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Volatile metabolites of *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno and their toxicity to brinjal mealybug *Coccidohystrix insolita* (G)

P. Sreeja and O. P. Reji Rani

Department of Entomology, College of Agriculture, Kerala Agricultural University, Vellayani, Thriruvananthapuram, Kerala, India 695 522. Email:sreejaranjit1@gmail.com

ABSTRACT: *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno is a versatile indigenous entomopathogenic fungus with high speed of kill on hemipteran insects. Investigations were carried out to explore the volatile metabolites of *L. saksenae* and bioefficacy of its crude toxin to different life stages of brinjal mealybug, *Coccidohystrix insolita.* GCMS spectrum of crude toxin extracted from cultures grown in potato dextrose broth and Czapak Dox medium revealed the presence of 25 compounds each. The major secondary metabolites identified were 2,6 pyridine dicarboxylic acid (dipicolinic acid), n-hexadecanoic acid, octadecanoic acid, harmine, dl- mevalonic acid lactone, 2-piperidinone, 4H-pyran-4-one 2,3-dihydro-3,5dihydroxy-6methyl, acetamide,N-(2-phenylethyl), pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro. The biological properties of these compounds include insecticidal to nematicidal and antimicrobial activities. Bioefficacy studies with crude toxins revealed the toxicity of secondary metabolities to *C. insolita.* The dose dependent bioassay revealed 100 per cent moratality at a higher concentration of 1000 ppm at 72 and 96 h after treatment on nymphs and adults respectively. Results highlighted the role of secondary metabolites in the pathogenicity of *L. saksenae* and pave way to the utilization of its biocide molecules in safer pest management.

KEY WORDS: Dipicolinic acid, volatile metabolites, *Lecanicillium saksenae* , GCMS

INTRODUCTION

Entomopathogenic fungi constitute the largest taxa of insect pathogens with approximately 750 to 1000 species distributed over 100 genera placed under the order Hypocreales and Entomophthorales (St. Legar and Wang, 2010). They are known to synthesize different secondary metabolites that act as toxins in insects resulting in a series of symptoms such as convulsions, lack of coordination, behaviour alteration, feeding cessation, paralysis and death. The first systematic study of toxin production by fungal entomopathogens *in vitro* was conducted on *Metarhizium anisopliae* which lead to the discovery of two novel insecticidal substances destruxin A and destruxin B (Kodaira, 1961). The genera *Beauveria* and *Lecanicillium* are also known to produce toxic metabolites *in vitro* and

* Author for correspondence

few of them have been characterized for their metabolites.

The genus *Lecanicillium* includes various species which are pathogenic to a broad range of pests such as insects, phytophagous mites, plant parasitic nematodes, and plant pathogens as well. *L. saksenae* ITCC - LsVs 1-7714 is an indigenous isolate from Kerala, India (Rani *et al*., 2015). It is pathogenic to sucking pests including the true bugs of Heteroptera (Sankar and Rani, 2018). *L. saksenae* which is speculated to be a potent toxin producer, as evidenced by the knock down action in rice bug *Leptocorisa acuta* Thunberg warrants investigation on its metabolite profile. An insight into the identification and bioassay of extracellular metabolites produced by *L. saksenae* would pave way for the development of novel biomolecules with potent insecticidal activity. Recent advances in the analytical techniques facilitate easier separation, identification and structural determination of biomolecules. Among different analytical techniques, GCMS is an authoritative tool for identification and quantification of volatile molecules. In this study, we attempted to profile the volatile secondary metabolites produced by *L. saksenae* by GCMS analysis and assessed their bioefficacy on one of its homopteran host *viz*. brinjal mealybug, *C.insolita.*

MATERIALS AND METHODS

Fungal culture and extraction of metabolites

Culturing was carried out in 250 ml Erlenmayer flasks containing 75 ml of growth media. *L. saksenae* (ITCC Accession No: LsVs1 -7714) was inoculated into two different media *viz.* potato dextrose broth (P^H 5.6) and Czapak Dox medium (P^H 7) with conidial suspension @ $1x10^7 mL^{-1}$. These cultures were incubated at 27°C in an incubator shaker with 150 rpm for 12 days. They were then centrifuged at 4°C for 10 min at 10000 rpm, to remove the mycelia and spores and the supernatants were filtered through millipore filters of pore size 0.45 µm. The filtrates were then divided into 100 mL aliquots and then extracted thrice with equal quantity of ethyl acetate for 10 min at 250 rpm. Ethyl acetate fractions from two different growth media were collected separately and concentrated under vacuum rotary evaporator at 40°C. Dried extracts were reconstituted with methanol and subjected to chromatographic analysis.

Gas chromatography - mass spectrometry (GCMS) analysis

The ethyl acetate extracts of *L. saksenae* cultured in PDB and Czapak Dox media were analysed separately in a Perkin Elmer (CLARUS SQ8C) system equipped DB-5 MS capilary standard non – polar column (dimension: 30mts, Id: 0.25 mm, film: 0.25 µm). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}; split ratio of 10 : 1; mass scan 50-600 Da; ionization energy,70 eV; ion source temperature, 240^{0}C and injector temperature, 250^{0}C. The oven temperature was programmed initially at 60^0 C for 2 min, raising by 10^0 C min^{-1} to 300 ^{0}C and then held isothermally for 6 min at 300^{0}C, with a total run time of 35 min. The chemical compounds were identified and characterized based on their retention time (RT). The mass spectral data retrieved from GC-MS was computer matched with those of the standards available in the the database of National Institute of Standards and Technology (NIST) 08 mass spectrum library, having more than 62,000 patterns.

Bioassay

Bioassay was carried out in different life stages of *C. insolita*. The test insects were reared on sprouted potato tubers under controlled conditions of 27°C (Mani and Shivaraju, 2016). The crude toxin extracted from PDB was dissolved in sterile distilled water and centrifuged at 10000 rpm for 5 minutes. The supernatant were made up to the test concentrations of 10, 50, 100, 250, 500 and 1000 ppm, by mixing it with distilled water containing 0.03 per cent Tween 80. Third instar nymphs and adults of *C. insolita* (30 each) of uniform age were transferred separately using a fine camel brush on to brinjal leaves kept inside Petri dishes lined with moist tissue paper. The tests insects were sprayed uniformly with different test concentrations using an atomizer. The treatment with extract of blank medium in distilled water containing 0.03 per cent

tween 80 served as control. The treated insects were air dried and the Petri plates were sealed with parafilm and kept at 27°C. Mortality was recorded at 24 h interval for a period of 96 h. The mortality was corrected using Abbott's formula and subjected to statistical analysis.

RESULTS AND DISCUSSION

GCMS chromatogram of crude toxin extracted from PDB and Czapak Dox cultures are depicted in Fig.1 and 2 respectively. Chromatogram 1 represents the extracellular metabolites produced in PDB. It revealed the presence of 25 compounds of varying chemical groups. Table 1, section A reveals the dominating bioactive compounds including two fatty acid compounds, octadecanoic acid, n-Hexadecanoic acid and an organic acid 2,6 pyridine dicarboxylic acid (dipicolinic acid- DPA). Chromatogram II depicts 25 compounds detected in Czpak dox medium which included alkaloids, terpenoides, fatty acids, cyclic esters and ketones. Chemical names of eight major bioactive metabolites with RT values are detailed in Table 1, section B. Biological activity of the metabolites isolated from *L. saksenae* is illustrated in Table 2, with reference to Dr. Duke's phytochemical and ethanobotanical database (Duke, 2013).

The present study is the first report of the volatile insecticidal metabolites of *L. saksenae*. The paralysis and death of insects treated with the culture filtrates on the same day of treatment as observed in the preliminary studies by Rani *et al*. (2015), Jasmy (2016) and Sankar (2017) was the real instinct to investigate its metabolite profile. As

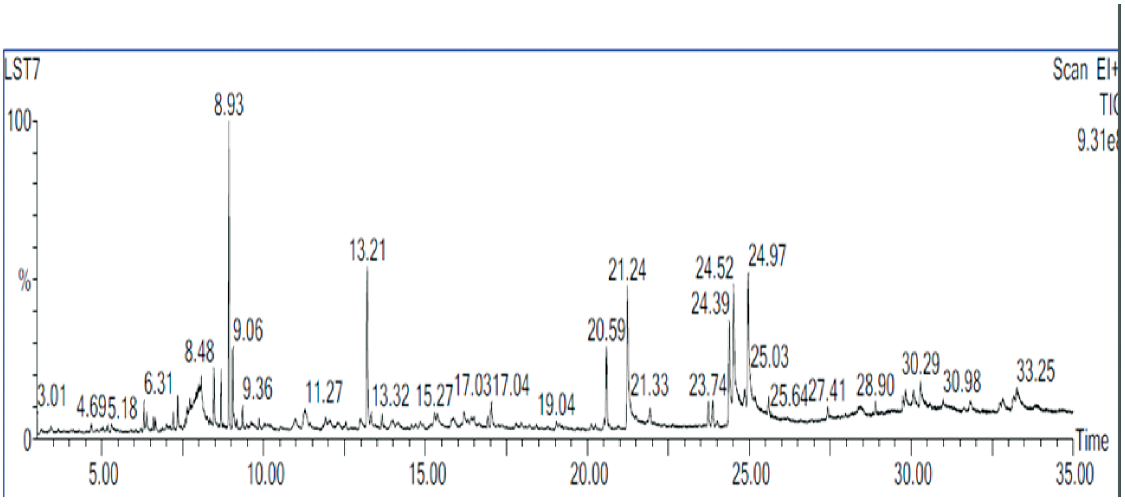

Fig. 1. Chromatogram of ethyl acetate fraction of *L. saksenae* in PDB medium

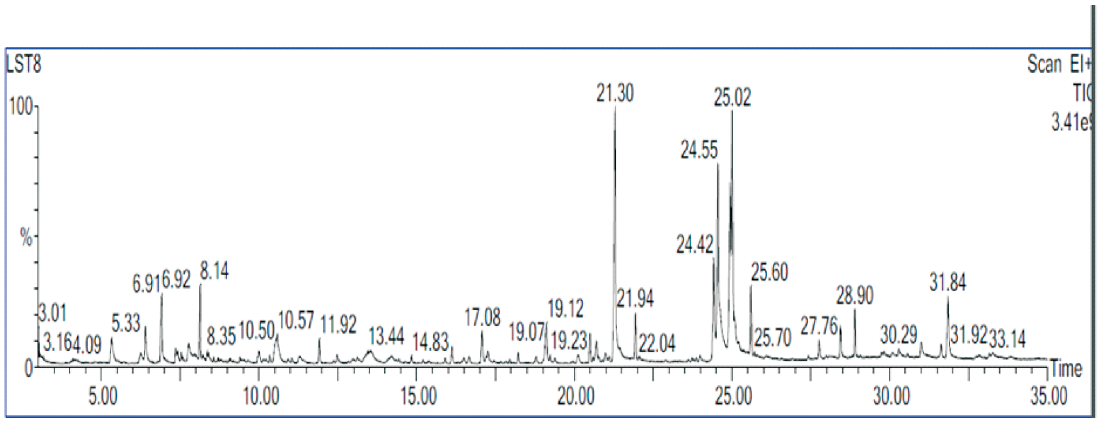

Fig. 2. Chromatogram of ethyl acetate fraction of *L. saksenae* in Czapak Dox medium

Table1. Bioactive volatile compounds detected from *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno

Sl No	RetentionTime	Chemical Group	Name of compound	Molecular Formula
A			Potato Dextrose Broth	
1	13.208	Organic acid	2,6 pyridine dicarboxylic acid	$C_7H_5NO_4$
2	20.591	Fatty acid	N-hexadecanoic acid	$C_{16}H_{32}O_2$
3	24.967	Fatty acid	Octadecanoic acid	$C_{18}H_{36}O_2$
B			Czapek Dox medium	
1	6.400	Ketone	4Hpyran-4 –one,2,3 dihydro3, 5-dihydroxyl- 6- methyl	$C_6H_8O_4$
2	6.925	Alkaloid	2- piperidinone	$C_9H_{19}BrNO$
3	8.140	Terpenoid	Dl- mevalonic acid lactone	$C_6H_{10}O_3$
4	11.917	Amide	Acetamide,N-(2-phenylethyl)	$C_{10}H_{13}NO$
5	13.462	Cyclic ester	3- deoxy-d- mannoic lactone	$C_6H_{10}O_5$
6	17.079	Diketopiperazine	Pyrrolo[1,2a] pyrazine1, 4-dione, hexahydro	$C_7H_{10}N_2O_2$
7	21.301	Fatty acid	N-.hexadecanoic acid	$C_{16}H_{32}O_2$
8	24.942	Alkaloid	Harmine	$C_{13}H_{12}N_2O$
9	25.017	Fatty acid	Octa decanoic acid	$C_{18}H_{36}O_2$

Table 2. Bioactivity* of volatile compounds detected from *L. saksenae*

Sl. No	Insecticidal compounds	Nematicidal compounds	Antimicrobial compounds
1	Dipicolinic acid	Octadecanoic acid	Acetamide,N-(2-phenylethyl), Harmine,
2	2-piperidinone	Harmine	Dl- Mevalonic acid lactone
3	Harmine	N-hexadecanoic acid	4H-pyran-4- one-2,3- dihydro- 3,5 di hydroxy-6 methyl
4	Dl- mevalonic acid lactone		3- Deoxy-d- mannoic lactone
5	Hexadecanoic acid		

* Dr.Dukes Phytochemical and Ethnobotanical Database (Duke, 2013)

per the Duke's database, the metabolites detected from PDB *viz* 2, 6 pyridine dicarboxylic acid (Dipicolinic acid-DPA) and octadecanoic acid are with insecticidal and nematicidal properties respectively. The fatty acid compound, n-hexadecanoic acid possesses both insecticidal and nematicidal properties. Among the metabolites detected from Czapak Dox medium 2-piperidinone and n-hexadecanoic acid are insecticidal, acetamide,N-(2 phenylethyl) and 3-deoxy-d-mannoic lactone, 4H-pyran-4-one 2,3- dihydro-3,5-di hydroxy 6-methyl are antimicrobial in nature. The metabolite harmine possesses insecticidal, antimicrobial and nematicidal properties, whereas dl- mevalonic acid lactone exhibits both insecticidal and antimicrobial properties.

Reports of toxins produced by *Lecanicillium* dates back to those by Suzuki *et al.* (1977) and Kanaoka *et al*. (1978) who detected the bioactive compound, bassianolide from *L.lecanii* . Claydon and Grove (1982) detected DPA and Soman *et al*. (2001),

detected the presence of vertilecanin-A, decenedioic acid and 10-hydroxy-8-decenoic acid. Jasmy (2016) detected the presence of DPA in *L. saksenae* through High Performance Thin Layer Chromatography (HPTLC) to the extent of 0.044 per cent.

Many authors have reported secondary metabolites similar to those identified from *L. saksenae* from different species of entomopathogenic fungi (Moragae and Vey, 2004, Asaff *et al.,* 2005). The major bioactive compounds identified in the ethyl acetate fraction of mycelia of *Beauveria bassiana* were n- hexadecanoic acid, 9,12, octadecadienoic acid, squalene, and octadecanoic acid (Ragavendran *et al* ., 2017). Vivekanandhan *et al.* (2018) reported hexa decanoic acid from mycelia of *B. bassiana* 28 as the major compound responsible for its pathogenicity. Recently, Ragavendran *et al*. (2019) identified mosquitocidal compounds *viz* 1-octadecene, 1-nonadecene, 9-octadecenoic acid and cyclobutane through GCMS analysis of *Penicillium* sp.

Bioactive metabolites are also reported from microbes other than fungi. The insecticidal alkaloid compound, harmine detected from *L. saksenae* is widely distributed among different medicinal plants (Siddiqui *et al.,* 1987). Similarly Pyrrolo (1,2) pyrazine1,4- dione hexahydro with antibiotic property was isolated from a marine bacterium *B. teqquilensis (*Kiran *et al.,* 2018*)*

Results of bioefficacy studies presented in Table 3, revealed the insecticidal potential of secondary metabolites of *L. saksenae*. A higher mortality of 95.98 per cent was observed with 1000 ppm toxin in the third instar nymphs, 48 HAT (Hours after treatment). The corresponding mortality in adults was 85.51 per cent. It took 72h to cause 100 per cent mortality of nymphs while in adults, it was 96 h. Lower concentrations of 100 ppm resulted in 62.83 per cent mortality, after 72 h exposure. In adults, it did not bring about significant mortality (2.5 to 31.08 per cent).

Bioactivity of secondary metabolites isolated from *L. saksenae* in the present study such as 2,6, pyridine dicarboxylic acid and hexadecanoic acid were previously reported from *L. lecanii* and *B. bassiana* respectively. Clydon and Grove (1982) observed insecticidal property of 2,6,pyridine

Table 3. Insecticidal activity of crude extract of *L. saksenae* on *C. insolita*

Concentration (ppm)	*Mean mortality of nymphs at 24 h interval (% ± SE)			
	24	48	72	96
10	2.63 ± 3.04	3.95 ± 7.89	5.56 ± 6.42	6.25 ± 0.00
50	25.44 ± 11.30	37.56 ± 11.60	43.06 ± 10.79	46.88 ± 10.83
100	41.74 ± 8.12	54.02 ± 7.72	62.83 ± 7.32	73.44 ± 10.67
250	60.82 ± 5.03	72.26 ± 4.12	84.40 ± 4.98	95.31 ± 5.98
500	78.29 ± 7.82	95.75 ± 5.35	100.00 ± 0.00	100.00 ± 0.00
1000	85.23 ± 4.74	95.98 ± 5.07	100.00 ± 0.00	100.00 ± 0.00
*Mean mortality of adults at 24 h interval (% ± SE)				
10	2.50 ± 2.89	2.50 ± 5.00	4.02 ± 5.07	5.56 ± 4.54
50	7.57 ± 6.42	7.63 ± 8.61	8.19 ± 3.05	9.90 ± 2.43
100	22.96 ± 6.02	26.29 ± 3.80	27.34 ± 3.85	31.08 ± 8.62
250	67.89 ± 5.21	73.79 ± 5.34	75.22 ± 7.52	79.86 ± 4.17
500	71.84 ± 6.30	76.29 ± 3.14	86.26 ± 3.38	100.00 ± 0.00
1000	83.36 ± 4.86	85.51 ± 5.04	89.04 ± 4.55	100.0 ± 0.00

* Mean of 4 replications

dicarboxylic acid (dipicolinic acid) from seven different strains of *L. lecanii.* Gindin *et al.* (1994) reported the toxicity of methanolic extract of mycelia of *L. lecanii* on the nymphs of *Bemisia tabaci* Gennadius and recorded mortality of 33.6 and 90 per cent at 0.1 and 0.5 per cent concentration respectively. The insecticidal activity of the extract was attributed to the phospholipid entity. Asaff *et al.* (2005) isolated the most abundant insecticidal metabolite of *Paecilomyces fumosoroseus* and characterized it as dipicolinic acid. LD_{50} value was 44.5 +_2.5, mgL^{-1} on brine shrimp *Artemia salina* Linnaeus. They suggested DPA, as the main active compound along with some other compounds might have contributed to the insecticidal activity. Crude toxins of *L. lecanii* were also reported to have ovicidal, repellant and antifeedant activities on *B. tabaci* (Wang *et al*., 2007). N. hexa decanoic acid from *B. bassiana* had a promising larvicidal and pupicidal activity on *Culex quinquefasciatus* Say (Raghavendran *et al.,* 2017).

Role of secondary metabolites in insect pathogenesis and mortality had been documented in many studies. Hunt and Ginsburg (1981) suggested the role of dipicolinic acid as an enzyme inhibitor responsible for the removal of essential ions from metalloenzymes especially Zn. Paterson (2008) suggested that DPA inhibits prophenoloxidase system during melanin synthesis in insects and interfere in the innate immune system. Involvement of secondary metabolites in various biological reactions such as pathogenicity, competition and defence as well as their antibiotic, antifungal, insecticidal and nematicidal properties have been reported by Xu *et al*. (2009).

Cao *et al*. (2016) reported that secondary metabolites inhibit multifunctional enzymes *viz.,* glutathione S transferase, α and β esterase that detoxify insecticides and endogenous compounds leading to enhanced death rate in insects. Raghavendran *et al.* (2019) reported the acetyl cholinesterase inhibition activity of the crude toxin containing different secondary metabolites extracted from *Pencillium* sp on fourth instar larvae of *Aedes aegypti* Linnaeus. The activity was attributed to the monoterpenoid fractions in crude extracts which are inhibitory to AchE activity. According to Elbanhawy *et al.* (2019), fungal metabolites are also reported to interfere with the normal physiological functions in insect. The methanol extract of *Purpureocilium lilacinum* caused reduction in the activity of aspartate aminotransferase, alanine aminotransferase, glutathione S. transferase and α and β esterase in *Aphis gossypii* Glover.

GCMS analysis of the ethyl acetate fraction of culture filtrate has thrown light into the chemical nature of the metabolites of *L saksenae*. The insecticidal, antimicrobial and nematicidal compounds detected in the present studies, cleary supported the findings of Goettel (2008) who suggested *Lecanicillium* as a multipurpose microbial agent for the control of arthropods, plant parasitic nematodes and plant pathogenic fungi. Insecticidal property exhibited by crude extract of *L. saksenae* may be due to the activity of more than one metabolite which includes the major metabolites dipicolinic acid, hexadecanoic acid and other nonvolatile compounds. The study established the role of secondary metabolites in the pathogenicity of *L. saksenae.*

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