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Production of Secondary nematicidal metabolites from *Purpureocillium lilacinum* using Karanja Deoiled Cake

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Abstract: *Karanja (Pongamia pinnata) deoiled cake was evaluated as an alternative substrate for the production of major nematicidal metabolite, leucinostatins, from Purpureocillium lilacinum 6029. Comparison between the chromatograms confirmed the enhanced production of leucinostatins in Karanja deoiled cake medium over commercially available Czapeck's Dox medium. Nematicidal activity assays showed that the fungal filtrate from Karanja deoiled cake medium was toxic (LT_{50} -1.46 h) killing 100 % Meloidogyne incognita larvae in 12 h. Our findings will help designing potent bionematicidal formulation comprising of biocontrol agent (P. lilacinum) and biodiesel by-product (Karanja deoiled cake) together in an environment-compatible approach.*

Keywords: *Karanja deoiled cake; Purpureocillium lilacinum; leucinostatins; Meloidogyne incognita*

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are the most difficult plant parasitic nematodes to control and most chemicals used as nematicides are toxic and/or volatile [1]. The problems associated with the use of nematicides, therefore, fuel the demand for alternative biological means of nematode control. Fungi are considered the most important biological control agents for phytonematodes [2].

Purpureocillium lilacinum is one of the important biocontrol fungi against *Meloidogyne incognita* [3, 4]. Various workers suggested that secretion of extracellular secondary metabolites viz. leucinostatins (also known as Paecilotoxins) by the fungus plays vital role in controlling nematode populations in soil [5, 6]. Leucinostatins are neutral straight peptides containing an unsaturated fatty acid and an amine residue in their N-terminus and C-terminus respectively and is quite similar to Peptaibols, synthesized mainly by *Trichoderma* species [7, 8].

Although *P. lilacinum* as a biocontrol agent has been extensively used against *M. incognita* in the field, limited success has been achieved so far. It is therefore, worthwhile and prudent to begin scientific exploration of various means to enhance the toxicity of *P. lilacinum* towards root-knot nematodes. In our previous research, we used biodiesel by-product i.e. Karanja (*Pongamia pinnata*) deoiled cake as an alternative substrate for the mass cultivation of nematophagous fungus *P. lilacinum* 6029 [9]. In the present investigation, we compared the production of secondary nematicidal metabolite i.e. leucinostatins by *P. lilacinus* 6029 cultured on Karanja deoiled cake medium and commercially available Czapeck's Dox medium. Secondly, we evaluated the nematicidal activity of *P. lilacinum* 6029 cultured on Karanja deoiled cake medium to correlate the toxicity of fungus with the substrate.

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MATERIALS AND METHODS

Microorganism and media preparation

Purpureocillium lilacinum 6029 was collected from Indian Type Culture Collection (ITCC), Indian agriculture research institute, New Delhi. The spores of the fungal strain were harvested from the slant surface by pouring sterile 0.1% Tween-80 to wash off the spores. One ml of spore suspension inoculated in the subsequent 250 ml flasks containing 100 ml of our optimized Karanja deoiled cake medium (KDM) and Czapeck's Dox broth (CDB) with culture conditions as described by Sharma *et al.* [9].

Detection of antimicrobial activity of P. lilacinum 6029 filtrate

Since the leucinostatins have antimicrobial activity against various Gram-positive bacteria, the toxin production was checked by a bioassay method using *Bacillus subtilis* as a test organism grown on nutrient agar medium. A 5-mm hole was made using cork borer aseptically in the center of 24 h-old culture plate of *B. subtilis* and vacuum-concentrated culture filtrate was pipetted into the well with three replications. Plates were then incubated at 37 °C up to 48 h. Antimicrobial activity was assessed by observing halo zone around hole. Uninoculated medium was treated as control.

Extraction and identification of leucinostatins

Leucinostatins were extracted using the method described by Mikami *et al.* [10]. Culture supernatants (100 ml) from KDM and CDB (control) were adjusted to pH 3.0 with 1 N HCl and extracted with the same volume of ethyl acetate. The extract was washed with 5% (w/v) $NaHCO_3$ and vacuum-concentrated. Crude leucinostatins fraction thus prepared was dissolved in a small amount of methanol. Each sample was passed through a 0.2-µm pore size teflon filter. The resulting extracted substance was analyzed by high-performance liquid chromatography (HPLC) in SGS lab, Gurgaon. All chromatograms were performed using a C18 column (Phenomenex Luna). The solvent used was methanol: 2-propanol: water: acetonitrile: diethylamine in a ratio 40:30:20:10:0.1. The flow rate was 1 ml min^{-1} and total running time was 20 min for each run. The chromatogram of *P. lilacinum* 6029 was compared with the published chromatogram of leucinostatins [11].

Nematicidal activity assay of P. lilacinum 6029 culture filtrate

Purpureocillium lilacinum 6029 was grown in Erlenmeyer flasks containing KDM and CDB for 15 days in the dark at 27 °C. The broth was filtered through Whatman no.1 filter paper to obtain culture filtrate. Uninoculated Karanja broth and CDB were also incubated for 15 days as controls. Mortality percentage of already hatched J_2 larvae of *M. incognita* was determined in 3, 6, 12 and 24 h [12].

All the results calculated are means of three replicates. The data for significant differences was analyzed by one-way analysis of variance (ANOVA) using Duncan's multiple range test 5 % level between treatments using SPSS software. LT_{50} was determined through probit analysis method using Stat-Plus 2009 software.

RESULTS

Antimicrobial activity of P. lilacinum filtrate

The effect of crude culture filtrate of *P. lilacinum* 6029 using Karanja deoiled cake (FKSM) applied in a hole of a *B. subtilis* plate is demonstrated in fig. 1. A visible effect on the growth of bacteria showing an 8 mm clearing (halo) zone around the hole was observed by the application of FKSM at 33 °C after 24 h of incubation. No halo was formed in control (Uninoculated medium) even after 48 h of incubation. The antibacterial activity by FKSM implied that *P. lilacinum* 6029 produced detectable levels of leucinostatins utilizing Karanja deoiled cake that inhibited the growth of Gram-positive bacteria.

HPLC analysis for the detection of leucinostatins by P. lilacinum

Figures 2a and 2b represent the HPLC chromatograms of *P. lilacinum* strain 6029 cultured on Karanja cake medium and CDB. Both chromatograms were compared with

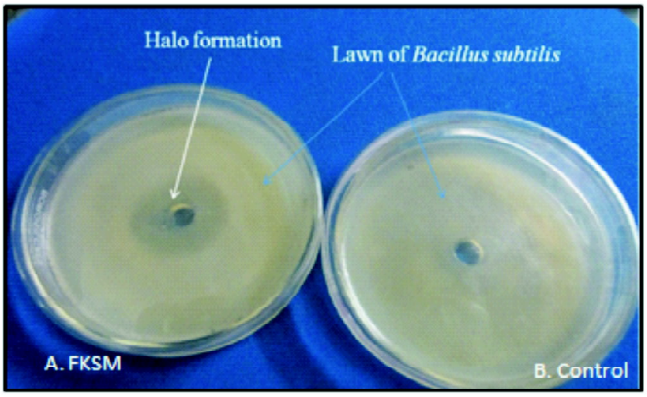

Figure 1: Antimicrobial assay of culture filtrate of *P. lilacinum* utilizing Karanja cake

chromatogram published for the crystallized leucinostatins from the Odashima strain by Khan *et al.* [11]. The three leucinostatins peaks viz. leucinostatins A, B and C labeled as A, B and C respectively appearing between 3.75 and 7.5 min, indicated that *P. lilacinus* strain 6029 produced detectable levels of leucinostatins by utilizing Karanja deoiled cake. It is noteworthy that culture filtrate from CDB had lower concentrations of leucinostatin C and no leucinostatin B. Our results, related to HPLC analysis are corroborated by earlier reports of production of leucinostatins by *P. lilacinum* [5, 10]. In contrast, Khan *et al.* [11] did not

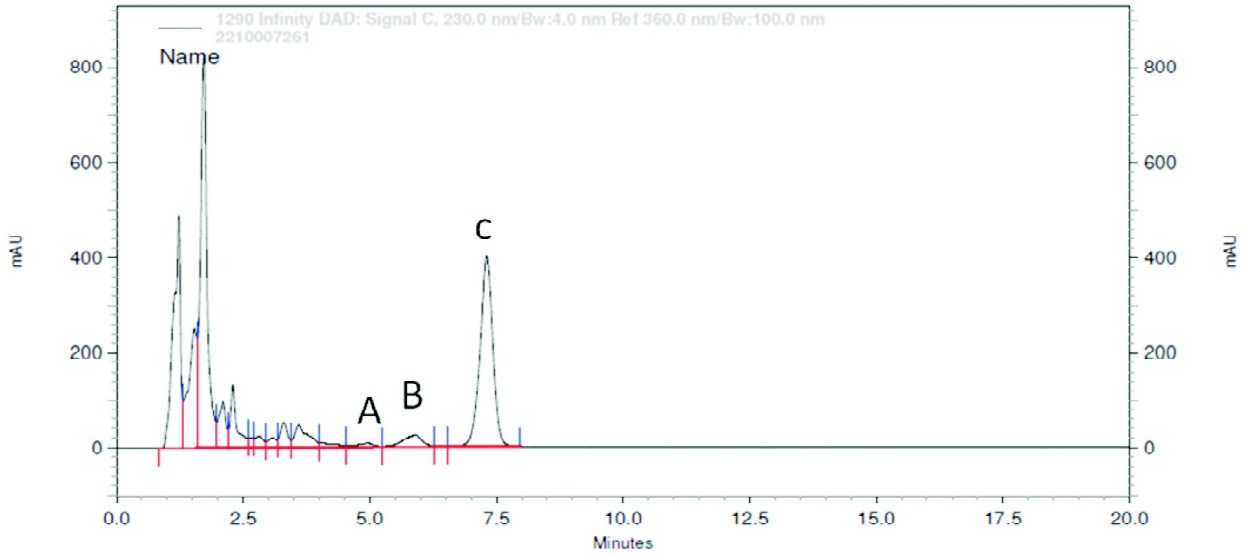

Figure 2a: Chromatogram of *P. lilacinum* 6029 from de-oiled Karanja cake medium. A, B and C indicates each toxin peak.

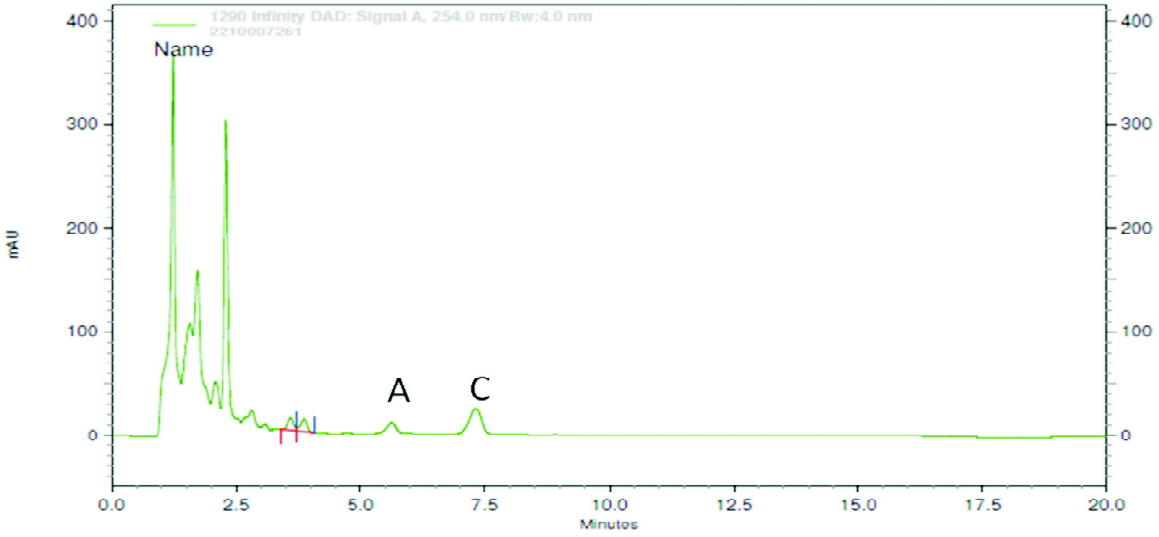

Figure 2b: Chromatogram of *P. lilacinum* 6029 from CDB. A and C indicates toxin peak

find any detectable levels of leucinostatins in *P. lilacinum* 251. The observations from our experiments also helped to explain the paradox of Karanja deoiled cake as an excellent alternative substrate not only for growth of *P. lilacinum* 6029 but also for the enhanced production of leucinostatins. In present study, production of leucinostatins C in KDM, but not in CDB, could have been possibly be due to the manipulation of carbon to nitrogen ratio and the pH of media with the introduction of novel nitrogen source in the form of Karanja deoiled cake [9].

Nematicidal activity assays

It is apparent from the data presented in fig. 3 that the pathogenicity of *P. lilacinum* 6029 differed significantly in different media. Comparing the nematicidal efficacy of culture filtrate from KDM (FKDM), CDB (FCDB) and uninoculated Karanja deoiled cake broth (KB) at 3 h of exposure, it was observed that no mortality was achieved by KB while FKDM exhibited significant toxicity causing death of 62.18% of juveniles of *M. incognita*. FCDB killed only 43.21% nematodes. After 12 h of exposure period, FKDM killed 100 % juveniles while only 78.28% were killed by FCDB. CDB alone did not play any role in killing nematodes (not shown in figure). LT_{50} value of FKDM (1.46 h) also demonstrated its enhanced toxicity against root-knot nematodes. FCDB showed less toxicity (LT_{50} =3.97 h) while KB had least toxicity with LT_{50} of 149.20 h. As a rule, the production of fungal metabolites is greatly influenced by the nature of substrates in culture medium [13] and in confirmation to this rule, the present results showed the enhanced toxic properties by KDM over CDB. Other researchers have also encountered this difference in toxicity due to nature of substrates. Cayrol *et al.* [14] demonstrated that highest toxicity of culture filtrate of same fungus against *M. arenaria* was obtained in malt extract broth followed by CDB and Mac Coy media.

CONCLUSION

Karanja deoiled cake used for *P. lilacinus* growth played important and decisive role in overall enhancement of its pathogenicity toward root-knot nematodes. This study, therefore, offers a very promising opportunity to design and develop a potential and cost-effective solid biopesticide of *P. lilacinum* using Karanja cake as a key component to control root-knot nematodes in an environment-compatible approach.

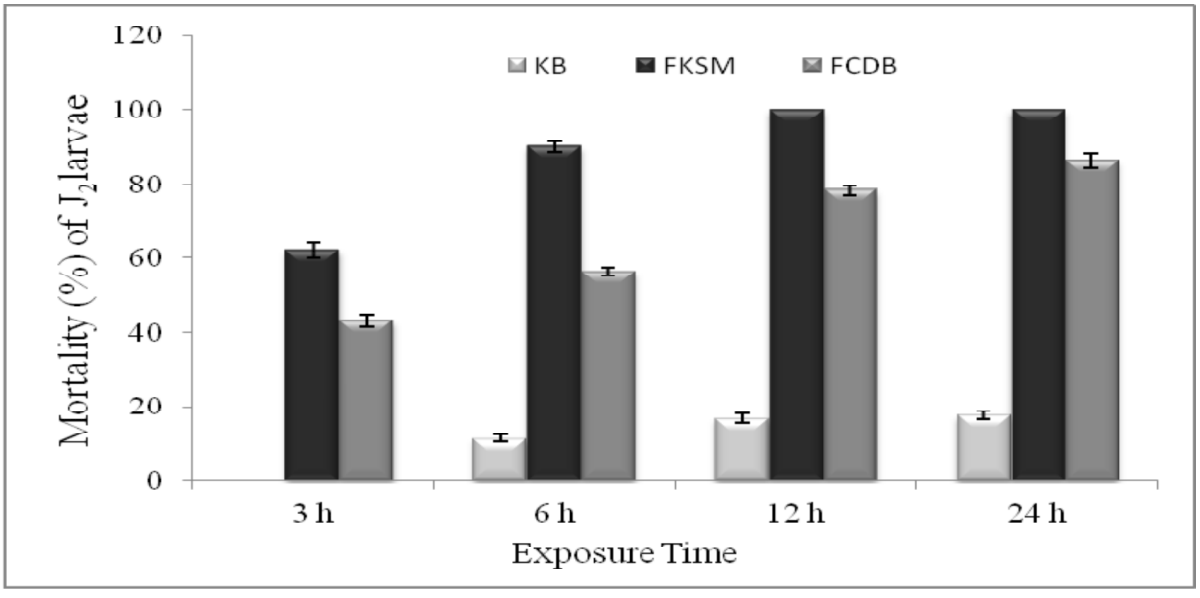

Figure 3: Comparative efficacy of fungal filtrate from deoiled Karanja cake, CDB and Karanja broth against *M. incognita* larvae at different exposure periods. KB: Undiluted Karanja broth incubated for 15 days; FKSM: Undiluted *P. lilacinum* filtrate from deoiled Karanja cake medium incubated for 15 days; FCDB: Undiluted *P. lilacinum* filtrate from CDB incubated for 15 days.

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