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PROFILING OF *TRICHODERMA KONINGII* IABT1252'S SECONDARY METABOLITES BY THIN LAYER CHROMATOGRAPHY AND THEIR ANTIFUNGAL ACTIVITY

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ABSTRACT
In the present study, the secondary metabolites were isolated from *Trichoderma koningii* IABT1252 and they were fractionated by TLC with four different organic solvent mixture. The acetone and chloroform in 1:1 ratio was able to separate total ten distinct spot on TLC plate. In mean time, the crude extract was tested for its bio-efficiency, against *Rhizoctonia solani, Fusarium oxysporum* and *Sclerotium rolfsii* pathogens. The isolated secondary metabolites were more effective against *S. rolfsii* compared to *R. solani* and *F.* oxysporum. The secondary metabolite concentration at 50 ppm inhibited 12.78 per cent growth of *S. rolfsii* while 1000 ppm showed maximum growth inhibition of 84.26 per cent. Whereas, the isolated secondary metabolites poorly inhibited the *R. solani i.*e. even at 1000 ppm concentration of secondary metabolites inhibited only 8.71 per cent. Bioassay of secondary metabolites against *F. oxysporum* showed that growth inhibition was ranged between 5.93 to 24.36 per cent from 50 ppm and 1000 ppm. The study indicated that isolated secondary metabolites were effective against *S. rolfsii* followed by *F. oxysporum* and least was against *R. solani*.

INTRODUCTION

Trichoderma is a soil dwelling filamentous fungi. This genus is well known for parasitisation of pathogenic fungi including *Scelrotium rolfsii, Rhizoctonia solani, Sclerotinia sclerotium, Heterobasidion parviporum, Phythium ultimum, Alertnaria species, Phytophthora erythroseptica, Mucor mucedo, Botrytis cinera, Botrytis fabae, Pseuperonospora cubensis, Fusarium, and Rhizopus oryzae* (Upendra *et al*., 2007, Rohini *et al*., 2011, Barakat *et al*., 2013, Elad, 2000, Ganesan *et al*., 2007, Manjula *et al*., 2004, Ritesh *et al*., 2012, Varadharajan *et al*., 2006, Sevugaperumal and Rajagobal, 2011, Mishra *et al*., 2011, Ashwani *et al*., 2011, Elad *et al*., 1980, Etebarian *et al*., 2000, Sunaina *et al*., 2013, Vizma *et al*., 2012, Hadar, *et al*., 1979, Hino and Endo, 1940, Howell, 2002, Liu and Baker, 1980, Sunil *et al*., 2007, Tronsmo and Dennis, 1977). Hence, this genus is being used as a biocontrol agent at commercial level (Harman *et al*., 2004). *Trichoderma* systematically parasitise the pathogen by releasing hydrolytic enzymes, chitinase secreted form *Trichoderma* is able to breakdown the chitin rich fungal cell wall (Sivan and Chet, 1989). Similarly, another hydrolytic enzyme alkaline protease PRB1 from *T. harzianum* IMI 206040 has been demonstrated to play an important role in biocontrol activity by destructing the pathogen proteins and fungal cell wall protein components (Benitez *et al*., 1998, Elad, 2000).

Apart from hydrolytic enzymes, the secondary metabolites such as, peptaibols, anthraquinones, daucanes, koninginins, trichodermamides, viridins, viridiofungins, nitrogen heterocyclic compounds, azaphilones, trichothecenes, setin-like metabolites, bisorbicillinoids, diketopiperazines, statins and acoranes (reviewd in Jose *et al*., 2008, Mei *et al*., 2012) play an important role in parasitizing the pathogen. The type and amount of secondary metabolite production is strain dependent and the confronting organism. These metabolites may be volatile *viz*., pyrones, butenolides, 6-(1'-pentenyl)-2H-pyran-2-one, *etc* or non-volatile metabolites *viz*., trichodermaol, koninginins A–E, viridian and *etc*. The 6-n-pentyl-2H-pyran-2-one (6PP) produced from *Trichoderma* species and inhibits the new cell wall biosynthesis by suppressing the enzymatic action of glycan synthase and avoid the cellulose glycan bond formation. These metabolites aid the *Trichoderma* to survive and mycoparasitise the host fungi. Various reports says the effectiveness of these secondary metabolites on control different pathogenic fungi such as *S. rolfsii, Rhizoctonia solani, Fusarium oxysporum, Phytophthora cinnamomi and Pythium middletonii* (reviewed in Jose, *et al*., 2008). Hence, the secondary metabolites are considered as one of the potential antagonistic property of *Trichoderma*.

These secondary metabolites are polar or non polar type and travel for short or long distance in soil (Aochi and Farmer, 2005). These can be effective even before contacting the pathogenic fungi. Hence, these metabolites are called as heterogeneous group and acts in a synergistic manner (Lorito *et al*., 2004). Therefore, there is a large scope to study this group of compound and use them in agriculture field to protect the crop against the pathogen infection.

Till now, several secondary metabolites were isolated from *Trichoderma* spp. and most of them were reported have antimicrobial activities which directly participate in host parasitisation process (Howell, 1998). Ghisalberti *et al*. (1990) isolated pyrone-like antibiotic from *Trichoderma harzianum* and the metabolite has strong growth inhibition action on *Gaeumannomyces graminis* var. Tritici. Vinale *et al*. (2005), isolated and purified secondary metabolites such harzianolide, T39butenolide and harzianopyridone, these molecules were applied on top of 5 mm agar plug containing young pathogen (*G. graminis* var. tritici, *R. Solani* and *P. ultimum*) mycelia and after 5 days the pathogen growth was strongly inhibited even at 200 µg concentration. Hence, it is an indicative that *Trichoderma* spp. has several secondary metabolites with agro-economic importance.

The secondary metabolites can be isolated from *Trichoderma* by various organic solvent systems like hexane, chloroform, ethyl acetate, benzene or even in different combination also. The extracted metabolites can be separated on Thin Layer Chromatography (TLC) plate. TLC is a basic technique commonly used for identifying components in a sample, and for preparative purposes. The technique comprised of two phase *viz*., Stationary and mobile phase. The movement of metabolites in crude mixer depend on their solubility in mobile phase and affinity to towards the stationary phase (Stahl, 1988). Vinale *et al*. (2008) extracted secondary metabolite from *T. harzianum* A6 isolate which was secreted in one-fifth strength of potato dextrose broth and observed about 10 spots on TLC plate developed by ethyl acetate: petroleum ether solvent mixer. Further, the separated secondary metabolite spots on TLC plate can be eluted and characterized by GC-MS or LC-MS to know the type of compound.

Based on all these studies the present work was directed to isolate and separate the secondary metabolite from *Trichoderma koningii* IABT1252 isolate and study the influence of these metabolite to suppress the growth of three pathogenic.

MATERIALS AND METHODS

Chemicals and reagents

The nutrient composition of Potato Dextrose Agar (PDA) such as dextrose, yeast extract powder and agar agar type I were purchased from Himedia, the HPLC grade solvents such as hexane, ethyl acetate, chloroform, acetone and methanol were purchased from SD Fine Chemicals Ltd. The conical flask, separating funnel and beakers were purchased from Borosil, India.

Fungal culture and their maintenance

Biocontrol agent

The biocontrol agent *Trichoderma koningii* IABT1252 was carried from previous studies (Chidanand *et al*., 2015) based on its superior performance under *in vitro* experiments. The culture was maintained in Department of Biotechnology, University of Agricultural Sciences, Dharwad, Karnataka, India and it was being sub-culturing on PDA medium periodically.

Phytopathogenic fungi

The three potent plant pathogenic fungi *viz*., *Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium oxysporum* were selected. The *S. rolfsii* was collected from Main Agricultural Research Station field, UAS Dharwad and remaining two pathogens were collected from Department of Plant Pathology, UAS Dharwad. The cultures were maintained on PDA at 28 ± 1ºC and were sub-cultured prior to use as inoculum.

Inoculation of *T. koningii* IABT1252 and extraction of secondary metabolites

The secondary metabolites were isolated from *T. koningii* IABT1252 as per the procedure described by Dubey *et al*. (2011). The five mm agar plug was collected from young growing mycelia of *T. koningii* IABT1252 and inoculated in 1/5th strength of PDB in a 2L flask. After incubation period at 28 ± 1ºC for 30 days in a non shaking condition, the broth was filtered through whatman No. 1 filter paper. To the filtrate equal volume of ethyl acetate was added and stirred well for 15 min at room temperature. Later the mixture was transferred to separating funnel and upper aqueous layer was transferred to round bottom flask for evaporation of ethyl acetate in roto-evaporator at 40 ºC. The solid material collected at the bottom which is of red brown in colour was collected and stored at -20 ºC in a dark coloured 2 ml eppendorf tube.

Thin layer chromatography

The isolated secondary metabolites were separated on silica gel plate based on their polarity. TLC was performed on 200 μm thick aluminium TLC plate (TLC silica gel 60 $F_{254,-}$ Merck, India). The plates were developed using four different organic solvents *viz*., ethyl acetate, hexane, acetone, chloroform and methanol. These solvents mixed in four different combination such as ethyl acetate and hexane, hexane and acetone, acetone and chloroform chloroform and methanol in 1:1 ratio (Vivek *et al.,* 2013). The plates were visualized under UV light and by iodine vapours. The separated compounds were marked and retention factor (Rf) was calculated as per the formula given below (Fried and Sherma, 1982).

$$\text{Retention factor} = \frac{\text{Distant travelled by substance}}{\text{Distant travelled by solvent front}}$$

Bioassay of secondary metabolites

The efficacy of secondary metabolite that was collected from *T. koningii* IABT1252 was used for bioassay against *S. rolfsii, R. solani* and *F. oxysporum* in its crude form. The different concentration of secondary metabolite *viz*., 50 ppm, 100 ppm, 200 ppm, 600 ppm, 800 ppm and 1000 ppm was prepared in ethyl acetate and used for bioassay. The 5 mm agar plug of young growing pathogen culture was placed at the centre of PDA plate and above the agar plug 10 μl of diluted crude secondary metabolite was placed but in case of control treatment only ethyl acetate was placed above the pathogen agar plug (Almassi *et al*., 1991, Vinale *et al.,* 2008). All the plates were kept at 28 ± 1 ºC, after 6 days of incubation the observation were recorded and the per cent growth inhibition was calculated by below formula. The experiment was conducted with three replication in a CRD design.

$I = [(C-T)/C] \times 100$

Where, I - Per cent growth inhibition of pathogen

C - Growth of pathogen in control plate

T - Growth of pathogen in treatment plate.

RESULTS AND DISCUSSION

Thin layer chromatography

Each secondary metabolites of crude extract were resolved and formed clear spots without any smear or streak patterns. Totally eight secondary metabolite spots were observed in different combinations of three organic solvents (ethyl acetate and hexane; hexane and acetone; chloroform and methanol) used. Whereas, in combination of acetone and chloroform resolved totally ten spots from the crude extract of secondary metabolite on TLC plate. The distance travelled by different secondary metabolites were ranged between 1.3 cm to 6.8 cm, 1.5 cm to 8.0 cm, 2.0 cm to 7.8 cm and 0.7 to 7.5 cm in ethyl acetate : hexane (Fig. 1A and Fig. 1B), hexane : acetone (Fig. 2), chloroform : methanol (Fig. 3) and acetone : chloroform (Fig. 4) (1:1 ratio) respectively, while in each combination the distance travelled by the solvent front was 8.4 cm. The overall Rf value ranged from 0.08 to 0.95 (Table 1). Lowest Rf value was in acetone and chloroform combination and maximum was in hexane plus acetone combination. The similar separation pattern was observed with secondary metabolite isolated from *Trichoderma* isolates (Vinale *et al*., 2008) during its phytopathogenic fungi confrontation (Dubey *et al*., 2011).

Bioassay of crude extract of secondary metabolites

The secondary metabolites were significantly inhibited the growth of *S. rolfsii*, *R. solani* and *F. oxysporum* whereas in control plates these pathogens were grown and covered the plate completely. The isolated secondary metabolites were

Table 1: Retention factor values of thin layer chromatography

Spot No.	Distant travelled (cm)				Rf values			
	1	2	3	4	1	2	3	4
1	1.3	1.5	2	0.7	0.154	0.178	0.238	0.083
2	1.8	3	2.7	1.5	0.214	0.357	0.321	0.179
3	2.4	3.5	3.9	2	0.285	0.416	0.464	0.238
4	4.8	5.5	4.6	2.7	0.571	0.654	0.547	0.321
5	5.6	6.5	6	3.3	0.666	0.773	0.714	0.393
6	6	7	6.6	3.9	0.714	0.833	0.785	0.464
7	6.3	7.5	7	5	0.75	0.892	0.833	0.595
8	6.8	8	7.8	5.7	0.809	0.952	0.928	0.679
9	-	-	-	7.1	-	-	-	0.845
10	-	-	-	7.5	-	-	-	0.893
Solvent front	8.4	8.4	8.4	8.4				

Legend - 1-Ethyl acetate : Hexane (1:1 ratio);2-Hexane : Acetone (1:1 ratio);3-Chloroform : Methanol (1:1 ratio);4- Acetone : Chloroform (1:1 ratio)

Figure 1A: Secondary metabolites extracted from *T. koningii* IABT1252 were visualized by iodine vapors, the TLC plate was developed by ethyl acetate and Hexane in 1:1 ratio.

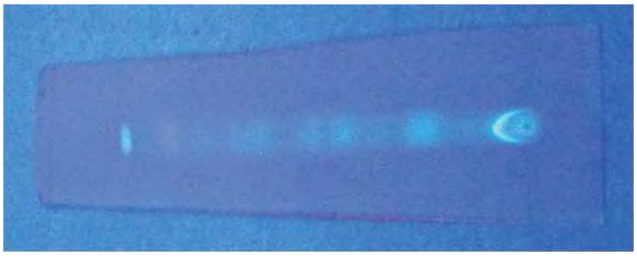

Fig.1B: Secondary metabolites extracted from IABT1252 were observed under UV light, the TLC plate was developed by ethyl acetate and Hexane in 1:1 ratio

Table 2: Per cent growth inhibition of *S. rolfsii* by secondary metabolites isolated from *T. koningii* IABT1252

S. No.	Secondary metabolite concentration (ppm) applied per plub	Per cent growth inhibition
1	50	12.78^{e}
2	100	35.56^{d}
3	200	35.74^{d}
4	600	59.44^{c}
5	800	75.37^{b}
6	1000	84.26^{a}
7	Ethyl acetate	0^{f}
	S.Em ±	0.40
	C.D. (5%)	1.22

* Supersrcipt alphabet letter indicate DMRT test at 5% level of significance.

Table 3: Per cent growth inhibition of *R. solani* by secondary metabolites isolated from *T. koningii* IABT1252.

S. No.	Secondary metabolite concentration (ppm) applied per plub	Per cent growth inhibition
1	50	2.61^{e}
2	100	4.58^{d}
3	200	4.79^{d}
4	600	5.45^{c}
5	800	7.19^{b}
6	1000	8.71^{a}
7	Ethyl acetate	0^{f}
	S.Em ±	0.13
	C.D. (5%)	2.85

* Supersrcipt alphabet letter indicate DMRT test at 5% level of significance.

Table 4: Per cent growth inhibition of *F. oxysporum* by secondary metabolites isolated from *T. koningii* IABT1252

S. No.	Secondary metabolite concentration (ppm) applied per plub	Per cent growth inhibition
1	50	5.93[e]
2	100	11.65[d]
3	200	17.79[d]
4	600	22.68[c]
5	800	23.09[b]
6	1000	24.36[a]
7	Ethyl acetate	0[f]
	S.Em ±	0.44
	C.D. (5%)	1.32

* Supersrcipt alphabet letter indicate DMRT test at 5% level of significance.

Figure 2: Secondary metabolites extracted from IABT1252 were visualized by iodine vapors, the TLC plate was developed by Hexane and acetone in 1:1 ratio

Figure 3: Secondary metabolites extracted from IABT1252 were visualized by iodine vapors, the TLC plate was developed by chloroform and methanol in 1:1 ratio.

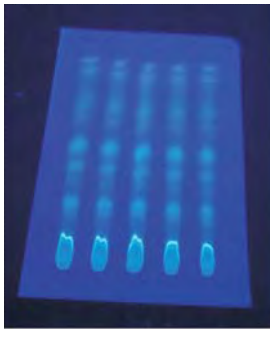

Figure 4: Secondary metabolites extracted from IABT1252 were observed under UV light, the TLC plate developed by acetone and chloroform in 1:1 ratio

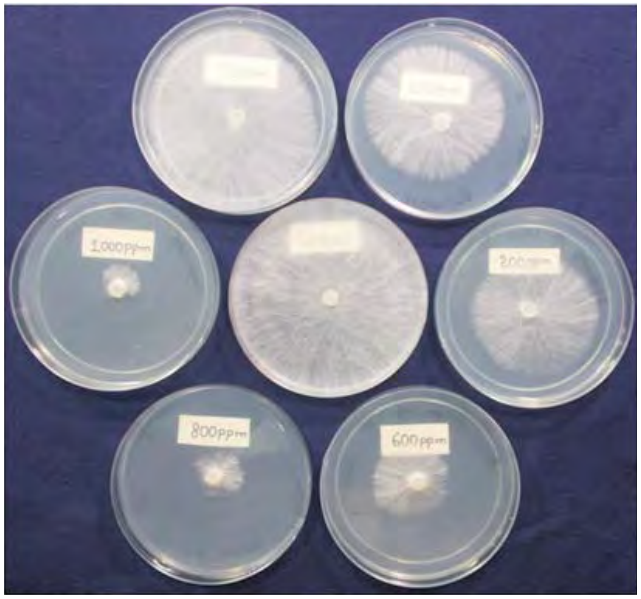

Figure 5: Secondary metabolite bioassay against *S. rolfsii*

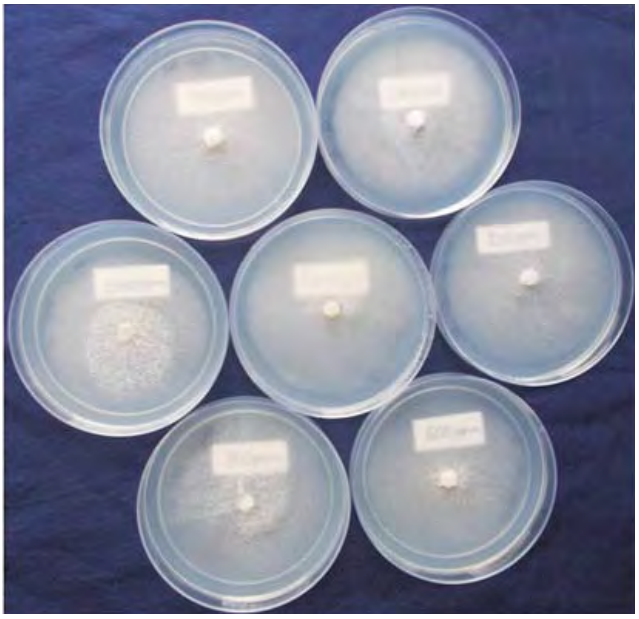

Figure 6: Secondary metabolite bioassay against *R. solani*

more effective against *S. rolfsii* compared to *R. solani* and *F. oxysporum*. The secondary metabolite concentration at 50 ppm inhibited 12.78 per cent growth of *S. rolfsii* while 1000 ppm showed maximum growth inhibition of 84.26 per cent (Table 2, Fig. 5). Similarly, Yin *et al*. (2010) reported that secondary metabolites from *T. harzianum* by using ethyl acetate highest inhibition of *Botrytis cinerea*. Several other research shown ethyl acetate as one of the potent solvent to extract the secondary metabolite from *Trichoderma* isolates (Jantamas and Dusanee, 2010, Vivek *et al*., 2013). In the present study the isolated secondary metabolites poorly inhibited the *R. solani* i.e. even at 1000 ppm concentration of secondary metabolites inhibited only 8.71 per cent (Table 3,

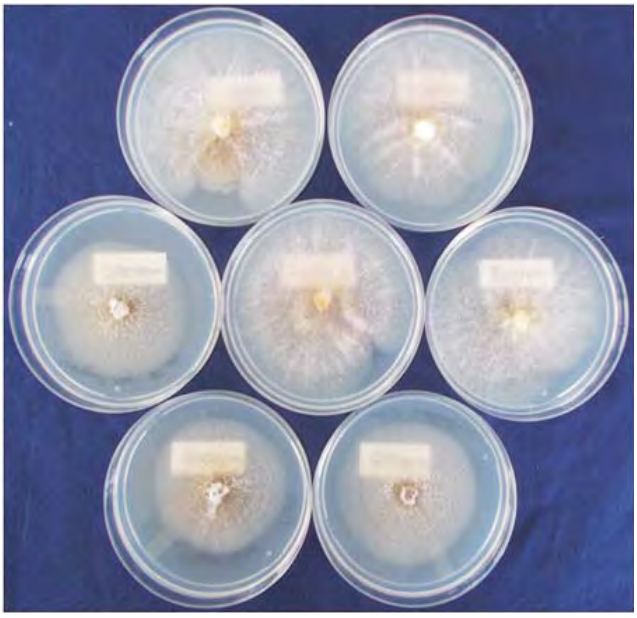

Figure 7: Secondary metabolite bioassay against *F. Oxysporum*

Fig. 6). Bioassay of secondary metabolites against *F. oxysporum* showed that growth inhibition was ranged between 5.93 to 24.36 per cent at 50 ppm and 1000 ppm (Table 4, Fig. 7). The study indicated that isolated secondary metabolites were effective against *S. rolfsii* followed by *F. oxysporum* and least was against *R. solani*. This may be due to the produced secondary metabolites were more specific to *S. rolfsii* than *F. oxysporum* and *R. solani*. Similarly, Vivek *et al*. (2013) reported that secondary metabolites isolated from *T. koningii* (T-8) extract shown 62.50% growth inhibition of *R. solani* but the same extract shown 76.97% growth inhibition of *F. oxysporum*. This is very clearly indicative that the mode of action and efficiency of secondary metabolites varies with the type of pathogen used for testing.

In conclusion, the solvent mixture acetone and chloroform (1:1 ratio) found good to be to resolve the secondary metabolites of *T. koningii* IABT1252 on TLC silicon plate. The total ten spots were observed on TLC plate, which was developed with acetone and chloroform (1:1 ratio) indicates that minimum ten diverse secondary metabolite groups are present in *T. koningii* IABT1252. The secondary metabolites are highly potential to inhibit the *S. roflsii* growth than *R. solani* and *F. oxysporum,* in other words *S. rolfsii* is susceptible and later two pathogens has resistant mechanism for secondary metabolites secreted from *T. koningii* IABT1252.

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