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Metabolic Profiling of Plant Growth Promoting Microorganisms (PGPMs) from Chickpea Rhizosphere and Their Antagonistic Activity against Dry Root Rot Pathogen *Rhizoctonia bataticola*

Gururaj Sunkad, Meghana S. Patil, S.T. Yenjeerappa,
Sunil A. Kulkarni, Satyanarayana Rao, Nagaraj M. Naik

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ABSTRACT

Background: Dry root rot (DRR) caused by *Rhizoctonia bataticola* is the major threat to chickpea production globally. Recently, upsurge in disease incidence is observed due to unexpected increase in temperature and soil moisture stress resultant of changing climatic scenarios. To combat this necrotrophic phytopathogenic fungus, the present study was conducted with the aim of managing the disease by using potential rhizospheric PGPMs.

Methods: Twenty-six isolates of soil-borne fungal PGPMs were isolated from rhizosphere of chickpea in North Eastern Karnataka zone and screened for bio-active compounds production against *Rhizoctonia bataticola* by inverted plate technique. Using ITS genes BLAST analysis, the putative rhizospheric fungal PGPM was identified at the molecular level. Finally, chemical characterization of the bio-active compounds and metabolic profiling was carried out using Gas Chromatography Mass Spectrometry (GC-MS) technique.

Result: All the isolates showed significant differences in radial growth of mycelium at five days after incubation. SFPGPM-13 isolate produced maximum concentration of bio-active compounds with per cent mycelial inhibition of 62.83 (52.43). Molecular identification and BLAST analysis showed the isolate SFPGPM-13 was identified as *Trichoderma harzianum*. GC-MS analysis resulted that 62 compounds were present in the extract of SFPGPM-13 at different retention time and mass to charge (m/z) ratio.

Key words: Dry root rot, GC-MS, Metabolic profiling, *Rhizoctonia bataticola*, *Trichoderma harzianum.*

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is third most important pulse in the world after common bean and field pea (Pooran, 2020). India is the largest producer of chickpea in the world followed by Australia, Myanmar and Ethiopia. Among chickpea producing states in India, Madhya Pradesh ranks first in production. In Karnataka, the major chickpea growing districts are Kalaburagi, Bidar, Dharwad, Gadag, Bellary and Raichur (Department of Agriculture and Cooperation, GOI, 2020-21).

However, chickpea cultivation is under threat due to abiotic and biotic stresses. It is affected by 172 plant pathogens which cause soil, seed borne and foliar diseases (Manjunatha *et al*. 2011). Among root diseases, chickpea is majorly affected by dry root rot, caused by phytopathogenic fungus *Rhizoctonia bataticola* (Taub.) Butler (Pycnidial stage: *Macrophomina phaseolina* (Tassi) Goid. Dry root rot in chickpea was first reported from India by Mitra (1931). Typical root symptoms of the disease includes scattered necrotic brownish to black lesions in roots, progressing to rotting and withering lateral roots, accompanied by prematurely dried, straw-colored foliage. Many black microsclerotia of *M. phaseolina* are visible in the root bark, cortex and pith regions. Foliar symptoms start with gradual yellowing from the base to the top leaves (Sharma *et al*. 2015). Under severe infection, the entire chickpea field shows prematurely dried plants, accounting for 60 to 80% yield loss (Rai *et al*., 2022).

Department of Plant Pathology, University of Agricultural Sciences, Raichur-584 104, Karnataka, India.

Corresponding Author: Meghana S. Patil, Department of Plant Pathology, University of Agricultural Sciences, Raichur-584 104, Karnataka, India. Email: meghanasp2@gmail.com

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At present, boosting agricultural productivity relies heavily on the use of chemicals, which cause negative impacts on environment, plant growth and yield. Therefore, to increase global agricultural production in a more economically and environmentally sustainable way, there is need to use plant growth-promoting microorganisms (PGPMs) which are advantageous for improving crop productivity and food quality in more eco-friendly manner (Etesami, 2020).

The rhizosphere and endophytic fungal and bacterial community can harbor beneficial organisms known as PGPMs. Based on the interaction of roots with plants, PGPMs includes organisms present in the soil *i.e*., plant

growth promoting rhizobacteria (PGPR) as well as plant growth promoting fungi (PGPF). Non-pathogenic soil borne filamentous fungi are classified under PGPF. PGPF belongs to the genus like *Fusarium, Penicillium*, *Phoma*, *Trichoderma*, *Pythium*, *Aspergillus etc*. (Mitra *et al*., 2019).

Literature indicates that microbes are good source for extraction of biologically active compounds due to their ease of isolation, growth and inability to impact negatively on the environment. Therefore, the study on extraction, identification, screening of bio-active compounds responsible for inhibition of *Rhizoctonia bataticola* was undertaken.

MATERIALS AND METHODS

Isolation and purification of the pathogen

Plants showing typical disease symptoms of dry root rot were collected from chickpea fields of UAS, Raichur during *rabi* 2020-21 cropping season and the pathogen was isolated from the infected portions and cultured on potato dextrose agar (PDA) medium using hyphal tip isolation method. Then pathogenicity was proved by Koch's postulates.

Collection of chickpea rhizospheric fungal PGPMs

Soil samples from healthy rhizosphere of chickpea were collected during *rabi* cropping season 2020, for collection of isolates of fungal PGPMs. The isolates were collected from 11 different districts of northern Karnataka *viz*., Bagalkot, Bellary, Bidar, Dharwad, Gadag, Haveri, Kalaburagi, Koppal, Raichur, Vijayapur and Yadgir.

Isolation of rhizospheric fungal PGPMs

Isolation of 26 rhizospheric fungal PGPMs was carried out by serial dilution technique (Waksman, 1922) in the Department of Plant Pathology, UAS Raichur, during 2020-21. The plates were then incubated at 25±2°C for 4-10 days for growth of the fungi. After successful growth of fungi, they were characterized for their cultural characters.

Screening and antagonistic activity of bio-active compounds on *Rhizoctonia bataticola*

The test for inhibitory bio-active compounds production by fungal PGPMs was carried out by inverted plate technique. Three replications were maintained for each test PGPM and the plates were incubated at 28±1°C for five days. The colony diameter of the pathogen was measured in test pathogen plate as well as in control. The per cent inhibition was calculated by using formula of Vincent (1947).

$$I = \frac{C - T}{C} \times 100$$

Where,
I = Per cent inhibition in growth of test pathogen.
C = Radial growth (mm) in control.
T = Radial growth (mm) in treatment.

Molecular identification of potential rhizospheric fungal PGPM

The experiment was carried out in A.R.S., Gangavathi in 2021-22. The potential soil fungal PGPM isolate was identified based on ITS genes. The total genomic DNA of potential PGPM was extracted by using the Cetyl Trimethylammonium Bromide (CTAB) method. ITS genes were amplified from fungal genomic DNA using fungal universal primers; ITS1-F (CTTGGTCAT-TTAG AGGAA GTAA) and ITS4-R (TCC TCCGCT-TATTGATATGC) was used for characterization of fungal isolates. Primer sequences were synthesized at commercial facilities (Eurofins, Bangalore, India). Sequencing was carried out by Sanger's dideoxy chain-termination method and aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

Extraction of bio-active compounds from PGPMs

The experiment was carried out in Pesticide Residue and Food Quality Analysis Laboratory (PRFQAL), UAS, Raichur, Karnataka. The efficient fungal PGPM was grown in 500 ml of potato dextrose broth (PDB) and flask was incubated at 28 ±1°C for 21 days to produce the bio-active compounds. The culture filtrate was obtained by straining through the muslin cloth to obtain a cell-free supernatant. Compounds were extracted by solvent extraction method into ethyl acetate (EtOAc) at the ratio of 1:1 (v/v). Then the content was transferred into separatory funnel. The upper organic phase containing bio-active compounds, were collected through separating funnel into conical flasks. Ethyl acetate was evaporated from the collected upper phase by using rotary evaporator at 35°C under reduced pressure. Finally, the residue obtained in the rotary evaporator was resuspended in solvent (acetone) (Fig 1). Further identification of compounds was carried out by GC/MS analysis.

RESULTS AND DISCUSSION

Biology of pathogen

The *R. bataticola* pathogen produced whitish abundant mycelium of fungus on PDA at 4 days after incubation. Later pathogen become black, brown to grey coloured mycelium and darker with age. The young hyphae were thin, hyaline, septate and dichotomously branched and later produce typical black sclerotia. The characteristic features of *R. bataticola* were right angle branching of the mycelium and constriction of the branch near the point of origin. The sclerotia formed were black, smooth, varying from spherical through oblong to irregular shapes.

Isolation and identification of PGPMs

Twenty-six isolates of rhizospheric fungal PGPMs were collected and isolated by serial dilution technique using PDA and incubated for 7 days at 28 ±1°C. After the incubation, all the isolates showed typical greenish to greenish white mycelial growth on PDA which were culturally similar to *Trichoderma* spp. The isolates were designated as SFPGPM-1 to SFPGPM-2. The obtained of PGPMs isolates were maintained on PDA slants for further studies. The cultural characters of all the isolates were recorded after 7 days of incubation at 28 ±1°C. The growth of mycelium was

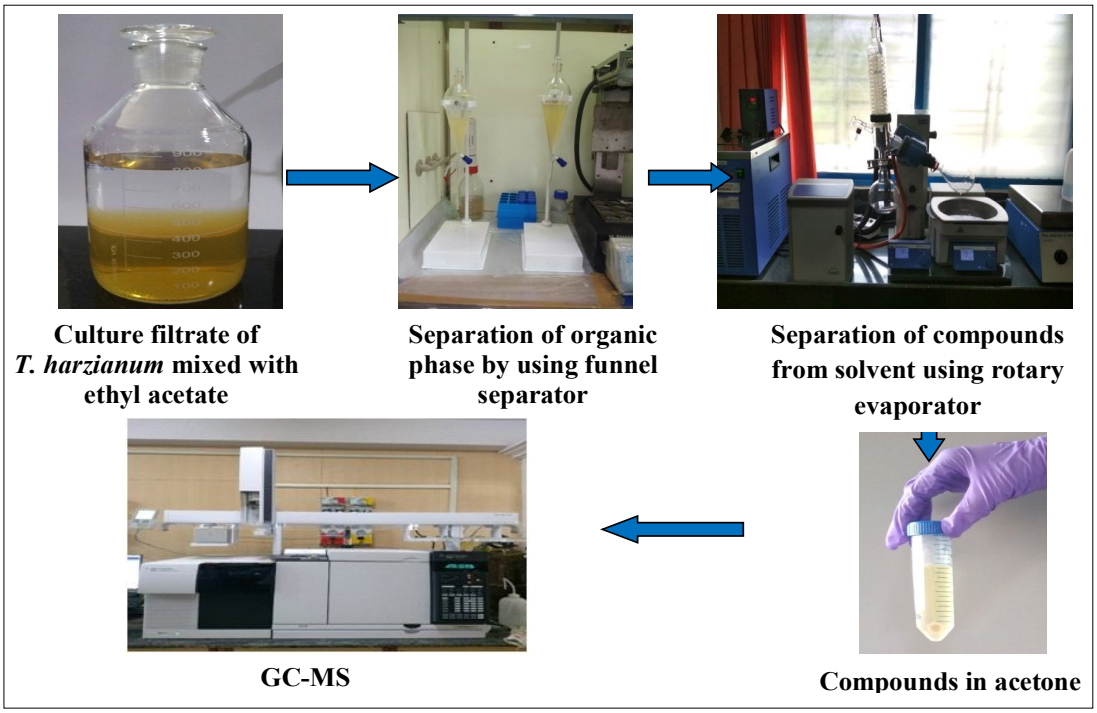

Fig 1: Schematic representation of extraction and identification of bio-active compounds from *T. harzianum* (SFPGPM-13).

Table 1: Cultural characteristics of rhizospheric fungal PGPMs on PDA after 5 days of incubation.

Isolate code	Colony colour	Colony shape	Type of growth	Type of margin	Colony growth (mm)
SFPGPM-1	Dark Green	Irregular	Fluffy and raised	Wavy	81.67
SFPGPM-2	Dark Green	Irregular	Fluffy and raised	Smooth	88.33
SFPGPM-3	Dark Green	Circular	Fluffy and raised	Smooth	89.00
SFPGPM-4	Dark Green	Circular	Fluffy and raised	Smooth	90.00
SFPGPM-5	Whitish green	Irregular	Fluffy and raised	Wavy	90.00
SFPGPM-6	Dark Green	Irregular	Fluffy and raised	Wavy	79.00
SFPGPM-7	Whitish green	Irregular	Fluffy and raised	Smooth	90.00
SFPGPM-8	Whitish green	Irregular	Fluffy and raised	Wavy	88.33
SFPGPM-9	Whitish green	Irregular	Fluffy and raised	Wavy	89.78
SFPGPM-10	Dark Green	Irregular	Fluffy and raised	Smooth	90.00
SFPGPM-11	Dark Green	Irregular	Fluffy and raised	Smooth	90.00
SFPGPM-12	Whitish green	Irregular	Uniform fluffy	Smooth	90.00
SFPGPM-13	Whitish green	Irregular	Fluffy and raised	Wavy	86.23
SFPGPM-14	Dark Green	Irregular	Fluffy and raised	Smooth	87.33
SFPGPM-15	Whitish green	Irregular	Fluffy and raised	Wavy	89.80
SFPGPM-16	Whitish green	Irregular	Fluffy and raised	Wavy	86.67
SFPGPM-17	Whitish green	Circular	Uniform fluffy	Smooth	90.00
SFPGPM-18	Dark Green	Irregular	Fluffy and raised	Wavy	89.00
SFPGPM-19	Dark Green	Irregular	Fluffy and raised	Smooth	78.33
SFPGPM-20	Light green	Circular	Uniform fluffy	Smooth	90.00
SFPGPM-21	Dark Green	Circular	Fluffy and raised	Smooth	88.00
SFPGPM-22	Dark Green	Circular	Fluffy and raised	Smooth	88.00
SFPGPM-23	Whitish green	Circular	Fluffy and raised	Smooth	87.33
SFPGPM-24	Whitish green	Irregular	Fluffy and raised	Smooth	88.00
SFPGPM-25	Whitish green	Irregular	Uniform fluffy	Smooth	90.00
SFPGPM-26	Whitish green	Circular	Uniform fluffy	Smooth	90.00

varied from 78.33 to 90.00 mm. The color and growth of the colony was very distinct (Table 1). Similarly, Shruthi (2017) isolated eleven *Trichoderma* spp. At 72 h of incubation, the maximum radial growth of mycelium (90 mm) was recorded. All the isolates were showed fluffy and raised colony to appraised flat type of colony growth, margin of colony varies from smooth and uniform to irregular margin.

Screening and antagonistic activity of bio active compounds on *Rhizoctonia bataticola*

All twenty six isolates produced considerable amounts of bio-active compounds which varied among them. Higher concentration of bio-active compounds was produced by isolate SFPGPM-13 with inhibition per cent of 62.83 followed by SFPGPM-9 with inhibition per cent of 62.50. All the isolates have shown significant difference in mycelial growth inhibition when compared to the control (Table 2). The results are in trend with Ranjana (2020) who tested twenty native *Trichoderma* isolates for volatile compounds production in inverted plate technique against *F. oxysporum* f. sp. *ciceris*. The results showed that all the isolates produced considerable amounts of volatile compounds which varied among isolates. Higher concentration of volatile compounds was produced by isolate TR-14 (72.22%) followed by TR-19 (66.67%), TR-9 (65.93%) and lower concentration of volatile compounds was produced by TR-18 (2.59%). All the isolates have shown significant difference in mycelial growth inhibition when compared to the control. Nagamani *et al.* (2017) isolated twenty *Trichoderma* isolates from chickpea rhizosphere soil and screened for their efficacy against soil borne plant pathogens namely *R. bataticola*, *F. oxysporum ciceri* and *S. rolfsii* by using invert plate technique. All the *Trichoderma* isolates produced toxic volatile metabolites having significant effect in reducing the radial growth of test pathogens.

Molecular identification of potential soil fungal PGPM

Potential PGPM isolate (SFPGPM-13) was identified based on ITS genes. Based on the results of ITS gene sequencing, SFPGPM-13 was subjected to BLAST analysis at NCBI for searching the closest phylogenetic relatives. Based BLAST analysis, the isolate was closely related to *T. harzianum* with 100% of similarity with accession no. ON514140.

Extraction and identification of bio-active compounds in *Trichoderma harzianum*

After solvent extraction method, the ethyl acetate extract of *T. harzianum* dissolved in acetone was subjected to GC-MS analysis. The GC-MS analysis revealed that the extract showed the presence of 62 compounds at different retention time ranging from 3.464 to 29.818 min. The mass to charge (m/z) ratio of the compounds were ranging from 43.6 to 191.9. The chromatogram of compounds showed different peaks (Fig 2). However, among 62 compounds, 20 compounds have antimicrobial property based the previous reports such as Pyridine, 3-ethyl (Marinescu and Popa, 2022), Butane-2-one, 3-methyl-3-(2-oxopropylamino)- (Al-Wathnani *et al*., 2012), 1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)- and 1,3-Propanediamine, N,N-diethyl-N′-methyl- (Culler *et al*., 1979), Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)- (Bhalla *et al*., 2021), Carbonic acid, 2-ethylhexyl nonyl ester and 4-Nitrobenzoic acid, 3-pentyl ester (Chowdhury *et al.* 2017), N- [Dimethylaminomethyl] aziridine and N-[3-[N-Aziridyl]propyl]-3-dimethylaminopropylamine (Kowalczyk *et al*., 2017) (Table 3). Hence, these compounds are involved in the defence mechanism against *R. bataticola*. Among 20 compounds, Pyridine, 3-ethyl- was found with maximum area of 747799 and retention time of 3.464 as compared to other compounds found (Fig 3). The structure of compound is shown in Fig 4. The results are also in accordance with Avinash (2017) who screened thirty five isolates of *Trichoderma* species for production of volatile compounds, two isolates *viz*., Tri-4 (*T. viridae*) and Tri-12 (*T. hamatum*) performed better under *in vitro* against major fungal pathogens *viz*., *Fusarium oxysporum* f. sp. *udum*, *Rhizoctonia bataticola*, *Sclerotium rolfsii* and *Alternaria alternata* and extracted and characterized secondary metabolites from isolate Tri-4 (*T. viridae*), LCMS result

Table 2: Effect of bio-active compounds produced by rhizospheric fungal PGPMs on mycelial inhibition of *R. bataticola.*

Isolate code	Colony growth* (mm)	Per cent mycelial inhibition*
SFPGPM-1	35.75	60.28 (50.92)
SFPGPM-2	37.15	58.72 (50.02)
SFPGPM-3	34.40	61.78 (51.81)
SFPGPM-4	45.45	49.50 (44.71)
SFPGPM-5	45.50	49.44 (44.68)
SFPGPM-6	45.40	49.56 (44.74)
SFPGPM-7	45.50	49.44 (44.68)
SFPGPM-8	45.35	49.61 (44.77)
SFPGPM-9	33.75	62.50 (52.23)
SFPGPM-10	43.50	51.67 (45.95)
SFPGPM-11	45.50	49.44 (44.68)
SFPGPM-12	45.55	49.39 (44.64)
SFPGPM-13	33.45	62.83 (52.43)
SFPGPM-14	44.90	50.11 (45.06)
SFPGPM-15	42.40	52.89 (46.65)
SFPGPM-16	45.15	49.83 (44.90)
SFPGPM-17	45.40	49.56 (44.74)
SFPGPM-18	45.45	49.50 (44.71)
SFPGPM-19	42.60	52.67 (46.52)
SFPGPM-20	45.50	49.44 (44.68)
SFPGPM-21	42.60	52.67 (46.52)
SFPGPM-22	40.65	54.83 (47.77)
SFPGPM-23	40.75	54.72 (47.70)
SFPGPM-24	45.30	49.67 (44.80)
SFPGPM-25	45.40	49.56 (44.74)
SFPGPM-26	42.50	52.78 (46.59)
Control	90.00	0.00 (0.00)
S. Em ±	-	0.34
CD at 1%	-	0.94

*Mean of three replications.

Table 3: Bio-active compounds with anti microbial property identified in acetone extract of rhizospheric fungi *T. harzianum* in GC-MS/MS analysis.

Formula	Name	RT	m/z ratio	Area
C_7H_9N	Pyridine, 3-ethyl-	3.464	91.7	747799
$C_8H_{15}NO_2$	Butane-2-one, 3-methyl-3-(2oxopropylamino)-	5.453	43.6	57446
$C_6H_{14}O3$	1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-	6.952	56.6	98983
$C_5H_{12}N_2$	N- [Dimethylaminomethyl] aziridine	10.586	57.8	586563
$C_8H_{20}N_2$	1,3-Propanediamine, N,N-diethyl-N'methyl-	10.825	43.6	42940
$C_{18}H_{36}O_3$	Carbonic acid, 2-ethylhexyl nonyl ester	11.383	70.6	70937
$C_8H_{20}N_2$	1,3-Propanediamine, N,N-diethyl-N'methyl-	14.132	71.6	46345
$C_8H_{20}N_2$	1,3-Propanediamine, N,N-diethyl-N'methyl-	14.27	71.7	104595
$C_8H_{20}N_2$	1,3-Propanediamine, N,N-diethyl-N'methyl-	16.368	57.6	45906
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	17.581	57.6	38609
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	17.733	71.6	74039
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	17.9	71.6	38481
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	18.265	71.7	37961
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	18.927	69.6	166704
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]- 3dimethylaminopropylamine	19.256	69.6	217322
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	19.479	69.6	197288
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	19.68	69.7	170545
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	26.45	69.6	49962
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	27.272	57.6	51901
$C_{10}H_{23}N_3$	N-[3-[N-Aziridyl]propyl]-3dimethylaminopropylamine	29.818	57.6	46015

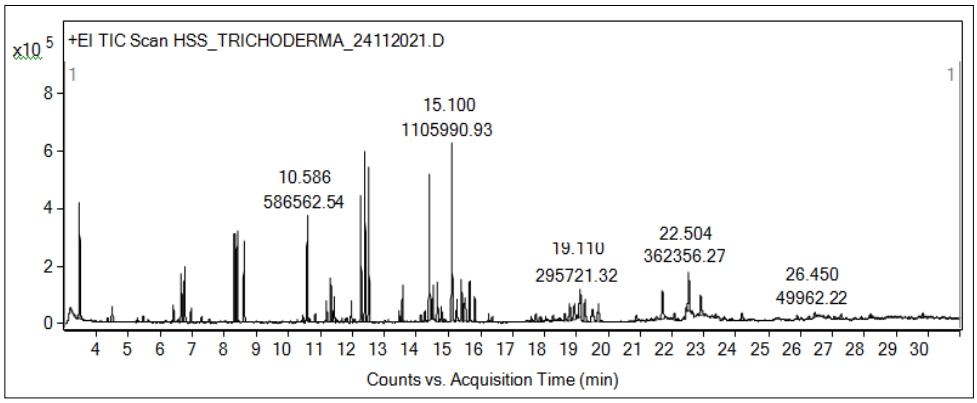

Fig 2: Chromatogram of compounds of *T. harzianum* extract obtained from GC-MS/MS analysis.

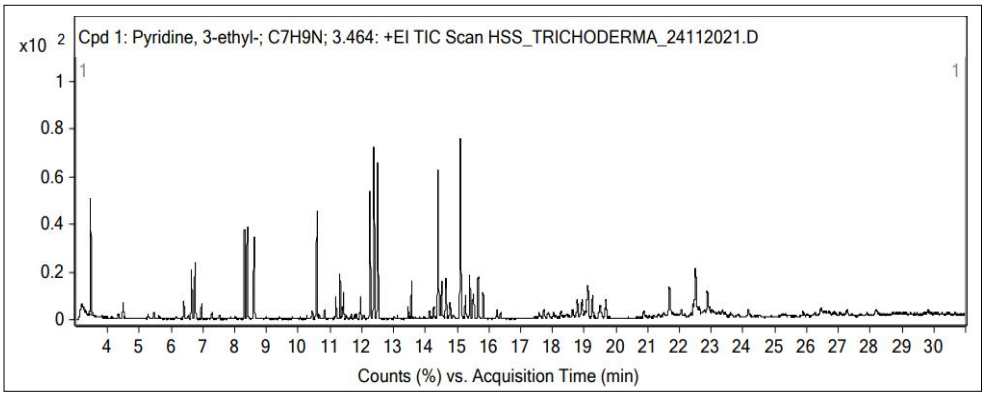

Fig 3: Mass spectrum of Pyridine, 3-ethyl- obtained from *T. harzianum* in GC-MS/MS analysis.

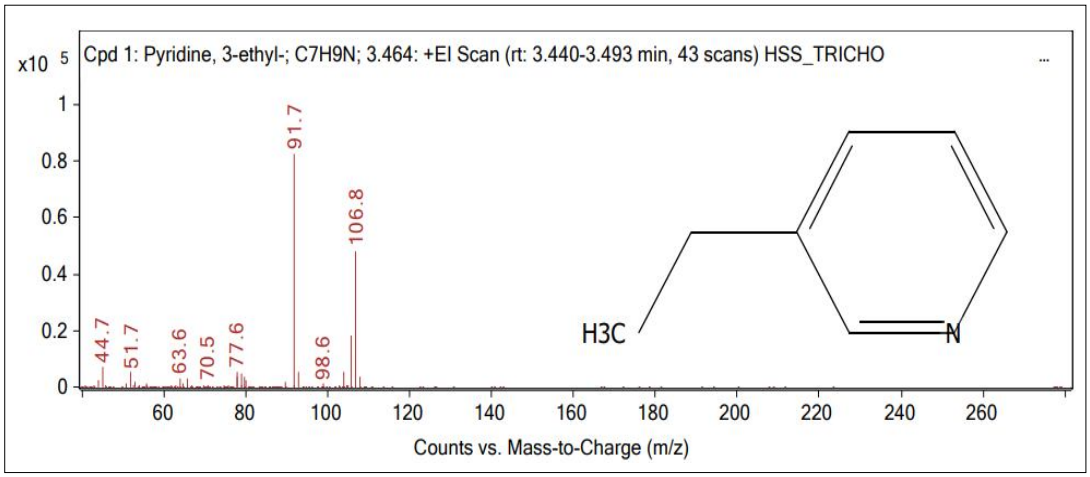

Fig 4: Mass fragmentation and structure of Pyridine, 3-ethyl- obtained from *T. harzianum* in GC-MS/MS analysis.

confirmed the presence of antifungal metabolites *viz*., ferulic acid, harzianic acid and viridofungin A. Apart from chickpea, the other legumes such as pea, groundnut, soybean, butter bean *etc*., reported to contain bio-active compounds like carotenoids, phenolic compounds, phytic and oxalic acid, phytosterols and saponins (Saini *et al*., 2019).

CONCLUSION

These findings open-up the perspective of using PGPMs with their potentiality for plant growth promotion and induction of bio active compounds production against pathogen as a sustainable approach. From our investigation, we found that chickpea rhizosphere does harbor diverse types of fungal PGPMs and all of them had potency as antagonistic activity against pathogen *R. bataticola* by producing 62 various bio active compounds. Further studies on quantification of the individual compounds responsible for antimicrobial activity and studies on the optimum conditions for producing this compound need to be explored.

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Conflict of interest: None.

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