nymphs and adults. 100 per cent mortality of adults was recorded at 72 HAT. White growth of the fungus over the cadavers was observed 24 h after death (Plate 1B).

4.1.1.3. B. tabaci

The treated flies, both the nymphs and adults were actively flying about for the first 12HAT, but were sluggish at the end of 24 h. Death observed in nymphs was 90.33 per cent and that in adults 80.33 per cent at 24 HAT. Later the death rate increased to 100 and 90.33 per cent respectively in the case of nymphs and adults at 48 HAT. By the end of 72 HAT all the adults were dead. Upon death, the flies turned stiff, and mycelia emerged first at the head region. Gradually the entire body was covered with fluffy mycelial growth (Plate 1C).

4.1.1.4. A. biguttula biguttula

Nymphs as well as the adult hoppers were found to be actively moving as in the case of untreated insects, for the first 12 HAT. Gradually they became immobile and ceased feeding. After 24 h, 85.33 per cent of the nymphs and 75.33 per cent of the adults were found dead. The mortality noted was 95.33 and 85.33 per cent respectively in the case of nymphs and adults at the end of 48 h. 100 per cent mortality was recorded at 72 HAT in the case of nymphs and 96HAT in the case of adults. The body was fully covered with white fluffy mycelia two days later (Plate 1D).

4.1.1.5 L. acuta

The bugs were normal only for a few hours after treatment. 85.66 per cent of the nymphs and 75.33 per cent of the adults were found dead after 24 h. Thereafter, the death rate increased to 95.66 and 80.33 per cent nymphs and adults respectively by 48 h. None of the nymphs were alive after 72 HAT and none of the adults lived after 96 h. In both the cases, profuse mycelial growth was observed first from the joints of the appendages and from the intersegmental regions of thorax and abdomen (Plate 2A) which eventually covered the entire body.

4.1.1.6. R. pedestris

Though the bugs were actively flying for about 12 to 14 h, 60.33 per cent of the nymphs and 55.33 per cent of the adults died after 24 h. The mortality observed was 65.33 and 60.66 per cent respectively in the case of nymphs and adults. Ninety per cent mortality of nymphs and adults were recorded at 120 HAT respectively. No fungal growth was noticed on the cadavers even after they were transferred to a moist chamber (Plate 2B).

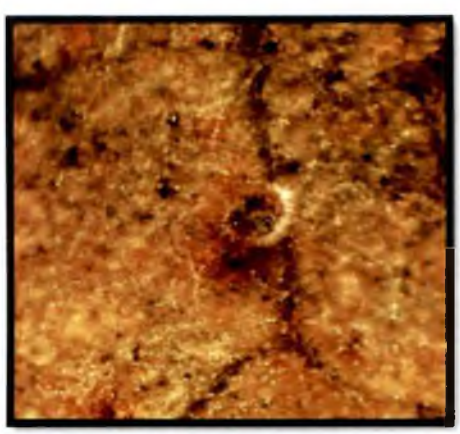
4.1.1.7 Lecanium sp.

By the end of 24 HAT, both the nymphs and adultsof the scale insect were found to be inactive even after they were disturbed and viewed under a microscope. 80.66 per cent of the nymphs and 70.33 per cent of the adults were found dead. The mortality observed in the case of nymphs and adults was 90.33 and 85.33 per cent respectively at 48 HAT. 100 per cent mortality of nymphs and adults was recorded at 72 HAT and 96 HAT respectively. Mycelia were seen to emerge from the lateral walls of the body eventually covering the entire body surface (Plate 2C).

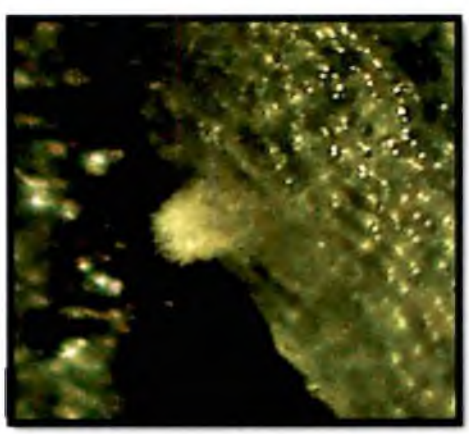
A) *Leptocorisa acuta*

B) *Riptortus pedestris*

C) *Lecanium* sp.



D) *Tetranychus* sp.



E) *Polyphagotarsonemus latus*

Plate 2. Symptom of mycosis produced by *Lecanicillium saksenae*

4.1.1.8 Tetranychus sp.

The mites treated with the spore suspension were observed to be pale and inactive after 24 h when observed under a microscope with 75.33 per cent mortality. By the end of 48 HAT, mortality observed was 85.66 per cent. Hundred per cent mortality was noted 96 HAT. Mycelial strands originated from the body margin gradually covering the whole body (Plate 2D).

4.1.1.9 P.latus

In the case of chilly mite, the movements were found to be arrested and body was completely shrunken prior to death. The mortality noted was 75.33 per cent,80.33 per cent and 100 per cent at 24, 48 and 72 HAT respectively. The body was covered with fluffy mycelia two days later (Plate 2E).

4.1.2 Effective Dose of *L. saksenae*

Effective dose was determined by working out the dose mortality response of test organisms, the results of which are presented in Tables 1 - 9.

4.1.2.1 A. craccivora

The dose dependent mortality of *A. craccivora* revealed that the spore concentration of 10^8 resulted in 96.66 per cent mortality of nymphs within 24 HAT (Table 1). The corresponding mortality observed in adults was 90.33 per cent. At 48 HAT there was 100 per cent mortality of both the nymphs and adults. When the concentration of the spore tested was 10^7, the mortality decreased to 85.66 per cent in nymphs and 75.66 per cent in adults. At 48 h, mortality recorded was 100 per cent in nymphs and 90.66 per cent in adults. At a lower concentration of 10^6 the mortality still decreased to 75.66 and 60.33 respectively in nymphs and adults after 24 h and to 80.33 and 70.33 at 48 h. After 72 h, the mortality recorded with the same concentration was 90.33 in nymphs and 80.33 in adults. The mortality ranged from 50 to 60 per cent

Table 1. Dose - mortality response of *A. craccivora* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=50)				
		24	48	72	96	120
Nymph	10^8	96.66	100	100	100	100
	10^7	85.66	100	100	100	100
	10^6	75.66	80.33	90.33	100	100
	10^5	50.33	60.33	70.33	77.77	88.09
	10^4	45.66	50.33	65.66	66.66	71.42
	10^3	0	0	30.33	33.33	40.47
Adult	10^8	90.33	100	100	100	100
	10^7	75.66	90.66	100	100	100
	10^6	60.33	70.33	80.33	84.09	88.37
	10^5	50.66	60.66	65.66	72.72	76.74
	10^4	40.66	50.66	60.33	65.90	76.74
	10^3	0	0	30.33	27.27	30.23

* Mean of three replications

during 24 to 48 h in the case of nymphs as well as adults when the test dose was 10^5 and it was 40 to 50 per cent when the dose was 10^4. The lowest dose tested (10^3), did not result in mortality till the end of 48 h and the death rate noted at the end of experimental period was negligible (40.47 per cent in nymphs and 30.23 per cent in adults).

4.1.2.2 C. insolitus

The mortality of *C. insolitus* treated with different doses of *L. saksenae* is furnished in Table 2. The highest test dose, 10^8 resulted in 95.66 per cent mortality of nymphs within 24 HAT whereas the corresponding mortality in adults was 90.33. Hundred per cent mortality of both the nymphs and adults was observed at 48 HAT. At a concentration of 10^7, after 24 h, the mortality observed was 90.33 and 85.66 per cent respectively in nymphs and adults and at 48 HAT there was 100 per cent mortality of nymphs and 95.33 per cent mortality in adults. Lower concentration of 10^6 decreased the mortality to 80.33 per cent and 70.33 per cent respectively in nymphs and adults after 24 h and to 85.33 per cent and 80.33 per cent at 48 HAT. With the same concentration, the mortality recorded was 90.33 per cent and 100 per cent respectively in nymphs and adults, 72 HAT. When the concentration was reduced to 10^5, the resultant mortality ranged from 60 to 70 per cent in nymphs and adults after 24h and 75 to 80 per cent after 48 h. The mortality of nymphs reduced to 30 per cent when the spore concentration was 10^4. None of the adult mealy bugs were found dead at this concentration, during the first 24 h. The same dose resulted in a death rate of 40 per cent nymphs and 30 per cent adults at the end of 48 h. The corresponding mortality observed at the end of 120 h was 62.22 per cent and 54.54 per cent respectively in the case of nymphs and adults. At the lowest concentration 10^3, there was no mortality at all even at the end of 72 HAT and the rate was negligible ranging from 20 to 30 per cent at 120 HAT.

Table 2. Dose - mortality response of *C. insolitus* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=50)				
		24	48	72	96	120
Nymph	10^8	95.66	100	100	100	100
	10^7	90.33	100	100	100	100
	10^6	80.33	85.33	90.33	100	100
	10^5	60.33	75.66	80.33	89.13	100
	10^4	30.33	40.33	50.33	56.52	62.22
	10^3	0	0	0	23.91	33.33
Adult	10^8	90.33	100	100	100	100
	10^7	85.66	95.33	100	100	100
	10^6	70.33	80.33	90.33	100	100
	10^5	60.33	70.33	80.33	95.55	100
	10^4	0	30.33	40.33	44.44	54.54
	10^3	0	0	0	11.11	20.45

* Mean of three replications

4.1.2.4. B. tabaci

Mortality recorded in the treated white flies is furnished in Table 3. At 24 HAT with 10^8concentration, the mortality observed was 95.66 per cent nymphs and 94.33 per cent adults. All the treated nymphs and adults were dead after 48 h. Spore concentration of 10^7 mL^{-1}resulted in 90.33 per cent mortality in nymphs and 80.33 per cent in adults after 24 h. Hundred per cent mortality of nymphs occurred after 48 h and in adults it was recorded after 72 h. The mortality observed with 10^6 spores $mL^{-1}$was 80.33 per cent in nymphs and 70.33 per cent in adults after 24 h. After 48 h, 90.33 per cent nymphs and 80.33 per cent adults were found dead. The death rate was 100 per cent in nymphs at 72 HAT and in adults 100 per cent was recorded at 96 HAT. Lower dose of 10^5spores mL^{-1} resulted in 50.33 to 60.33 per cent mortality after 24 h and 60.33 to 70.33 per cent in 48 h. The maximum mortality observed was 88.09 per cent in nymphs and 87.80 per cent in adults after 120 h. With 10^4 spores mL^{-1}, the mortality recorded was 52.38 per cent nymphs and 51.21 per cent adults, at the end of 120 h. The lowest dose tested (10^3), did not result in mortality till the end of 48 h and the maximum death rate observed was 40.47 per cent nymphs and 26.82 per cent adults at the end of experimental period.

4.1.2.4 A. bigutulla bigutulla

The dose dependent mortality of *A. bigutulla bigutulla* treated with varying spore concentrations is presented in Table 4. It was observed that 90.33 per cent of nymphs were dead within 24 h when they were treated with 10^8 spores mL^{-1}. The mortality observed in adults was 80.33 per cent. At 48 h, 100 and 90.33 per cent mortality was recorded for nymphs and adults respectively. The spore concentration of 10^7 decreased the mortality to 85.33 per cent in nymphs and 75 per cent in adults. Complete mortality was observed in nymphs at 72 HAT and in adults at 96 HAT. At a lower concentration of 10^6, after 24 h, resultant mortality was 70.33 per cent and 60.33 per cent respectively in nymphs and adults. The mortality recorded at 48 HAT was 80.33 per cent in nymphs and

Table 3. Dose - mortality response of *B. tabaci* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=50)				
		24	48	72	96	120
Nymph	10^8	95.66	100	100	100	100
	10^7	90.33	100	100	100	100
	10^6	80.33	90.33	100	100	100
	10^5	50.33	60.33	66.66	72.72	88.09
	10^4	0	40.33	44.44	50	52.38
	10^3	0	0	22.22	31.81	40.47
Adult	10^8	94.33	100	100	100	100
	10^7	80.33	90.33	100	100	100
	10^6	70.33	80.33	88.88	100	100
	10^5	60.33	70.33	77.77	81.39	87.80
	10^4	0	30.33	33.33	41.86	51.21
	10^3	0	0	0	18.60	26.82

* Mean of three replications

Table 4. Dose - mortality response of *A. bigutulla bigutulla* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=50)				
		24	48	72	96	120
Nymph	10^8	90.33	100	100	100	100
	10^7	85.33	95.33	100	100	100
	10^6	70.33	80.33	85.33	88.88	100
	10^5	0	0	0	22.22	30.23
	10^4	0	0	0	22.22	30.23
	10^3	0	0	0	11.11	18.60
Adult	10^8	80.33	90.33	100	100	100
	10^7	75.33	85.33	90.33	100	100
	10^6	60.33	70.33	80.33	88.63	100
	10^5	0	0	30.33	31.81	41.86
	10^4	0	0	0	26.13	30.23
	10^3	0	0	0	9.09	13.95

* Mean of three replications

70.33 per cent in adults. There was 100 per cent mortality of nymphs and adults at 120 HAT. The lower spore concentrations, 10^5, 10^4 and 10^3 did not result in mortality till 72 HAT, and by the end of experimental period the death rate recorded was 18.60 per cent in nymphs and 13.95 per cent in adults

4.1.2.5 L. acuta

Rice bugs treated with *L. saksenae* spore suspension showed dose dependent mortality (Table 5). The highest concentration of 10^8 spores mL^{-1} resulted in 90.66 per cent mortality of nymphs and 85.33 per cent mortality of adults within 24 HAT. After 48 h, there was 100 per cent mortality of nymphs and 95.33 per cent mortality of adults. The mortality decreased to 85.66 per cent in nymphs and 75.33 per cent in adults with a spore concentration of 10^7spores mL^{-1} at 24 HAT. Hundred per cent mortality was recorded after 72 h in the case of nymphs, and after 96 h in the case of adults. Spore concentrations of 10^6 resulted in 70.33 per cent and 40.33 per cent mortality respectively in nymphs and adults after 24 h. Corresponding mortality observed after 48 h were 80 per cent and 50.66 per cent. After 72 h the mortality recorded with the same concentration was 90.66 per cent in nymphs and 60.33 per cent in adults. Hundred per cent mortality of nymphs was noted at 96 HAT. The lower doses tested 10^5, 10^4 and 10^3 did not result in mortality till the end of 48 h. Thereafter, mortality recorded was zero to 23.91 per cent in the case of nymphs and 14.89 per cent for adults at the end of 120 h.

4.1.2.6 R. pedestris

Table 6 represents the observations on mortality of pod bugs treated with *L. saksenae*. The spore concentration of 10^8 spores mL^{-1} resulted in 70.33 per cent mortality of nymphs within 24 HAT. The corresponding mortality observed in adults was 75.33 per cent. After 48 h, the mortality observed in the case of nymphs was 80.66 per cent and that in adults was 85.66 per cent. Hundred per cent mortality was recorded at 96 HAT in case of both the nymphs and adults. At

Table 5. Dose - mortality response of *L. acuta* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=20)				
		24	48	72	96	120
Nymph	10^8	90.66	100	100	100	100
	10^7	85.66	95.66	100	100	100
	10^6	70.33	80	90.66	100	100
	10^5	0	0	30.33	37.50	45.65
	10^4	0	0	0	0	23.91
	10^3	0	0	0	0	0
Adult	10^8	85.33	95.33	100	100	100
	10^7	75.33	80.33	90.66	100	100
	10^6	40.33	50.66	60.33	73.95	78.72
	10^5	0	0	0	27.08	36.17
	10^4	0	0	0	0	14.89
	10^3	0	0	0	0	0

* Mean of three replications

Table 6. Dose - mortality response of *R. pedestris* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=20)				
		24	48	72	96	120
Nymph	10^8	70.33	80.66	90.33	100	100
	10^7	60.33	65.33	71.67	85.33	92.66
	10^6	40.33	50.33	60.66	75.66	85.66
	10^5	0	0	30.66	35.66	40.66
	10^4	0	0	0	0	20.33
	10^3	0	0	0	0	0
Adult	10^8	75.33	85.66	90.66	100	100
	10^7	55.33	60.66	75.66	80.33	90.66
	10^6	30.66	40.66	50.66	60.33	70.33
	10^5	0	0	0	20.66	30.33
	10^4	0	0	0	0	20.66
	10^3	0	0	0	0	0

* Mean of three replications

a concentration of 10^7, after 24 h, the mortality observed was 60.33 per cent in nymphs and 55.33 per cent in adults and it was 65.33 per cent nymphs and 60.66 per cent adults at 48 HAT. There was 71.67 per cent mortality of nymphs at 72 HAT. The corresponding mortality in adults was 75.66 per cent. At the end of 120 h the mortality rate observed was 92.66 per centand 90.66 per cent for nymphs and adults respectively. At a concentration of 10^6 the mortality of nymphs ranged from 40.33 to 85.66 per cent and that of adults from 30.66 to 70.33 per cent during24 to 120 h. Lower concentration of 10^5, did not cause any mortality of nymphs or adults till 48 h. There onwards the mortality recorded ranged from 30.66 per cent to 40.66 per cent in nymphs and 20.66 to 30.33 in adults during the experimental period. The concentration 10^4 did not result in mortality for first 96 h. There after the mortality recorded was negligible ranging from zero to 20.33 per cent in the case of nymphs and to 20.66 per cent for adults. The lowest dose of 10^3 did not result in mortality till the end of the observational period.

4.1.2.7 Lecanium sp.

The observations on *Lecanium* sp. revealed that the spore concentration of 10^8 spores mL^{-1} resulted in 90.66 per cent mortality of nymphs within 24 HAT (Table 7). The corresponding mortality observed in adults was 85.33 per cent. At 48 HAT there was 100 per cent mortality for nymphs and 90 per cent mortality in adults. It caused 100 per cent mortality of nymphs and adults at 96 HAT. When the concentration of the spore tested was 10^7, the mortality decreased to 80.66 per cent in nymphs and 70.33 per cent in adults. At a lower concentration of 10^6, the mortality decreased to 75.33 per cent in nymphs and adults after 24 h and to 80.33 per cent after 48 h. At 72 h, the mortality recorded with the same concentration was 88.88 per cent in nymphs and 94.44 per cent in adults. Hundred per cent mortality recorded at 96 HAT for both nymphs and adults. At 96 h the mortality recorded with the same concentration was 100 per cent in nymphs and 88.88 per cent in adults. Concentration of 10^5 resulted in 51, 60.63, 66.66, 76.74 and 87.8 per cent mortality of nymphs over a period of 24 to 120 HAT. The corresponding

Table 7. Dose - mortality response of *Lecanium* sp. to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N=50)				
		24	48	72	96	120
Nymph	10^8	90.66	100	100	100	100
	10^7	80.66	90.33	100	100	100
	10^6	75.33	80.33	88.88	100	100
	10^5	51	60.33	66.66	76.74	87.80
	10^4	0	0	11.11	24.41	26.82
	10^3	0	0	0	6.976	8.53
Adult	10^8	85.33	90	100	100	100
	10^7	70.33	85.33	88.88	100	100
	10^6	75.33	80.33	94.44	100	100
	10^5	0	0	33.33	40.47	51.21
	10^4	0	0	0	16.66	26.82
	10^3	0	0	0	0	0

* Mean of three replications

values in adults ranged from zero to 51.21 per cent. Resultant mortality in 10^4 was 26.82 per cent both in nymphs and adults. The lowest dose 10^3, did not result in mortality of nymphs till 72 HAT and there was no mortality in adults till the end of experimental period.

4.1.2.8 Tetranychus sp.

Table 8 represents the mortality observed in mite population treated with spore suspension. The spore concentration of 10^8 spores mL^{-1} resulted in 80.33 per cent mortality of adults within 24 HAT. A mortality per cent of 90.33 per cent was recorded after 48 h and 100 per cent after 72 h. When the concentration of the spore tested was 10^7, the mortality decreased to 75.33 per cent at 24 h and 85.66 per cent at 48 h. Hundred per cent mortality was observed at 96 HAT. At 10^6 the mortality decreased to 50.33 (24 h) and to 65 per cent (48 h). After 72 h the mortality recorded with the same concentration was 70.33 per cent. The lower dose 10^5, did not result in mortality till 48 h and there after a less percentage of 30.33 to 42.24 per cent was noted during 72 to 120 HAT. At 10^4 the mortality recorded was zero during the first 72 h and there after a negligible death rate of 11.84 to 19.37 was noted during 96 to 120 HAT. There was no mortality at all with the spore concentration of 10^3.

4.1.2.9. P. latus

Similar observations were recorded in chilly mite also (Table 9). The spore concentration of 10^8 resulted in 80.33 per cent mortality of adults within 24 HAT, 95.33 per cent after 48 h and 100 per cent after 72 h. When the concentration of the spore tested was 10^7, the mortality decreased to 75.33 per cent at 24 h, 80.33 per cent at 48 h and 100 per cent at 96 HAT. At 10^6 the mortality decreased to 50.66 per cent at 24 h and to 60.33 per cent at 48 h and 70.66 per cent after 72 h. The lower dose 10^5, did not result in mortality till 48 h and there after a less percentage of 20.66 to 30.61 was noted during 72 to 120 HAT. There was no mortality at all with the spore concentrations 10^4 and 10^3.

Table 8. Dose-mortality response of *Tetranychus* sp. to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval (N= 50)				
		24	48	72	96	120
Adult	10^8	80.33	90.33	100	100	100
	10^7	75.33	85.66	95.33	100	100
	10^6	50.33	65	70.33	78.14	88.75
	10^5	0	0	30.33	33.7	42.24
	10^4	0	0	0	11.84	19.37
	10^3	0	0	0	0	0

* Mean of three replications

Table 9. Dose-mortality response of *P. latus* to *L. saksenae*

Stage of insect	Dose (spores mL^{-1})	* Per cent mortality at 24 h interval N=50				
		24	48	72	96	120
Adult	10^8	80.33	95.33	100	100	100
	10^7	75.33	80.33	90.33	100	100
	10^6	50.66	60.33	70.66	72.22	77.51
	10^5	0	0	20.66	22.58	30.61
	10^4	0	0	0	0	0
	10^3	0	0	0	0	0

* Mean of three replications

4.1.3 Egg Parasitism on Root Knot Nematode

The eggs of *Meloidogyne incognita* (Kofoid and White) Chit. was not parasitized when inoculated with the suspension of 10^8 spores mL^{-1} of *L. saksenae.*

4.2 BIOSAFETY OF *L.saksenae*

4.2.1 Cross Infectivity to Crop Plants

Soil as well as leaf inoculation tests with spore suspension of 10^8 spores mL^{-1}, revealed that none of the crops tested *viz.*, cow pea, bhindi, brinjal and tomato were infected by *L. saksenae.*

4.2.2 Safety to Non Target Organisms

4.2.2.1 Predators

The results revealed that the fungus is not pathogenic to neither the larval and adult stages of coccinellids and syrphids, nor to the spider species tested. All the treated grubs of coccinellids and maggots of syrphids kept under observation pupated normally and emerged as healthy adults and oviposited normally as in the case of untreated insects. The spiders did not develop any symptoms of mycosisnor did they die till the end of experimental period.

4.2.2.2 Parasitoids

4.2.2.2.1 Bracon brevicornis F.

The adult parasitoids of *B. brevicornis* treated with spore suspension @10^8spores mL^{-1} did not exhibit any behavioural abnormalities nor developed any symptoms of mycosis. No fungal growth was observed when dead insects were surface sterilized and inoculated in PDA.

4.2.2.2.2 *Goniozus nephantidis* Muesebeck.

The adult parasitoids of *G. nephantidis* treated with spore suspension @10^8 spores mL^{-1} did not exhibit any behavioural abnormalities nor developed any symptoms of mycosis. No fungal growth was observed when dead insects were surface sterilized and inoculated in PDA.

4.2.2.3. Trichogramma spp

The adult emergence percentage of *Trichogramma japonicum* Ashmead and *T. chilonis* treated with the spore suspension (10^8 spores mL^{-1}) is presented in Table 10. Observations recorded at 24 h interval revealed that the adult emergence percentage did notvary in treated and untreated cards.

4.2.2.3 Pollinators

None of the carpenter bee *Xylocopa* sp. and wasp *Vespula* sp. treated with spore suspension of 10^8 spores mL^{-1}develop symptoms of mycosis. There was no mortality even at 10 DAT.

4.2.2.3 Beneficial Insects

The honey bee such as *Apis cerana indica* F, *A. mellifera.* and stingless bee *Tetragonula iridipennis* Smith treated with *L. saksenae* spores@10^8spores mL^{-1} did not develop any symptoms of mycosis till 72 HAT. No death was recorded even after 10 days of treatment.

4.3 CHITIN ENRICHED SUBSTRATES FOR MASS PRODUCTION

Nitrogenous polysaccharides like chitin and chitosan are reported as sporulation inducers in *Lecanicillium lecanii* (Zimmermann) Zare and Gams (Nithya, 2015). To test the suitability of these amendmends in inducing sporulation of *L. saksenae*, solid and liquid substrates mentioned in para 3.5 were tested.

Table 10. Effect of *L. saksenae* on *Trichogramma* eggs

Parasitoid sp.	Adult emergence (%)		
	24 h	48 h	72 h
T. japonicum treated with *L. saksenae*	87.5	91.95	92.78
T. japonicum (untreated)	80.33	90	93
T. chilonis treated with *L. saksenae*	87.20	91.95	91.83
T. chilonis (untreated)	86	90.33	95.33

Table 11. Spore count of *L. saksenae* in chitin and chitosan enriched solid substrates

Sl. No.	Substrates	Mean* spore count (10^8 mL^{-1})		
		14 DAI	21 DAI	28 DAI
1.	RB + chitin (5%)	4.27^c	3.32^b	1.64^b
2.	WB + chitin (5%)	5.50^b	2.62^b	1.86^b
3.	RB + chitosan (5%)	7.27^a	5.16^a	3.94^a
4.	WB + chitosan (5%)	6.88^a	4.72^a	3.36^a
5.	RB (check)	0.83**	0.13**	0.09**
6.	WB(check)	0.88**	0.68**	0.10**
	CD(0.05)	0.834	0.882	0.681

*Mean of three replications, DAI - Days after Inoculation, RB - Rice Bran, WB – Wheat Bran, ** Not included in the statistical analysis. Values with different letters are significantly different from each other by LSD at 5% level

4.3.1 *Solid Substrates*

The effect of enriching the substrate with cost effective and naturally available materials was assessed based on the spore count (mL^{-1}), number of colony forming units (mL^{-1}) and per cent mortality of test insect.

4.3.1.1 Effect of Enriched Solid Substrates

4.3.1.1.1. On Spore Yield

The spore count of *L. saksenae* cultured in solid substrates enriched with chitin 5 per cent and chitosan 5 per cent showed significant variation (Table 11).

At 14 days after inoculation (DAI), maximum spore yield of 7.27 x 10^8 spores mL^{-1} was obtained from rice bran (RB) + chitosan which was on par with the yield (6.88 x 10^8 spores mL^{-1}) from wheat bran (WB) + chitosan (Plate 9). This was followed by WB + chitin (5.50 x 10^8 spores mL^{-1}). The spore yield recorded from RB + chitin (4.27 x 10^8 spores mL^{-1}) was significantly lower than that in WB + chitin. In the non enriched substrates the spore yield was significantly lower. It was 0.83 x 10^8 spores mL^{-1} in RB and 0.88 x 10^8 spores mL^{-1} in WB.

A similar trend was noted at 21 DAI also. Chitosan enriched RB and chitosan enriched WB were the superior substrates with spore count, 5.16 x 10^8 spores mL^{-1} and 4.72 x 10^8spores mL^{-1} respectively. This was followed by chitin enriched RB with spore count, (3.32 x 10^8 spores mL^{-1}) and chitin enriched WB with yield of 2.62 x 10^8 spores mL^{-1}. Significantly lower spore count was noted in the non enriched RB (0.13 x 10^8 spores mL^{-1}) and WB (0.68 x 10^8 spores mL^{-1}).

At 28 DAI also, chitosan enriched substrates were superior to chitin enriched substrates. The spore yield from RB + chitosan was 3.94 x 10^8 spores mL^{-1} and that from WB + chitosan was 3.36 x 10^8spores mL^{-1}. It was preceded by chitin enriched substrates, the spore yield 1.86 x 10^8 spores mL^{-1} in WB + chitin

Table 12. Viability of *L. saksenae* in chitin and chitosan enriched solid substrates

Sl. No.	Substrates	*Mean number of cfu (10^7 mL^{-1})		
		14 DAI	21 DAI	28 DAI
1.	RB + chitin (5%)	5.89^b	3.10^b	1.47^c
2.	WB + chitin (5%)	3.46^c	1.41^c	0.90^d
3.	RB + chitosan (5%)	9.10^a	7.40^a	3.41^a
4.	WB + chitosan (5%)	3.31^c	2.20^c	1.82^b
5.	RB (check)	1.82^d	1.50^c	0.80^e
6.	WB (check)	0.97^e	0.50^d	0.27^f
	CD (0.05	0.251	0.166	0.023

*Mean of three replications, DAI - Days after inoculation, RB - Rice Bran, WB – Wheat Bran, Cfu - Colony forming units. Values with different letters are significantly different from each other by LSD at 5% level

Table 13. Efficacy of *L. saksenae* cultured in chitin and chitosan enriched solid substrates

SI. No.	Substrates	Mean mortality of *C. insolitus* (%) at 24 h interval		
		24	48	72
1.	RB + chitin (5%)	92.33 (9.61)^b	100 (10.00)^a	100 (10.00)^a
2.	WB + chitin (5%)	90.33 (9.51)^c	100 (10.00)^a	100 (10.00)^a
3.	RB + chitosan (5%)	95.66 (9.78)^a	100 (10.00)^a	100 (10.00)^a
4.	WB + chitosan (5%)	94.66 (9.73)^a	100 (10.00)^a	100 (10.00)^a
5.	RB (check)	62.33 (7.89)^d	92 (9.59)^b	100 (10.00)^a
6.	WB (check)	57.33 (7.57)^e	88.66 (9.42)^c	100 (10.00)^a
	CD (0.05)	0.07	0.06	NS

RB - Rice bran, WB - Wheat bran, Figures in parentheses are values after $\sqrt{x} + 1$ Transformation. Values with different letters are significantly different from each other by LSD at 5% level .

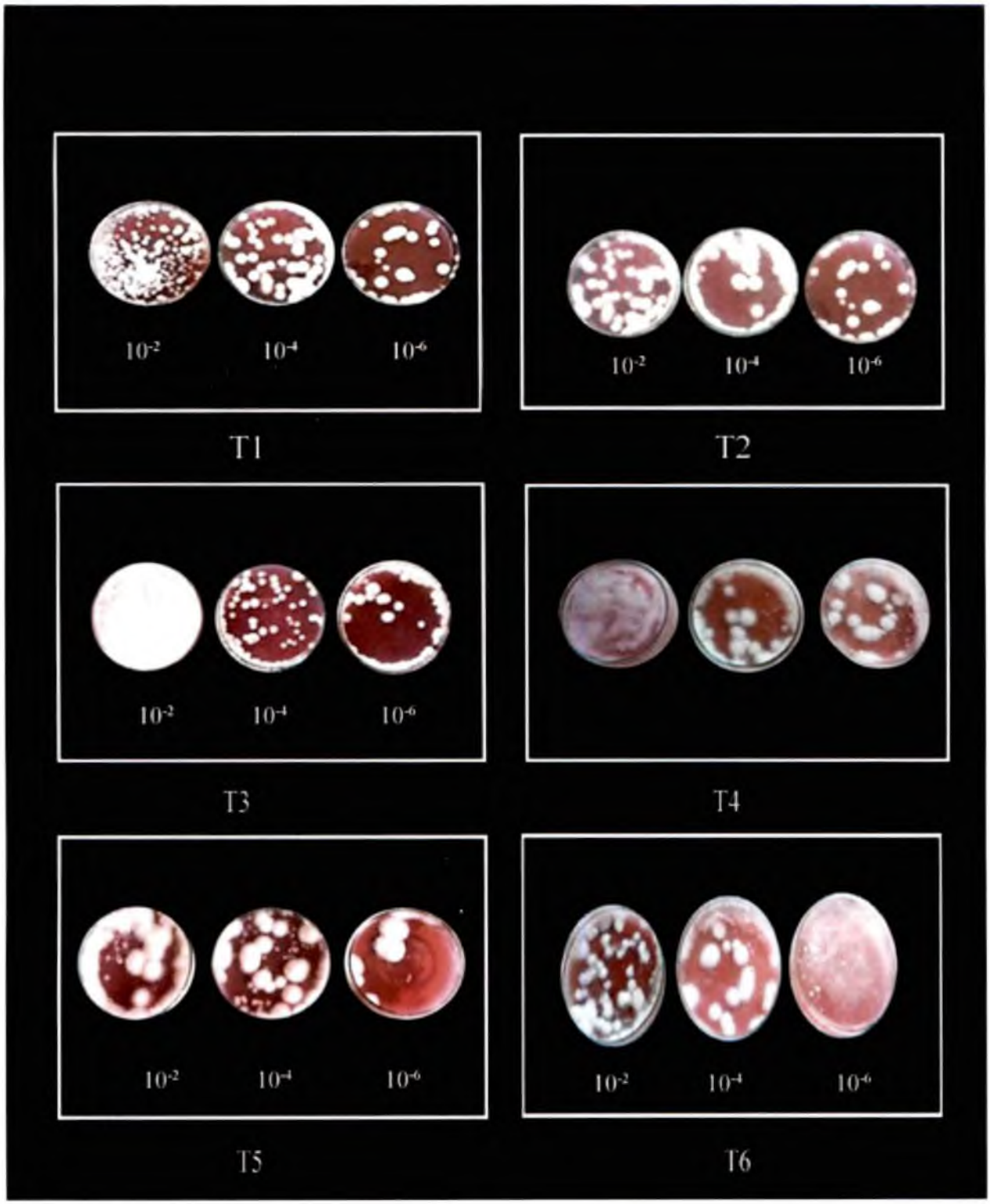


Plate 3. Comparison of number of viable colonies on 6 th day after inoculation in enriched and non enriched solid substrates

T1 : Rice bran + chitin T2 : Wheat bran + chitin T3 : Rice bran + chitosan, T4 : Wheat bran + chitosan T5 : Rice bran T6: Wheat bran

was on par with that of RB + chitin (1.64 x 10^8spores ml^{-1}). Least spore count was observed in WB (0.10 x 10^8 spores mL^{-1}) and RB (0.09 x 10^8 spores mL^{-1}).

4.3.1.1.2. On Number of Colony Forming Units

The viability of spores in enriched and non enriched substrates was compared by enumerating the cfu at 10^{-6}dilution, the results of which are presented in Table 12.

At 14 DAI, maximum number of cfu was observed in RB + chitosan. The number of cfu recorded was 9.10 x 10^7 mL^{-1}(Plate 3). RB + chitin ranked second (5.89 x 10^7 mL^{-1}) which was followed by WB + chitin (3.46x 10^7 mL^{-1}) and WB + chitosan (3.31 x 10^7 $mL^{-1)}$. Less number of cfu was recorded with RB (1.82 x 10^7 mL^{-1}) and least yield was from WB (0.97 x 10^7 mL^{-1}).

At 21 DAI, RB + chitosan wasfound to be superior with a maximum number of cfu, 7.40 x 10^7 mL^{-1}. This was followed by RB + chitin with 3.10 x 10^7 mL^{-1}cfu. The number of cfu recorded in WB + chitosan was 2.20 x 10^7 mL^{-1} which was significantly superior to WB + chitin (1.41 x 10^7 mL^{-1}) and the non enriched RB (1.50 x 10^7 mL^{-1}). The least number of cfu was recorded with WB (0.50 x 10^7 mL^{-1}).

The number of cfu observed at 28 DAI also revealed that the viability of spores in RB + chitosan was maximum (3.41 x 10^7 mL^{-1}) and significantly higher than that in rest of the tested substrates. The number of cfu observed with WB+ chitosan was 1.82 x 10^7 mL^{-1}which was significantly superior to RB + chitin (1.47 x 10^7 mL^{-1}), WB + chitin (0.91 x 10^7 mL^{-1}) and RB (0.81 x 10^7 mL^{-1}), each one being significantly different from the other. The number of viable colonies was least in WB (0.27 x 10^7 mL^{-1}).

4.3.1.1.3. On Virulence

Efficacy of the *L. saksenae* cultured on chitin and chitosan enriched solid substrates was evaluated for its virulence on *C. insolitus*. The data on mortality

recorded at 24 h interval are given in Table 13. After 24 h, mortality was very high varying from 57.33 to 95.66 per cent in different media. RB + chitosan showed highest mortalityof 95.66 per cent and was statistically on par with WB + chitosan with 94.66 per cent mortality. This was followed by RB + chitin with 92.33 per cent mortality. The mortality recorded with WB + chitin was 90.33 per cent which was superior to RB (62.33 per cent). Mortality recorded with spores harvested from WB was the least (57.33).

At 48 h, 100 per cent mortality was noticed with spore suspensions obtained from all the enriched media and the corresponding values in the non enriched media were 92 per cent in RB and 88.66 per cent in WB.

After 72 h, spores from the non enrichedmedia also attained 100 per cent mortality.

4.3.2 Liquid substrates

Various naturally available liquid substrates such as coconut water and rice water was enriched with 0.3 per cent chitin and chitosan to study the effect of the amendments on sporulation, number of viable spores and virulence of the spores.

4.3.2.1 Effect of Enriched Liquid Substrates

4.3.2.1.1. On Spore Yield

The spore count of *L. saksenae* cultured in liquid substrates enriched with chitin 0.3 per cent and chitosan 0.3 per cent showed significant variation among treatments.

The spore countassessed at weekly intervals varied significantly in different substrates (Table 14). At 14 DAI, Sabouraud Dextrose Broth (SDB) + chitosan was found to be the significantly superior to all the natural substrates with a spore count of 2.63 x 10^8 spores mL^{-1}. Among the natural substrates Rice water (RW) + chitosan ranked first (9.22 x 10^7 spores mL^{-1}) and RW+chitin, the

second (7.80 x 10^7 spores mL^{-1}). The spore yield from Coconut water (CW) + chitosan was 7.55 x 10^7 spores mL^{-1} which was significantly lower than RW based enriched media, SDB + chitin (6.56 x 10^7 spores mL^{-1}), CW + chitin (6.44 x 10^7 spores mL^{-1}), SDB (1.68 x 10^7 spores mL^{-1}) and RW (0.50 x 10^7 spores mL^{-1}), each one being significantly different from the other. The spore count noted was least in CW (0.37 x 10^7 spores mL^{-1}).

At 21 DAI also all the above treatments were significantly different from each other. The synthetic standard media SDB enriched with chitosan ranked first with 1.55 x 10^8spores mL^{-1}. Among the natural substrates RW + chitosan ranked first (6.98 x 10^7spores mL^{-1}), CW + chitosan, the second (5.94 x 10^7 spores mL^{-1}). The spore count recorded for CW + chitin was5.46 x 10^7 spores mL^{-1} which was superior than SDB + chitin (5.13 x 10^7 spores mL^{-1}). RW + chitin yield, 4.23 x 10^7 spores $mL^{-1}$was found to be superior than SDB alone (1.27x 10^7 spores ml^{-1}). The spore yield observed in RW was 0.40 x 10^7 spores $mL^{-1}$and the least yield was noted in CW (0.31 x 10^7 spores mL^{-1}).

A more or less similar trend was noted at 28 DAI. Maximum spore count was recorded for SDB + chitosan (8.47 x 10^7 spores mL^{-1}) followed by RW + chitosan (4.48 x 10^7 spores mL^{-1}). SDB + chitin was superior (4.19 x 10^7 spores mL^{-1}) to RW + chitin (3.90 x 10^7 spores mL^{-1}), CW + chitin (3.84 x 10^7 spores mL^{-1}). CW + chitosan (3.82 x 10^7 spores mL^{-1}) which were on par with each other and was superior to SDB (1.33 x 10^7 spores mL^{-1}). The CW yield was lesser (0.26 x 10^7 spores mL^{-1}) and the lowest count was from RW (0.13x 10^7 spores mL^{-1}).

Among the liquid substrates tested maximum sporulation was noted for SDB + chitosan. However among the natural substrates, RW + chitosan was the best. Chitin enriched media were superior to non enriched media.

4.3.2.1.2. On Number of Viable Spores

As observed in the case of spore count, number of viable colonies was also maximum in SDB + chitosan at 14 DAI, cfu being 2.94 x 10^8 mL^{-1} (Table 15)

Table 14. Spore count of *L .saksenae* in chitin and chitosan enriched liquid substrates

Sl. No.	Substrates	Mean* spore count (10^7 mL^{-1})		
		14 DAI	21 DAI	28 DAI
1.	RW + chitin (0.3%)	7.80^{c}	4.23^{f}	3.90^{d}
2.	CW + chitin (0.3%)	6.44^{f}	5.46^{d}	3.84^{d}
3.	RW + chitosan (0.3%)	9.22^{b}	6.98^{b}	4.49^{b}
4.	CW + chitosan (0.3%)	7.55^{d}	5.94^{c}	3.82^{d}
5.	RW (check)	0.50**	0.40**	0.12**
6.	CW (check)	0.37**	0.31**	0.26**
7.	SDB + chitin (0.3%) (check)	6.56^{e}	5.13^{e}	4.12^{c}
8.	SDB + chitosan (0.3%) (check)	26.27^{a}	15.50^{a}	8.47^{a}
9.	SDB (check)	1.68^{g}	1.13^{g}	1.13^{e}
	CD (0.05)	0.080	0.100	0.081

*Mean of three replications, DAI - Days after inoculation, RW - Rice Water, CW - Coconut Water, SDB- Sabouraud Dextrose Broth, ** Not included in the statistical analysis. Values with different letters are significantly different from each other by LSD at 5% level

Table 15. Viability of *L. saksenae* in chitin and chitosan enriched liquid substrates

Sl. No.	Substrates	* Mean number of cfu (x 10^7 mL^{-1})		
		14 DAI	21 DAI	28 DAI
1.	RW + chitin (0.3%)	2.20^{e}	1.37^{e}	0.80^{d}
2.	CW + chitin (0.3%)	0.37^{g}	0.18^{h}	0.14^{f}
3.	RW + chitosan (0.3%)	5.32^{d}	4.14^{c}	1.93^{c}
4.	CW + chitosan (0.3%)	0.57^{fg}	0.37^{g}	0.18^{f}
5.	RW (check)	0.88^{f}	0.48^{f}	0.37^{e}
6.	CW (check)	0.27^{g}	0.17^{h}	0.12^{f}
7.	SDB + chitin (0.3%) (check)	5.94^{c}	3.84^{d}	1.93^{c}
8.	SDB + chitosan (0.3%) (check)	29.46^{a}	10.40^{a}	5.40^{a}
9.	SDB (check)	8.88^{b}	6.23^{b}	2.20^{b}
	CD(0.05)	0.311	0.057	0.078

*Mean of three replications, DAI - Days after inoculation, RW - Rice Water, CW - Coconut Water, cfu - colony forming units. Values with different letters are significantly different from each other by LSD at 5% level

(Plate 5). The number of cfu obtained from unamended SDB was 8.88 x 10^7 mL^{-1} which was significantly lower than the first. This was followed by SDB + chitin (5.94 x 10^7 mL^{-1}) which in turn was superior to RW + chitosan (5.32 x 10^7 mL^{-1}) (Plate 4). The number of viable colonies recorded in RW + chitin was 2.20 x 10^7 mL^{-1}. It was followed by CW + chitosan (0.57 x 10^7 mL^{-1}) which was on par with non enriched RW (0.88 x 10^7 mL^{-1}). Lowest yield was recorded from CW + chitin (0.37 x 10^7 mL^{-1}), CW + chitosan (0.57 x 10^7 mL^{-1}) and CW (0.27 x 10^7 mL^{-1}).

A similar trend was observed at 21 DAI also. Maximum number of cfu was noticed with SDB + chitosan (1.04 x 10^8 mL^{-1}) followed by SDB with a cfu value of 6.23 x 10^7 mL^{-1}. The treatments RW + chitosan (4.14 x 10^7 mL^{-1}) was found superior to SDB + chitin (3.84 x 10^7 mL^{-1}). The number of cfu recorded for RW + chitin was (1.37 x 10^7 mL^{-1}), which was significantlyhigher than that in RW (0.48 x 10^7 mL^{-1}) and CW + chitosan (0.37 x 10^7 mL^{-1}). The number of cfu observed in CW + chitin was on par with unamended CW. The values being 0.18 x 10^7 mL^{-1} and 0.17 x 10^7 mL^{-1} respectively.

As that of earlier, at 28 DAI also maximum number of cfu was recorded for SDB + chitosan with (5.40 x 10^7 mL^{-1}). This was followed by SDB (2.20 x 10^7 mL^{-1}). The number of cfu recorded in RW + chitosan (1.93 x 10^7 mL^{-1}) was statistically on par with SDB + chitin (1.93 x 10^7 mL^{-1}). RW + chitin (0.80 x 10^7 mL^{-1}) ranked fourth which was followed by RW (0.37 x 10^7 mL^{-1}). The number of cfu observed in CW + chitosan (0.18 x 10^7 ml^{-1}), CW + chitin (0.14 x 10^7 mL^{-1}) and CW (0.18 x 10^7 mL^{-1}) were statistically similar.

The observations based on cfu revealed that the chitosan enriched SDB media was the best for better viability of spores. Chitosan enriched RW was the better option among natural substrates.

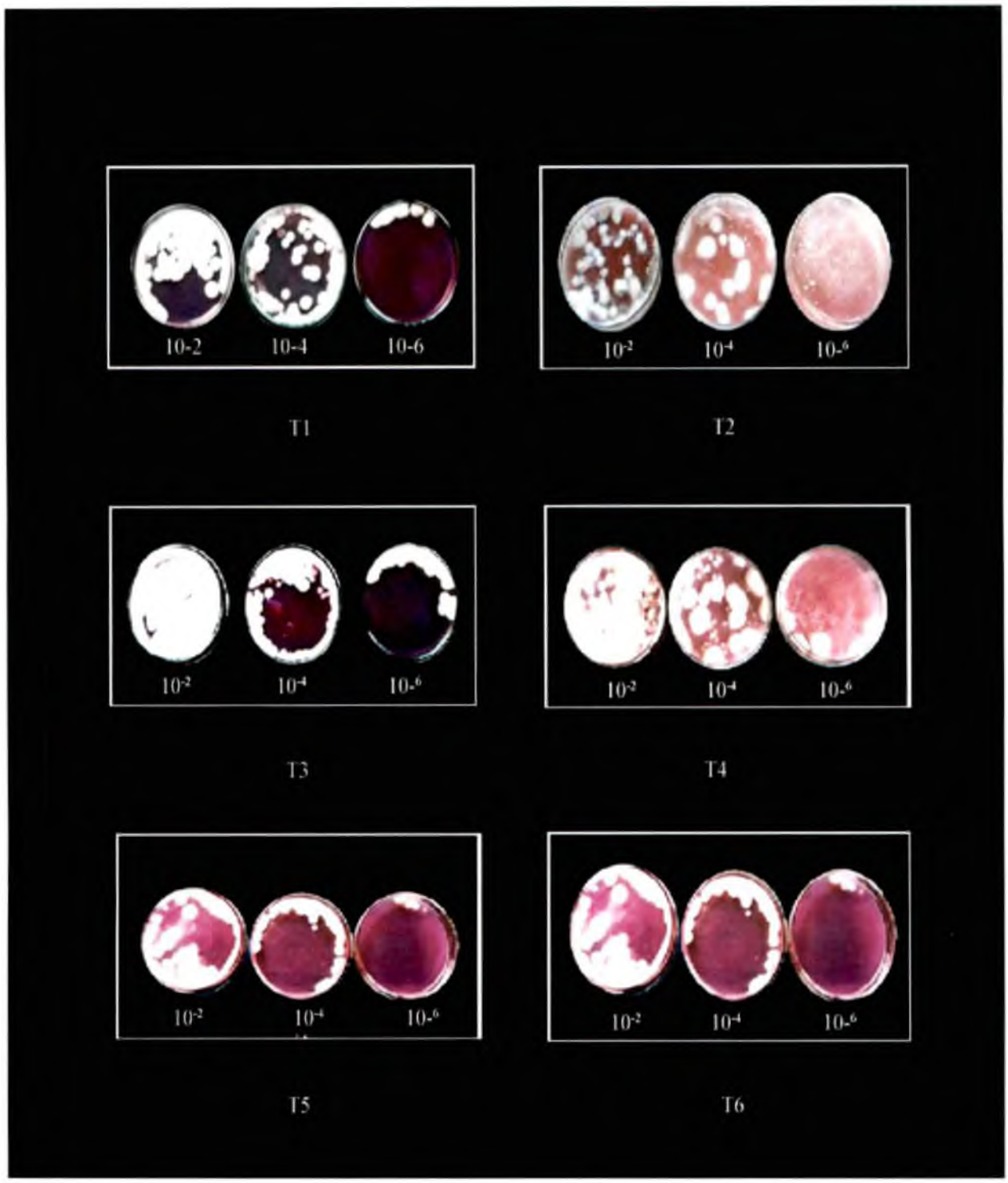


Plate 4. Comparison of number of viable colonies on 6 th day after inoculation in enriched and non enriched liquid substrates

T1 : Rice water + chitin T2 : Coconut water + chitin T3 : Rice water + chitosan
T4 : Coconut water + chitosan T5 : Rice water T6 : Coconut water

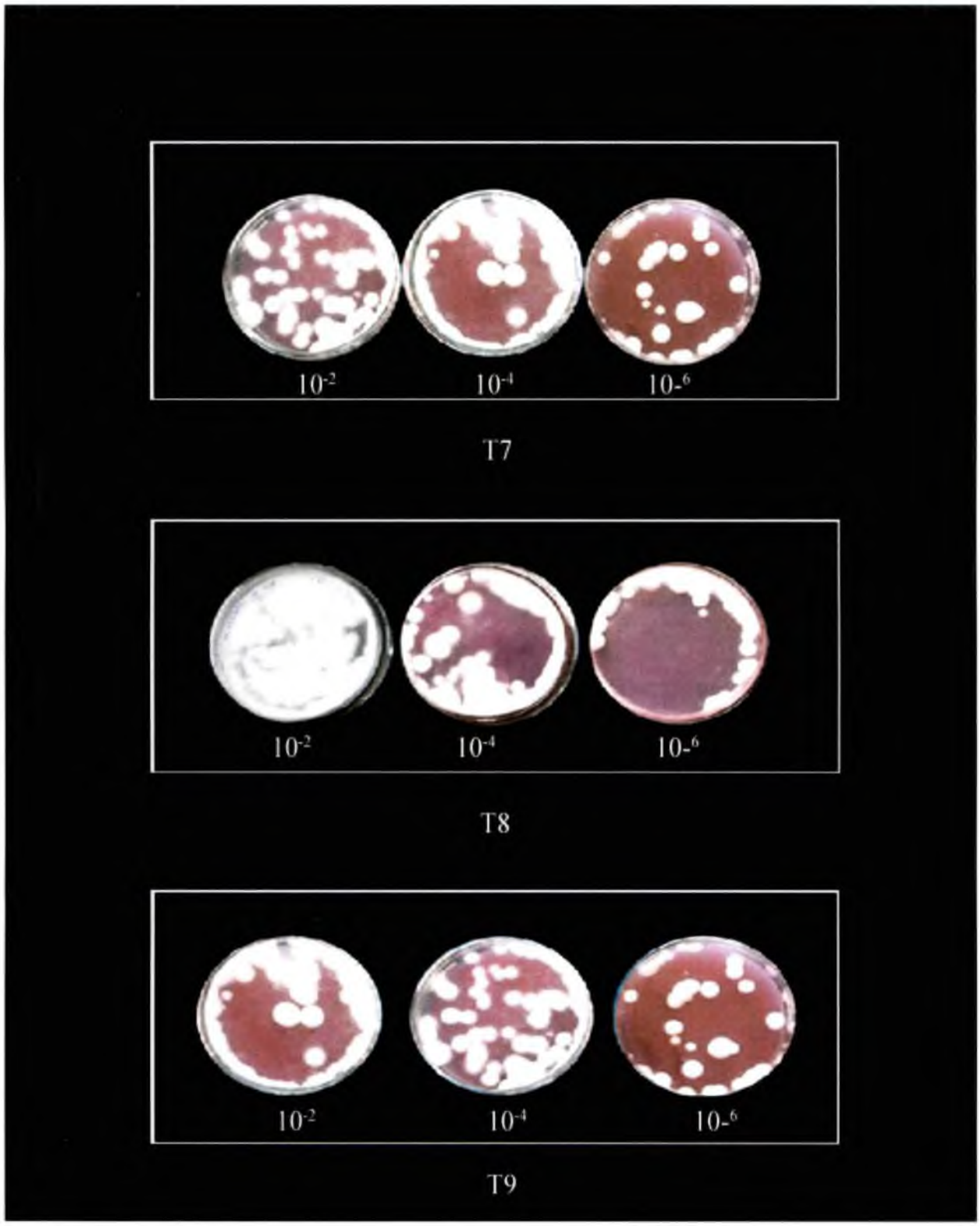


Plate 5. Comparison of number of viable colonies on 6 th day after inoculation in enriched and non enriched Sabouraud Dextrose Broth (SDB).

T7 : SDB + chitin T8 : SDB + chitosan T9 : SDB

4.3.2.1.3. On Virulence

Efficacy of the spores harvested from different substrates was evaluated on for their virulence by assessing the mortality caused in the test insect, *C. insolitus*, the data of which are presented in Table 16.

A very high mortality of 95.33 per cent was noted after 24 h, with spores obtained from SDB +chitosan. The death rate observed with spores obtained from SDB + chitin was 93.66 per cent which was statistically on par with that of RW + chitosan (93.33 per cent). The mortality recorded with CW + chitosan (92 per cent) was superior to RW + chitin (90.66 per cent). CW + chitin recorded 85.66 per cent mortality which was statistically on par with that of SDB (85.33 per cent). A lower death rate was recorded with spores from CW (54.33 per cent) and the least with RW (53.33 per cent).

After 48 h, 100 per cent mortality could be attained with spores obtained from chitin and chitosan enriched SDB, RW and CW. This was followed by CW with 72.66 per cent mortality. Lowest mortality was recorded in the case of spores obtained from RW (70.33 per cent).

At 72 h, 100 per cent mortality observed for all substrates except RW and CW with 98.66 and 97.33 per cent respectively.

By the end of 96 h, spores from all the substrates resulted in 100 per cent mortality.

The mortality achieved in media amended with chitin and chitosan enriched media resulted 100 per cent mortality within 48 h and was found to be superior than the un amended substrates. In general, chitosan enriched media resulted higher per cent mortality followed by chitin enriched substrates *ie*, mortality per cent increased with increased sporulation. Both chitosan and chitin enriched solid as well as liquid substrates resulted 100 per cent mortality within 48 h after treatment.

Table 16. Efficacy of *L. saksenae* cultured on chitin and chitosan enriched liquid substrates

Sl. No.	Substrates	Mean mortality (%) of *C. insolitus* at 24 h interval			
		24	48	72	96
1.	RW + chitin (0.3%)	90.66 (9.52)^{d}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
2.	CW + chitin (0.3%)	85.66 (9.26)^{e}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
3.	RW + chitosan (0.3%)	93.33 (9.66)^{b}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
4.	CW + chitosan (0.3%)	92 (9.59)^{c}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
5.	RW (check)	53.33 (7.30)^{g}	70.33 (8.39)^{c}	98.66 (9.93)^{b}	100 (10.00)^{a}
6.	CW(check)	54.33 (7.37)^{f}	72.66 (8.53)^{b}	97.33 (9.87)^{c}	100 (10.00)^{a}
7.	SDB + chitin (0.3%)	93.66 (9.66)^{b}	100 (10.00)^{a}	100 (10.00)^{a}	100^{a} (10.00)
8.	SDB + chitosan (0.3%)	95.33 (9.76)^{a}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
9.	SDB (check)	85.33 (9.24)^{e}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
	CD (0.05)	0.061	0.028	0.024	NS

RW- Rice Water, CW- Coconut Water, SDB - Sabouraud Dextrose Broth , NS - Non significant, Figures in parentheses are values after $\sqrt{x + 1}$ transformation. Values with different letters are significantly different from each other by LSD at 5% level

4.3.3. Cost of Substrates

Among the different solid substrates used for mass production, RB and WB were the least expensive ones (Table.17). Cost Kg^{-1} being Rs. 20/- . The amount incurred for RB or WB + chitin five per cent was Rs. 30 Kg^{-1} and that for RB or WB + chitosan five per cent was Rs. 50 Kg^{-1} *.ie,* Additional cost of Rs. 10 Kg^{-1} and Rs. 30 Kg^{-1} was incurred respectively for enriching with chitin and chitosan. The spore yield obtained with RB or WB was 10^7 spores mL^{-1} and those within chitin or chitosan was 10^8 spores mL^{-1}.

Liquid substrates such as CW and RW are obtained free of cost. Production of CW or RW + 0.3 per cent chitin incurred an additional cost of Rs. 59 L^{-1} and that with chitosan 0.3 per cent incurred an additional amount of Rs. 79 L^{-1}. The spore yield obtained from RW alone was 10^7 spores mL^{-1} and that with CW was 10^7 spores mL^{-1}. In enriched liquid media RW + chitin and CW + chitin, there was no increase in yield (10^7 spores mL^{-1}).

Comparing the cost of substrates and spore yield, mass production in RB or WB with chitin is cost effective. The standard check SDB with spore yield 10^7 spores mL^{-1} incurred the highest cost of Rs. 84 L^{-1}.

4.4. ENZYMES PRODUCED BY *L. saksenae*

To elucidate the main cuticle degrading enzymes produced by the fungus and to study the role of culture media in secretion of enzymes each of the amended and unamended media were subjected to both qualitative and quantitative essay.

Table 17. Comparison of cost of substrates Kg^{-1} and spore yield mL^{-1}

Sl. No.	Substrates	Cost Kg^{-1} (Rs.)	Spore yield (mL^{-1})
Solid substrates			
1	RB	20	10^7
2	WB	20	10^7
3	RB + chitin (5%)	30	10^8
4	WB + chitin (5%)	30	10^8
5	RB + chitosan (5%)	50	10^8
6	WB+ chitosan (5%)	50	10^8
Liquid substrates			
7	RW	-	10^6
8	CW	-	10^6
9	RW + chitin (0.3 %)	59	10^7
10	RW + chitosan (0.3%)	79	10^7
11	CW + chitin (0.3%)	59	10^7
12	CW + chitosan (0.3%)	79	10^7
13	SDB	84	10^7
14	SDB + chitin (0.3%)	143	10^7
15	SDB + chitosan (0.3%)	163	10^8

RB - Rice bran, WB - Wheat bran, RW- Rice water, CW- Coconut water, SDB- Sabouraud dextrose broth

4.4.1. Qualitative Assay

4.4.1.1 Chitinase Acticity

Chitinase activity of *L. saksenae* in different substrates were assessed by measuring the halo diameter (Plate 6) and working out the enzyme index as mentioned in para 3.6.1.2.1. To detect the peak activity period seven day old and 14 day old cultures were observed on the second and fourth DAI.

Seven day old culture varied in the diameter of halo produced, depending on the substrate in which they were originally cultured (Table 18). At two DAI, the largest halo (2.07cm) was detected in SDB + chitosan which was on par with that of SDB + chitin (2.00 cm). This was followed by the diameter (1.73 cm) observed in the case of media in which chitosan was the sole carbon source (Peptone + chitosan). SDB produced a lesser halo diameter of 1.53 cm which was superior to Peptone + chitin (1.23 cm).

The halo diameter measured was maximum at four DAI, in all the substrates. The largest halo was produced by SDB + chitosan (2.77 cm) followed by SDB + chitin (2.37cm). The halo produced in SDB (2.07 cm) was statistically on par with that produced in Peptone + chitosan (2.00 cm). Peptone + chitin resulted the smallest halo diameter of 1.53 cm.

With 14 day old culture, halo diameter observed was less (0.07cm to 1.53 cm) at two DAI (Table 19). The diameter observed in SDB + chitosan was the highest (1.53 cm) which was statistically superior to all other treatments. SDB + chitin and SDB were on par with each other (1.17 cm). Peptone + chitosan (1.03cm) ranked third. The smallest halo was detected in Peptone + chitin (0.70 cm).

On the fourth day also, the largest halo was produced by SDB + chitosan (1.87 cm) which was on par with SDB + chitin (1.81 cm). This was followed by Peptone + chitosan (1.53 cm) and SDB alone (1.5 cm). The least halo was noticed in Peptone + chitin (1.33 cm).

Table 18. Enzyme activity of seven day old *L. saksenae* cultured in chitin and chitosan enriched substrates

Sl. No.	Substrates	Halo diameter (cm)					
		Chitinase		Protease		Lipase	
		2 DAI	4DAI	2 DAI	4 DAI	2 DAI	4 DAI
1	SDB	1.53^{c}	2.07^{c}	2.77^{c}	3.10^{c}	0.87^{c}	1.67^{c}
2	SDB + chitin (0.3%)	2.00^{a}	2.37^{b}	3.00^{b}	3.57^{b}	1.03^{b}	1.87^{b}
3	SDB + chitosan (0.3%)	2.07^{a}	2.77^{a}	3.43^{a}	4.03^{a}	1.57^{a}	2.07^{a}
4	Peptone + chitin (2%)	1.23^{d}	1.53^{d}	1.33^{e}	1.60^{d}	0.77^{c}	1.53^{d}
5	Peptone + chitosan (2%)	1.73^{b}	2.00^{c}	1.53^{d}	1.73^{d}	0.87^{c}	1.80^{b}
	CD(0.05)	0.094	0.094	0.094	0.430	0.105	0.094

DAI - Days after inoculation, SDB - Sabouraud Dextrose Broth,*Mean of three observations. Values with different letters are significantly different from each other by LSD at 5% level

Table 19. Enzyme activity of seven day old *L. saksenae* cultured in chitin and chitosan enriched substrates (14 day old)

SI .No.	Substrates	Halo diameter (cm)					
		Chitinase		Protease		Lipase	
		2 DAI	4DAI	2DAI	4 DAI	2DAI	4 DAI
1.	SDB	1.17^{b}	1.53^{b}	2.00^{c}	2.10^{c}	0.33^{c}	0.50^{e}
2.	SDB + chitin (0.3%)	1.17^{b}	1.81^{a}	2.10^{b}	2.53^{b}	0.53^{b}	0.88^{b}
3.	SDB + chitosan (0.3%)	1.53^{a}	1.87^{a}	2.77^{a}	2.67^{a}	1.03^{a}	1.23^{a}
4.	Peptone + chitin (2%)	1.03^{c}	1.33^{c}	1.07^{e}	1.33^{e}	0.43^{bc}	0.57^{d}
5.	Peptone + chitosan (2%)	0.70^{d}	1.53^{b}	1.27^{d}	1.53^{d}	0.53^{b}	0.73^{c}
	CD (0.05)	0.094	0.096	0.081	0.094	0.105	0.049

DAI - Days after inoculation, SDB - Sabouraud Dextrose Broth,* Mean of three observation. Values with different letters are significantly different from each other by LSD at 5% level

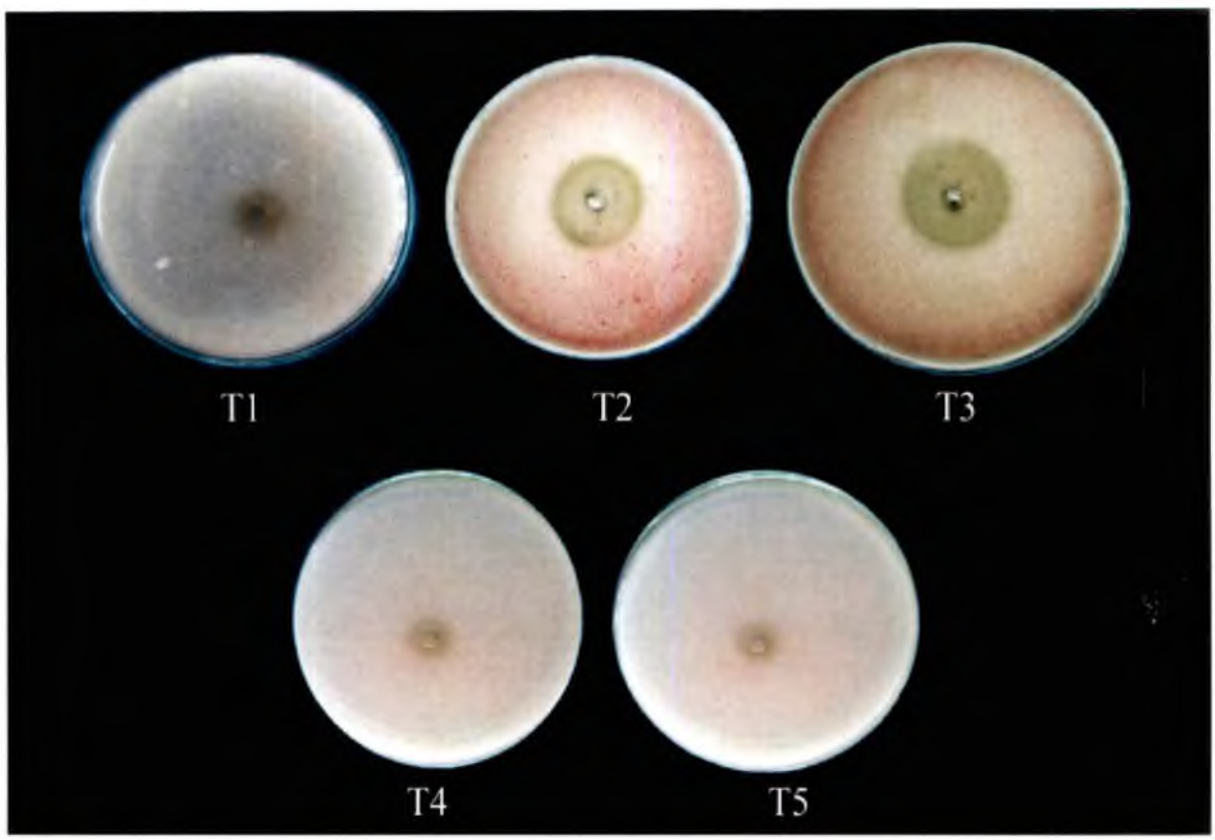


Plate 6. Chitinase activity of seven day old *L. saksenae* as observed on the fourth day

T1 : SDB T2 : SDB + chitin T3 : SDB + chitosan
T4 : Peptone + chitin T5 : Peptone + chitosan

Observations noted on sixth day showed that there was no increament in halo diameter after fourth day, in the case of seven as well as 14 day old cultures.

The enzyme index of chitinase in seven day old culture is represented in Fig. 1. The index ranged from a minimum of 2.37 in Peptone + chitin to a maximum of 4.13 in SDB + chitosan on the second day from 3.07 to 5.57 on the fourth day. The corresponding index in 14 day old culture (Fig. 2) varied from 2.06 to 3.13 on the second day and 2.57 to 3.77 on the fourth day.

4.4.1.2. *Protease Acticity*

Protease activity was determined by measuring the blue coloured zone formed around the well as seen in Plate 7 and calculating the enzyme index thereby. Both seven day old and 14 day old cultures were used to detect its activity on second and fourth DAI

The coloured zone measured, on the second and fourth day with seven day old culture exhibited statistical variation among themselves. The diameter ranged from 1.33 cm to 3.43 cm after two DAI and 1.60 cm to 4.03 cm after four DAI (Table 18).

SDB + chitosan produced the largest halo of 3.43 cm. This was followed by SDB + chitin (3cm) which was found superior to SDB (2.77 cm). Peptone + chitosan produced a lesser diameter of 1.53 cm. The smallest diameter was observed in the case of Peptone + chitin (1.33 cm).

The halo reached the maximum on four DAI with the same trend among treatments. The halo diameter was maximum in SDB + chitosan (4.03 cm) followed by SDB + chitin (3.57 cm). SDB alone was ranked third (3.10 cm) which was superior to those in which chitin or chitosan served as the sole carbon source, the values being 1.73 cm in Peptone + chitosan and 1.60 in Peptone + chitin.

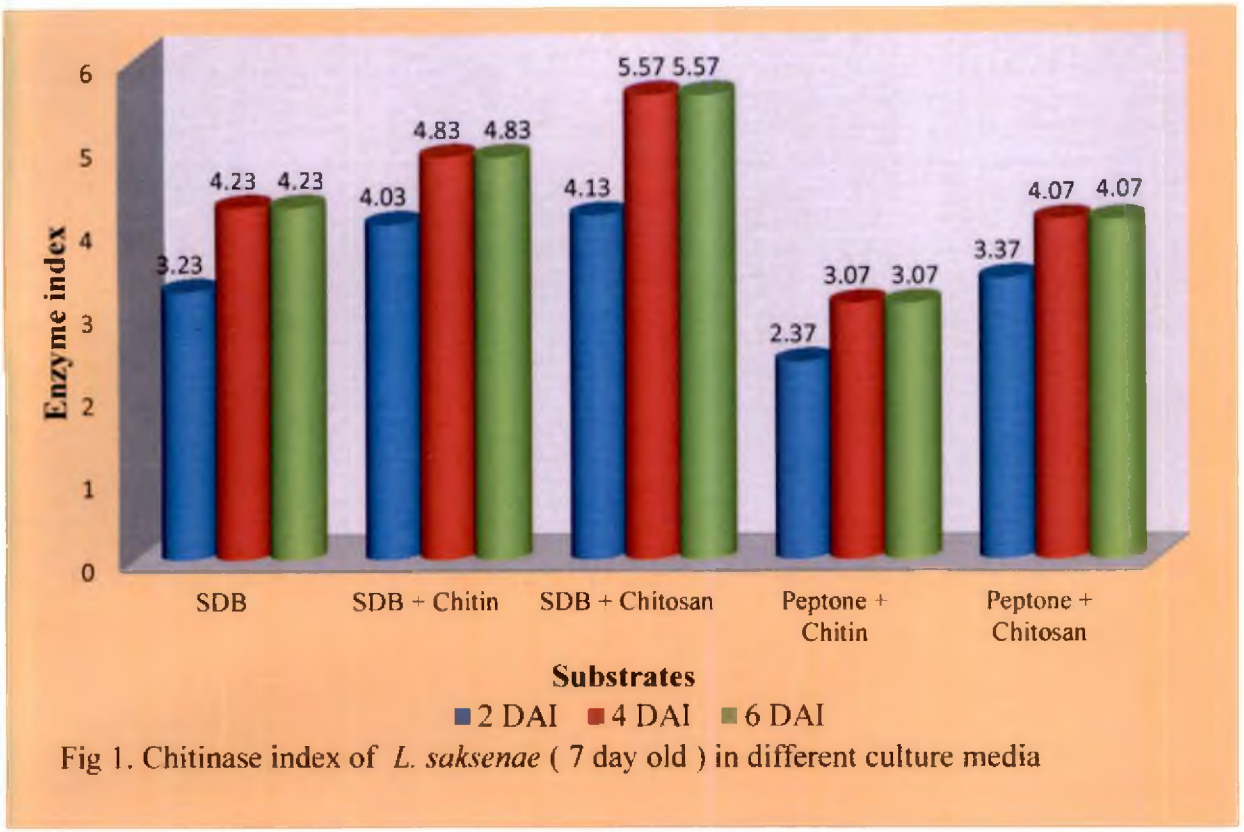


Fig 1. Chitinase index of *L. saksenae* (7 day old) in different culture media

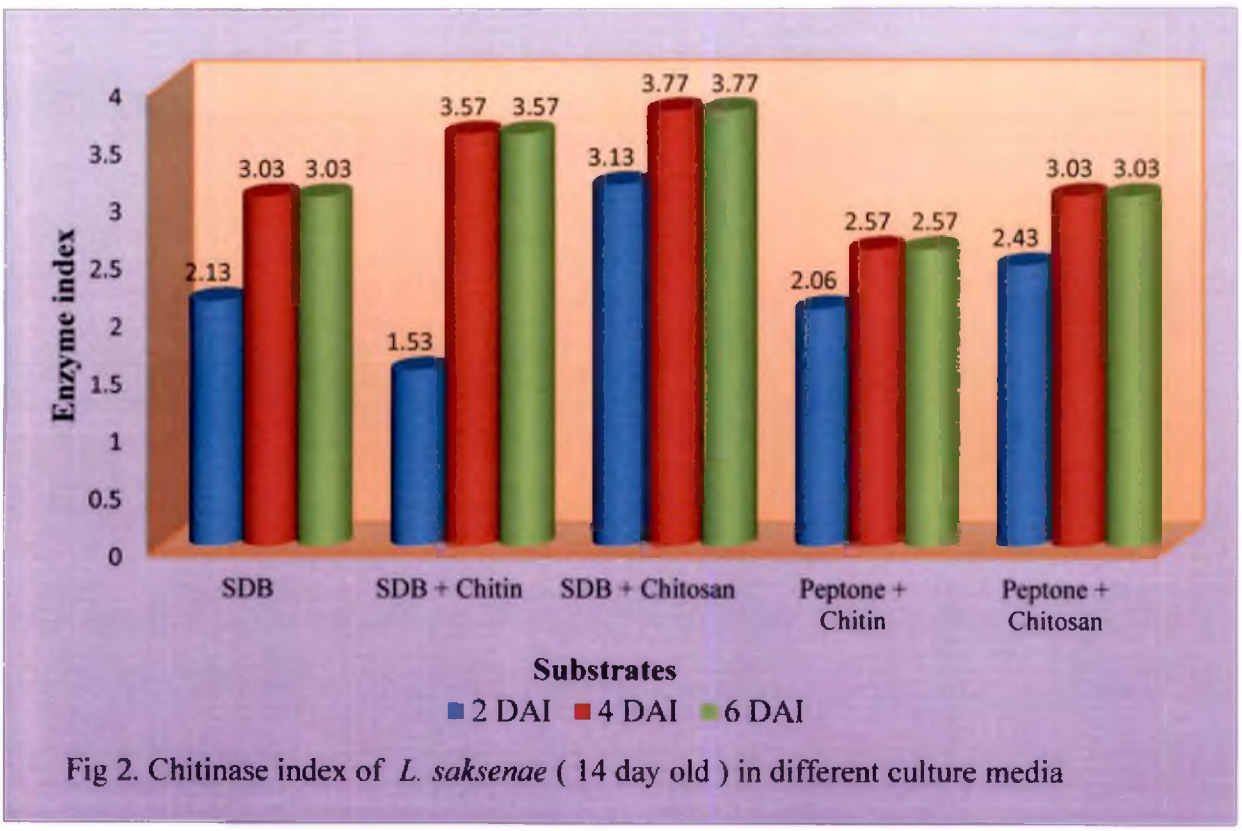


Fig 2. Chitinase index of *L. saksenae* (14 day old) in different culture media

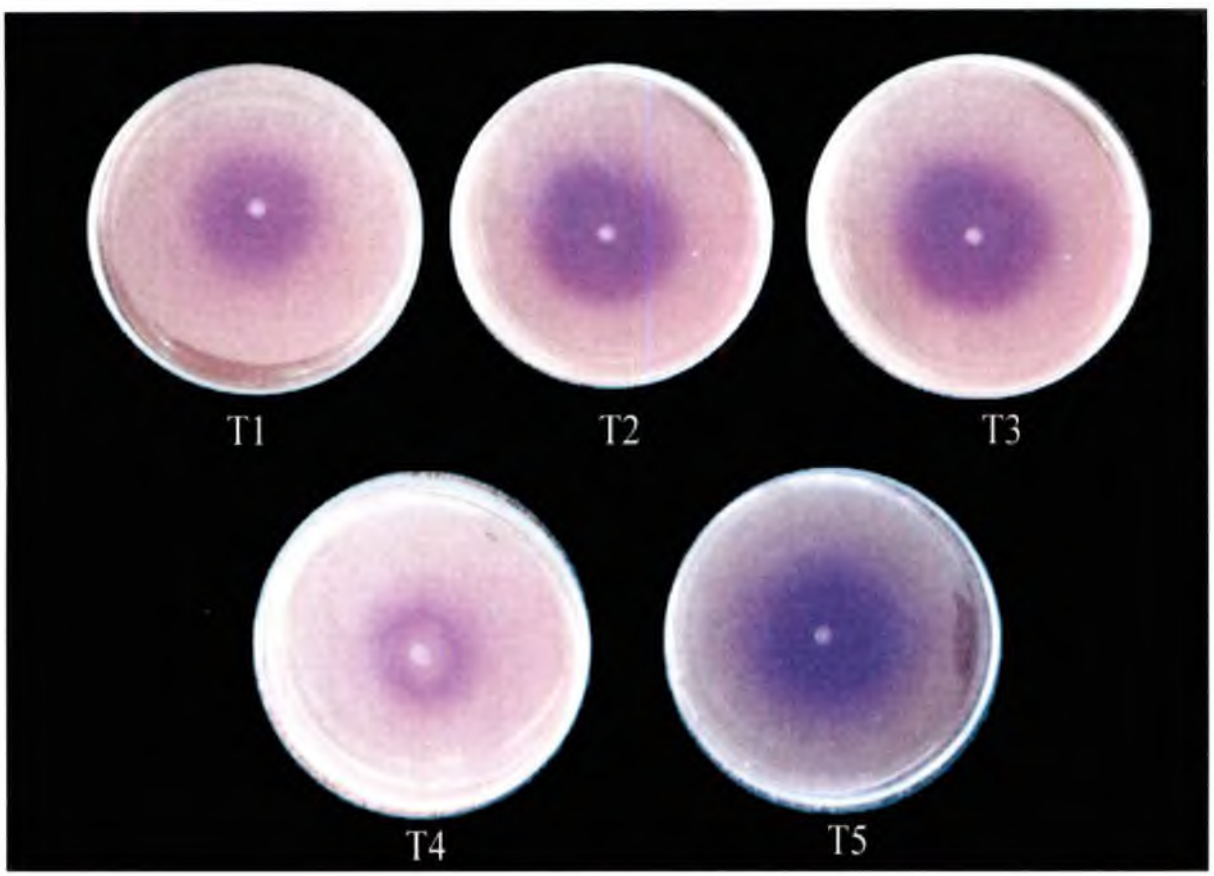


Plate 8. Lipase activity of seven day old *L. saksenae* as observed on the fourth day

T1 : SDB T2 : S DB + chitin T3 : SDB + chitosan

T4 : Peptone + chitin T5 : Peptone + chitosan

In the case of 14 day old culture also it was SDB + chitosan that produced maximum diameter of 2.77 cm at two DAI (Table 19). The sequential superioriority of other treatments were in the order SDB + chitin (2.10 cm), SDB (2 cm), Peptone + chitosan (1.27 cm) and Peptone + chitin (1.07 cm). On the fourth day the halo reached its maximum size where a same trend was observed among treatments. SDB+chitosan produced a maximum halo of 2.67 cm followed by SDB + chitin (2.53 cm), SDB (2.10 cm), Peptone +chitosan (1.53 cm) and Peptone + chitin (1.33 cm).

Observations noted on sixth day showed that there was no increament in halo diameter after fourth day, in the case of seven as well as 14 day old cultures.

The protease index varied from a minimum of 2.53 in Peptone + chitin to a maximum of 7.03 in SDB + chitosan in seven day old culture two DAI (Fig 3). At 4 DAI, theindex increased from 3.37 in Peptone + chitin to 8.03 in SDB + chitosan. A similar trend was observed with 14 dayold culture also (Fig 4). At two DAI, the corresponding values ranged from 2.03 to 5.43 and at four DAI it ranged from 2.63 to 5.63.

4.4.1.3 Lipase Activity

Lipase activity of seven and fourteen day old culture was studied by measuring the red coloured halo (Plate 8). The enzyme index was calculated using the same method followed for chitinase and protease.

In the case of seven day old culture, SDB + chitosan produced a halo of 1.57 cm diameter which was superior to SDB + chitin with 1.03 cm (Table 18). Peptone + chitosan and SDB produced a halo of 0.87 cm each, which was on par with Peptone + chitin (0.77 cm). After four days of incubation the halo reached its maximum size. SDB + chitosan produced a halo diameter of 2.07 cm which was followed by that of SDB + chitin (1.87cm) and Peptone + chitosan (1.80 cm). SDB produced a halo of 1.67 cm and was found superior to Peptone + chitin (1.53 cm).

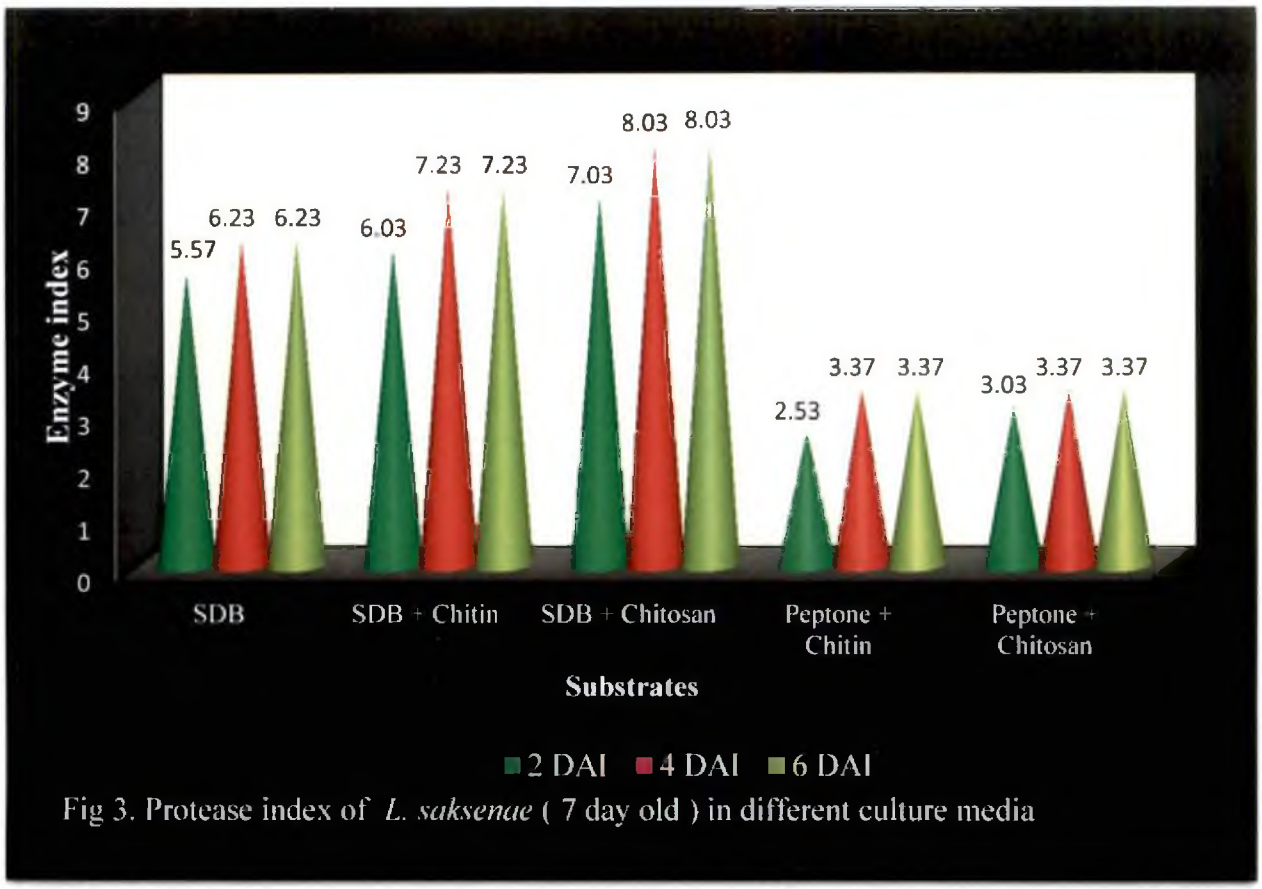


Fig 3. Protease index of *L. saksenae* (7 day old) in different culture media

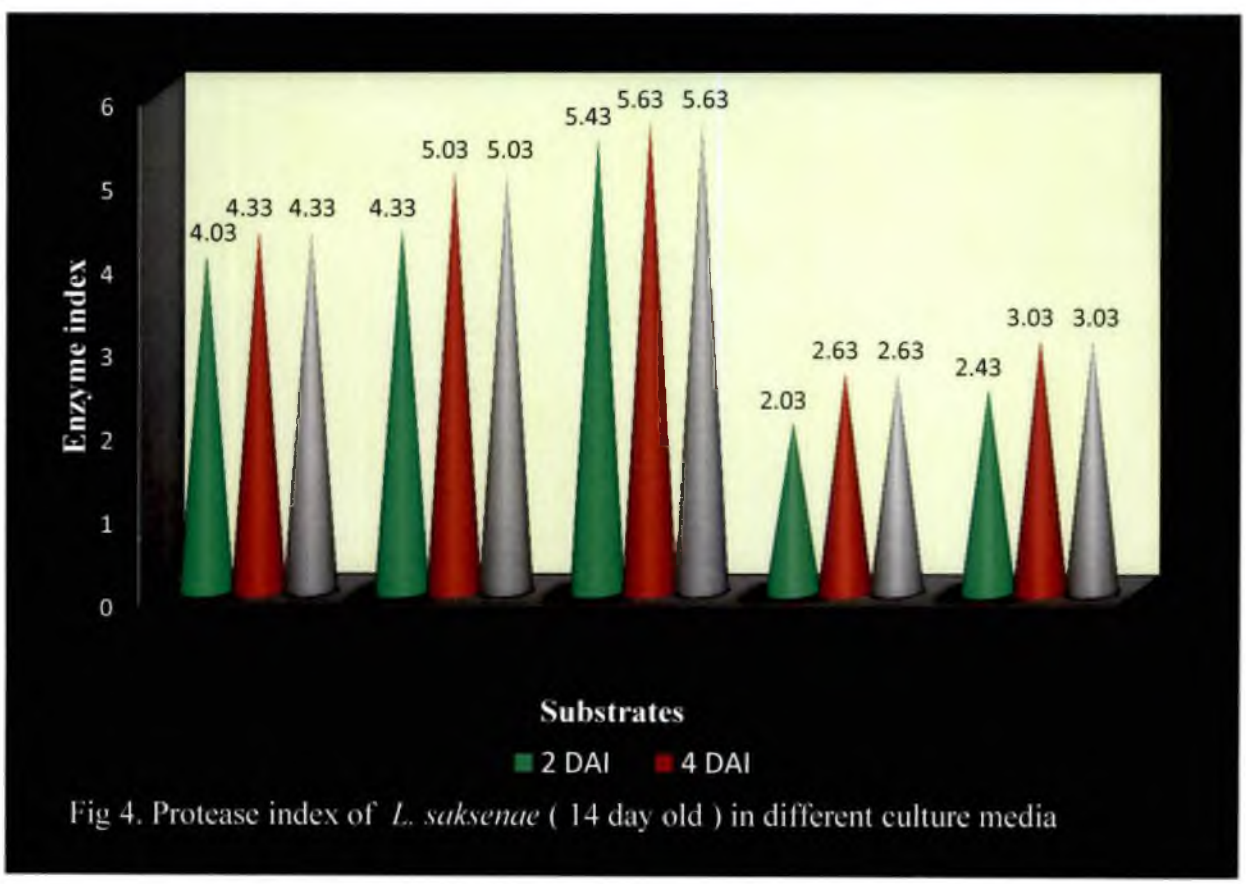


Fig 4. Protease index of *L. saksenae* (14 day old) in different culture media

Fourteen day old culture showed lesser values, where SDB + chitosan recorded largest diameter of 1.03 cm after two DAI (Table 19). This was followed by SDB + chitin (0.5 cm), Peptone + chitosan (0.5 cm) and Peptone + chitin (0.43 cm). SDB produced a halo of 0.33 cm which was on par with Peptone + chitin (0.43 cm).

After four DAI, SDB + chitosan produced the largest halo diameter of 1.23 cm. This was followed by SDB + chitosan (0.88 cm), Peptone + chitosan produced a halo of 0.7 cm followed by Peptone + chitin (0.57 cm). The least halo was observed for SDB (0.50 cm).

Observations noted on sixth day showed that there was no increament in halo diameter after fourth day, in the case of seven as well as 14 day old cultures.

The lipase index worked with seven day old culture (Fig. 5) was maximum in, SDB + chitosan (3.07) two DAI. The index was least in Peptone + chitin (1.53). At 4 DAI also the index was maximum in SDB + colloidal chitosan (4.07) and minimum in Peptone + chitin (3.03).The same trend was noticed with 14 day old culture which showed the maximum index of 2.03 in SDB + chitosan and a minimum index of 0.65 in Peptone + chitin, at two DAI. The corresponding index after 4 DAI was 0.67 to 0.12.

4.4.2 Quantitative Assay

Quantity of cuticle degrading enzymes produced by *L. saksenae* was assayed using standard procedures and their role in pathogenicity to insects (test insect *C. insolitus*) was determined by working out the correlation coefficients.

4.4.2.1 Chitinase Production

Chitinase production of the fungus was compared in both the enriched and non enriched media. Both seven day old and 14 day old cultures were used for

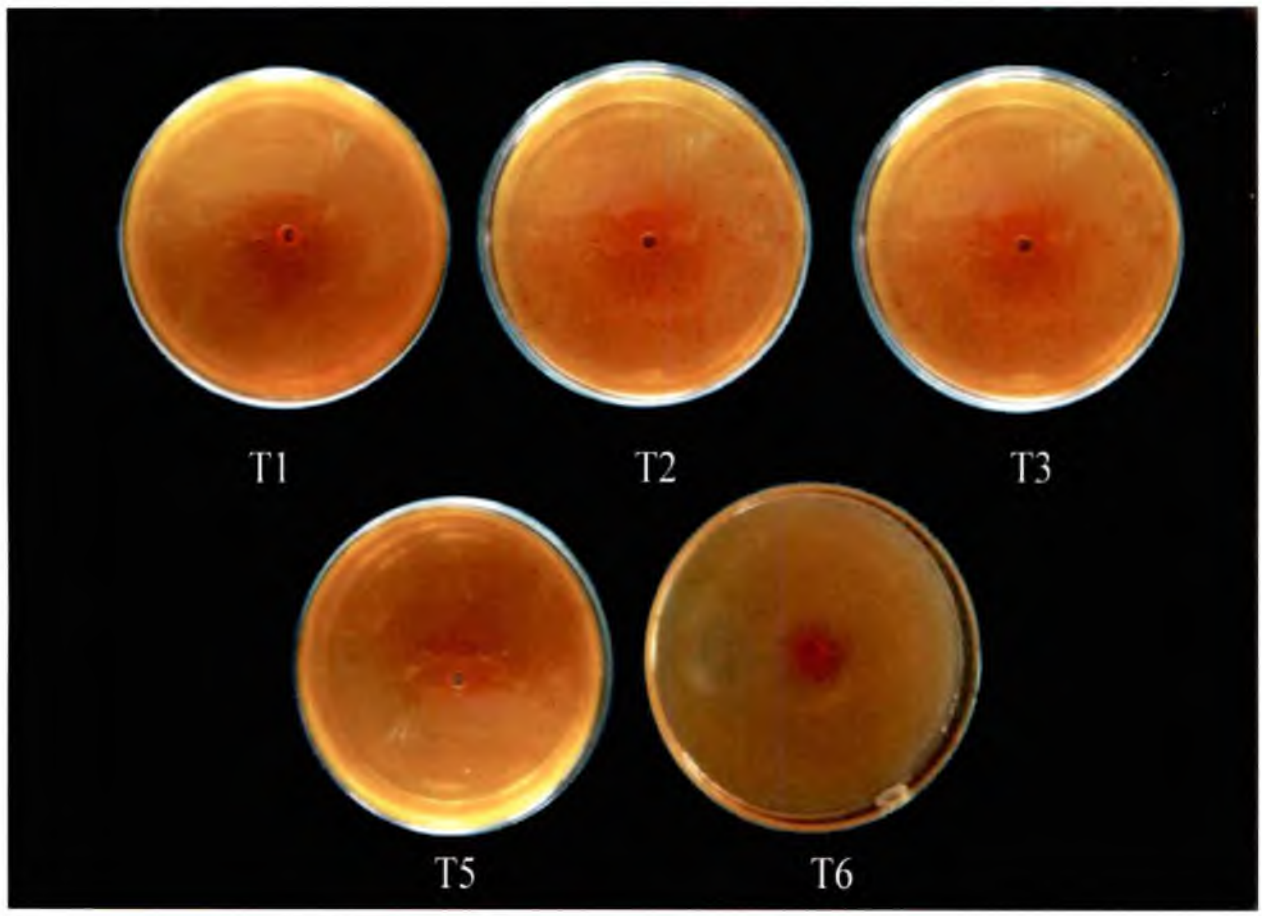


Plate 8. Lipase activity of seven day old *L. saksenae* as observed on the fourth day

T1 : SDB T2 : SDB + chitin T3 : SDB + chitosan
T4 : Peptone + chitin T5 : Peptone + chitosan

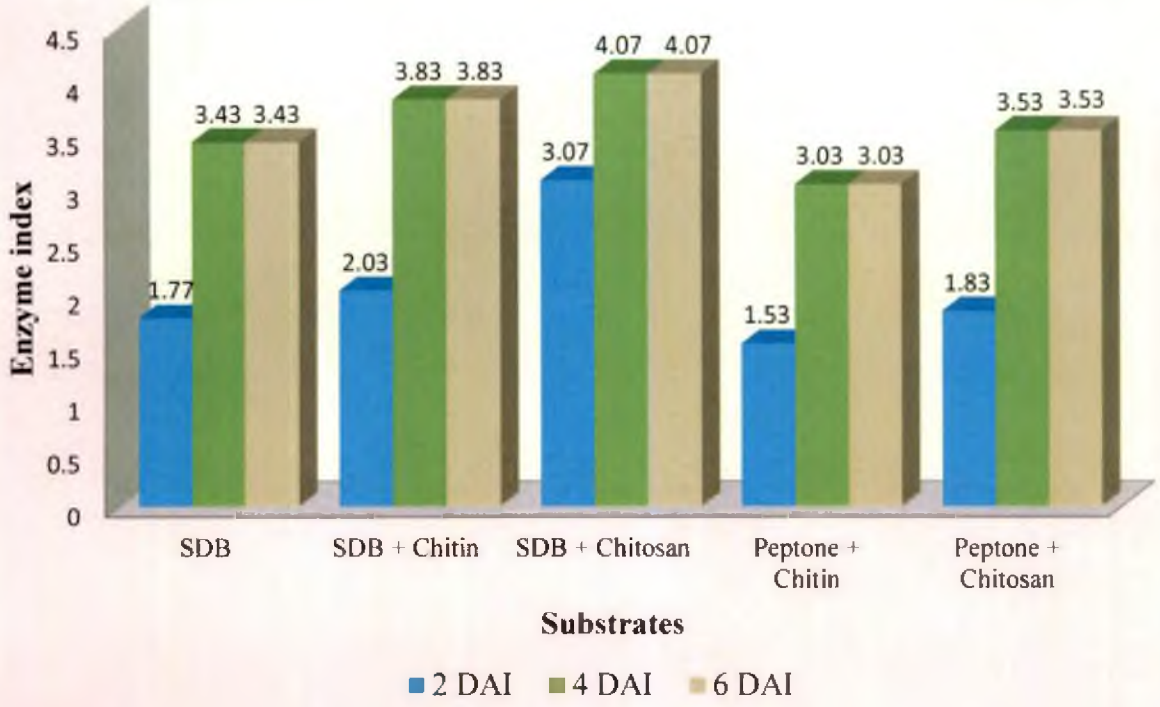


Fig 5. Lipase index of *L. saksenae* (7 day old) in different culture media

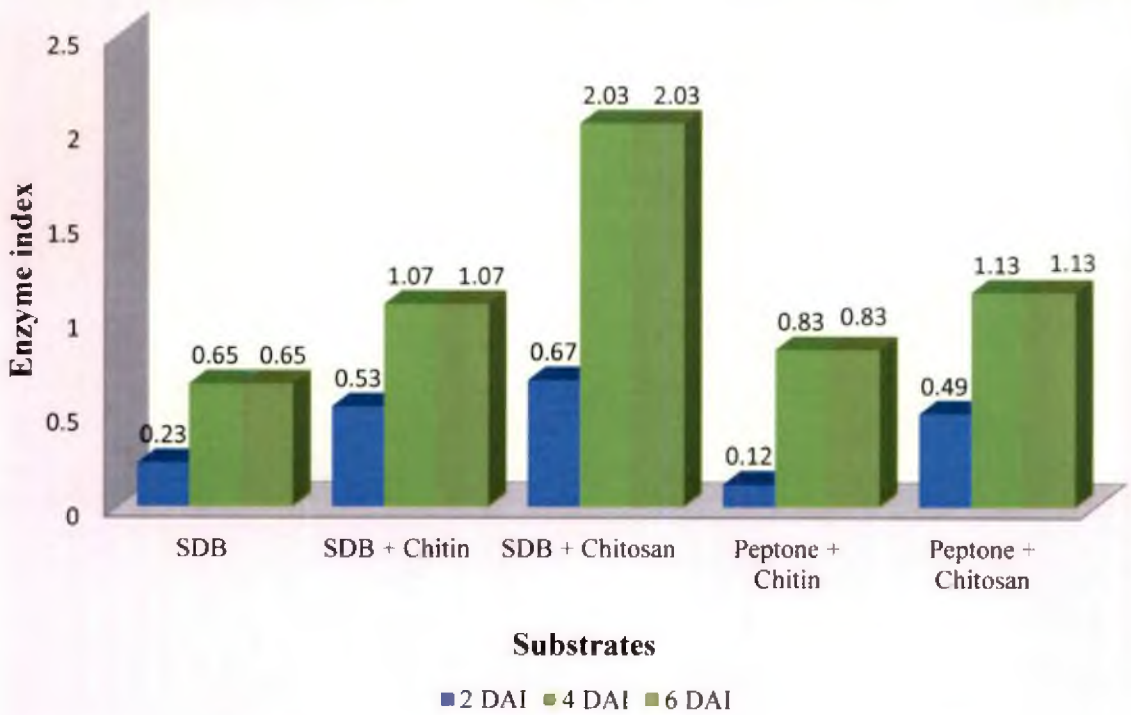


Fig 6. Lipase index of *L. saksenae* (14 day old) in different culture media

the study. The results indicated significant variation among the media as well as the age of the culture (Table 20 and 21).

In the case of seven day old culture, maximum chitinase production (0.36 UmL^{-1}) was recorded in chitosan enriched medium, SDB + chitosan. Chitin enriched medium, SDB + chitin ranked second (0.26 mL^{-1}). This was followed by Peptone + chitosan (0.16 mL^{-1}). Non enriched medium, SDB, ranked fourth with 0.13 UmL^{-1} and the least amount was detected in Peptone + chitin (0.08 UmL^{-1}).

In 14 day old culture also maximum chitinase production was observed with enriched media, SDB+ chitosan (0.28 U mL^{-1}) which was followed by SDB + chitin (0.26 U mL^{-1}). SDB (0.12 U mL^{-1}) ranked third. The media in which chitosan was the sole carbon source *ie.* Peptone + chitosan (0.09 UmL^{-1}) was found to be superior to Peptone + chitin (0.05 UmL^{-1}).

4.4.2.2 Protease Production

In the case of seven day old culture, highest protease content of 6.52 U mL^{-1} was observed in the culture filtrate of SDB + chitosan followed by SDB + chitin (4.36 U mL^{-1}). The protease content in SDB (3.45 U mL^{-1}).was on par with that of Peptone + chitosan (3.27 U mL^{-1}) which in turn was on par with Peptone + chitin (2.87 U mL^{-1}).

With 14 day old culture also, SDB+ chitosan recorded highest protease content of 4.88 U mL^{-1} which was followed by Peptone + chitosan (2.80 U mL^{-1}). SDB + chitin (2.37 U mL^{-1}) was found to be on par with Peptone + chitin (2.47 U mL^{-1}). The lowest content was recorded in SDB (2.00 U mL^{-1}).

4.4.2.3 Lipase Production

Significant difference was observed in the lipase content of culture filtrates obtained from different substrates.In the case of seven day old culture SDB + chitosan had highest lipase content of 1.55 UmL^{-1}. The content in SDB + chitin

Table 20. Cuticle degrading enzymes produced by *L. saksenae* (7 day old) in chitin and chitosan enriched media

Sl. No.	Substrates	Chitinase (U mL^{-1})	Protease (U mL^{-1})	Lipase (U mL^{-1})
1.	SDB	0.13^{d}	3.45^{c}	0.63^{d}
2.	SDB + chitin (0.3%)	0.26^{b}	4.36^{b}	1.28^{b}
3.	SDB + chitosan (0.3%)	0.36^{a}	6.52^{a}	1.55^{a}
4.	Peptone + chitin (2%)	0.08^{e}	2.87^{d}	0.24^{e}
5.	Peptone + chitosan (2%)	0.16^{c}	3.27^{cd}	1.13^{c}
	CD (0.05)	0.015	0.477	0.084

SDB - Sabouraud Dextrose Broth. Values with different letters are significantly different from each other by LSD at 5% level

Table 21. Cuticle degrading enzymes produced by *L. saksenae* substrates (14 day old in chitin and chitosan enriched

Sl. No.	Substrates	Chitinase (U mL^{-1})	Protease (U mL^{-1})	Lipase (U mL^{-1})
1.	SDB	0.12^{c}	2.00^{d}	0.43^{c}
2.	SDB + chitin (0.3%)	0.26^{b}	2.37^{c}	0.86^{b}
3.	SDB + chitosan (0.3%)	0.28^{a}	4.88^{a}	1.17^{a}
4.	Peptone + chitin (2%)	0.05^{e}	2.47^{c}	0.23^{d}
5.	Peptone + chitosan (2%)	0.09^{d}	2.80^{b}	0.90^{b}
	CD (0.05)	0.015	0.102	0.121

SDB - Sabouraud Dextrose Broth. Values with different letters are significantly different from each other by LSD at 5% level

was 1.28 U mL^{-1} which was superior to that of Peptone + chitosan (1.13 UmL^{-1}). Lipase content in SDB (0.63 UmL^{-1}) ranked fourth and that of Peptone + chitin (0.24 UmL^{-1}) ranked last.

A more or less similar trend was noted with 14 day old cultures too. Highest lipase content (1.17 UmL^{-1}) was observed in SDB + chitosan which was followed by Peptone + chitosan (0.90 UmL^{-1}) and SDB + chitin (0.86 UmL^{-1}). SDB (0.43 UmL^{-1}) ranked third. The lipase content was minimum in Peptone + chitin (0.23 UmL^{-1}).

4.4.3 Role of Cuticle Degrading Enzymes on Virulence

The efficacy of cuticle degrading enzymes in determining the virulence of the fungus was assessed based on the mortality caused by the culture filtrates obtained from various enriched and non enriched media on the test insect, *C. insolitus* nymphs. Both seven day old and 14 day old cultures were used to test the mortality (Table 22 and 23).

In the case of seven day old culture, significant difference was observed among the mortality caused by the fungus grown in different substrates recorded at different intervals of time. After 24 h of treatment, SDB + chitosan showed maximum mortality of 56.66 per cent. It was followed by SDB + chitin (45.33 per cent). Peptone + chitosan (34.33 per cent) was found to be on par with SDB (34.33 per cent). The least mortality was exhibited by Peptone + chitin (24.33 per cent)

After 48 h, a similar trend was noticed with a maximum mortality of 78.33 per cent with the culture filtrate from SDB + chitosan. SDB + chitin (72.66 per cent) ranked second. It was followed by SDB (47.33 per cent). Peptone + chitosan resulted in 45.33 per cent mortality. The lowest mortality was observed for Peptone + chitin (33.33 per cent).

At 72 h also, a similar trend was observed. SDB + chitosan resulted in maximum mortality of 89.66 per cent followed by that recorded with SDB + chitin (83.33 per cent). Lower death rate was observed with SDB (63.66 per cent) followed by that of Peptone + chitosan (54.66 per cent). Least mortality was exhibited by Peptone + chitin (46.66 per cent).

After 96 h, mortality increased from 65.33 per cent (Peptone + chitin) to 95.66 per cent (SDB + chitosan). SDB+ chitin (94.33 per cent) ranked second. This was followed by SDB (91.33 per cent). The mortality exhibited in Peptone + chitosan was 73.33 per cent with least mortality in Peptone + chitin (66.50 per cent).

Hundred per cent mortality was observed after 120 h in the case of SDB + chitosan and SDB + chitin. SDB alone resulted in 94.33 per cent while the mortality exhibited in Peptone + chitosan was 80.33 Least mortality was exhibited by Peptone + chitin (70.33 per cent).

When 14 day old culture was tested a similar trend was noted. After 24 h maximum mortality was recorded with culture filtrates of SDB + chitosan (95.33 per cent), followed by SDB + chitin (90.33 per cent). Non enriched SDB resulted in 84.33 per cent mortality. The mortality recorded with culture filtrate of Peptone + chitosan was 62.33 per cent. Least death rate was recorded with Peptone + chitin (31.33 per cent).

After 48 h of treatment, 100 per cent mortality was observed with SDB + chitosan and SDB + chitin. The trend observed with other treatments was more or less the same as those recorded with seven day old culture filtrate at the same point of time. The corresponding values were 94.33 per cent (SDB), 72 per cent (Peptone + chitosan); and 45.33 per cent (Peptone + chitin).

At 72 h, SDB, SDB + chitin and SDB + chitosan resulted 100 per cent mortality. This was followed by Peptone + chitosan (82.33 per cent). Lowest mortality was exhibited by Peptone + chitin (50.33 per cent).

Table 22. Efficacy of culture filtrates *L. saksenae* (7 day old) cultured in chitin and chitosan enriched substrates

Sl. No.	Substrates	Mean mortality (%) of *C. insolitus* at 24 h interval				
		24	48	72	96	120
1	SDB	34.33 (5.86)^{c}	47.33 (6.88)^{c}	63.66 (7.98)^{c}	91.33 (9.56)^{c}	94.33 (9.71)^{b}
2	SDB + chitin (0.3%)	45.33 (6.73)^{b}	72.66 (8.53)^{b}	83.33 (9.13)^{b}	94.33 (9.71)^{b}	100 (10.00)^{a}
3	SDB + chitosan (0.3%)	56.66 (7.53)^{a}	78.33 (8.85)^{a}	89.66 (9.47)^{a}	95.66 (9.78)^{a}	100 (10.00)^{a}
4	Peptone + chitin (2%)	24.33 (4.93)^{d}	33.33 (5.77)^{e}	46.66 (6.83)^{e}	65.33 (8.08)^{e}	72.33 (8.50)^{d}
5	Peptone + chitosan (2%)	34.33 (5.86)^{c}	45.33 (6.73)^{d}	54.66 (7.39)^{d}	73.33 (8.56)^{d}	80.33 (8.96)^{c}
	CD (0.05)	0.087	0.074	0.066	0.058	0.045

Figures in parentheses are values after $\sqrt{x} + 1$ transformation, SDB - Sabouraud Dextrose Broth. Values with different letters are significantly different from each other by LSD at 5% level

Table 23. Efficacy of culture filtrates of *L. saksenae* (14 day old) cultured in chitin and chitosan enriched substrates

Sl. No.	Substrates	Mean mortality of *C. insolitus* (%) at 24 h interval				
		24	48	72	96	120
1.	SDB	84.33 (9.189)^{c}	94.33 (9.71)^{b}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
2.	SDB + chitin (0.3%)	90.33 (9.50)^{b}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
3.	SDB + chitosan (0.3%)	95.33 (9.76^{a})	100^{a} (10.00)	100 (10.00)^{a}	100 (10.00)^{a}	100 (10.00)^{a}
4.	Peptone + chitin (2%)	31.33 (6.00)^{e}	45.33^{d} (6.73)	50.33 (7.10)^{c}	55.33 (7.44)^{c}	64.55 (8.02)^{b}
5.	Peptone + chitosan (2%)	62.33 (7.90)^{d}	72 (8.49)^{c}	82.33 (9.07)^{b}	94.33 (9.71)^{b}	100 (10.00)^{a}
	CD (0.05)	0.067	0.064	0.042	0.040	0.029

Figures in parentheses are values after $\sqrt{x} + 1$ transformation, SDB - Sabouraud dextrose Broth. Values with different letters are significantly different from each other by LSD at 5% level

After 96 h of treatment, the mortality observed with other treatments was found to increase to 94.33 per cent in Peptone + chitosan and 55.33 per cent in Peptone + chitin.

Hundred per cent mortality was noted in all the treatments except Peptone + chitin (64.55 per cent) at the end of experimental period.

4.4.4 Correlation of Cuticle Degrading Enzymes with Virulence

The correlation coefficient worked out using the enzyme content in UmL^{-1} and the mortality of test insect, revealed that all the three cuticle degrading enzymes *viz.* chitinase, protease and lipase had a strong positive correlation with virulence, the values of which are presented in Table 24. Maximum correlation was obtained with chitinase followed by protease and lipase. The values were 0.978, 0.889, 0.874 at 48 h and 0.752, 0.695 and 0.634 respectively.

4.5. TOXIN PRODUCED BY *L. saksenae*

4.5.1 Detectionof Toxin

The chromatogram developed on thin silica plates sprayed with 0.051 M cerium ammonium sulphate as dye detected an yellow spot (Plate 9) similar to the spot developed by dipicolinic acid spotted on the same plate. This proved the presence of dipicolinic acid in the culture filtrate.

4.5.2 Effect of Crude Toxin on Virulence of the Fungus

Effect of crude toxin on virulence of the fungus was assessed by applying it at varying concentrationsof1000 ppm, 500 ppm, 100 ppm and 10 ppm on *C. insolitus* topically.

Observations on mortality recorded at 24 h interval is presented in Table 25. At 1000 ppm concentration, 100 per cent mortality was recorded after 72 h. When 500 ppm solution was applied, the time taken for 100 per cent

Table 24. Correlation between quantity of enzymes and pathogenicity to *C. insolitus*

Sl. no	Parameters	Correlation coefficients	
		48 h (N = 15)	96 h (N = 15)
1	Chitinase	0.978**	0.752**
2	Protease	0.889**	0.695**
3	Lipase	0.874**	0.634**

** Significant at 1% level

Table 25. Mean mortality of *C. insolitus* treated with different concentrations of crude toxin of *L. saksenae*

SI no	Concentration of toxin (ppm)	Hours after treatment				
		24	48	72	96	120
1	1000	91.33	96.33	100	100	100
2	500	80.33	85.33	94.33	100	100
3	100	21.33	38.66	49.66	58.33	60.33
4	10	0	0	20.33	28.33	32.33
5	1 ppm chloroform	0	0	0	25.22	30.33
6	Fungal spore (10^7 spores mL^{-1})	90.33	100	100	100	100

mortality was 96 h. The mortality recorded with 100 ppm was 60.33 per cent at the end of the experimental period (120 h). Lesser concentrations of 10 ppm crude toxin and 1 ppm chloroform did not cause any mortality till 48 h and at the end of 120 h it recorded 30 per cent mortality. The fungal spore suspension at a concentration of 10^7 spore mL^{-1} resulted 100 per cent mortality at 48 HAT

4.5.3 Quantification of Dipicolinic acid

The chromatogram obtained from High Performance Thin Layer Chromatography (HPTLC) indicates the presence of nine fractions in the crude toxin with dipicolinic acid as one of them. The Rf value of dipicolinic acid noted was 0.26. (Fig. 7)

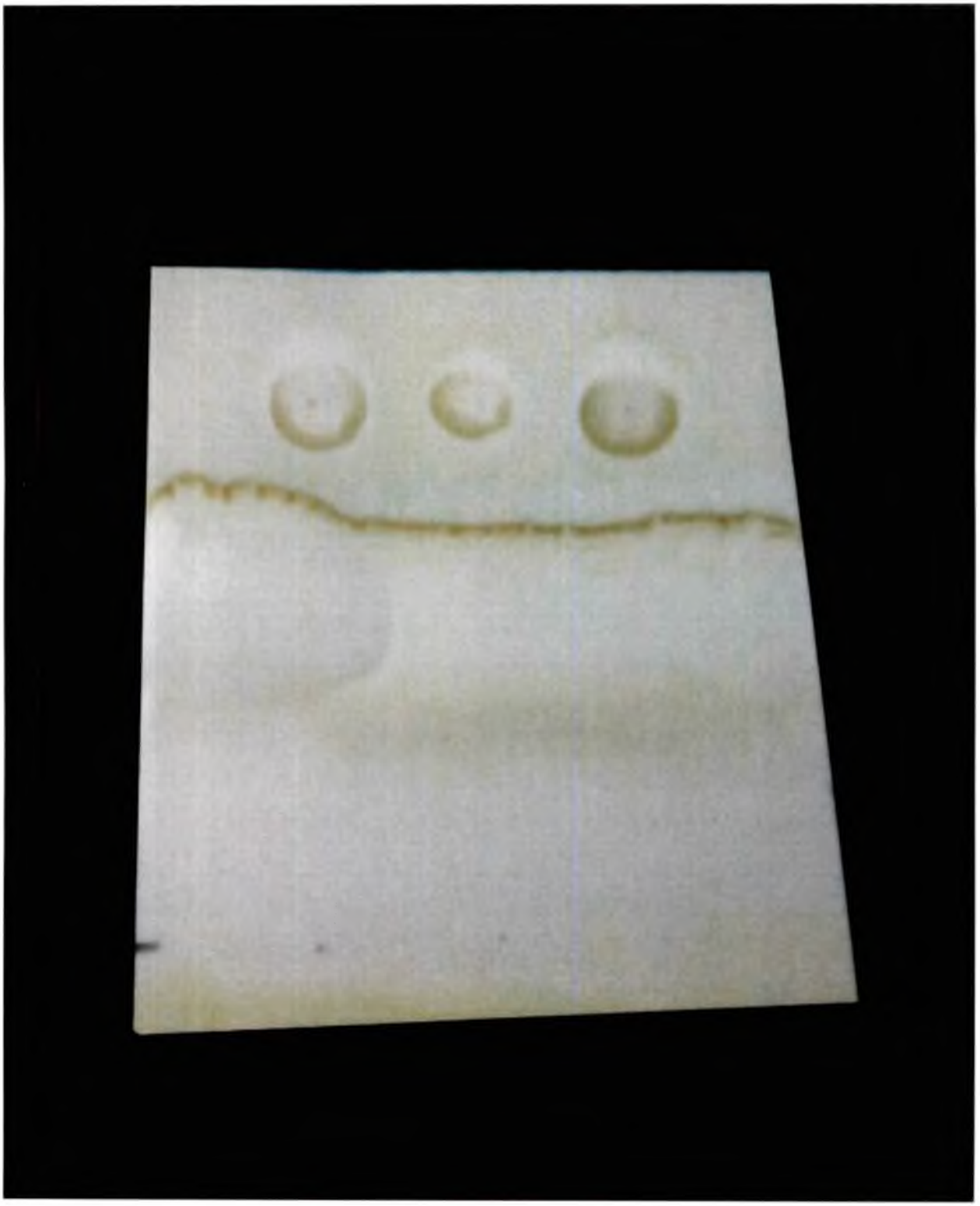


Plate 9. Detection of Dipicolinic acid on Thin Layer Chromatography

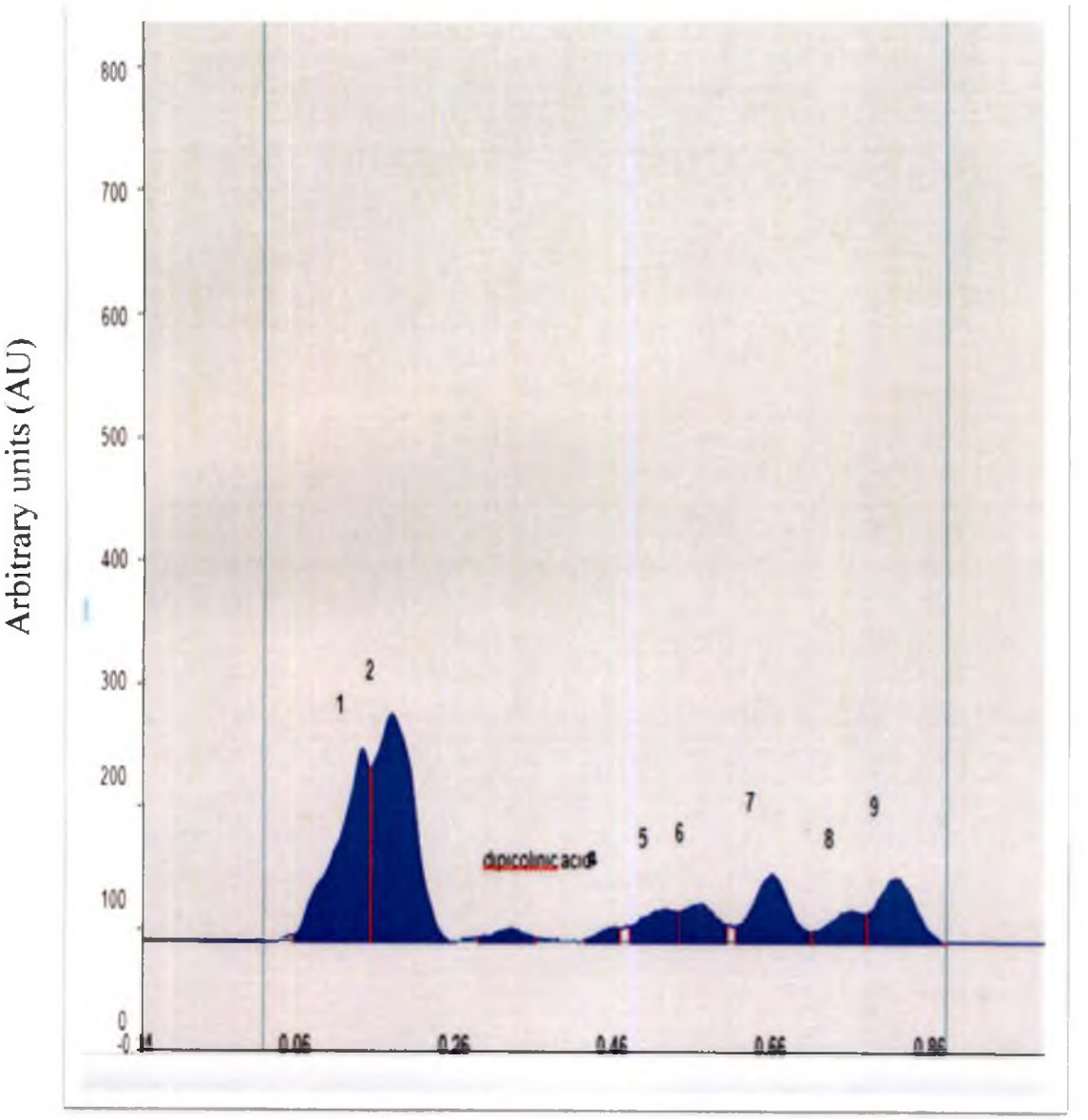


Fig 7. Detection of Dipicolinic acid using High Performance Thin Layer Chromatography

Discussion

5. DISCUSSION

Worldwide interest in the use and exploitation of entomopathogenic fungi (EPF) for biological control of insects and other arthropod pests, paved way to intensified research in the predominant genera *Beauveria*, *Lecanicillium*, *Metarhizium* and *Paecilomyces*. Geographical variation and strain specificity can alter the biological and biochemical traits of EPF. *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno obtained from soils of Vellayani was found to be a promising isolate (ITCC No: Ls.Vs.1-7714) based on the preliminary investigations carried out at Biocontrol Laboratory, College of Agriculture, Vellayani. It was found to be more potent than *L. lecanii* (isolate Ll8) obtained from National Bureau of Agricultural Insect Resources (NBAIR). This new isolate was found to infect the sucking pest complex in vegetable ecosystem (Rani *et al.*, 2014). Development of this fungus as a new biopesticide needs basic investigation on its biological traits such as pathogenicity, cross infectivity, safety to non target organisms, ideal substrate for mass production and also its biochemical properties such as enzyme activity and role of toxins in pathogenicity.

PATHOGENICITY TO INSECT PESTS AND MITES

Pathogenicity is a biological trait that refers to the qualitative ability of a pathogen to cause disease. Genus *Lecanicillium* is known for its pathogenicity to insects, mites as well as nematodes infesting various crop plants. *Lecanicillium* species exhibits a wide host range including insects, mites and nematodes.

The present study revealed that *L. saksenae* is pathogenic to homopteran pests such as black pea aphid, *Aphis craccivora* Koch, the brinjal mealy bug *Coccidohysterix insolitus* Green, the jassid *Amrasca biguttula biguttula* Ishida, the white fly *Bemisia tabaci* Gennadius and the brown soft scale *Lecanium* sp. and to heteropterans such as the rice bug *Leptocorisa acuta* Thunberg and the pod bug

Riptortus pedestris F. It was also infective to the red spider mite *Tetranychus* sp. and the chilly mite *Polyphagotarsonemus latus* Banks. However, it was non infective to some of the heteropteran insects such as the green shield bug *Nezara virudula* L., the red cotton bug *Dysdercus cingulatus* F. and the leaf footed bug *Leptoglossus phyllopus* L. Though the genus *Lecanicillium* is typically a pathogen of soft bodied hemipterans, its pathogenicity to hard bodied hemipterans such as *R. pedestris* and *L. acuta* indicates a wider host spectrum of the fungus within the order Hemiptera that has not been reported earlier. The spectrum of action of this new species makes it an ideal candidate in the management of sucking pest complex including heteropterans too. Such variation in host range among the species may be a natural phenomenon owing to its geographical variation and difference in habitat. Similar observations on the difference in host specificity and inherent virulence within the genus *Lecanicillium* has been reported by Sugomoto and Koike (2002) while working with 44 isolates obtained from different geographical areas.

High level pathogenicity of the most common species *Lecanicillium lecanii* Zimmermann (Zare) and Gams to a wide range of sucking pests has been reported worldwide by several workers. Its first report in India dates back to 1976 when Easwaramoorthy and Jayaraj found it as a pathogen of coffee green scale, *Coccus viridis* Green, infecting various crops. Thereafter, several workers reported its pathogencity to a number of sucking pests. Kanakaratnam *et al.* (1982) reported infectivity of *L. lecanii* to white fly *Trialeurodes vaporariorum.* Westw. while Raheem *et al.* (2009) reported its infectivity to *B. tabaci.* Saranya *et al.* (2010) reported its pathogenicity to aphid *A. craccivora.* Banu and Gopalakrishnan (2012) reported its infectivity to mealy bug *Paracoccus marginatus* Williams and Granara de Willink. Its infectivity to the mite *Tetranychus urticae* L. was reported by Amjad *et al.* (2012). From Kerala, pathogenicity of *L. lecanii* isolate Ll 8 was recently reported by Lokesh (2014) to *A. craccivora, B. tabaci* and *P. latus* and by Nithya (2015) to

A. craccivora, A. biguttula biguttula, B. tabaci, Ferrisa virgata Cockerell, *Lecanium* sp. and *Tetranychus* sp.

L. saksenae was first described as a new species of *Verticillium* by Kushwaha (1980). He reported it as a keratin degrading fungus, isolated from soils of Madhya Pradesh. But, literature pertaining to this fungus are sparse. Its report from Indonesia was as an epiphyte from soil arthropod of Kalimantan Province (Sukarno *et al.*, 2009). Later in 2012 Pinto reported it as a pesticide degrader. Its entomopathogenicity was first reported by Rani *et al.* (2015) who isolated it from soils of Vellayani, Kerala and found it to be pathogenic to the homopterans *A. craccivora, A. gossypii , B. tabaci, A. biguttula bigutulla, C. insolitus* and also to the heteropteran *R. pedestris* and mite *P. latus*.

Symptoms due to the infection of *L. saksenae* were more or less similar in all the treated insects and mites except in *C. insolitus*, where a distinct symptom of shedding of mealy outgrowth on the same day of treatment was observed besides mortality within 24 hours after treatment (HAT). Such an immediate response in the treated insect shows that, *C. insolitus* is the most vulnerable insect to *L. saksenae*. Generally, it takes two to three days for the death of mycosed insects as it occurs after the germination of conidia and penetration of the insect cuticle by the appressorium of the fungus. Occurrence of death within 24 h noted in this case clearly indicates the role of some other factors involved in the pathogenicity of the fungus other than the role of its conidia alone.

Unlike *L. lecanii*, this new indigenous species was found to be pathogenic to *L. acuta* and *R. pedestris* too. This report is thus in concurrence with that of Rani *et al.* (2015).

Though mortality was noted in *R. pedestris* there was no fungal growth over the cuticle. Here, the death or complete paralysis which was noted as death may be

attributed to the action of mycotoxins before the invasion by the fungus. Ferron (1998) opined that the death of insects infected by EPF is accounted by mechanical pressure exerted by excessive fungal growth as well as by the action of mycotoxins produced by it. This was further explained by Brodeur (2012) who reported that the variability in *Lecanicillium* sp. is due to a complex set of events, including various molecular events during the host recognition, enzyme production and production of secondary metabolites.

Inoculam level is an important factor which affects the performance of an entomopathogen. It is a general trend that higher doses give higher mortality, but in order to avoid wastage of inoculam the effective dose is to be determined. Effective dose of *L. saksenae,* determined based on dose-mortality response in various insects revealed that the doses 10^8 and 10^7 spores mL^{-1} were equally effective resulting in more than eighty per cent mortality of both the nymphs and adults of *A. craccivora, C. insolitus, B. tabaci, A. bigutulla bigutulla, Lecanium* sp. *L. acuta* and the adults of plant parasitic mites such as *Tetranychus* sp. and *P. latus.* In the case of *R. pedestris*, a higher concentration of 10^8 spores mL^{-1} was needed to attain the same level of mortality. The susceptibility varied slightly among different species, the time taken for 100 per cent mortality being 48 h in *A. craccivora, C. insolitus, B. tabaci, A. bigutulla bigutulla, L. acuta* and *Lecanium* sp., whereas it took 72 h in the case of mites *Tetranychus* sp. and *P. latus.* In *R. pedestris* there was delay in mortality, the time taken being 96 h. This variation in dose - mortality response among the pests is because of the fact that the defense mechanisms exhibited by each species may vary and even within the species it may vary from habitat to habitat.

Though the effective dose of EPF like *Metarhizium* and *Beauveria* are generally fixed as 10^8 spores mL^{-1}, in the present study the effective dose of *L. saksenae* was determined as 10^7spores mL^{-1} which could result in 100 per cent death within 48 to 72 h after treatment. A comparatively faster action of

Lecanicillium species even with a dose lower than 10^8 spores mL^{-1}, on sucking pests was earlier revealed by Nirmala *et al.* (2006) who reported 80.80 per cent mortality of *A. craccivora* with *L. lecanii* @ 10^7 spore mL^{-1}. In white fly *B. tabaci* also, the best dose of *L. lecanii* was determined as 10^7spores mL^{-1} by Raheem *et al.* (2009). Lokesh (2014) and Nithya (2015) reported that the spore doses of 10^7 spores mL^{-1} and 10^8 spores mL^{-1} of *L. lecanii* were equally effective in the susceptible insects *A. craccivora,*, *Lecanium* sp., *B. tabaci* and *Tetranychus* sp.

In this study, it was generally observed that the nymphs were more susceptible than adults. Stage of host insect usually plays an important role in success of an entomopathogen. Not all stages are equally susceptible to fungal infection. Such stage dependent susceptibility of different sap feeders to *L. lecanii* was earlier reported by various workers. Steenberg and Humber (1999) reported *L. lecanii* (10^7 spores mL^{-1}) to be more infective to the nymphal stage of whiteflies *B. tabaci* than adult. Gindin *et al.* (2000) found that nymphal stages of silver leaf white fly, *Bemisia argentifolii* Bellows and Perring was more susceptible to *L. lecanii* than the adults. Shinde *et al.* (2010) observed that the nymphs of *A. gossypii* were more susceptible to *L. lecanii* than adults.

The observation that *L. saksenae* was non parasitic to the root knot nematode *Meloidogyne incognita* (Kofoid and White) Chit. is in contradiction with the earlier reports on parasitism of *L. lecanii* on the same nematode species as revealed in the work carried out by Meyer (1999). Nguyen *et al.* (2007) reported parasitism of another species *Lecanicillium antillanum* (Castaneda and Arnold) Zare and Gams on the root knot nematode. The difference noted here may be attributed to the variation in fungal species which alters the host specificity.

BIOSAFETY OF *L. saksenae*

A "safe" fungus with respect to invertebrates is essentially the one with a restricted host range. Fungi with narrow host range pose the least threat to non target organisms. Though *Lecanicillium* is one such species with a comparatively less host range, assessment of the possible adverse effects on non target organisms such as crop plants, parasites, predators and other beneficial insects is highly warranted. The work carried out to evaluate its infectivity to crop plants revealed that the fungus at 10^8 spores mL^{-1} did not show any disease symptoms on crop plants *viz.*, cowpea (*Vigna unguiculata* L.), bhindi (*Abelmoschus esculentus* L.), brinjal (*Solanum melongena* L.) and tomato (*Lycopersicum esculentum* L.) in which *Verticillium* diseases were reported. Similar line of works carried out by Gurulingappa *et al.* (2010) revelaed the safety of *Beauveria bassiana* (Balsam) Vuillemin and *L. lecanii* to the crop plants, cotton (*Gossypium hirsutum* L.), wheat (*Triticum aestivum* L.), bean (*Phaseolus vulgaris* L.), corn (*Zea mays* L.), tomato (*L. esculentum*), and pumpkin (*Cucurbita maxima* L). Vidal and Jaber (2015), revealed that *B. bassiana, Metarhizhium anisopliae* (Metsch.) Sorokin and *L. lecanii* live as endophytes in various field crops without any ill effects on crop plants.

Furthermore, *L. saksenae* was also found to be non pathogenic to the coccinellid predators, *Chilomenes sexmaculata* F. and *Coccinella septumpunctata* L. the syrphids, *Ischiodon scutellare* F. and *Xanthogramma scutellare* F. as well as to the spiders, *Tetragnatha maxillosa* Thorell and *Oxyopus* sp. Its non infectivity to the parasites *Bracon brevicornis* F, *Goniozus nephantidis* Muesebeck, *Trichogramma. japonicum* Ashmead and *T. chilonis* and the pollinators *Xylocopa* sp. and wasp *Vespula* sp makes it an ideal candidate in pest management programme.

Various studies on the safety of the genus *Lecanicillium* to the predators and parasites has been carried out earlier. Rondon *et al.* (1982), found that *L. lecanii* was

safe to the aphid predators *Cycloneda sanguinea* L., *Oxyptamus gastrostacus* L. and *Zelus* sp., while Wang *et al.* (2005) reported its safety to the coccinellid *Delphastus catalinae* Horn. It was also found to be safe to the predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Koike *et al.*, 2005). Safety of *L .lecanii* to parasitoids was reported by Kim *et al.* (2005), who found that the fungus at a concentration of 10^8 spores mL^{-1} was non pathogenic to the aphid parasitoid *Aphidius colemani* Viereck.

Investigation on the safety aspects to the beneficial insects such as honey bees proved that *L. saksenae* will not cause any adverse effects on the honey bees *Apis cerana indica* F., *A. mellifera* L., and *Tetragonula iridipennis* Smith. Similar observations are there as reported by Shaw *et al.* (2002), who observed that *L. lecanii* at a concentration of 10^8spores mL^{-1} was non pathogenic to *A. mellifera*. Safety of *L. saksenae* to the pollinators, *Xylocopa* sp. and *Vespula* sp. is supported by the findings of Hall (1982) and Landa (1984) who reported the safety of *L. lecanii* to the beneficial wasp, *Encarsia formosa* Gahan.

CHITIN ENRICHED SUBSTRATES FOR MASS PRODUCTION

Development of an EPF as biopesticide depends on its amenability to mass production. Identification of cost effective substrates that could sustain conidial viability and virulence is the key factor for its mass production. A formulation can meet the prescribed standards only if the spore yield can be maximized at the production level. Apart from identifying the basic substrates on which the fungus can be cultured manipulation of cultural and nutritional parameters can be attempted to favour the production of infective propagules. Culture media and production conditions not only affect the growth and sporulation of the fungi but also the spore viability and virulence (Kmitowa and Popowska - Nowak, 1995;

Kleespiess and Zimmermann, 1998). Optimization of culture media is thus necessary for supporting maximum mycelial growth which should be followed by conidiation.

Experiment carried out to evaluate the solid substrates suitable for *L. saksenae* revealed that agricultural byproducts like wheat bran and rice bran can support its growth and sporulation under ordinary culture conditions with spore yield to the tune of 10^7spores mL^{-1}. A number of research results show that the EPF such as *Metarhizium* and *Beauveria* can be well grown in rice bran (RB) and wheat bran (WB) resulting in a spore yield to the tune of 10^8 spores mL^{-1} (Dorta *et al.*, 1990; Vimaladevi and Prasad, 1996; Puzari *et al.*, 1997; Burges, 1998; Feng, 2000; Vu *et al.*, 2008)

Among the liquid substrates tested, maximum sporulation was noted in enriched media than in non enriched ones. It was Sabouraud Dextrose Broth (SDB) + chitosan 0.3 per cent (2.63×10^8 spores mL^{-1}) that yielded most. However, among the natural substrates, rice water (RW) + chitosan 0.3 per cent was the best (9.22×10^7 spores mL^{-1}) compared to coconut water (CW) + chitosan 0.3 per cent (7.55×10^7 spores mL^{-1}). Chitin (0.3 per cent) enriched media were superior to non enriched media but inferior to chitosan enriched media. Among these RW + chitin (0.50×10^7 spores mL^{-1}) was better than CW + chitin (0.37×10^7 spores mL^{-1}).

The suitability of rice water and coconut water for mass production of various EPF was attempted by several researchers. Rani (2000) reported that the EPF *Fusarium pallidoroseum* (Cooke) Sacc. sporulates well in rice water with a spore yield of 10^8 spores mL^{-1}. Rachappa *et al.* (2005) found that *M. anisopliae* sporulates well in rice water with the spore yield ranging to the tune of 10^8 spores mL^{-1}. Amenability of *B. bassiana* and *L. lecanii* to sporulate in rice water was reported by Sahayaraj and Namasivayam in 2008.

Though, coconut water was reported as an ideal liquid medium for *B. bassiana* (Feng *et al.*, 1994), *M. anisopliae* (Danger *et al.*, 1991, 1999), and *F. pallidoroseum* (Rani, 2000), *L. lecanii* (Sahayaraj and Namasivayam, 2008), attempts to mass produce them in these substrates enriched with chitin and chitosan has not been tried earlier.

More often than not, *Lecanicillium* is a less sporulating fungus with the average yield of 10^7spores mL^{-1} under normal culture conditions, but as observed in this study, enrichment of culture media with nitrogenous polysaccharides like chitin and chitosan enhanced the spore yield by tenfold. Maximum spore yield was obtained in chitosan enriched substrates followed by chitin enriched substrates. Scope of enriching substrates with naturally available sporulation enhancers such has chitosan and chitin was explored by various workers. In *Lecanicillium psalliotae* (Treschew) Zare and Gams, *B. bassiana*, *M. anisopliae, Paecilomyces. lilacinus* (Thom) Samson and *Pochonia chlamydosporia* (Goddard) Zare and Gams, addition of two per cent chitosan added to corn meal agar (CMA) medium profoundly increased conidiation from five to 60 per cent of which *P. lilacinus* showed the highest promotion of conidiation (Palma-Guerrero *et al.*, 2010). Nithya (2015) reported an increase in conidial yield of *L. lecanii* when cultured in SDB amended with five per cent chitosan. She could obtain a tenfold increase in yield (10^8 spores mL^{-1}) as against the yield of 10^7spores mL^{-1} in unamended medium.

In this study, chitin (five per cent) enriched solid substrates too boosted the conidial yield of *L. saksenae* to the tune of 10^8 spores mL^{-1}. Chitin was reported as a sporulation enhancer in *M. anisopliae, B. bassiana, B. brongniartii* and *P. lilacinus.* Higher yield was reported in *B. bassiana* by Hegedus *et al.* (1990), Khachatourians *et al.* (1990), Liu *et al.* (1990), Sun and Liu (2006) and Gerding-Gonzalez *et al.* (2007). In *M. anisopliae,* by Wu *et al.* (2010), in *B. brongniartii* by Sreekanth and Santhalekhmi, (2012), in *P. lilacinus* by Agus *et al.* (2015). In *L. lecanii*, Reyez -

Hernandez *et al.* (2014) found that chitin enriched Sabouraud Dextrose Agar (4g L^{-1}) resulted in higher conidiation of *L. lecanii* isolate ATCC26854, the spore yield being 5.3 x 10^9 spores mL^{-1}. Nithya (2015) obtained a tenfold increase in conidial production by amending SDB with five per cent chitin.

Chitin as a source of carbon probably plays a considerable role in conidiation. However, factors contributing to the onset of conidiation of *Lecanicillium* are not seen elucidated as in the case of *Beauveria* and *Metarhizium*.

The conidial viability of an EPF is greatly influenced by the culture conditions including biotic and abiotic factors. Among the abiotic factors composition of the medium is of prime importance. Carbohydrate and protein content are the essential constituents that determine the viability.

While evaluating the effect of substrates on viability of *L. saksenae* conidia harvested from solid substrates it was noted that maximum number of colony forming units (cfu) of 9.10 x 10^7 mL^{-1} was recorded when it was grown in Rice bran (RB) + chitosan five per cent, followed by RB + chitin five per cent (5.89 x 10^7 mL^{-1}). Without adding chitosan or chitin the number of cfu was significantly low (1.82 x 10^7 mL^{-1}) in RB and Wheat bran (WB) (0.97 x10^7 mL^{-1}). Viability in the case of conidia harvested from WB + chitin (3.46 x 10^7 mL^{-1}) was more than that in WB + chitosan (3.31 x 10^7 mL^{-1}) as well. Thus, from this experiment it is evident that solid substrates such as rice bran and wheat bran enriched with chitosan and chitin can improve the conidial viability of *L. saksenae*.

Assessment of cfu revealed that the chitosan (0.3 per cent) enriched SDB was superior among the liquid substrates tested, in terms of conidial viability (2.95 x 10^8 mL^{-1}) whereas chitosan enriched RW (5.32 X 10^7 mL^{-1}) was the better option among natural substrates.

Although Palma - Guerrero (2010) reported that addition of chitosan (2 mg mL^{-1}) to CMA did not affect the conidial viability of *B. bassiana,* Nithya (2015) proved the suitability of these biopolymers in enhancing the germination level of *L. lecanii* (L1 8) which was attributed to their carbohydrate and protein content. Chitin is a natural polymer composed of simple sugar molecule and chitosan, a derivative of N-acetyl-D-Glucosamine. The potential of chitin and chitosan in increasing the viability of the EPF, *Penicillium* sp. has been earlier reported by Agus *et al.* (2015) who observed 83.32 per cent increase in germination.

Even if it is a known fact that *Lecanicillium* grows well in synthetic media such as SDA and SDB, natural and cost effective substitutes can replace this synthetic media. While working out the cost of production it was observed that natural media such as RB and WB were less expensive (Rs. 20 Kg^{-1}) than SDB (Rs. 84 L^{-1}), both giving an yield of 10^7 spores mL^{-1}. Additional cost incurred for enriching the solid media with chitin five per cent is Rs. 10 /- and with chitosan was Rs. 30/- . To enrich the liquid media, the additional amount incurred for enriching with colloidal chitin 0.3 per cent is Rs. 59 /- and with colloidal chitosan 0.3 per cent was Rs. 79 /-. Taking into account the spore yield, the enriched solid media could provide spore yield to the tune of 10^8 spores mL^{-1} where as that in enriched liquid media and non enriched media is 10^7 spores mL^{-1}. Hence, mass production can be recommended on natural substrates like rice bran or wheat bran enriched with chitin or chitosan. The additional cost incurred for enrichment is justified with a tenfold increase in spore yield.

Prasad and Pal (2014), while analyzing the cost factor for mass production of *L. lecanii* concluded that the spore yield obtained from SDB (1.80×10^8 spores mL^{-1}) was on par with the that from Farm yard manure - FYM (1.85×10^8 spores mL^{-1}). They worked out that there is a cost gain of Rs. 18 Kg^{-1} when FYM is used for mass production.

Conidia are the infective propagules which ultimately lead to mortality of insects and their virulence is generally evaluated in terms of mortality to host insects. Observations recorded on mortality of insects treated with the spores harvested from different media revealed that spores obtained from chitin and chitosan enriched media, both solid and liquid, resulted in 100 per cent mortality of the test insect, *C. insolitus* within 48 h and was found to be superior than the non enriched substrates. It was chitosan enriched media that resulted in higher per cent mortality than chitin enriched ones. However, Kim *et al.* (2013) observed that when *B. bassiana* Bb 08 was cultured in Adamek's medium with and without chitin (one per cent), the resultant spore suspensions did not vary in the mortality (91.7 per cent) of green peach aphid *Myzus persicae* Sulzer.

The ability of chitosan to speed up the death event in treated insects was well explained by Palma - Guerrero *et al.* (2009). They stated that chitosan has the ability to penetrate the cells of insects, and interact with intracellular structures and molecules causing cellular damage and thereby hasten mortality. Their theory was further substantiated by the finding of Nithya (2015) that chitin and chitosan at all the tested concentrations increased the conidiation as well as pathogenicity of *L. lecanii* with the retention of conidial viability. She observed 100 per cent mortality of *A. craccivora*, within 72 HAT when media were amended with chitin or chitosan.

Entry of EPF into the host involves both the enzymatic degradation of cuticle barrier as well as mechanical pressure. An array of cuticle degrading enzymes (CDE) plays a vital role in the interaction between EPF and host insect. Enzyme secretion is thus believed to be a key factor for its virulence (Mustafa and Kaur, 2009).

Qualitative assay carried out to elucidate the enzyme profile of the new indigenous isolate *L. saksenae* showed a positive response with respect to three CDE *viz.*, chitinase, lipase and protease. The pale red colour halo developed by

L. saksenae around the detection well is due to the degradation of chitin present in the media by the enzyme chitinase secreted by the fungus. This halo development is due to the colour change that occurs as a result of degradation of chitin. Agrawal and Kosthane (2012) attributed the halo development in terms of colour change to the change in pH caused by the chitinase activity. In the case of lipase the halo detected was deep red in colour which indicated the degradation of lipids present in the culture filtrate.

Protein degradation by protease was detected by the presence of a blue halo formed due to the degradation of milk protein in the pH indicator medium (pH 5.2). St. Leger *et al.* (1999) while investigating the influence of pH in extracellular enzyme production of *M. anisopliae,* detected a subtilisin and trypsin like protease in the culture media. A comparable halo was detected in the case of *M. anisopliae* by Sharma *et al.* in 2001, which they expressed as a clear zone formed by degradation of lipids by the enzyme, lipase.

The above observations demonstrate how an EPF degrades the essential constituents of insect cuticle such as chitin, lipids and proteins with the help of CDE.

A comparison of substrates used for mass production, with and without addition of chitin and chitosan was made by calculating the enzyme indices so as to reveal the impact of these biopolymers in inducing the fungus in its CDE secretion. Obviously, the indices were elevated in the case of culture filtrates obtained from chitosan and chitin enriched media than in non enriched medium indicating their role in inducing enzyme secretion.

Among the different substrates tested, chitosan enriched SDB indicated high enzyme indices of 4.13, 3.07 and 7.03 respectively for chitinase, lipase and protease on the second day followed by chitin enriched SDB with the indices 4.03, 2.03 and 6.03 respectively. In non enriched SDB the corresponding indices were 3.23, 1.77

Fig. 8 Chitinase index of *L. saksenae* cultured on chitin and chitosan enriched substrates

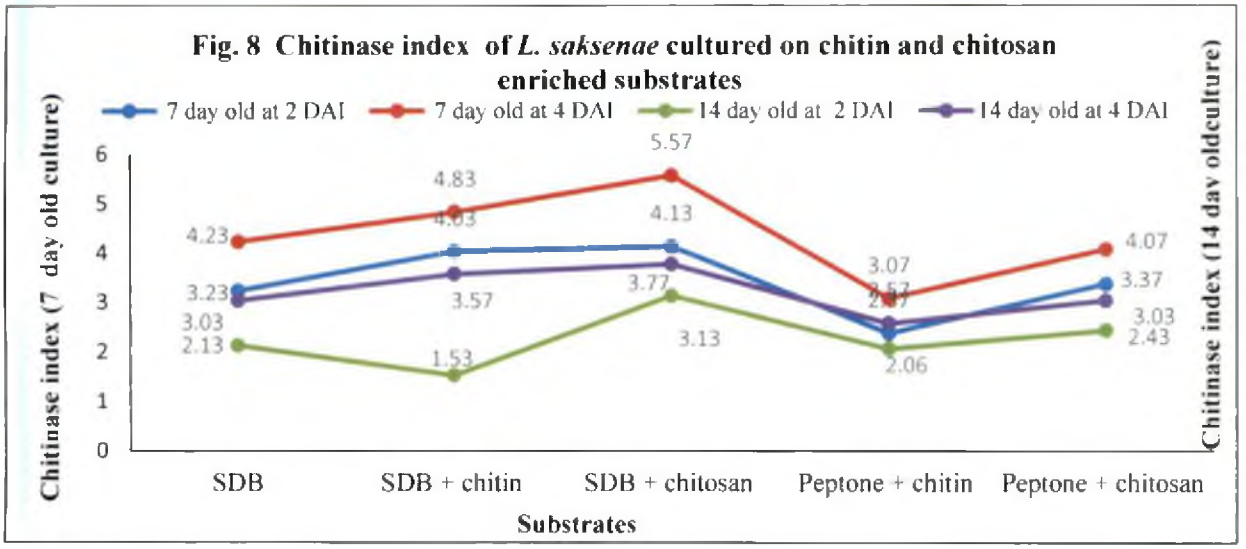


Fig. 9 Proteasee index of *L. saksenae* cultured on chitin and chitosan enriched substrates

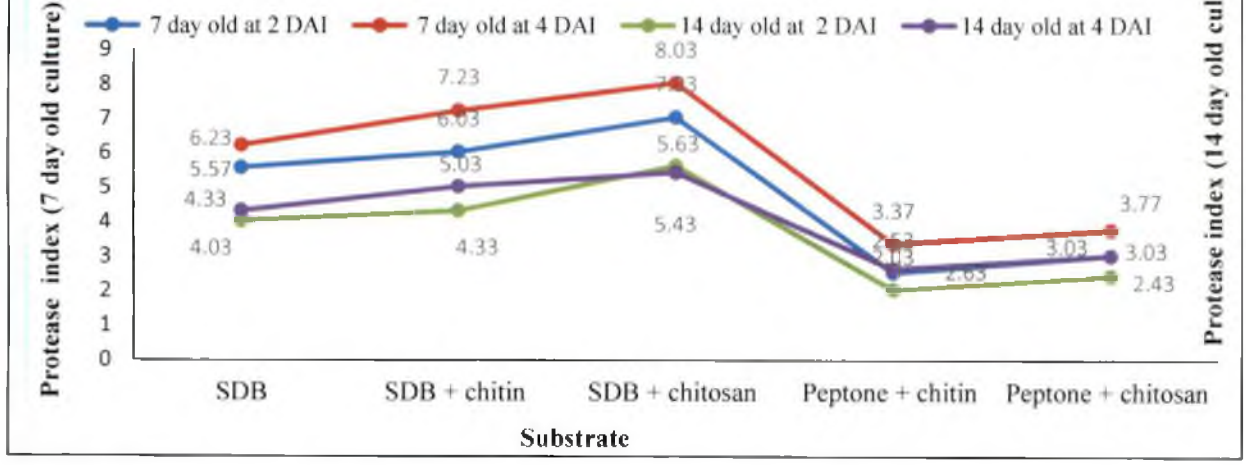
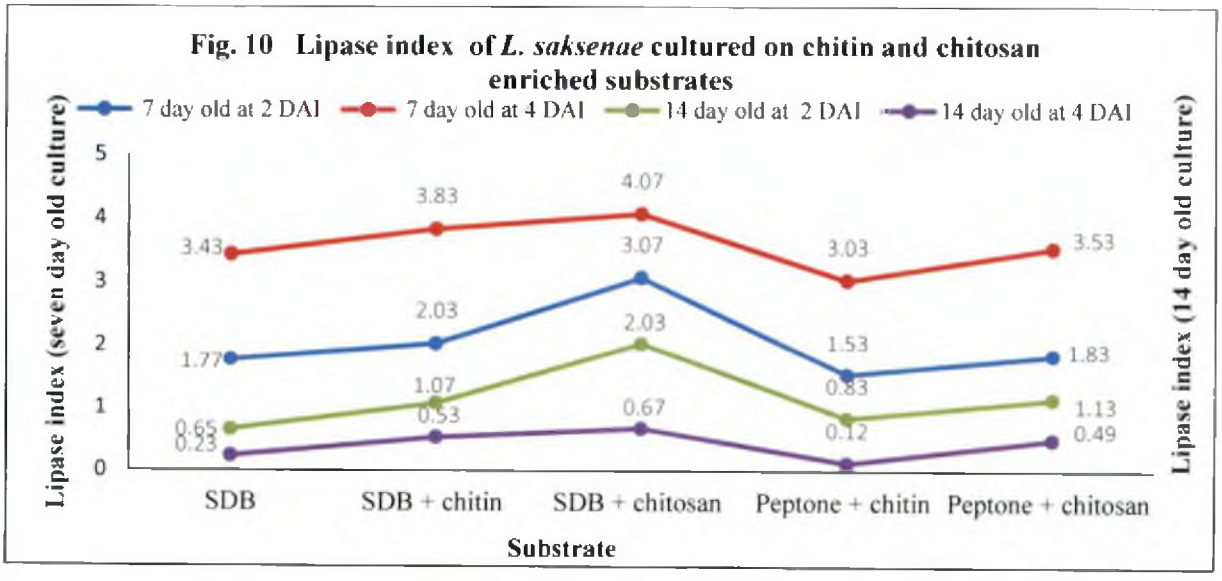


Fig. 10 Lipase index of *L. saksenae* cultured on chitin and chitosan enriched substrates



and 5.57. It also revealed that maximum enzyme production was on the fourth day (Fig. 8 – 10), the indices being 5.57, 4.07 and 8.03 respectively for chitinase, lipase and protease in SDB + chitosan, 4.83, 3.83 and 7.23 in SDB + chitin and 4.23, 3.43 and 6.23 in SDB. These results clearly indicate the dominance of protease among the cuticle degrading enzymes in *L. saksenae*. This was further confirmed by the quantitative assay carried out using spectrophotometry that recorded the CDE content. It was maximum in chitosan enriched SDB (0.36 U mL^{-1} of chitinase; 1.55 U mL^{-1} of of lipase and 6.52 U mL^{-1} of protease). The corresponding content in chitin enriched SDB was 0.26 U mL^{-1} chitinase, 1.28 U mL^{-1} lipase and 4.36 U mL^{-1} protease. The enzyme content in non enriched medium was lower, the values being 0.13, 0.63 and 3.45 U mL^{-1} respectively. It is hereby evident that, among the three enzymes, protease content was more followed by lipase and chitinase.

However, Nahar *et al.* (2004) quantified the chitinase content of *M. anisopliae* to the tune of 0.01 to 0.039 U mL^{-1} and protease content, 0.01 to 0.02 U mL^{-1}, where they did not observe any significant variation in the content of chitinase, lipase and protease. Bai *et al.* (2012), while studying the potential of nine isolates of *M. anisopliae* cultured in Potato dextrose agar - yeast medium found that the chitinase indices ranged from 1.5 to 2.2, lipase from 1.15 to 7.1 and protease from 1.2 to 3.3, wherein they did not observe any significant variation in levels among the enzymes. While quantifying the CDE content they observed that the content varied in different isolates of *M. anisopliae*. It was 0.525 to 1.560 U mL^{-1} of chitinase, 0.153 to 0.500 U mL^{-1} of protease and 0.020 to 0.114 U mL^{-1} of lipase. Hasan *et al.* (2013) studied the production of extracellular enzymes of *L. lecanii* grown in Sabouraud maltose agar yeast and detected the presence of lipase, protease and amylase. The indices worked out by them were 2.14 for lipase, 2.19 each, for protease and amylase on the seventh day. Here also there was no significant difference in the levels among the three different enzymes. On comparison of the enzyme indices worked out with those of Hasan *et al.* (2013) it is seen that, *L. saksenae* has higher levels of protease

(6.23) in non enriched SDB , 8.03 in chitosan enriched SDB and 7.23 in chitin enriched SDB, on the fourth day, while the corresponding levels of lipase observed were 4.07, 3.83 and 3.43. The dominance of protease secretion exhibited by *L. saksenae* as observed in the present study is in corroboration with the observations of St. Legar *et al.* (1987) who reported a rapid production of proteases (Pr-1 and Pr-2) by *M. anisopliae* and of Lopez Llorca *et al.* (1999) who reported that among the various enzymes produced by *L. lecanii,* protease showed the highest activity. Dominance and importance of Protease Pr-1 in the reports of Shinde *et al.* (2010) indicated that it served as major cuticle degrading enzyme in *L. lecanii*. They also reported that protease concentration increased at the site of penetration peg in comparison to enzymes and that it is essential for penetration of the procuticle of host insect. The susceptibility of the host insect to the CDE may vary with the innate defense mechanism exhibited by the species which in turn may be influenced by a complex of biological and environmental factors.

An analysis of the enzyme studies carried out by different researchers cited as above, shows that the levels of all the enzymes secreted by *L. saksenae* is higher than those reported in *M. anisopliae* and *L. lecanii*. This may be due to the fact that in the present study enzyme assay was carried out using the culture filtrates obtained from chitin and chitosan amended media. Nevertheless, their levels were also found to be high in non enriched media when compared to those of other species such as *M. anisopliae* and *L. lecanii*. This indicates the potential of this new species as a fast acting entomopathogen and it warrants the characterization of the genes encoding for CDE production, especially the protease.

One of the reasons for the high level pathogenicity of *L. saksenae* observed in the pathogenicity tests may be due its capacity to produce high levels of cuticle degrading enzymes. This was further established with the observation that *L. saksenae* took lesser time for causing death in target insects, than *L. lecanii*. This

conclusion is in consensus with that of St. Leger *et al.* (1986, 1987), Goettel (1989) and Khachatorians (1991) who demonstrated that expression of protease, lipase and chitinase, potentiate the virulence of entomopathogenic fungi against the target pests.

Literature on evaluation of substrates for their ability to induce secretion of CDE was found to be meager. However, a few workers have pointed out the significance of enriching the substrate with skimmed milk, colloidal chitin and tributyrin to enhance CDE secretion in EPF. St. Leger *et al.* (1999) and Djamel *et al.* (2009) found that during the assay studies addition of one per cent skimmed milk in the detection well contributes to increased protease secretion. Abirami (2012) stated that addition of two per cent colloidal chitin enhanced chitinase production and Sulochana *et al.* (2014) observed that tributyrin in the detection well enhances lipase production during assay. Comparison of age of the culture with enzyme activity, it was noted that seven day old culture showed peak activity of CDE. The indices of 14 day old culture showed a declining trend. This finding is in accordance with that of Hasan *et al.* (2013), who noted that the enzyme activity of *L. lecanii* was highest on seventh day of inoculation and thereafter it decreased by fourteenth and twenty first day.

Studies on bioefficacy of CDE on the test insect *C. insolitus* also revealed that chitosan and chitin enriched culture filtrates resulted in hundred per cent mortality after 120 h while the effect was profoundly increased when 14 day old cultures were used where it took only 72 h. The correlation coefficient worked out using the enzyme content in units mL^{-1} and the mortality of *C. insolitus* revealed that all the three CDE *viz.,* chitinase, protease and lipase had a strong positive correlation with the virulence. Results of the study conducted by Askary *et al.* (1997) showed that the enzymatic action would favour the penetration of *L. lecanii* in the host and further saprophytic exploitation of host tissues. Kaur and Padmaja (2009) reported that there is certain correlation between pathogenicity and enzymes production which resulted

cuticular structure weakening or slimming. Moreover they cause damages to the peritrophic membrane in the intestine which leads to its death (Binod, et *al.*, 2007). Perusal of literature revealed limited studies on correlation coefficient between enzyme levels of EPF and mortality of insects.

EPF are prolific producers of bioactive secondary metabolites which operate as either defensive or offensive chemical weapon against pests. It helps the fungus in its virulence towards target insects. Although much of the research works related to pathogenicity and virulence have been focused towards the initial steps of invasion through cuticle penetration, and invasion of haemocoel, a few researchers have directed their study to learn the role of toxins in fungal pathogenesis. The high level of pathogenicity observed for *L. saksenae* had been a real instinct to investigate its metabolite profile. Apart from the role of conidia and enzyme activity, the role of toxins present should also be explicated . Crude toxin @ 2 g L^{-1} could be extracted from *L. saksenae* through silica coated chromatographic column using phosphate buffer as eluent. The TLC performed using 0.051 M cerium ammonium sulphate, detected an yellow spot indicating the presence of dipicolinic acid (DPA), with an Rf value of 0.26. It is noteworthy that the bioefficacy studies conducted on nymphs of *C. insolitus* using the crude toxin revealed that the crude toxin when applied topically @ 1000 ppm resulted in 91.33 per cent mortality at 24 HAT. In this context, it may be noted that spore suspension @ 10^7 spores mL^{-1} caused 90.33 per cent mortality at the same point of time and the culture filtrate which is devoid of spore resulted in 34.33 per cent mortality. Investigation using High Performance Thin Layer Chromatography (HPTLC) confirmed the content of DPA in crude toxin. Nevertheless, the content was low to the extent of 0.044 per cent.

While studying the effect of crude toxin, Wang *et al.* (2007) reported that the crude toxin extracted from *L. lecanii* strain V3450 and Vp28 had ovicidal, repellant and antifeedant activities on *B. tabaci* and the nymphal stage was more susceptible.

Chun Li *et al.* (2010) observed a strong pathogenicity of crude toxin extracted from *L. lecanii* strain MZ041024 to the second instar larvae of *Spodoptera exigua* Hubner. Karthikeyan and Selvanarayanan (2011) also reported that the crude toxin at 2500 ppm extracted from *L. lecanii* resulted in hundred per cent mortality of *A. gossypii* and *B. tabaci* on second day after treatment.

Reports of toxins produced by *L. lecanii* dates back to those by Suzuki *et al.* (1977) and Kanaoka *et al.* (1978) who detected the bioactive compounds such as beuvericin and bassianolide; Claydon and Grove (1982) who detected DPA and Soman *et al.* (2001), who obtained vertilecanin-A1, decenedioic acid and 10-hydroxy-8-decenoic acid. The low yield of DPA (0.044 g L^{-1}) as observed in this study was similar to that noted by Assaf *et al.* (2005) who reported that the yield of DPA was low in *P. lilacinus* and the maximal concentration was only 0.041 g L^{-1}. Detection of eight other peaks in the chromatogram developed through HPTLC performed as a part of this study indicates the presence of eight other components other than DPA. Peaks higher than that of DPA indicate the presence of some other strong toxic metabolites produced by *L. saksenae* which necessitate further investigation on its metabolite profile.

The present study establishes the pathogenicity and virulence of the indigenous isolate of *L. saksenae* (ITCC Accession no: Ls.Vs.1-7714) through a chronological investigation on the symptoms developed, host range, cuticle degrading enzymes and mycotoxin production. It also throws light to its cost effective production methods as well as the ways and means to improve its virulence by enhancing conidiation and inducing enzyme secretion. The investigation indicates the possible utilization of metabolites such as DPA as environment friendly plant protectants. Characterization of gene that encode protease secretion and detection of unknown metabolites other than chitinase, lipase, protease and DPA are the future areas to be addressed.

Summary

6. SUMMARY

The genus *Lecanicillium* comprises of entomopathogens effective against sucking pests. An indigenous isolate of *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno (ITCC No: Ls.Vs.1-7714) obtained from the cultivated soils of Vellayani was found to be more pathogenic than the popular *L. lecanii* (L18) isolate of National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. The present work was undertaken with an aim to study its biological and biochemical properties that would establish its virulence to insect, mite and nematode pests. The research thus included a sequential investigation of its pathogenicity to insect, mite and nematode pests, cross infectivity to crop plants, safety to predators, parasitoids, pollinators and other beneficial insects. The study standardized the cost effective substrates for mass production of the bioagent and the methods to enhance sporulation and enzyme activity during the mass production. Qualitative and quantitative assay of the cuticle degrading enzymes, extraction of crude toxin, its bioefficacy and finally the detection of toxic components produced by the fungus were also explicated in this investigation.

Pathogenicity studies revealed that the fungus at a concentration of 10^7 spores mL^{-1} is infective to homopteran pests such as the aphid *Aphis craccivora* Koch, the brinjal mealy bug *Coccidohysterix insolitus* Green, the jassid *Amrasca biguttula biguttula* Ishida, the whitefly *Bemisia tabaci* Gennadius, the brown soft scale *Lecanium* sp, and also to the heteropteran pests such as the rice bug *Leptocorisa acuta* Thunberg and the pod bug *Riptortus pedestris* F. It was also found infective to plant parasitic mites *viz*., red spider mite *Tetranychus* sp. and chilly mite *Polyphagotarsonemus latus* Banks. Neither the lepidopteran nor the coleopteran pests tested were found to be susceptible to the fungus. It was non parasitic to the root knot nematode *Meloidogyne incognita* (Kofoid and White) Chit.

Symptoms of infection noticed in the susceptible organisms such as *A. craccivora, A. biguttula biguttula, B. tabaci, Lecanium* sp., *Tetranychus* sp. and *P. latus* were more or less similar. The treated insects became inactive and pale within 24 HAT. A higher per cent mortality was observed within 24 HAT. The cadavers showed fluffy white mycelial growth within two days after death. Of the various organisms tested, *C. insolitus* was most susceptible and produced distinguishable symptoms. The infected mealy bugs became naked and brownish by shedding the mealy outgrowth within 24 HAT and later the body was covered with white mycelial growth. Interestingly, *R. pedestris* was immediately paralysed with no mycelial growth indicating the presence of some other factors other than spores, responsible for paralysis and death.

Dose mortality response revealed that as the spore concentration increased, the time taken for mortality reduced. Two doses, 10^8 and 10^7 spores mL^{-1} resulted in more than ninety per cent mortality of the nymphs as well as adults of *A. craccivora, C. insolitus, B. tabaci, A. bigutulla bigutulla, Lecanium* sp. and *L. acuta* at 48 HAT. In the case of mites, *Tetranychus* sp. and *P. latus* it took 72 h. Therefore 10^7 spores mL^{-1} can be selected as the effective dose.

Cross infectivity studies ruled out the chances of this EPF being harmful to those crop plants in which *Verticillium* diseases were reported. Leaf as well as soil inoculation of 10^8 spores mL^{-1} did not cause any disease symptoms on cowpea, bhindi, brinjal and tomato. *L. saksenae* was found safe to the coccinellid predators, *Chilomenes sexmaculata* F. and *Coccinella septumpunctata* L., the syrphid predators, *Ischiodon scutellaris* F. and *Xanthogramma scutellare* F. and spiders such as *Tetragnatha* sp. and *Oxyopus sp.* It was also found to be safe to parasitoids such as *Bracon brevicornis* F., *Goniozus nephantidis* Muesebeck, *Trichogramma japonicum* Ashmead and *T. chilonis* Ishii. The treated organisms completed their normal life cycle and the trichocards showed normal rate of adult emergence. The common

pollinator *Xylocopa* sp. and *Vespula* sp., as well as the productive insects such as Indian bee, *Apis cerana indica* F., the Italian bee *A. mellifera* and also the stingless bee *Tetragonula iridipennis* Smith were found unaffected by the fungus.

Experiment that evaluated the cost effective and enriched solid substrates for mass production, disclosed the suitability of natural substrates such as rice bran or wheat bran, which could further be enriched with the natural biopolymers like chitin and its derivative chitosan for increasing the spore yield. The spore yield could be increased by tenfold by enriching rice bran with five per cent chitosan followed by rice bran enriched with five per cent chitin. Considering the number of viable spores also, rice bran + chitosan was superior while rice bran + chitin ranked second. Among the liquid substrates tested, rice water + chitosan was the best in terms of spore yield and number of viable colonies followed by chitin enriched rice water. The efficacy of *L. saksenae* spores harvested from different culture media revealed that the biopolymers chitin and chitosan can increase the virulence of conidia. Conidia harvested from chitosan and chitin enriched substrates resulted in 100 per cent mortality within 48 h whereas those from non enriched media took 72 h.

Considering the cost factor, price per kilogram of medium was high in the case of synthetic medium SDB (Rs. 84/-), compared to bran (Rs. 20/-). When the bran was enriched with chitosan it incurred an additional amount of Rs. 30/- and when enriched with chitin it incurred an additional amount of Rs. 10/-. In the case of liquid substrates, enrichment with colloidal chitosan needs an extra expenditure of Rs. 79/- and that with colloidal chitin needed an extra cost of Rs. 59/- . The marginal increase in cost of raw material for enrichment was negligible considering the ten fold increase in spore yield.

The experiment to elucidate the main cuticle degrading enzymes (CDE) secreted by the fungus, revealed the presence of chitinase, lipase and protease in its

culture filtrates. When different media were tested for their suitability in inducing chitinolytic enzymes, it was observed that SDB + 0.3 per cent chitosan was the best as indicated by the high enzyme index of 4.13, 3.07, and 7.03, respectively for chitinase, lipase and protease on the second day after inoculation. SDB + chitin ranked next and SDB alone ranked last. Highest value recorded was always for protease.

Quantitative assay using spectrophotometric analysis revealed that there was a significant variation in all the three cuticle degrading enzymes produced in different substrates. The content was maximum in SDB + 0.3 per cent chitosan followed by SDB + 0.3 per cent chitin. The quantity calculated was 6.5 U mL^{-1} of protease, 1.55 U mL^{-1} of chitinase and 0.36 U mL^{-1} of lipase. It is also worth mentioning that the enzyme assay studies revealed that the enzyme activity was more in seven day old cultures than 14 day old ones and also the activity of enzymes reached its peak on the fourth day and that protease was the dominant cuticle degrading enzyme of *L. saksenae.*

Bioefficacy study of seven day old culture filtrates from the above mentioned media revealed that those obtained from chitosan and chitin enriched media resulted in hundred per cent mortality of *C. insolitus* nymphs after 120 HAT. The time taken for the same was profoundly decreased to 72 h when 14 day old culture filtrate was used. Correlation coefficient worked out using the enzyme content in units mL^{-1} and the mortality of *C. insolitus* revealed that all the three cuticle degrading enzymes *viz.*, chitinase, protease and lipase had a strong positive correlation with the virulence of the fungus.

The fungus produced crude toxin @ 2 g L^{-1} as detected through silica coated chromatographic column using phosphate buffer as eluent. The presence of dipicolinic acid (DPA) was detected as a yellow spot by Thin Layer Chromatography

(TLC) using 0.051 M cerium ammonium sulphate as dye. The Rf value noted was 0.26. On evaluating its bioefficacy on the test insect at varying concentrations the highest concentration of 1000 ppm resulted in 91.33 per cent mortality, 24 h after topical application while the corresponding mortality with spore suspension was 90.33 per cent.

High performance thin layer chromatography performed to quantify the DPA could separate out DPA @ 0.044 per cent of the total crude toxin extracted. The chromatogram revealed the presence of eight dominant metabolites other than DPA which needs further investigation.

It is concluded that *L. saksenae* is a potent entomopathogen effective to hemipteran pests, and plant parasitic mites @ 10^7 spores mL^{-1}. It is safe to non target organisms such as crop plants, insect predators, parasitoids, pollinators and productive insects. Rice bran or wheat bran enriched with chitosan or chitin is an ideal cost effective substrate for improving spore yield, conidial viability as well as virulence of the fungus. *L. saksenae* isolate secreted three cuticle degrading enzymes namely chitinase, lipase and protease of which protease was the dominant one. Addition of chitin or chitosan to the culture medium could improve its enzyme activity. The fungus secretes an array of toxic compounds of which DPA alone caused 91.33 per cent mortality to the test insects, when applied topically @ 1000 ppm. Eight other higher peaks noted in the chromatogram indicated the presence of some other strong toxic metabolites produced by the fungus, which warrants further investigation.

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PATHOGENICITY AND BIOCHEMICAL PROPERTIES OF ENTOMOPATHOGENIC FUNGUS *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno

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ABSTRACT

The investigation entitled "Pathogenicity and biochemical properties of entomopathogenic fungus *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno" was conducted at College of Agriculture, Vellayani during 2014 -2016. The objective was to study the pathogenicity and biochemical properties of *L. saksenae* in order to establish its virulence to insect, mite and nematode pests.

L. saksenae is an indigenous isolate from the soils of Vellayani. Pathogenicity studies revealed infectivity of the fungus to the hemipteran pests, *Aphis craccivora* Koch, *Coccidohysterix insolitus* Green, *Amrasca biguttula biguttula* Ishida, *Bemisia tabaci* Gennadius, *Lecanium* sp, *Leptocorisa acuta* Thunberg and *Riptortus pedestris* F. Apart from this it was pathogenic to the plant parasitic mites, *Tetranychus* sp. and *Polyphagotarsonemus latus* Banks . On the other hand, neither the lepidopteran nor the coleopteran pests tested were found to be susceptible to the fungus. It was non parasitic on eggs of root knot nematode, *Meloidogyne incognita* (Kofoid and White) Chit. The effective dose for pathogenicity was determined as 10^7 spores mL^{-1} for all the above pests except for *R. pedestris*, where it was 10^8 spores mL^{-1}.

The fungus was non pathogenic at a spore concentration of 10^8 spores mL^{-1} to crop plants *viz.*, cowpea, bhindi, brinjal and tomato in which *Verticillium* diseases were reported. It was safe to the coccinellid predators, *Chilomenes sexmaculata* F. and *Coccinella septumpunctata* L., the syrphids, *Ischiodon scutellare* F. and *Xanthogramma scutellare* F. and spiders, *Tetragnatha maxillosa* Thorell. and *Oxyopus* sp., the parasitoids *Trichogramma. japonicum* Ashmead , *T. chilonis*, *Bracon brevicornis* F., *Goniozus nephantidis* Muesebeck as well as to the pollinators *Xylocopa* sp. and *Vespula* sp. The beneficial insects *Apis cerana indica* F.. *A. mellifera* and *Tetragonula iridipennis* Smith were found to be unaffected by the fungus.

Among the various substrates tested chitosan (five per cent) enriched rice bran was the best solid substrate for mass multiplication with a maximum spore yield (7.27 x 10^8 spores mL^{-1}), number of viable colonies (9.10 x 10^7 cfu mL^{-1}) and mortality to *C. insolitus* (95.66 per cent) at 14 DAI . Of the liquid substrates, though RW + chitosan

(0.3 per cent) was superior with 9.22 x 10^7spores mL^{-1} and 5.32 x 10^7 cfu mL^{-1}, considering the cost factor and yield, Rice bran or Wheat bran enriched with chitosan 0.3 per cent is the best that supports maximum spore yield and number of viable colonies.

Qualitative assay revealed the presence of cuticle degrading enzymes chitinase, protease and lipase, the activity of which was maximum in Sabouraud Dextose Broth + chitosan, the indices being 5.57, 8.03 and 4.07 respectively. Quantitative assay indicated the dominance of protease (6.52 mL^{-1}) followed by lipase (1.55 U mL^{-1}) and chitinase (0.36 mL^{-1}). The experiment conducted to study the bioefficacy of enzymes on the pathogenicity of the fungus revealed a strong positive correlation (0.978, 0.889 and 0.874) between all the cuticle degrading enzymes *viz.*, chitinase, protease and lipase and the pathogenicity of the fungus.

L. saksenae produced crude toxin @ 2 g L^{-1} which resulted in 91.33 per cent mortality of *C. insolitus* at 1000 ppm after 24 h. The chromatographic separation of crude toxin revealed the presence of dipicolinic acid on a thin silica plate with Rf value 0.26. Confirmation and quantification using HPTLC detected dipicolinic acid at a concentration of 0. 044 per cent along with eight other higher peaks indicating the presence of an array of strong unknown metabolites.

The investigation concludes that *L. saksenae* is an efficient entomopathogen to hemipteran pests @ 10^7spores mL^{-1}, and it is safe to crop plants and non target organisms. It multiplies well in rice bran with chitosan (0.3 per cent) as a sporulation enhancer. Among the cuticle degrading enzymes protease is the dominant one. Dipicolinic acid present in crude toxin resulted in 91.33 per cent mortality of test insect. A detailed metabolite profile of the fungus and identification of genes responsible for hike in protease can tap the potential of *L. saksenae* to its maximum.