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Research Article

# Arms Race of Melanogenic Actinobacteria *Actinoalloteichus cyanogriseus* against Mulberry Root Rot Pathogens

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## Abstract

Mulberry (*Morus indica* L.), is an astounding multipurpose woody, deciduous crop grown all over the world. Due to pathogens that cause root rot diseases in mulberry have a major impact on intensive crop cultivation and commercial cocoon production. Notably, it has been found that these pathogens affect healthy mulberry plantations regardless of their age, variety that grown in wide-ranging soil and agro-climatic conditions. To manage the pathogens, two potent melanogenic actinobacteria *Actinoalloteichus cyanogriseus* isolated from mulberry rhizosphere with few extremophilic characteristics were identified in the previous study. Their antagonism towards these pathogens exhibited through a variety of phenomena. The chemical fingerprints of bioactive isolates revealed the presence of more than 30 compounds for each. Advantageously, smaller molecules were found to be the majority of them. Important bioactive inhibitory compounds including, 2,4-DTBP, binapacryl, decanoic acid groups, 1-hydroxy-6-methylphenazine, *etc*. were identified through GC-MS. In addition to evidence of antifungal metabolites there were also found traces of anti-bacterial, allelopathic compounds with other antioxidants and flavonoid compounds. The current work thus sheds light on the antifungal potency of melanogenic isolates, which has been unexplored/ poorly analyzed.

**Keywords:** Anti-fungal metabolites, GC-MS, Melanogenic actinobacteria, Mulberry root rot

## Introduction

Sericulture engrosses mulberry cultivation for silkworm rearing and proteinaceous cocoon production. It is a highly remunerative, agro-based enterprise providing livelihood for rural, semi-urban community from more than 26 states of India with cultivated area around 2.53 L ha and silk production of 36,582 metric tonnes (CSB, 2023). Out of 150 known species of *Morus*, varieties derived from species like *M. indica*, *M. alba*, *M. lavigata*, *M. multicaulis* are highly sought after for global silkworm rearing (Ramesh *et al*., 2014).

Disease is the major factor affecting quantity and quality of mulberry yield. Due to many pathogenic microbes that may result in endemic or epidemic or pandemic or sporadic diseases. Apart from saprobes nine types of root rot causing pathogens were found in mulberry. Besides, in mulberry like perennial crops, once the soil gets contaminated with anyone of these pathogens, it continued to survive/ endure in variable forms even without hosts leading to desertion of cultivable fields. However, disease intensification in mulberry gardens was influenced by soil, climate and environmental factors (Chowdary, 2006). Notably, it has been found that these pathogens affect healthy mulberry plantations regardless of their age, variety that grown in wide-ranging soil and agro-climatic conditions (Saratha *et al*., 2021).

Pathogens evolved various strategies to survive in the cropping environment even during adverse conditions. Now it is mandatory to identify bio-agents with surviving ability in extreme conditions in order to use them effectively against phytopathogens. The previous study focused on isolating melanogenic actinobacteria for the reason that microbial melanogenesis had reported as ‘armour’ as it

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aids in improvising surviving and competitive abilities of the organism in adverse conditions (Shivlata and Sathyanarayana, 2015). Screening of bioagent alone couldn't help to manage these omnipresent pathogens. Further the elaborative study of various novel mechanisms involved in antagonism need to be done to manipulate for maximum efficacy.

Former reports showed that the inhibition/ antibiosis were closely associated to the diffusible volatile/ non-volatile antimicrobial compounds produced by antagonists for pathogen suppression (Franks *et al*., 2006; Patil and Senthilraja, 2021). Actinobacteria are multicellular organisms with intricate life cycle and renowned for exceptional array of bioactive metabolites production including alkenes, alcohols, ketones, esters, terpenes, aldehydes and sulfur/ phthalate containing compounds in both volatile and non-volatile forms (Claessen *et al*., 2014). These metabolites were unique to the specific isolate and reported to have multifaceted application in soil health, plant growth promotion and protection (Garbeva *et al*., 2014; Hagai *et al*., 2014). Further, in the peculiar actinobacterial life cycle, potential bioactive metabolite production is associated with their sporulation and it was increased during stress (Kunova *et al*., 2016).

The results corroborated with earlier reports and accordingly, antibiosis effect increased during sporulation (Saratha *et al*., 2022). Additionally, there were many reports available for antibacterial potency of melanogenic actinobacteria (Manivasagan *et al*., 2013; Prajapati *et al*., 2016) whereas their antifungal potency is unexplored/ poorly studied. This renewed our interest in identification of bioactive compounds from the potent melanogenic actinobacteria.

## Materials and Methods

*Potent Melanogenic Actinobacterial Isolates*

Previously, *Actinoalloteichus cyanogriseus* (M11 and M12) isolated from the healthy mulberry rhizosphere were identified with maximum inhibitory effect against mulberry root rot pathogens and extremophilic traits were used for the current study (Saratha *et al.*, 2022). Mulberry root rot samples and soil samples were collected from traditional districts of Tamil Nadu. Both the previous and current experiments were carried out in Tamil Nadu Agricultural University, Coimbatore during the year 2019-20 with the aim of exploring the antifungal ability of melanogenic actinobacteria against various fungal pathogens associated with mulberry root rot disease.

*Extraction of Secondary Metabolites of Potent Melanogenic Actinobacterial Isolates*

For collection of extracellular secondary metabolites of potent melanogenic isolates (M11 and M12) were grown in starch casein (SC) broth at 30±2 °C. The cell free supernatant (CFS) of 14 days old actinobacteria grown broth was collected after centrifugation for 10 min at rpm. The filtrate subjected to ethyl acetate based solvent extraction (1:1 v/v) and kept overnight shaking for extraction completely. Later the organic phase containing metabolites was separated and concentrated *in-vacuo* using a rotary flash vacuum evaporator (Roteva, Equitron, Mumbai) at 45 °C and 100 rpm. The residues were weighed and re-suspended (defatted) in metahnol to obtain crude extract (Hemashenpagam, 2011; Ahmad *et al.*, 2017) and preserved at -20°C for further studies.

*Gas Chromatography-Mass Spectrometry (GC-MS) of Crude Metabolites*

For GC-MS analysis, the crude metabolites of potential isolates (M11 and M12) were re-suspended in HPLC grade methanol (Ahmad *et al.*, 2017). The sample crude extract (3 µl) was analysed in GC with (DB-5 MS) capillary standard non-polar column (Perkin Elmer Clarus SQ 8C GC-MS, USA) and helium as a carrier gas. Initial column temperature of 110 °C for 9 min and temperature of 250 °C for 36 min for injecting sample were maintained. Electron impact (EI) energy fixed at 70 eV and its mass scan (m/z) was recorded in the range of 45-450 AMU. From the chromogram results, the compounds with probability more than 80% identified from NIST Mass Spectral Library for interpretation.

## Results and Discussion

Various actinobacteria performed well as sources of plant growth promoters, antibiotics, bio-insecticides, bio-weedicides and cytotoxic compounds. Barka *et al.* (2015) discussed antibiotic mediated pathogens' inhibition as primary focus to mitigate plant diseases. Moreover, kasugamycin, polyoxins, validamycin, *etc*. were identified and marketed as antifungal actinobacterial metabolite to mitigate phytopathogens.

*Extraction of Secondary Metabolites of Potent Melanogenic Actinobacterial Isolates*

The fermented SC broth of M11 and M12 isolates was processed as above and yielded the dull red tinged residue which was re-suspended in HPLC grade methanol.

*GC-MS Profile of Bioactive Metabolites*

Microbial screening which produces bioactive compounds is highly interested in identifying novel compounds to combat a variety of phytopathogens. To identify those compounds and their structure, different methods were employed including LC-MS, GC-MS, NMR, and so on (Tiwari *et al*., 2015). For isolation of bioactive compounds found in secondary metabolites GC-MS is widely adopted. Further it could be used to separate volatile and semi-volatile molecules with lower molecular masses (Snyder *et al*., 1997). The major volatile component of *Streptomyces* TH23-7 was identified by GC-MS as 2,2-dimethyl-4-(3-methyl but-2-enyl)-6-methylidene cyclohexyl methanol reported to the cause for morphological deformations in *L. theobromae* (Ruangwong *et al.*, 2022).

Melanogenic *A. cyanogriseus* isolates (M11 and M12) of the study exhibited wide array of bio active compounds in GC-MS profile using ethyl acetate as solvent. Managamuri *et al*. (2017) isolated *Streptomyces sparsus* from sea sediments and extracted its bioactive components using ethyl acetate fraction. Further their chemical fingerprints showed the existence of diverse compounds that were proved to have antibacterial, antifungal and antioxidant activities.

Among many solvents used in previous studies, ethyl acetate extract had a broad antibiotic range against bacterial and fungal diseases (Khamna *et al.*, 2009). Four solvents were used to extract the fermentation broth of *A. kerguelensis* VLRK 09 in order to assess the antibacterial efficacy. Ethyl acetate extract showed the highest antibacterial efficacy, whereas the other *viz.*, methanol, acetone, chloroform extracts showed moderate to low inhibition (Munaganti *et al.*, 2015).

The broth of *Streptomyces* strain proved to be effective against a variety of plant diseases, including *R. solani*, *Sclerotinia sclerotiorum*. The active fraction was obtained by solvent extraction and two potential antifungal compounds were identified through GC-MS were eicosane and dibutyl phthalate (Ahsan *et al.*, 2017). In this study, many of the constituents documented for antimicrobial, antioxidant, anticancerous, pesticidal, herbicidal properties including phenols, terpenes, amines, esters, fatty acids, flavonoids (Table 1, 2) and few compounds of unknown function.

Diverse anti-fungal metabolites with different functional groups including 2,4-di-tert-butyl phenol (2,4-DTBP), 2',4'-dihydroxy-3'-methylacetophenone, 1-nonadecene, tetra, penta and hexadecanoic acid (Figure 1a, Table 1) were present in the ethyl acetate fraction of M11. Similarly, the M12 strain showed the presence of binapacryl as a major antifungal and acaricidal compound followed by 2,4-DTBP, Dibenzo(b,f)oxepin-3-amine, 2',4'-dihydroxy-

Table 1: GC-MS volatile profile of ethyl acetate fraction of M11

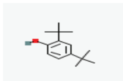
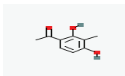
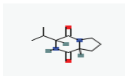
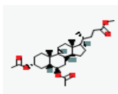
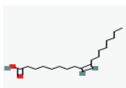
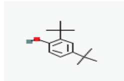
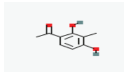
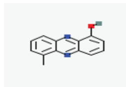
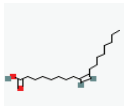
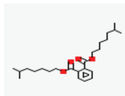
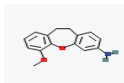
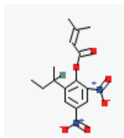
| Sl. No. | Retention time (min) | Peak area (%) | Compound name | Molecular weight (g mol$^{-1}$) | Molecular formula | Molecular structure | Biological function | References |
|---|---|---|---|---|---|---|---|---|
| 1. | 12.842 | 5.572 | 2,4-Di-tert-butylphenol (2,4 DTBP) | 206.32 | $C_{14}H_{22}O$ |  | Antifungal, anti-oxidant | Varsha *et al.* (2015) |
| 2. | 16.314 | 0.497 | 2',4'-Dihydroxy-3'-methylacetophenone (III- fungicide) | 166.17 | $C_9H_{10}O_3$ |  | Antifungal | Shi *et al.* (2016) |
| 3. | 16.834 | 0.855 | Dodecyl acrylate | 240.38 | $C_{15}H_{28}O_2$ |  | Antimicrobial | Joo *et al.* (2010) |
| 4. | 18.199 | 1.475 | Tetradecanoic acid | 221.37 | $C_{14}H_{28}O_2$ |  | Antioxidant, Antimicrobial, Weedicide | Yayli *et al.* (2006); Ross *et al.* (2004) |
| 5. | 18.965 | 1.085 | 1-Nonadecene | 266.5 | $C_{19}H_{38}$ |  | Antifungal, antioxidant, | Premathilaka and Silva (2016) |
| 6. | 21.531 | 2.167 | Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 154 | $C_{10}H_{16}N_2O_2$ |  | Antioxidant, antimicrobial and algicidal | Tan *et al.* (2018); Kadhim *et al.* (2016) |
| 7. | 20.24 | 0.411 | Pentadecanoic acid | 242.4 | $C_{15}H_{30}O_2$ |  | Antibacterial and antifungal | Agoramoorthy *et al.* (2007) |
| 8. | 22.311 | 10.466 | n-Hexadecanoic acid | 256.42 | $C_{16}H_{32}O_2$ |  | Nematicide, antibacterial and antifungal | Kumar *et al.* (2010); Chandrasekaran *et al.* (2011) |
| 9. | 23.471 | 31.755 | Diisooctyl phthalate | 390.6 | $C_{24}H_{38}O_4$ |  | Algicide, antioxidant, allelopathic, antimicrobial | Huang *et al.* (2021) |
| 10. | 24.557 | 0.156 | Cholan-24-oic acid, 3,6-bis(acetyloxy)-, methyl ester, (3à,5á,6à)- | 490.7 | $C_{29}H_{46}O_6$ |  | Antifungal, antibacterial | Cazar *et al.* (2005); Salem *et al.* (2016) |
| 11. | 25.572 | 6.646 | Oleic acid | 282.5 | $C_{18}H_{34}O_2$ |  | Antibacterial | Awa *et al.* (2012) |

Table 2: GC-MS volatile profile of ethyl acetate fraction of M12

| Sl. No. | Retention time (min) | Peak area (%) | Compound name | Molecular weight (g $mol^{-1}$) | Molecular formula | Molecular structure | Biological function | References |
|---|---|---|---|---|---|---|---|---|
| 1. | 13.072 | 0.743 | 2,4-Di-tert-butylphenol (2,4 DTBP) | 206.32 | $C_{14}H_{22}O$ |  | Antifungal, antioxidant | Varsha *et al.* (2015) |
| 2. | 16.549 | 0.881 | 2',4'-Dihydroxy-3'-methylacetophenone (III- fungicide) | 166.17 | $C_9H_{10}O_3$ |  | Antifungal | Shi *et al.* (2016) |
| 3. | 17.074 | 0.241 | Dodecyl acrylate | 240.38 | $C_{15}H_{28}O_2$ |  | Antimicrobial | Joo *et al.* (2010) |
| 4. | 18.389 | 0.257 | Tetradecanoic acid | 221.37 | $C_{14}H_{28}O_2$ |  | Antioxidant, Antimicrobial, Weedicide, | Yayli *et al.* (2006); Ross *et al.* (2004) |
| 5. | 19.73 | 0.573 | Pentadecanoic acid | 242.4 | $C_{15}H_{30}O_2$ |  | Antibacterial and antifungal | Agoramoorthy *et al.* (2007) |
| 6. | 21.756 | 1.552 | n-Hexadecanoic acid | 256.42 | $C_{16}H_{32}O_2$ |  | Nematicide, antibacterial and antifungal | Kumar *et al.* (2010); Chandrasekaran *et al.* (2011) |
| 7. | 22.736 | 10.69 | 1-Hydroxy-6-methylphenazine | 210.23 | $C_{13}H_{10}N_2O$ |  | Antimicrobial | Norman *et al.* (2004) |
| 8. | 23.967 | 0.315 | Oleic Acid | 282.5 | $C_{18}H_{34}O_2$ |  | Antibacterial | Dilika *et al.* (2000) |
| 9. | 24.462 | 0.957 | Diisooctyl phthalate | 390.6 | $C_{24}H_{38}O_4$ |  | Algicide, antioxidant, allelopathic, antimicrobial | Huang *et al.* (2021) |
| 10. | 25.992 | 6.809 | Dibenzo[b,f] oxepin-3-amine, 10,11-dihydro-6-methoxy- | 241.28 | $C_{15}H_{15}NO_2$ |  | Antibacterial, antifungal | Limban and Chifiriuc (2011) |
| 11. | 26.763 | 39.904 | Binapacryl- Ester of dinoseb | 322.31 | $C_{15}H_{18}N_2O_6$ |  | Fungicide, miticide | Lewis *et al.* (2006) |

3'-methylacetophenone, 10,11-dihydro-6-methoxy, tetra, penta and hexadecanoic acid in the ethyl acetate fraction.

Specific production of some metabolites by the particular *Streptomyces* isolates was reported by Cordovez *et al.* (2015). This supported the present finding of binapacryl production by M12 isolate alone even though M11 and M12 were identified as same organism. The isolates had common metabolites; however, the production of binapacryl by M12 might be responsible for significant difference in their antifungal activity.

The GC-MS profile of both *A. cyanogriseus* isolates showed the production of 2,4-di-tert-butyl phenol (2,4-DTBP), which corroborated with the results of Dharni *et al.* (2014) whom reported that 2,4-DTBP from *Pseudomonas monteilii* (PsF84) was responsible for inhibiting spore germination and hyphal growth of *F. oxysporum*. Bacterial strain *Flavobacterium johsoniae* GSE09 inhibited the pepper pathogen, *Phytophthora capsici* by the production of 2,4-DTBP, indolic compounds, biofilms and biosurfactants (Sang and Kim, 2012).

Besides *A. cyanogriseus* isolates (M11 and M12) revealed the production of 2',4'-dihydroxy-3'-methylacetophenone, which classified as III generation fungicide had showed *in-vitro* antifungal activities against five potential phytopathogens *viz.*, *Glomerella cingulate*, *Cytospora* sp., *Botrytic cinerea*, *Alternaria solani* and *Pyricularia oryzaecar* (Shi *et al.*, 2016). Gideon (2015) studied the antimicrobial role of different forms of decanoic acids and was supported well with the present results.

Additionally, M12 was found to produce other potential antimicrobial compounds including phenazine-5,10-dioxide (PDO), dichloroacetic acid, 3-pentadecyl ester, octadecanoic acid, 1,3-diamino-5,6-dihydro-7-methoxybeno[f]quinazoline and phenanthridine-6,10-diol-10-acetyl-2-methyl-7,8,9,10-tetrahydro (Figure 1b, Table 2).

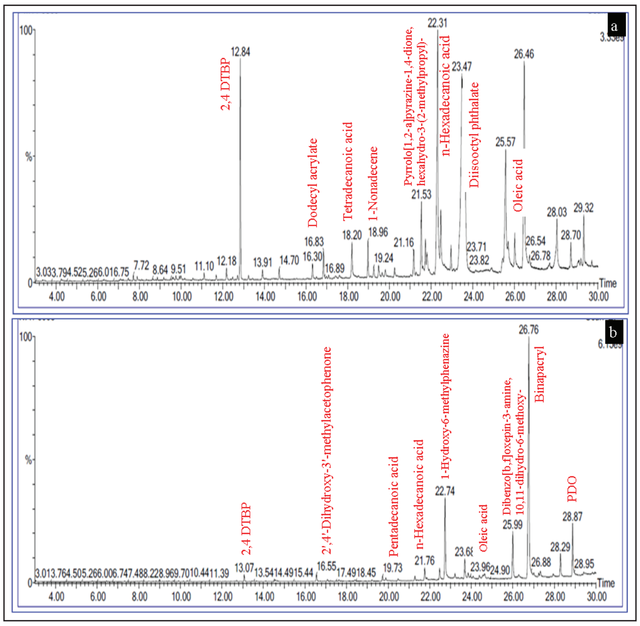



Figure 1: GC-MS volatile profile: (a) M11 ethyl acetate fraction, (b) M12 ethyl acetate fraction

Both the isolates produced an array of antimicrobials such as dodecyl acrylate, dodecanoic acid, 1-hydroxy methylphenazine; anti-bacterials including oleic acid; allelopathic compounds like diisooctyl phthalate with other antioxidants and flavonoid compounds. Phthalate derivatives were also produced by melanogenic isolates *A. cyanogriseus* coinciding with the report of Boudjelal *et al.* (2011), whom confirmed this as a natural product which had antibiotic effect. *Actinoalloteichus* sp. AH97 of their study produced two bioactive chemicals, were purified by HPLC and identified as aminoglycosidic molecule (hydrophilic) and dioctyl phthalate (hydrophobic).

Similarly, *A. cyanogriseus* 12A22 isolated from deep sea known to produce the various compounds including cyclo-(L-Pro-D-Pro-L-Tyr-L-Tyr) and 2-hydroxyethyl-3-methyl-1,4-naphthoquinone showed inhibition against pathogens such as *F. oxysporum* f. sp. *cucumerinum*, *Setosphaeria turcica*, *Botrytis cinerea* and *B. subtilis* (Zhang *et al.*, 2021). Apart from this many bioactive metabolites had been discovered from the *Actinoalloteichus* genus, including, cyclopentenone, caerulomycins (A, F-K), dioctyl phthalate, neomaclafungins A-I, *etc*.

GC-MS profile of melanogenic isolates revealed the presence of more than 30 compounds for each (Figure 1a, 1b). Most of them were identified as small molecules (<900 Daltons), which could act faster than antimicrobial polymers/ macromolecules. Organic compounds with low molecular weight might inhibit/ disrupt protein interactions and many drugs were small molecules (Arkin and Wells, 2004).

## Conclusion

Actinobacterial bioactive products, however, remained the maximum potential source of novel bioactive compounds, despite the need for innovative ways to increase the efficiency of mass production and extraction.

Microbial screening which produces bioactive compounds is highly interested in identifying novel compounds to combat a variety of phytopathogens. Thence, these extracellular metabolites production of the potent melanogenic isolates (M11 and M12) strengthened the inhibitory interaction against soil-borne fungal pathogens associated with the root rot disease of mulberry.

Identifying strong, long-lasting, broad-spectrum antifungal agents is essential for controlling phytopathogens. Thus, bioinoculums and its natural antimicrobial compounds may reduce the downbeats of chemical formulae and improve agroecology.

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