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Characterization of Antifungal Volatile Organic Compounds Produced by Bacterial Endophytes against *Fusarium oxysporum* through GC-MS Analysis

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Authors' contributions

This work was carried out in collaboration among all authors. Authors PM and ST designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PM and PS managed the analyses of the study. Author PS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Banana (*Musa* spp.) is one of the most important fruit crops cultivated throughout the world. *Fusarium* wilt caused by the fungus, *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen (Foc) causes significant threats to banana production. Endophytic bacteria play a significant role in plant protection against soil borne plant pathogens and promote overall productivity of agricultural ecosystems. Secondary metabolites produced by several species of *Bacillus* have been found to possess antibacterial or antifungal activity against different isolates of *Fusarium oxysporum* f. sp. *cubense.* These were identified by crude tests using agar gel diffusion and GC-MS analysis of the fungal crude extracts. Understanding of the metabolites produced by endophytes is also important in the context of biological control of soil-borne diseases of banana. GC-MS analysis of crude antibiotic extracts of bacterial endophytic isolates *viz*., GNBS3, PVBS3, NPBS4, KVBS4, BS1, revealed DL-Proline,5 Oxo as the major compound. Having both antifungal and antibacterial activity.

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Keywords: Banana; Bacillus; panama wilt; secondary metabolites; GC-MS analysis.

1. INTRODUCTION

Banana (*Musa* spp.) is grown in more than a hundred tropical and subtropical countries and provides staple food for hundreds of millions of people. Bananas are highly rich source of vitamins A, B and minerals like potassium and calcium [1]. Panama wilt is known to be one of the most important threatening diseases. The disease is ranked as one of the top six important plant diseases in the world [2]. India contributes 24% of the world banana production and it ranks second next to Brazil. In India, banana is cultivated in Tamil Nadu, West Bengal, Kerala, Maharastra, Gujarat, Karnataka, Assam, Andhra Pradesh and Bihar. Overall, Tamil Nadu, Maharastra and Gujarat together contribute 62% of the country's production. Fungal diseases cause remarkable losses in world-wide agriculture. The causal organism of banana wilt disease, *Fusarium oxysporum* f. sp. *cubense* is a highly variable soil borne fungus [3]. In India, it has been reported that the disease is highly destructive on variety of Rasthali [4]. Panama wilt is known to be one of the most important diseases threatening banana. It is especially serious in Central and South America, parts of Africa, Sri Lanka, Burma, Thailand, Indonesia and the Philippines, which results in great economic losses every year [2]. The typical distinguishable internal symptoms are discoloration of vascular tissues (in roots, corm and pseudostem) varying from light yellow to dark brown, which appear first in the outer leaf sheath, then extend up to the pseudostem. Generally, infected plants will not produce any bunches and if produced, the fruits are very small with only few fingers. The fruits ripen irregularly and the flesh is acidic. Finally whole plant dies [5]. *Fusarium* wilt is a ubiquitous disease in several agricultural and horticultural crops [6].

Endophytes can be defined as microorganisms inhabiting plants by forming a symbiotic relation with their host without causing any inherent negative effects. These endophytes not only form symbiotic association with their host, they also secrete secondary metabolites which protects plants against the invading pathogenic microorganism and play a vital role in plant defense against disease. Endophytic bacteria can not only promote plant growth and act as biocontrol agents, but can also produce natural products to control plant diseases [7] by reducing disease severity. Management of Fusarium wilt in banana has mostly resulted from eco-friendly approaches like bio control agents and resistant cultivars [8. Biological control of soil borne disease caused especially by *Fusarium oxysporum* is well documented. Biological control offers an alternative management strategy for several *Fusarium* wilt diseases [9]. Under these circumstances, at least one biological control mechanism is likely to be functional under the varied environmental conditions faced by the released biocontrol agents. Use of biocontrol agents to protect and promote plant growth is generally considered as a potentially safe approach for management of plant diseases. Endophytic bacteria associated with medicinal plants possess unique strategies that enhance growth and survival of host plants, many of which are mediated by distinctive secondary metabolites. These bacteria and their secondary metabolites are important subjects for both basic and applied research aimed at sustainable agriculture. Strikingly, most of the research carried out so far on bacterial volatiles mostly dealt with the response of *Fusarium* species. Secondary metabolites produced by several species of *Bacillus* have been found to possess antibacterial or antifungal activity against different phytopathogens [10]. Many of the bacterial endophytes like *Bacillus* and *Pseudomonas* sp. produce several secondary metabolites. Recent advancement in the field of biological control of plant diseases includes release of antimicrobial volatile organic compounds (VOCs) based formulation in the field. Therefore, the objectives of the present study were: (1) to screen a diverse collection of endophytic bacteria for activity against different isolates of *Fusarium oxysporum* f. sp*. cubense in vitro*; (2) to extract the antifungal secondary metabolites from potential endophytic bacterial isolates; and (3) to identify prevalent volatile organic compounds (VOCs) produced by endophytes, which are likely to be among the elicitor of the antifungal properties.

2. MATERIALS AND METHODS

2.1 Isolation of Endophytic Bacteria

Banana plants were manually uprooted and brought to the laboratory. Root and rhizome samples (2-3 cm long) were collected at different stages of crop growth of banana (3^{rd}, 5^{th} and 7^{th} month after planting). The root samples were taken just below the soil surface for younger

plants and 5-10 cm below the soil surface for older plants. Stem samples were taken 1-2 cm above the soil surface in younger plants and 10 cm above the soil surface in older plants. Stem samples were first weighed and surface sterilized with 1 per cent sodium hypochlorite mixed with 0.05 percent triton X100 for 10 min and rinsed four times in 0.02 M sterile potassium phosphate buffer pH 7.0. An aliquot of 0.1 ml was taken from the final buffer wash and transferred to 9.9 ml Tryptic Soy Broth (TSB) to serve as sterility check. Samples were discarded if growth was detected in the sterility check samples within 48 h. Each sample (0.5 g) was macerated with a sterile mortar and pestle in 9.5 ml of the final buffer wash. Serial dilution up to (10^{-10}) of the triturate was made in phosphate buffer. Each dilution of every sample was plated (0.1 ml) on three different media with three replications; Tryptic soy agar, Nutrient agar medium and King's B medium [11]. The plates were incubated at 28±2.0ºC for 48-72 h. One representative of *Bacillus subtilis* bacterium BS1, as evident from colony morphology was transferred to the plates containing fresh King's B medium to establish pure culture. The following pure cultures were used for the study *viz*., GNBS3-*Bacillus mojavensis* from Grand Naine, PVBS3-*Bacillus subtilis* sub sp. *spinnizi* from Poovan, NPBS4-*Bacillus malacitensis* from Ney Poovan, KVBS4-*Bacillus subtilis* from Karpooravalli and KVPf1- *Pseudomonas fluorescens* from Karpooravalli.

2.2 Extraction of Crude Antibiotics

The crude antibiotics of endophytic bacterial isolates were extracted as per the protocol described [12] with some modifications. A loopful of 24 h old bacterial isolates were inoculated into 300 ml sterilized King's B medium, and kept on shaker at 120 rpm for five days at room temperature. Culture broth was centrifuged at 10,000 rpm for 30 min. The cell-free supernatant was acidified to pH 2.0 with concentrated HCl, allowing the precipitate to form at 4ºC overnight. The precipitate was collected by centrifugation and re-suspended in one ml of 1N NaOH to adjust the pH to 7.0. The solution thus resulted was extracted twice with methanol using a separating funnel. The methanolic extract was mixed with an equal volume of ethyl acetate and kept on a shaker at 120 rpm for one hour. The ethyl acetate fraction was separated through separating funnel and condensed using a rotary evaporator at 80 rpm. The condensed form of the ethyl acetate fraction was dissolved in 1.5 ml of methanol: chloroform (1:1) and mixed thoroughly [13].

2.3 Bioassay of Crude Antibiotics against Mycelial Growth of *F. Oxysporum* F. Sp. *Cubense*

The agar well diffusion assay, as modified [14] was used to determine the antagonistic activity of purified antibiotic compounds. PDA medium (20 ml) was poured into each sterile petri plate, followed by placement of mycelial discs (5 mm diameter) of the tested pathogen at the center of the plates. A well (7 mm in diameter) was made by punching the agar with a sterile cork borer on the corner of the plate in four places with equal distance. Then purified crude antibiotic compounds from different strains of bacterial isolates were poured into the wells at the rate of 100 µl per well separately and incubated for 72 h at 28±2ºC. The inhibitory activity of each concentration was expressed as the percent growth inhibition, compared to the control (solvent only used in the wells), according to the formula,

$$\text{Growth inhibition \%} = (DC-DT)/DC \times 100$$

Where,

DC- Diameter of fungal colony in control
DT- Diameter of fungal colony with treatment

2.4 GC-MS Analysis of Crude Antibiotics

Detection of active bio-molecules present in the crude antibiotics of bacterial endophytes responsible for the suppression of *Fusarium* wilt in banana was carried out through GC-MS analysis (GC Clarus 500 Perkin Elmer). Volatile compounds were identified by GC/MS using a column Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 µm equipped with GC clarus 500 Perkin Elmer. The turbo mass-gold-Perkin-Elmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1, and injected volumes were 3µl. The column temperature was maintained initially at 110ºC at the rate of 10ºC/min - No holding followed by increases upto 280ºC at the rate of 5ºC /min and 9 min. The injector temperature was 250ºC and this temperature was held constant for 36 min. The electron impact energy was 70eV, Julet line temperature was set at 2000ºC and the source temperature was set at 200ºC. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 a MU range. Using computer searches on the

NIST Ver.2005 MS data library and comparing the spectrum obtained through GC/MS the compounds present in the crude sample were identified [15].

2.5 Statistical Analysis

The data were statistically analysed [16] and the treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute, Biometrics Unit, Philippines.

3. RESULTS AND DISCUSSION

In vitro efficacy of crude antibiotics from bacterial endophytic isolates from different cultivars against *Fusarium oxysporum* f. sp. *cubense.* The GNBS3 isolate (*Bacillus mojavensis*) recorded the maximum mycelial inhibition of 62.50 percent and an inhibition zone of 0.4 cm. The NPBS4 isolate (*Bacillus malacitensis*) recorded a maximum mycelial inhibition of 60.00 percent and an inhibition zone of 0.6 cm. The KVPf1 isolate (*Pseudomonas fluorescens*) recorded maximum mycelial inhibition of 56.66 per cent and an inhibition zone of 0.5 cm. The PVBS3 isolate (*Bacillus subtilis* sub sp. *spinnizi*) recorded maximum mycelial inhibition of 42.50 per cent and an inhibition zone of 0.8 cm. The KVBS1 isolate (*Bacillus* sp.) exhibited lowest maximal mycelial inhibition among those tested of 28.50 per cent over control (Fig. 1).

The volatile compounds from bacterial endophytic GNBS3 isolate were analyzed through GC-MS to detect novel compounds and secondary metabolites present in the bacterial endophytes. The compounds identified were confirmed through use of the NIST library 2005 AMDIS software programme. Totally, 50 compounds were identified, among these a few compounds were selected based on their unique nature and relative abundance of the peaks. The major compound present in the ethanol extracts was DL-Proline, 5 oxo with area percentage of (11.80%) followed by n-Hexa-decanoic acid with area percentage of (2.69%), Pyrrolidine,1-methyl (2.52%), Trans-2-decenoic acid (2.31%), 3-Decenoic acid,(E) (1.33%). 9-Decanoic acid (0.25%). These need to be broken into readable sentences! Better yet, make a table of the data! Also, be consistent with use of capital letters.

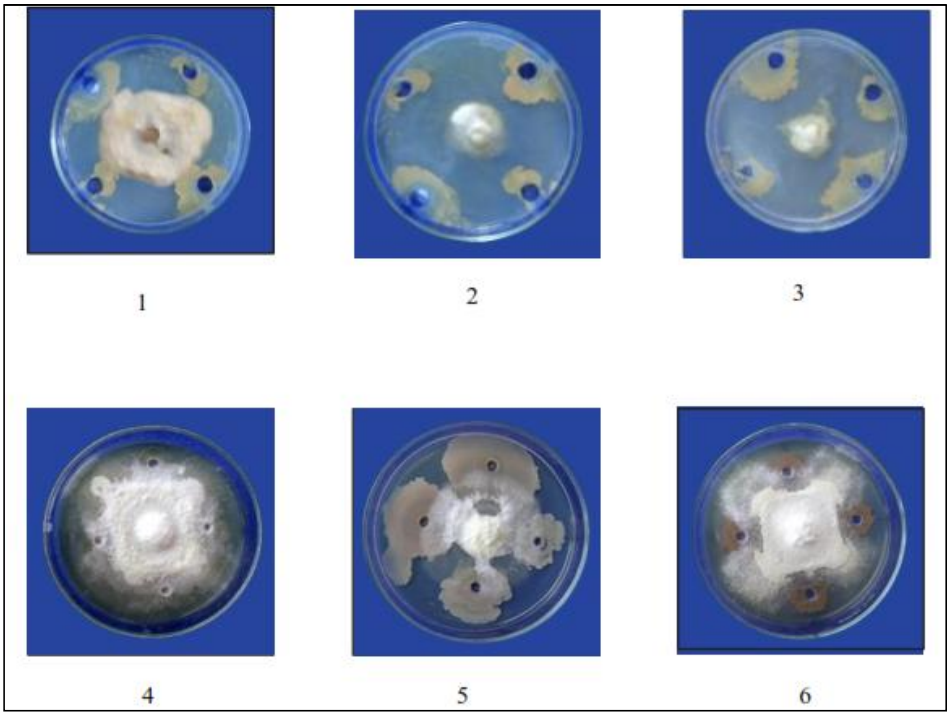

Fig. 1. *In vitro* efficacy of crude antibiotics from bacterial endophytic isolates *Fusarium oxysporum* f. sp. *cubense* (Agar well diffusion assay)
1. GNBS3; 2. NPBS4; 3. KPVF1; 4. BS1; 5. PVBS3; 6. KVBS1

Similarly, for GC-MS analysis of crude antibiotics of bacterial endophytic PVBS3 isolate, the major compound present in the ethanol extracts was Pyrrolo1,2-a}pyrazine-1,4-dione? Hexahydro-3 (2-methylpropyl) (15.83%) were found to be major component followed by 2,5 – Piperazinedione, 3,6-bis (2-methylpropyl) (8.14%), dihexylamine, N- nitro (5.5), 2(3H)-Furanone,5-acetyldihydro (2.85%) and 9,12-octadecadienoic acid (Z,Z)-methyl ester (0.17) component shows minimum level of area percentage. GC-MS analysis of crude antibiotics of bacterial endophytic NPBS4 isolate, the major compound present in the ethanol extracts of trans-13-octadecanoic acid, methyl ester 7 (15.11%) were found to be major component and followed by 9,12 Octadecadienoic acid (z,z)-methyl ester (8.77), 6-Octadecanoic acid, methyl ester,(z) (3.62%), butylated hydroxyl toluene (2.54%),Pentadecanoic acid (0.93%). 1-Decane (0.10%) component produce minimum level of are a percentage. GC-MS analysis of bacterial endophytic KVBS3 isolate, the major compound present in the ethanol extracts of DL-Proline,5 oxo (11.80%) were found to be major component and followed by 9,12-Octadecanoic acid (z,z)-methyl ester (7.60), n-Hexadecanoic acid (2.69%), Pyrrolidine,1-methyl (2.52%), Trans-2-decenoic acid (2.31), 3-Decenoic acid (1.33%). Butanoic acid,3-hydroxy-3-methyl (1.03%) component produce minimum area percentage. The major compound present in the ethanol extracts of *Pseudomonas fluorescens* KVPf1 are Pyrrolo{1,2-a}pyrazine1,4-dione, hexahydro-3 (2-methylpropyl) (15.94%) followed by Pyridine (6.59%), 2(3H)Furanone,5-acetyldihydro (2.01%) and Trans-2-decenoic acid (0.12%). Similarly, the major compounds present in the ethanol extracts of BS1 isolate are DL-Proline,5-oxo (17.72%), was found to be major component and followed byn-Hexadecanoic acid (8.40%), 9,12-Octadecadienoic acid (ZZ)-, methyl ester (4.19%), 6-octadecenoic acid, methyl ester(z-) (2.00%), Undecane (0.07%).

3.1 Discussion

Fusarium wilt and other diseases caused by fungi, are conventionally controlled using synthetic chemicals but, the indiscriminate use of these products has increased the resistance of pests to most of them, and has also generated adverse effects on the environment and human health [17]. The use of natural volatile compounds from biological extracts is a promising alternative for the control of phytopathogens. In this perspective, this research executed with the analysis of volatile organic antimicrobial compounds emitted by the beneficial antagonistic microflora of banana. Four endophytic *Bacillus* isolates (Grand Naine, Poovan, Ney Poovan and Karpooravalli), one endophytic *Pseudomonas fluorescens* isolate from Karpooravalli and standard isolate (BS1) were screened against *Fusarium oxysporum* f. sp. *cubense*. Among the bacterial endophytic isolates, GNBS3 *(Bacillus mojavensis*) isolate showed high level of suppression of Foc with minimum mycelial growth followed by GNPS1 isolate with minimum mycelial growth. The similar findings were reported by several workers [18]. The GCMS analysis results showed the presence of 10 different antimicrobial compounds present in the crude extracts. The major compound present in the ethanol extracts was DL-Proline, 5 oxo with high area percentage. Similar results obtained the components present in the ethanol extract of leaves of *Hugonia mystax* were identified by GC-MS analysis. The active principals with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the ethanol extract of leaves of *Hugonia mystax*. Thirteen components were detected in ethanol extract of *Hugonia mystax* leaves. The results revealed that 1,2-Benzenedicarboxylic acid and Diisooctyl ester (48.75%) were found to be major components followed by n- Hexadecanoic acid (13.52%), Phytol (9.25%), Squalene (6.41%), vitamin E (4.09%), Dianhydromannitol (3.56%), 9,12 – Octadecadienoic acid (Z,Z) – (3.20%) and 3,7,11,15 – Tetramethyl -2- hexadecen -1.ol (2.85%). Figs. 2,3,4,5 and 6 shows the mass spectrum and structure of Hexadecanoic acid, Ethyl ester, 11, 14, 17-Eicosatrienoic acid, Phytol, Squalene, and Vitamin E [19]. The prospective of numerous bacterial volatiles for growth inhibition of fungi liable for major crop losses in present day agriculture is profusely documented. Similar to the examination for growth-promoting volatiles in plants, that for the principle compounds responsible for the VOCs-mediated antifungal action of bacterial strains is very perplexing. Beyond inorganic volatile compounds such as ammonia (NH3) or HCN, isolating organic compounds, which constrain the growth of fungi when sprayed in concentrations related to the natural situation, has recognized a hard task.

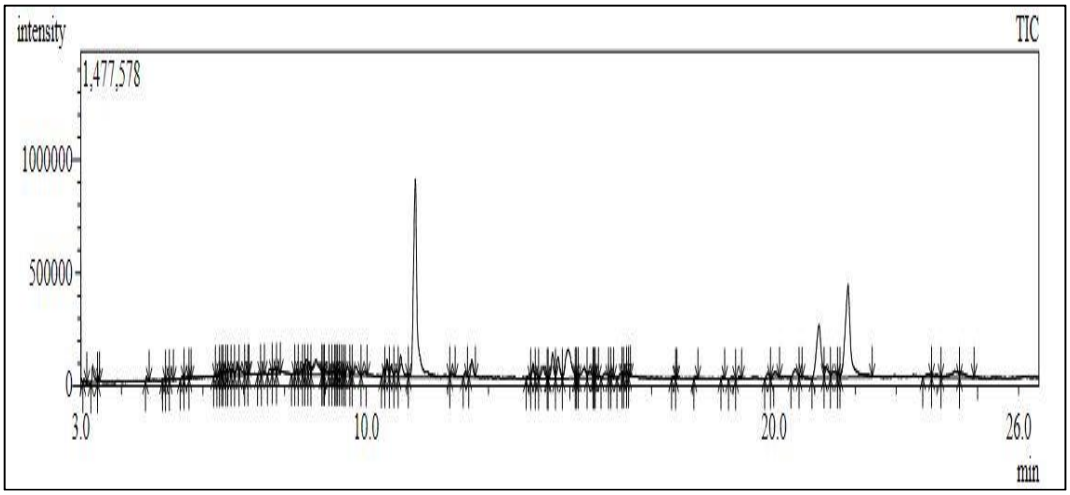

Fig. 2. GC-MS analysis of crude antibiotics of bacterial endophytic isolate of GNBS3 (*Bacillus mojavensis*)

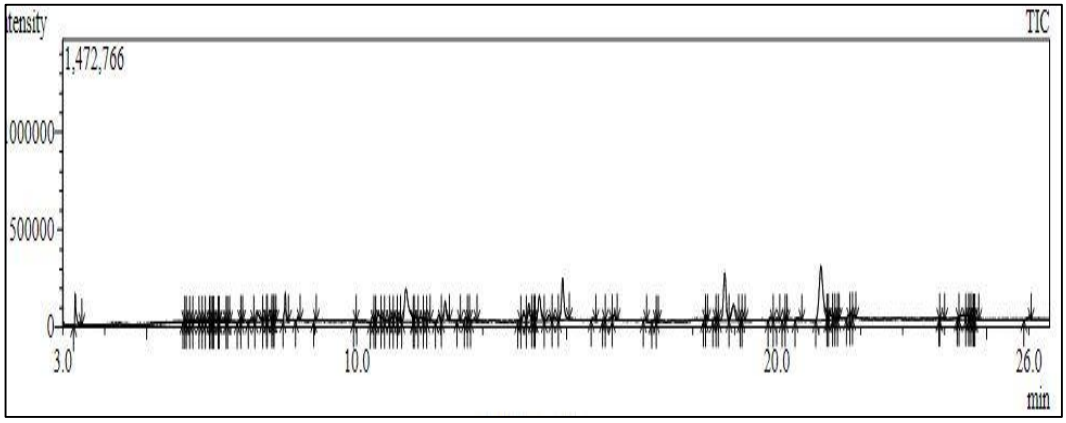

Fig. 3. GC-MS analysis of crude antibiotics of bacterial endophytic isolate of PVBS3 (*Bacillus subtilis* sub. sp. *spinnizi*)

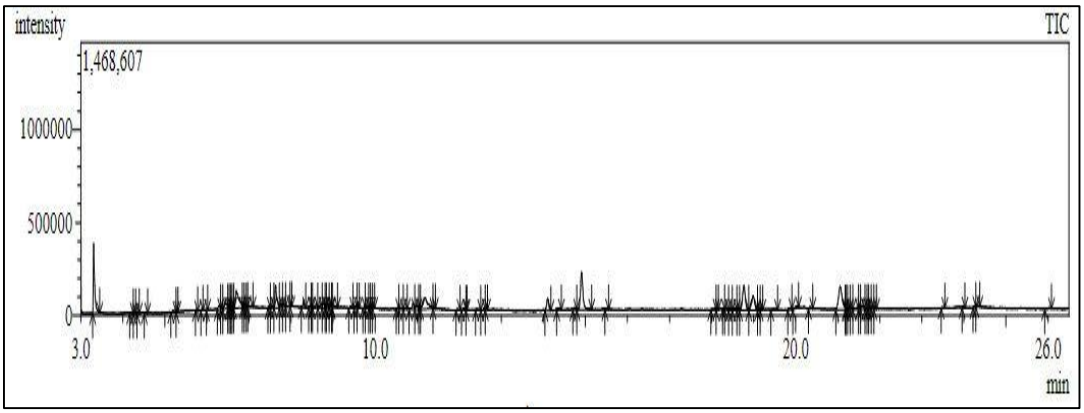

Fig. 4. GC-MS analysis of crude antibiotics of bacterial endophytic isolate of NPBS4 (*Bacillus malacitensis*)

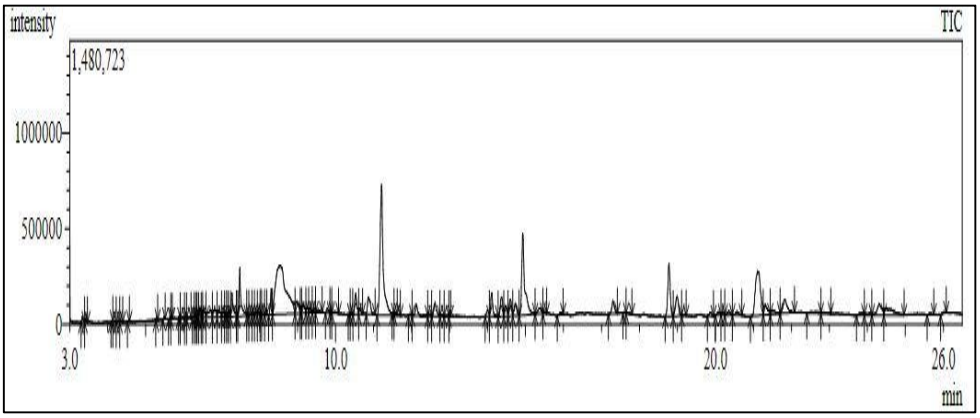

Fig. 5. GC-MS analyses of crude antibiotics of bacterial endophytic isolate of KVBS3 isolate (*Bacillus subtilis*)

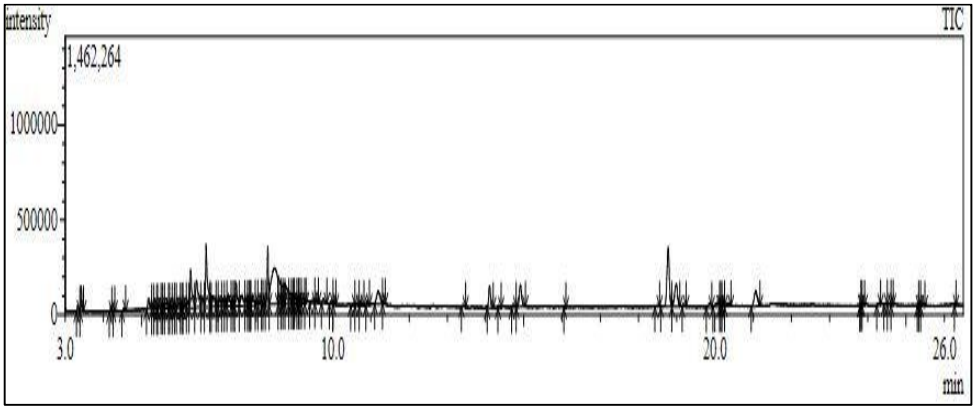

Fig. 6. GC-MS analysis of crude antibiotics of bacterial endophytic isolate of PF1 (*Pseudomonas fluorescens* 1)

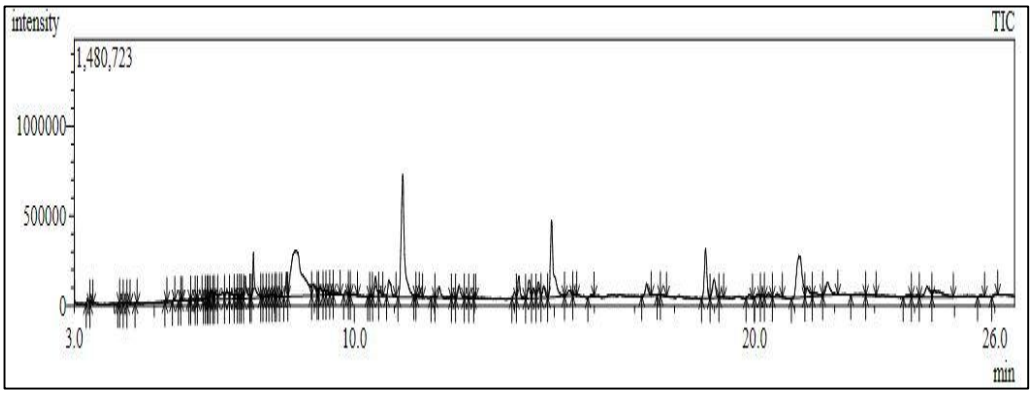

Fig. 7. GC-MS analysis of crude antibiotics of bacterial entophytic isolate of BS1 *(Bacillus subtilis* 1)

4. CONCLUSION

The antimicrobial biomolecules present in the effective isolate of GNBS3 (*Bacillus mojuvensis*), PVBS3 (*Bacillus subtilis* sub. sp. *spinnizi*), NPBS4 (*Bacillus malacitensis*), KVBS4 (*Bacillus subtilis*), BS1 (*Bacillus subtilis* 1) and Pf1 (*Pseudomonas fluorescens*), analysed through

the GCMS using crude antibiotics extraction. The GCMS analysis results showed the presence of ten different compounds with crude antibiotic properties. The major compound present in the ethanol extracts are DL-Proline, 5 oxo, the compound may having both antifungal and antibacterial activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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