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TRICHODERMA ASPERELLUM: A TREASURE HOUSE OF BIOACTIVE COMPOUNDS WITH ANTAGONISTIC ACTIVITY AGAINST *Rhizoctonia bataticola*, A CAUSAL AGENT OF DRY ROOT ROT IN CHICKPEA

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ABSTRACT

Dry root rot is a major threat to chickpea production globally. The disease is caused by *Rhizoctonia bataticola.* The recent rise in global temperature and worsening of drought spells has aggravated dry root rot outbreaks in chickpeas. To combat this necrotrophic pathogen, the present study was conducted to inhibit the pathogen by using potential plant growth-promoting microorganisms (PGPMs). Forty isolates of endophytic fungal PGPMs were isolated from healthy plant parts of chickpeas in a potato dextrose agar medium. Isolates were screened for antagonistic potential and bioactive compound production against *R. bataticola.* Using ITS genes BLAST analysis, the putative endophytic fungal PGPM was identified at the molecular level. Finally, the extraction of bio-active compounds and metabolic profiling was carried out by solvent extraction method and GC-MS/MS technique, respectively. Among forty isolates, FEPGPM-34 produced the maximum concentration of bio-active compounds with the highest percent mycelial inhibition of 74.61. Through molecular characterization and BLAST analysis, the isolateFEPGPM-34 was identified as *Trichoderma asperellum*. GC-MS/MS analysis of *T. asperellum* extract showed the presence of 65 compounds at different retention times and mass to charge (m/z) ratios with 13 compounds exhibiting antimicrobial properties. Hence, PGPMs can be exploited for managing dry root rot disease.

Keywords: Dry root rot, GC-MS, Metabolic profiling, *Rhizoctonia bataticola*, *Trichoderma asperellum*

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the earliest cultivated annual legumes of the family Fabaceae (Zohary and Hopf, 2000). Chickpea is a good source of important vitamins such as riboflavin, niacin, thiamin, folate and the vitamin A precursor β-carotene. As with other pulses, chickpea seeds also contain anti-nutritional factors (Jukanti *et al*., 2012). However, chickpea cultivation is affected by seed, soil-borne and foliar diseases (Manjunatha *et al*., 2011). Among them, the crop is severely affected by the disease dry root rot, caused by the necrotrophic phytopathogen *Rhizoctonia bataticola* (Taub.) Butler (Rai *et al.,* 2022). Dry root rot in chickpeas was first reported from India by Mitra in 1931. *R. bataticola* is a soil-borne, necrotrophic and polyphagous fungal pathogen. The characteristic morphological features of *R. bataticola* are right-angle mycelial branching, multinucleate septate mycelia, cross-wall formation at the beginning of new branching mycelia and partial hyphal fusion (Sharma *et al.,* 2016).

At present, boosting agricultural productivity relies heavily on the use of chemicals, which cause negative impacts on the environment, plant growth and yield. Therefore, to increase global agricultural production in a more economically and environmentally sustainable way (Mitra *et al.,* 2019), there is a need to use plant growth-promoting microorganisms (PGPMs) (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 include organisms present in the soil *i.e*., Plant Growth Promoting Rhizobacteria (PGPR) as well as Plant Growth Promoting Fungi (PGPF) and also organisms present inside the plant *i.e*., endophytes (Mitra *et al*., 2019). Most reported endophytes are *Pseudomonas* sp., *Bacillus* sp., *Burkholderia* sp. *Streptomyces* sp., *Actinoplanes* sp., *Alternaria* sp., *Trichoderma* sp., *Fusarium* sp., *etc*., (Mitra *et al.,* 2019). These microbes are a good source for the extraction of biologically active compounds due to their ease of isolation, growth and inability to impact negatively on the environment. The bioactive compounds may be growth promoters and resistance inducers (Msimbira and Smith, 2020). Thus, there is an urge to understand better and move forward with the knowledge that PGPMs holds great promise in

promoting productivity through synergistic interactions with host plants and can play role in disease management. Therefore, the present investigation aimed to identify the potential endophytic PGPMs which were antagonistic against *R. bataticola* and to reveal the bio-active compounds responsible for the inhibition of the pathogen.

MATERIALS AND METHODS

Isolation and purification of the pathogen: Plants showing typical dry root rot disease symptoms were collected from chickpea fields of UAS, Raichur during *Rabi,* 2020-21 and the pathogen was isolated from the infected portions on potato dextrose agar (PDA) medium by tissue isolation. The pieces were then transferred aseptically to Petri dishes containing sterilized Potato Dextrose Agar (PDA) and incubated at 28 ± 2 ºC under a BOD incubator. The Petri dishes were examined at regular time intervals for fungal growth radiating from the infected pieces. The pure culture of the pathogen was obtained from hyphal tip culture and such pure culture was used for further studies. Later, the pathogen culture was subjected to Koch's postulates.

Collection of endophytic fungal PGPMs: Plant parts of healthy chickpea plants were collected during *Rabi*, 2020-21 for collection of isolates of endophytic PGPMs. The isolates were collected from eleven different districts of northern Karnataka *viz*., Bagalkot, Bellary, Bidar, Dharwad, Gadag, Haveri, Kalaburagi, Koppal, Raichur, Vijayapur and Yadgir wherever chickpea is grown.

Isolation and maintenance of endophytic PGPMs: The isolation was carried out during 2020-2021 at the Bio-input Entrepreneurship Centre, College of Agriculture, Raichur. Isolation of forty fungal endophytic PGPMs from plant samples was carried out by tissue isolation method. Later, Petri dishes were incubated at 25 ± 2°C to obtain mycelial growth and the required cultures were purified in PDA media for further studies.

The antagonistic potential of fungal endophytic PGPMs against *Rhizoctonia bataticola*: To identify the antagonistic potential of PGPMs against *R. bataticola,* the antagonistic activity of endophytic PGPMs was tested by dual culture technique (Xu and Kim, 2014) under *in vitro.* Similar to Kamaruzzaman *et al*. (2021), the percent inhibition of mycelial growth of test pathogen was calculated using the formula,

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

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

Molecular characterization of potential endophytic fungal PGPMs: The experiment was carried out during 2021-22. The potential endophytic fungal PGPM isolates were characterized based on ITS genes. The total genomic DNA of fungi was extracted by using the Cetyl Trimethyl Ammonium Bromide (CTAB) method. ITS genes were amplified from fungal genomic DNA using fungal universal primers; ITS1-F (CTTGGTCAT-TTAGAGGAAGTAA) and ITS4-R (TCCTCCGCT-TATTGATATGC) (White *et al*., 1990). 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 and identification of bio-active compounds from potential fungal PGPM responsible for inhibition of *R. bataticola:* 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 the 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 the separatory funnel. The upper organic phase contained bio-active compounds and they were collected through the separating funnel into conical flasks. Ethyl acetate was evaporated from the collected upper phase by using a rotary evaporator at 35°C under reduced pressure. Finally, the residue obtained in the rotary evaporator was resuspended in solvent (acetone). Further, the identification of compounds was carried out by GC/MS analysis.

RESULTS

Isolation and purification of the pathogen: The pathogen was isolated successfully using the tissue isolation method. The pathogen produced whitish abundant mycelium on PDA after 4 days after incubation. Later, pathogen become black, brown to grey colored mycelium and darker with age. The young hyphae were thin, hyaline, septate and dichotomously branched and later produce typical black sclerotia.

Isolation and maintenance of endophytic PGPMs: Forty fungal endophytic PGPMs were isolated from the samples collected from eleven districts of north Karnataka and the isolates were assigned with codes such as FEPGPM-1 to FEPGPM-40. This indicated that the PGPMs co-evolve with the host.

Table 1: Antagonistic potential of endophytic fungal PGPMs against *R. bataticola* in dual culture technique

Sl. No.	Isolate code	Colony growth* (mm)	Percent mycelial inhibition*
1	FEPGPM- 1	63.00	30.00 (33.20)
2	FEPGPM- 2	66.67	25.93 (30.60)
3	FEPGPM- 3	75.33	16.30 (23.75)
4	FEPGPM- 4	63.33	29.63 (32.97)
5	FEPGPM- 5	38.00	57.78 (49.47)
6	FEPGPM- 6	87.33	2.96 (9.87)
7	FEPGPM- 7	62.67	30.37 (33.43)
8	FEPGPM- 8	70.33	21.85 (27.85)
9	FEPGPM- 9	71.33	20.74 (27.05)
10	FEPGPM- 10	59.50	33.89 (35.60)
11	FEPGPM- 11	31.33	65.19 (53.85)
12	FEPGPM- 12	52.33	42.96 (36.70)
13	FEPGPM- 13	64.33	28.52 (32.26)
14	FEPGPM- 14	59.50	33.89 (35.60)
15	FEPGPM- 15	57.00	36.67 (37.23)
16	FEPGPM- 16	34.67	61.48 (51.64)
17	FEPGPM- 17	65.33	27.41 (31.55)
18	FEPGPM- 18	82.33	8.52 (16.91)
19	FEPGPM- 19	73.00	18.89 (25.73)
20	FEPGPM- 20	71.00	21.11 (27.33)
21	FEPGPM- 21	62.17	30.93 (33.78)
22	FEPGPM- 22	66.33	26.30 (30.84)
23	FEPGPM- 23	82.67	8.15 (16.53)
24	FEPGPM- 24	33.33	62.96 (52.52)
25	FEPGPM- 25	71.00	21.11 (27.31)
26	FEPGPM- 26	61.33	31.85 (34.34)
27	FEPGPM- 27	23.33	69.63 (56.56)
28	FEPGPM- 28	35.00	61.11 (51.42)
29	FEPGPM- 29	55.33	38.52 (38.34)
30	FEPGPM- 30	52.00	42.22 (40.52)
31	FEPGPM- 31	66.67	25.93 (30.58)
32	FEPGPM- 32	83.33	7.41 (15.72)
33	FEPGPM- 33	71.33	20.74 (27.00)
34	FEPGPM- 34	12.17	86.48 (68.43)
35	FEPGPM- 35	27.33	74.07 (59.38)
36	FEPGPM- 36	65.67	27.04 (31.30)
37	FEPGPM- 37	70.33	21.85 (27.85)
38	FEPGPM- 38	70.00	22.22 (28.12)
39	FEPGPM- 39	66.33	26.30 (30.83)
40	FEPGPM- 40	77.00	14.44 (22.32)
	Control	90.00	00.00 (00.00)
	S. Em ±	**-**	**1.03**
	CD at 1%	**-**	**3.83**

*Mean of three replications, Figures in parentheses are arc sine transformed values

The antagonistic potential of endophytic fungal PGPMs: Results of the dual culture technique indicated that the percent of mycelial inhibition of the pathogen varied significantly among all the isolates (Table 1). The percent inhibition varied from 2.96-86.48 mm. However, the maximum percent inhibition of 86.48 was recorded in FEPGPM-34 followed by FEPGPM-35 (74.07). The efficacy of this isolate may be due to mycoparasitism where the overgrowth of the PGPM occurred by suppressing the growth of the pathogen.

Molecular characterization of potential endophytic PGPM: In molecular characterization, the primers produced an amplified product size of 500-650 bp. Further, the ITS sequence was BLAST searched and

results showed that isolate FEPGPM- 34 was identified as *T. asperellum*, with accession numbers ON514144.

Extraction and identification of bio-active compounds from *T. asperellum* for inhibition of *R. bataticola:* The GC-MS/ MS results of the ethyl acetate extract of *T. asperellum* showed the presence of sixty-five compounds at different retention times ranging from 3.166 to 30.91 min. The mass to charge (m/z) ratio of compounds was ranging from 42.7 to 281.8. The chromatogram of compounds showed different peaks (Fig. 1). Among sixty-five compounds identified, twelve compounds have antimicrobial properties (Table 2). Hence, these compounds were responsible for the inhibition of the pathogen and might process antifungal activity against the *R. bataticola.* Among twelve bio-active compounds, Butanal, 3-hydroxy- was found with a maximum area of 106059 and retention time of 3.166 as compared to other compounds. The chromatogram and structure of the compound are shown in Fig. 2.

Table 2: Bio-active compounds with the anti-microbial property identified in acetone extract of fungal endophyte *T. asperellum* in GC-MS/MS

Sl. No.	Formula	Name	RT	m/z ratio	Area	Percentage Peak area (%)
1	$C_4H_8O_2$	Butanal, 3-hydroxy-	3.166	42.7	106059	58.49
2	$C_3H_7NO_2$	D-Alanine	3.878	43.6	3047	1.68
3	$C_{10}H_{22}O$	2-Decanol	6.154	43.6	19531	10.77
4	$C_5H_{10}O$	Butanal, 3-methyl-	6.398	44.6	3662	2.01
5	$C_7H_8O_2$	1,2-Benzenediol, 4-methyl-	8.422	77.6	15548	8.57
6	$C_3H_7NO_2$	Propanamide, 2-hydroxy-	9.517	44.6	2688	1.48
7	$C_2H_7BO_2$	Boronic acid, ethyl-	16.362	44.6	4536	2.50
8	$C_{12}H_{20}N_2O$	1-iso-Propyl-3,6-diazahomoadamantan- 9-one	25.838	57.6	14258	7.86
9	$C_{16}H_{31}NSi$	2-Adamantylamine, TBDMS derivative	28.384	207.7	4325	2.38
10	$C_{16}H_{31}NSi$	2-Adamantylamine, TBDMS derivative	28.469	44.6	2131	1.17
11	$C_{16}H_{31}NSi$	1-Adamantylamine, N-tertbutyldimethylsilyl-	30.69	207.6	2260	1.24
12	$C_{16}H_{31}NSi$	2-Adamantylamine, TBDMS derivative	30.86	207.7	3271	1.80

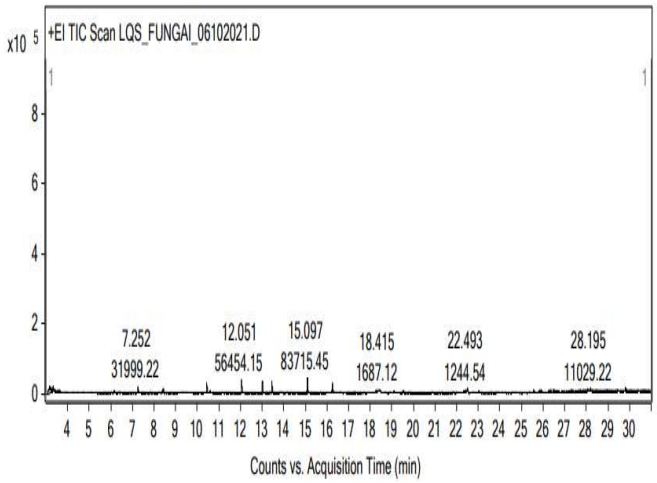

Fig. 1: Chromatogram of compounds of *T. asperellum* extract obtained from GC-MS/MS analysis

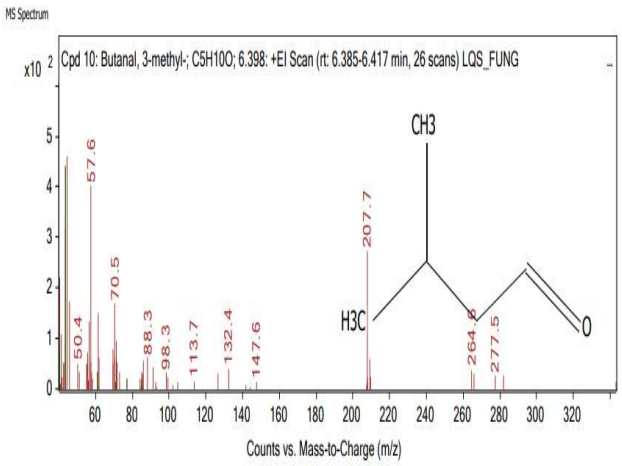

Fig. 2: Mass fragmentation and structure of Butanal, 3-hydroxy- obtained from *T. asperellum*in GC-MS/MS analysis

DISCUSSION

Isolation and purification of the pathogen: In the present investigation, 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.

Similarly, Chiranjeevi *et al*. (2020) isolated *R. bataticola* and observed morphological and cultural characters using the descriptions given by Barnett and Barry (1972). The mycelium was initially white in color which was later converted to dark brown to black in color. Production of aerial mycelium was also observed in some isolates. Branching occurred mostly at the right angle to parent hyphae, but branching at acute angles was also observed.

The antagonistic potential of endophytic fungal PGPMs: All the fungal endophytic PGPMs showed a significant percent of mycelial inhibition of *R. bataticola*. All the isolates showed significant differences over the control. Shirasangi and Hegde (2018) also reported that endophytes have antagonistic potential against *Rhizoctonia.* In their report, they collected a total of 26 fungal endophytes from the root, of different parts of healthy tomato samples from 30 locations in three districts of northern Karnataka. Among 26 fungal root endophytes evaluated against *R. solani* by dual culture technique, the isolate RFHKM-9 showed the maximum mycelial inhibition of 60.39 percent against *R. solani* which was followed by RFBBA- 23 (54.51 %) and RFDHE-10 (51.76) as noticed in the present investigation.

Molecular characterization of potential endophytic PGPM: In the present study, the efficient isolate was identified as *T. asperellum*. Similarly, in the molecular study conducted by Nagamani *et al.* (2017), sixteen *Trichoderma* isolates were isolated from different chickpea growing areas of Andhra Pradesh region and were identified by ITS sequencing as *T. asperellum*, *T. longibrachiatum, Trichoderma* spp.

Extraction and identification of bio-active compounds from *T. asperellum* for inhibition of *R. bataticola:* The twelve compounds which showed anti-microbial properties among 65 compounds in the study were reported by the previous studies conducted by Marcone *et al.* (2019), Togashi *et al*. (2007), Al-fatimi (2018), Guy *et al.* (2019), Osuntokun and Cristina (2019) and Al-Garaawi (2019). Hence these compounds were responsible for the inhibition of the pathogen in the dual culture and process antifungal activity against the *R. bataticola.*

Conclusions: The findings unplug the perspective of deployment of PGPMs with their potentiality for plant growth promotion and inhibition of pathogens as a sustainable approach. The present investigation reports that chickpea endophytes harbor diverse types of PGPMs and all of them had potency as antagonistic activity against pathogen *R. bataticola. Trichoderma asperellum* being more potential could able to inhibit the pathogen by producing thirteen various bioactive compounds.

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