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Profiling Secondary Metabolites of Cocoa (*Theobroma cacao* L.) Endophytic Fungi *Lasiodiplodia pseudotheobromae* PAK-7 and *Lasiodiplodia theobromae* TN-R-3 and Their Antimicrobial Activities

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MC, SV and VH designed the study. Author MC performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VJ and EA provided samples for analysis. Authors AR and VR managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the chemical composition of secondary metabolite of cocoa endophytic fungi *L. pseudotheobromae* PAK-7, *L. theobromae* TN-R-3 and their anti-oomycete activities.
Statistical Design: Multivariate analysis.

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Place and Duration of Study: Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu from April 2018 to December 2019.
Methodology: Lipophilic extracellular secondary metabolites were extracted using ethyl acetate as a solvent and their chemical composition was detected by Gas Chromatography-Mass Spectrometry (GC-MS) and identified by NIST library and Pub Chem databases.
Results: Metabolic profiling of cocoa endophytic fungi *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 showed the presence of eleven peaks representing nine compounds. The most abundant compound observed were Acetic acid, 3-methyl-6-oxo-9oxabicyclo[3.3.1]non-2-yl ester, 2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-, Melezitose, Ethyl à-d-glucopyranoside collectively representing 58.01% area. In comparison to *L. pseudotheobromae* PAK-7 GC-MS analysis of *L. theobromae* TN-R-3 exhibited the presence of 29 peaks. The most abundant compounds were dl-Mevalonic acid lactone, Methyl 6-O-[1-methylpropyl]-á-d-galactopyranoside, 2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-, Melezitose, Ethyl à-d-glucopyranoside, 1,6-Anhydro-á-d-talopyranose collectively representing 60.47% of the total area.
Conclusion: Chemical compositions and anti-oomycete activities of crude secondary metabolites of *L. pseudotheobromae* PAK-7, *L. theobromae* TN-R-3 differed entirely depending on the property and abundance of bioactive metabolites.

Keywords: Endophyte; cocoa; metabolic profiling.

1. INTRODUCTION

Microorganisms interact with many organisms in different ways. The interaction may be symbiotic or mutualistic or pathogenic depending on the nature of the host [1]. A large group of fungi are plant pathogens, and they live as endophytes, asymptomatically within the intracellular spaces of leaves, roots and stem tissues [2]. Endophytes are transmitted both vertically (systemic) and horizontally (nonsystemic). The interaction between host and endophytes varies, in vertical transmission, they exhibit mutualism and horizontally transmitted endophytes exhibit antagonism to the host [3]. The endophytic fungi become pathogenic upon senescence. The plants colonized with endophytic fungi are adapted to abiotic and biotic stresses [4]. The tolerance of plants against biotic stress attributed to the production of secondary metabolites by endophytic fungi [5]. The extracellular secondary metabolites produced by endopytic fungi are nonessential compounds that serve as an ecological advantage under certain environmental conditions [6] and possess antimicrobial activity [7]. *L. theobromae* and *L. pseudotheobromae* are cosmopolitan fungi having wide host range and exhibits pathogenic and saprophytic or endophytic nature [8,9, 10,11]. The metabolite produced by *L. pseudotheobromae* F2 strain and *L. theobromae* SPMKF 5 showed antibacterial activity [7,12]. Not much information is available on antimicrobial activity of metabolites produced by *Lasiodiplodia* sp. With this limited information, an attempt was made to characterize and screen secondary metabolites produced by endophytic *L. pseudotheobromae.* PAK-7 and *L. theobromae* TN-R-3 for their anti-oomycete activity.

2. MATERIALS AND METHODS

2.1 Fungal Culture and Growth

In cocoa, black pod rot is a major problem and it is caused by *Phytophthora palmivora*. The virulent isolate of *P. palmivora* AYLB3 was selected for this study. Endophytic fungal isolates, isolated from cocoa were screened for their antagonistic activity against *P. palmivora* (MN814835- unpublished data*)* under *in vitro* conditions. Among all tested isolates, *L. pseudotheobromae* PAK-7 (MN418007) and *L. theobromae* TN-R-3 (MN400974) showed a cent percent inhibition (unpublished result). To check whether they are having any effect on cell wall lysis, the fungal cultures were grown in a 250 ml conical flask containing 100ml of malt extract broth and incubated at 27±2℃ for 7 days inside a BOD incubator.

2.2 Secondary Metabolite Extraction

The ethyl acetate method of secondary metabolite extraction procedure was followed [13]. Endophytic fungal culture was grown in ME broth for seven days. The culture filtrate was collected by centrifuging at 6000 rpm for 20 min. An equal volume of culture filtrate and ethyl acetate was taken in a separating funnel and agitated for 10 min. The samples were extracted two to three times with ethyl acetate and

evaporated. A brown colored concentrate secondary metabolite found after evaporation was dissolved in 1ml HPLC grade methanol.

2.3 Agar Well Diffusion Assay

The anti-oomycete activity of crude secondary metabolite of *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 against *Phytophthora palmivora* AYLB3(MN814835- unpublished data*)* was evaluated by performing agar well diffusion method. Agar wells of 5 mm size were made using sterile cork-borer. Different concentration of secondary metabolites *viz.,* 10, 20, 30, 40 µl was poured into the wells and sterile water was used as control. The experiment was repeated three times and inoculated plates were kept at 28℃ for 48-72h [14]. The anti-oomycete activity was confirmed by the formation of a clear zone around the well. The experiment was repeated two times to confirm the cell wall lysis properties of crude metabolites of endophytic *Lasiodiplodia* isolates.

2.4 Gas Chromatography-Mass Spectro-photometry

The crude metabolite extracted from endophytic fungi was subjected to GC-MS analysis to identify the different bioactive compounds present in it. The Clarus SQ 8C Gas Chromatography-Mass Spectrometer from Perkin Elmer, was engaged for analysis. The DB-5 MS capillary standard non - polar column with 0.25 mm dimension (Agilent Co., USA) with Helium as carrier gas (1 ml/min) was used. The bioactive compounds present in crude metabolite extract were identified using NIST libraries MS search version 2.2. The spectrum of the known components was compared with the spectrum of the known components stored in the inbuilt library.

2.5 Statistical Analysis

Metabolic profile of *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 isolates were analyzed using JMP® software. Under the multivariate method, principal component analysis (PCA) was performed following an independent linear combination of factors. Identified PCs were expressed in a heat map using an online heat mapper website following the average linkage clustering method and spearman rank correlation distance measurement method.

3. RESULTS AND DISCUSSION

3.1 Agar Well Diffusion Assay

Crude secondary metabolites from endophytic fungal isolates *L. pseudotheobromae* PAK-7 and *L.theobromae* TN-R-3 were screened for their anti-oomycete activity following agar well diffusion method as described by Sharma et al. [15] (Fig. 1). The *L. theobromae* TN-R-3 extracellular secondary metabolite exhibited mycelia cell wall lysis at 30 µl and 40 µl concentrations whereas *L. pseudotheobromae* PAK-7 exhibited maximum mycelial cell wall lysis at 40 µl concentration. The anti-oomycete activity by secondary metabolites of endophytic *Lasiodiplodia* is not studied well. But few reports are there on antibacterial properties of secondary metabolites of *Lasiodiplodia* sp. Taufiq and Darah [14] reported that crude metabolite *of L. pseudotheobromae* IBRL OS-64 exhibited antibacterial activity against 13 human pathogenic bacteria. Among them, MRSA ATCC 33591, *Staphylococcus aureus* and *Streptococcus mutans* a gram-positive bacteria found most susceptible with an inhibition zone of ≥21 mm. Chagas et al. [16] reported antimicrobial activity of *L. theobromae* isolates FHS074, FHS075 and FHS076. These three isolates found effective against *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa.* Orlandelli et al. [17] employed a cup-plate method to screen secondary metabolite of *L. theobromae* an endophyte of the medicinal plant *Piper hispidum* Sw. against human pathogen *Enterococcus hirae*, *E. coli*, *Micrococcus luteus*, *Salmonella typhi* and *Staphylococcus aureus*. The crude metabolite of *L. theobromae* at 61.4 mg/ml concentration inhibited all tested human pathogens.

3.2 Metabolite Profiling of *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3

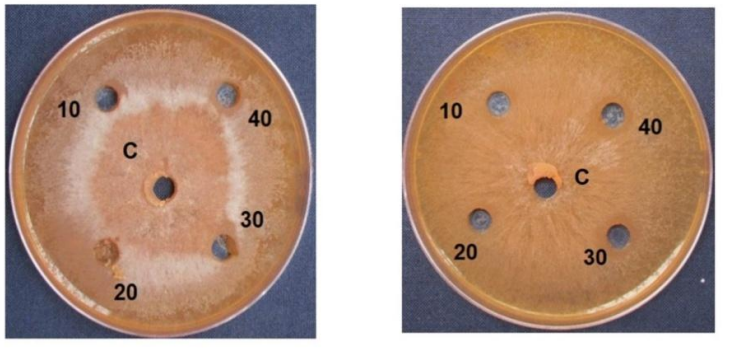
Crude metabolites extracted from endophytic fungal isolates were subjected to GC-MS analysis. On a total of 40, bioactive metabolites were detected and they were matched with NIST library compounds (Table 1, Fig. 2). The relative abundance of metabolites at different retention time (RT) was represented as peaks. Chemical analysis of *L. pseudotheobromae* PAK-7 revealed the presence of eleven peaks corresponds to the presence of nine compounds. The most abundant compound observed were

Acetic acid, 3-methyl-6-oxo-9oxabicyclo[3.3.1]non-2-yl ester, 2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-, Melezitose, Ethyl à-d-glucopyranoside collectively representing 58.01% area. In comparison to *L. pseudotheobromae* PAK-7 GC-MS analysis of *L. theobromae* TN-R-3 exhibited the presence of 29 peaks. The most abundant compounds were dl-Mevalonic acid lactone, Methyl 6-O-[1-methylpropyl]-á-d-galactopyranoside, 2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-, Melezitose, Ethyl à-d-glucopyranoside collectively representing 60.41% of the total area.

These two cocoa endophytic isolates belonged to the same genus, but their metabolic profiling is entirely different. Compounds like dl-Mevalonic acid lactone, Methyl 6-O-[1-methylpropyl]-á-d-galactopyranoside, 2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-, Melezitose, Ethyl à-d-glucopyranoside and Octadecanoic acid are common among them. Qian et al. [10] reported that *Lasiodiplodia* sp. ME4-2 endophytic fungi of *Viscum coloratum* produced five aromatic compounds which included cyclo-(Trp-Ala), indole-3-carboxylic acid (ICA), indole-3-carbaldehyde, mellein and 2-phenylethanol. This finding is in agreement with the current finding where isolate *L.theobromae* isolate TN-R-3 produced Cyclohexanecarboxylic acid, 2-hydroxy-, ethyl ester.

dl-Mevalonic acid lactone is a δ-lactone form of mevalonic acid, a precursor in the mevalonate pathway. Metabolites of the mevalonate (MVA) pathway are involved in signaling pathways that transduce endo-symbiotic microbial signals in the *Medicago truncatula* [18]. In the present study both isolates *i.e., L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 produced dl-Mevalonic acid lactone. This compound might have played a role in endophytic colonization. Antifungal activity of (R)-5,6-dihydro-6-pentyl-2H-pyran-2-one against *Penicillium* species was reported by Parker et al. [19]. The methanolic extract of *Solanum torvum* contained Methyl 6-O-[1-methylpropyl]-á-d-galactopyranoside, that showed cytotoxic effect on cancer cell lines [20].

Melezitose is a non-reducing tri-saccharide also known as melicitose. It is a common metabolite produced by both two isolates of *Lasiodiplodia* sp. This compound produced from the stem and leaves of *Alhagi maurorum Medik* (Camel thorn) reported to be used in ancient ayurvedic medicine for the treatment of anorexia, dermatosis, and leprosy [21]. Another compound common among *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 was Ethyl à-d-glucopyranoside. The synonyms for this compound is Ethyl hexopyranoside. Mahdi et al. [22] reported that fungus growing termite *Macrotermes bellicosus* is used in traditional medicine in Benin for the treatment of diarrhoea, pulmonary infection and infectious and inflammatory diseases and it's effective against pathogenic microbes. NMR spectra of a chemical compound from *Macrotermes bellicosus* revealed the presence of compounds *viz.,* benzohydroquinone, 2-methylhydroquinone, niacinamide, ethyl-hexopyranoside, 3,4-dihydroxyphenethyl glycol and N-[2-(3,4-Dihydroxyphenyl)ethyl]acetamide.



Lasiodiplodia pseudotheobromae PAK-7 *Lasiodiplodia theobromae* TN-R-3

Fig. 1. *In vitro* screening of crude secondary metabolites employing agar well diffusion method
Note: Zone of clearance indicates cell wall lysis of Phytophthora palmivora AYLB3

Table 1. Metabolic profiling of endophytic fungi *L. pseudotheobromae* PAK-7 and *L. theobromae* isolate TN-R-3

RT	Compound	%Area	
		PAK-7	TN-R-3
3.033	1-Nitro-2-acetamido-1,2-dideoxy-d-mannitol	0.285	0.000
3.249	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	0.212	0.000
3.444	ñ-Tetrahydro-3-furanmethanol	2.045	0.000
5.664	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6methyl	0.118	0.000
6.245	2-Cyclohexen-1-one, 4,5-dimethyl-	1.330	0.000
6.98	Fluroxypyr	0.660	0.000
7.295	1,2-Benzenediol, 3-methyl-	3.228	0.000
7.475	dl-Mevalonic acid lactone	0.389	7.470
7.575	1,2,4-Cyclopentanetrione, 3-butyl-	1.123	0.000
7.71	1,5-Dimethyl-6-oxa-bicyclo[3.1.0]hexane	1.510	0.000
7.875	Benzenemethanol, 4-hydroxy-	0.140	0.000
8.13	1,1-Cyclohexanedimethanol	0.390	0.000
8.266	2-Nitrohept-2-en-1-ol	1.251	0.000
8.406	Octan-2-one, 3,6-dimethyl-	1.355	0.000
8.546	1,4-Benzenediol, 2-methyl-	2.061	0.000
8.686	Oxirane, 2-(chloromethyl)-2-cyclobutyl-	0.810	0.000
8.766	3(2H)-Furanone, dihydro-5-isopropyl	2.439	0.000
8.941	Prenoxdiazine	0.236	0.000
9.256	Methyl 6-O-[1-methylpropyl]-á-d-galactopyranoside	0.691	14.773
9.381	2-Chloro-5-methylbenzene-1,3-diol	3.459	0.000
9.571	3-Methoxymethoxy-2,3-dimethylundec-1-ene	0.374	0.000
9.696	1,3-Cyclohexanediol, 2,5-dimethyl-2-nitro-	1.501	0.000
9.786	Formamide, N-[1-[(1-cyano-2methylpropyl)hydroxyamino]butyl]	1.041	0.000
10.181	Acetic acid, 3-methyl-6-oxo-9oxabicyclo[3.3.1]non-2-yl ester	26.487	0.000
10.561	Sucrose	0.222	0.000
10.792	2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-	6.234	10.706
11.467	Melezitose	17.713	9.256
13.348	Ethyl à-d-glucopyranoside	7.579	14.013
13.613	p-tert-Butylcatechol	1.367	0.000
14.918	Jasmonic Acid	0.522	0.000
14.988	Benzaldehyde, 3-ethoxy-	1.231	0.000
15.914	Tetradecanoic acid	0.203	0.000
19.92	n-Hexadecanoic acid	0.807	0.000
23.607	Octadecanoic acid	0.230	23.587
27.513	Gibberellic acid	0.201	0.000
27.698	Hexadecane, 1,1-bis(dodecyloxy)-	0.216	0.000
28.228	2-Hydroxy-4-methoxy-7-methyl-7,8,9,10,11,12,13, 14-octahydro-6-oxabenzocyclododecen-5-one	0.213	0.000
28.668	17-Pentatriacontene	0.319	0.000
3.208	Isomaltol	0.000	3.208
9.616	Benzeneethanol, 4-hydroxy-	0.000	0.909
15.33	Tetradecanoic acid	0.000	0.564
19.425	Dibutyl phthalate	0.000	0.393
3.053	1-Nitro-2-acetamido-1,2-dideoxy-d-mannitol	0.000	0.488
3.289	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	0.000	2.390
4.014	Erythritol	0.000	2.084
4.164	Ropinirole	0.000	1.795
4.474	Methanamine, N-methoxy-	0.000	1.536
4.704	1,2,4-Cyclopentanetrione, 3-methyl-	0.000	2.925
5.059	5-Aminolevulinic acid	0.000	0.776
5.649	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6methyl	0.000	1.044
6.065	o-Acetyl-L-serine	0.000	0.371

RT	Compound	%Area	
		PAK-7	TN-R-3
6.855	5-Hydroxymethylfurfural	0.000	1.926
7.335	2H-Pyran-2-one, 5,6-dihydro-6-propyl-	0.000	1.120
7.77	Indole	0.000	0.871
7.995	Ethanone, 1-(2-hydroxy-5-methylphenyl)-	0.000	0.631
8.13	Cyclohexanecarboxylic acid, 2-hydroxy-, ethyl ester	0.000	0.930
8.556	á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	0.000	0.447
11.822	à-d-6,3-Furanose, methyl-á-d-glucohexodialdo-1,4furanoside	0.000	0.700
14.198	1,6-Anhydro-á-d-talopyranose	0.000	3.139
14.418	Isopropyl palmitate	0.000	1.165
18.795	Geraldol	0.000	0.456
19.72	n-Hexadecanoic acid	0.000	0.990
24.922	Pyrrolo[1,2-a]pyrazine-3-propanamide, 2,3,6,7,8, 8a-hexahydro-1,4-dioxo	0.000	0.519

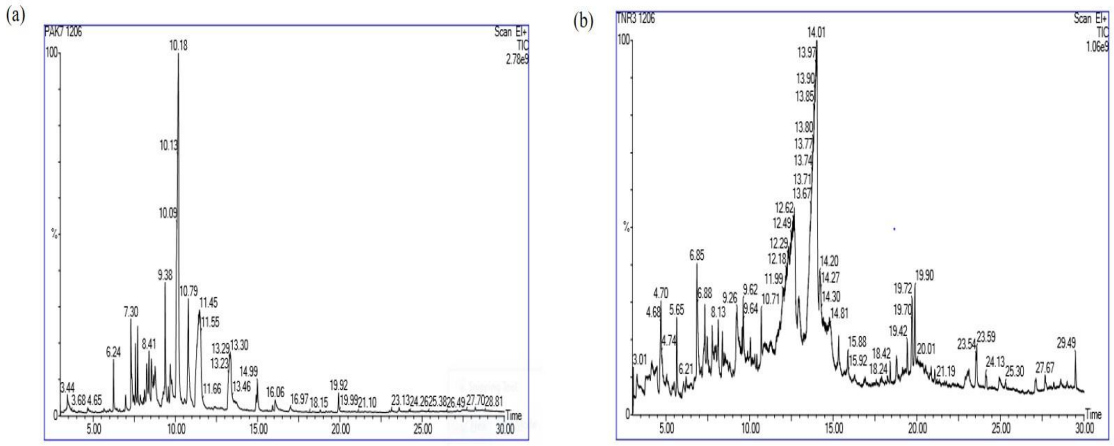


Fig. 2. GC-MS chromatogram showing different peaks representing % area
(a) *L. pseudotheobromae* PAK-7 (b) *L. theobromae* TN-R-3

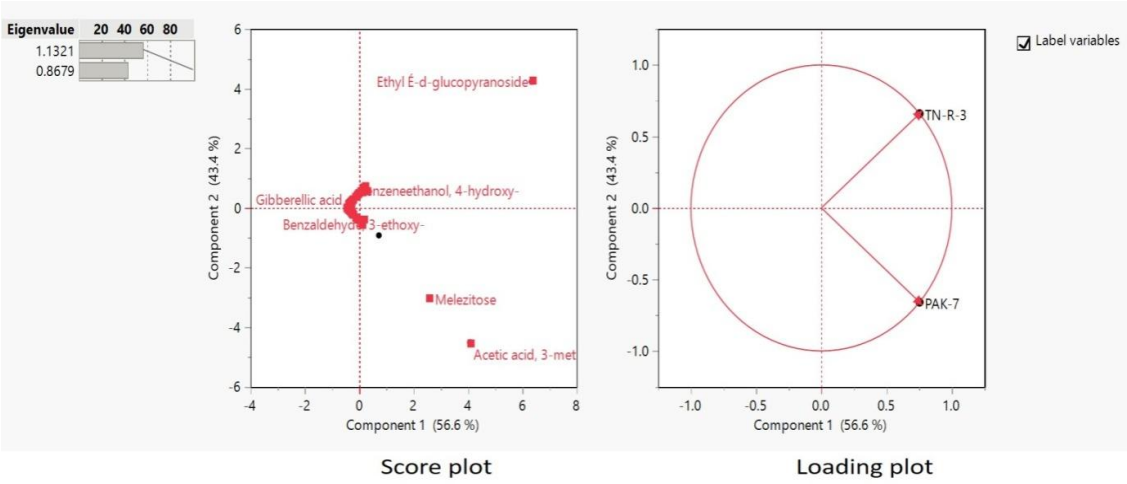


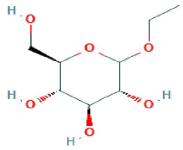
Fig. 3. PCA of extracellular secondary metabolites obtained by ethyl acetate fraction and identified by GC-MS in *L. pseudotheobromae.* PAK-7 and *L. theobromae* isolate TN-R-3

3.3 Principle Component Analysis

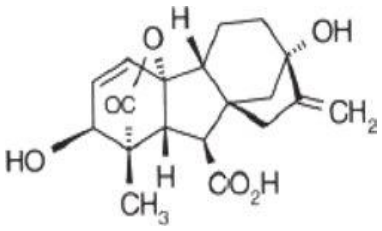
To identify compounds having antifungal activity in crude metabolite of endophytic *L. pseudotheobromae* PAK-7, and *L. theobromae* isolate TN-R-3 a multivariate analysis was carried out based on correlation [23]. The data-set consisted of a 2×40 matrix (Table 2). The row represented two isolates of *Lasiodiplodia* sp. and column was represented by compounds. The PCA was performed and the PCs having eigenvalue ≥1 were considered. The score plot and loading plot for secondary metabolites of *L. pseudotheobromae* PAK-7, and *L. theobromae* TN-R-3 were shown in Fig. 3. The first two main PCs accounted for 100% variance; 56.60% variance was attributed to PC1 and it was positively correlated with Benzaldehyde,3-ethoxy-, Melezitose, Acetic acid, 3-methyl-6-oxo-9oxabicyclo[3.3.1]non-2-yl ester and negatively correlated with Gibberellic acid. PC2 accounted for 43.40% of the total variable and it was positively correlated with Ethyl à-d-glucopyranoside and Benzeneethanol, 4-hydroxy- (Table 2, Figs. 3,4,5).

Table 2. Unique principle components identified in *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 isolates of cocoa

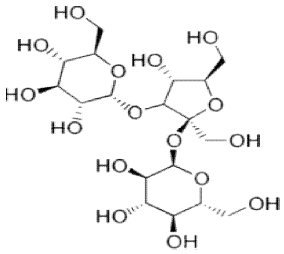
Sl. no	Compound	Pak-7	TN-R-3	Property	Reference
1	Ethyl à-d-glucopyranoside	+	+	Antibacterial	Mahdi et al. [22]
2	Gibberellic acid	-	+	Growth promotion and salinity tolerance	Waqas et al. [24]
3	Melezitose	+	-	Antidematosis	Duke et al. [21]
4	Acetic acid, 3-methyl-6-oxo-9oxabicyclo[3.3.1]non-2-yl ester	+	-	Antibacterial	Wali and Abed [25]
5	Benzaldehyde, 3-ethoxy-	+	-	Antifungal	Ahluwalia et al. [26]
6	Benzeneethanol, 4-hydroxy-	-	+	Antifungal	Ahluwalia et al. [26], Manilal et al. [27]



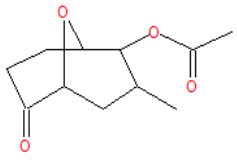
Ethyl E-d-glucopyranoside



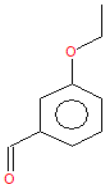
Gibberellic acid



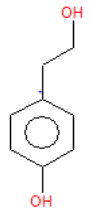
Melezitose



Acetic acid, 3-methyl-6-oxo 9 oxabicyclo[3.3.1]non-2-yl ester



Benzaldehyde, 3-ethoxy-



Benzeneethanol, 4-hydroxy-

Fig. 4. Chemical structure of principal components (PCs) identified in *L. pseudotheobromae* PAK-7 and *L. theobromae* isolate TN-R-3

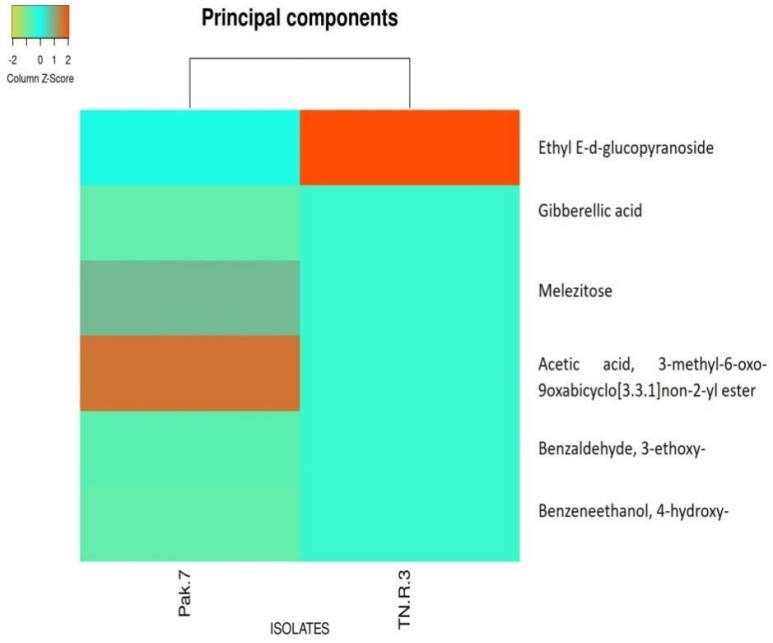


Fig. 5. Heat map for principle components identified in endophytic fungal isolates *L. pseudotheobromae* PAK-7 and *L. theobromae* isolate TN-R-3 (Clustering Method- Average linkage method, Distance Measurement Method-Spearman rank correlation)

4. CONCLUSION

In the present investigation secondary metabolic profiling and anti-oomycetes activity of crude extracellular metabolite of endophytic fungi *L. pseudotheobromae* PAK-7 and *L. theobromae* TN-R-3 was evaluated. Even though they are from the same genus their metabolic profiling differed. Agar well diffusion method and PCA showed that compounds present in crude metabolite of these two endophytic fungal isolates are having cell wall lysis property. There are reports on the production of signaling molecule *viz*., jasmonic acid by *Lasiodiplodia* sp. [28] but the antifungal/anti-oomycete activity of crude metabolites of *Lasiodiplodia* is not known or not studied. So an attempt was made to isolate and characterize secondary metabolites of endophytic fungal species of cocoa for their anti-oomycete activity. For further confirmation, investigations may be carried out to establish the anti-oomycete activity by the chemical compounds produced by these endophytic fungi.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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