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Characterization of Secondary Metabolites from *Rhizopus oryzae* and Its Effect on Plant Pathogens

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Secondary metabolites plays a vital role during plant-fungal interaction, in addition to the synthesis of secondary metabolites, several fungal species also have antifungal effects on other microbes. In the present study secondary metabolites of three different isolates of *Rhizopus oryzae* were extracted and screened for their antifungal properties against *Alternaira, Pythium, Phoma* and *Rhizoctonia* at 500 ppm. The isolate *R. oryzae* (15) showed maximum inhibition against *R. solani* (81.53 %). GC/MS analysis of the secondary metabolites in *R. oryzae* (6975) revealed the presence of Benzene, 1,2-dimethoxy-4-(2-propenyl) commonly known as methyl euginol which might be responsible for the antibiotic activity of *R. oryzae* and may also be due to the presence of compounds containing benzene and higher fatty acids group.

Introduction

Fungal secondary metabolites are low moelcualr weight, heterogeneous group of natural compounds mainly functioning on survival of the organsim under stress conditions, and also responsible for competition, symbiosis, metal transport, and differentiation (Demain and Fang, 2000). Apart from it, the compounds also used for medical, pharmaceutical, or agricultural purposes (Calvo *et al.,* 2001). This group includes antibiotics, of natural origin, that are capable of inhibiting the microbial growth, of various microbes during the processes of development and sporulation (Stone and Williams, 1992). Secondary metabolite production usually commences late in the growth of microbe, often upon entering the stationary or resting phase.

The macrocyclic polyketide metabolite rhizoxin produced by *R.* oryzae causes rice seedling blight. The phytotoxin exerts its destructive effect by binding to rice β-tubulin, which results in inhibition of mitosis, because of its strong antimitotic activity. Besides, rhizoxin is also used as a potential antitumour drug (Laila *et al.,* 2005). With the above back ground we have tested the effect of the secondary metabolites of *R. oryzae* on *Alternaria alternata, Pythium*

aphanidermatum, Rhizoctonia solani and *Phoma lingum*.

Materials and Methods

Fungal cultures belonging to *Rhizopus oryzae, Alternaria alternata, Pythium aphanidermatum, Rhizoctonia solani* and *Phoma lingum* were obtained from Indian Type Culture Collection (ITCC), Division of Plant Pathology, ICAR-IARI, New Delhi, India.

Liquid culture and metabolite production

Two 7-mm diameter plugs of *R. oryzae* obtained from actively growing margins of PDA cultures, were inoculated into 250 ml conical flasks containing 100 ml of sterile one-fifths strength potato dextrose broth (PDB). The stationary cultures were incubated for three weeks days at 25 °C. The cultures were filtered through filter paper (Whatman No. 4; Brentford, UK).

Extraction of the metabolites from the liquid culture

The filtered culture broth (2L) of *R. oryzae* was extracted exhaustively with equal volume of ethyl acetate twice. The combined organic fraction was dried (Na_2SO_4) and evaporated under reduced pressure at 35˚C using rotary evaporator (Heidolph, Germany). The red-brown residue was recovered and further used for GCMS analysis.

Antifungal assays

The organic crude extracts and the chromatographic fractions were tested against *A. alternata, P. aphanidermatum, R. solani* and *P. lingum* for their antifungal properties. Pathogen plugs (9-mm diameter) from growing edges of the colonies were placed at the centre of petri dishes containing one-fifths the strength of PDA. Ten microlitre of the crude extracts were applied on the top of each plug. Plates were incubated at 25˚C for 3 days. The pathogen growth was measured daily as colony diameter. Each treatment consisted of three replicates and the experiment was repeated twice.

In vitro seed germination assay

Isolates of *R. oryzae* were tested for the seed mitotic inhibitor activity by seedling vigour index by the standard roll towel method (ISTA, 1999). Twenty five mustard seeds treated with 500 ppm were kept over the presoaked germination paper. The seeds were held in position by placing another presoaked germination paper strip and gently pressed. The butter paper sheets along with seeds were then rolled and incubated in growth chamber for 15 days. Two replications with 25 seeds each per replication, were maintained. Root length and shoot length of individual seedlings were measured and the germination percentage of seeds was recorded.

Vigour Index = (Mean root length + Mean shoot length) x Germination %

GC/MS analysis of volatile compounds

The crude metabolites of *R. oryzae* (isolate 5) was analyzed through GC/MS (Thermo scientific Trace GC Ultra DSQ II) equipped with column (30mm×0.25mm×0.25µm) under the following conditions: carrier gas as Helium with flow rate at 1ml per minute and 1µl sample injection with pre injection of solvent by AI/AS 3000 Method; split-less mode injection with 30 sec of sampling time; the column temperature maintained initially at 60°C at the rate of 5°C/min and no hold was followed by increasing up to 240 °C. It was kept at the same temperature for 8 minutes hold; the electron impact energy was 70eV, Julet line temperature was set at 2000 °C and

the source temperature was set at 200 °C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range.

The total chromatogram was obtained for each sample. The base peak of each spectrum was compared with the base peak of the chemical components in the NIST Ver.2005 MS data library through on-line and the spectrum was compared through GC/MS for the identification of the secondary metabolites present in the crude toxin.

Results and Discussion

All filamentous fungi produce several low molecular weight bioactive compounds which are known as secondary metabolites. In fungi, the genes required for the biosynthesis of a secondary metabolite are clustered. *R. oryzae* produce ethanol, lactic acid, and fumaric acid in high quantities (Guo *et al.,* 2010; Vially *et al.,* 2010). Production of antimicrobial secondary metabolites has been reported in many fungal biocontrol agents (Sivasithamparam and Ghisalberti 1998; Vyas and Mathur 2002). However, in the present study four plant pathogens were screened with the secondary metabolites of three different *R. oryzae* isolates. The isolate *R. oryzae* (15) had the maximum inhibition against *R. solani* (81.53 %) followed by the same isolates against *Pythium* (50.00 %) (Table 1 and Figure 1).

The ethyl acetate extract of the cell culture of *R. oryzae* exhibited significant antifungal activity against *R. solani* indicating that the metabolies are effective against those fungi which multiplies through mitosis. Sohail *et al.,* (2014) showed that the extracts of *R. stolonifer* is quite effective in acetonitrile solvent as compared to results in n-hexane, minimum inhibitory concentration (MIC), of the extracts were in the range of 0.25 mg/ml. Mitotic inhibition activity among the three isolates was tested on seedling of *Brassica juncea*, maximum reduction in seed vigour index was 51.61 % with the isolate *R. oryzae* (isolate 15) as against untreated control. It was followed by *R. oryzae* (isolate 10) which indicates that the extract may contain herbicidal activity. However, further studies to isolate the bioactive compounds are required (Table 2 and Figure 2).

Table.1 *In vitro* antifungal activity of crude metabolites from *R. oryzae*

S. No	Isolate	*Pythium aphanidermatum*		*Rhizoctonia solani*		*Alternaria alternata*		*Phoma lingum*	
		Radial Growth (cm)	Inhibition over control (%)*	Radial Growth (cm)	Inhibition over control (%)*	Radial Growth (cm)	Inhibition over control (%)*	Radial Growth (cm)	Inhibition over control (%)*
1	*R. oryzae* 5	3.65	3.94[b]	1.05	67.69[b]	3.75	8.53[b]	4.2	-
2	*R. oryzae* 10	3.7	2.63[b]	1.2	63.07[b]	3.7	9.75[b]	4.2	-
3	*R. oryzae* 15	1.9	50.00[a]	0.6	81.53[a]	3.35	18.29[a]	4.2	-
4	Control	3.8		3.25		4.1		4.2	

* Mean of four replications

Table.2 Effect of crude metabolites at 500 ppm on the growth of mustard seeds

S. No	Isolates No.	Shoot length (cm) *	Root Length (cm)*	Reduction in Germination (%)	Reduction in vigour (%)
1	5	7.53	18.18	88	13.97[c]
2	10	6.04	17.62	68	38.82[b]
3	15	5.23	15.98	60	51.61[a]
4	Control	7.97	18.33	-	-

* Mean of twenty five mustard seeds in four replications

Table.3 Compounds identified from *R. oryzae* isolate 5 through GC/MS

S. No.	RT	Name of the compound
1	28.028	1-(tert-Butyl)-3-cyclohexylcarbodi
2	31.963	Phthalic acid, cycloheptyl ethyl ester
3	32.831	Hexadecanoic acid
4	36.073	9,12-Octadecadienoic acid, methyl ester,
5	44.139	Cyclodecasiloxane, eicosamethyl-
6	44.980	Pentanoic acid
7	45.652	2,4-diphenyl-5-(p-methoxybenzoyl)- 1,2,3-triazole

Fig.1 *In vitro* screening of ethyl acetate extract metabolites on the Growth of the pathogen @ 500 ppm

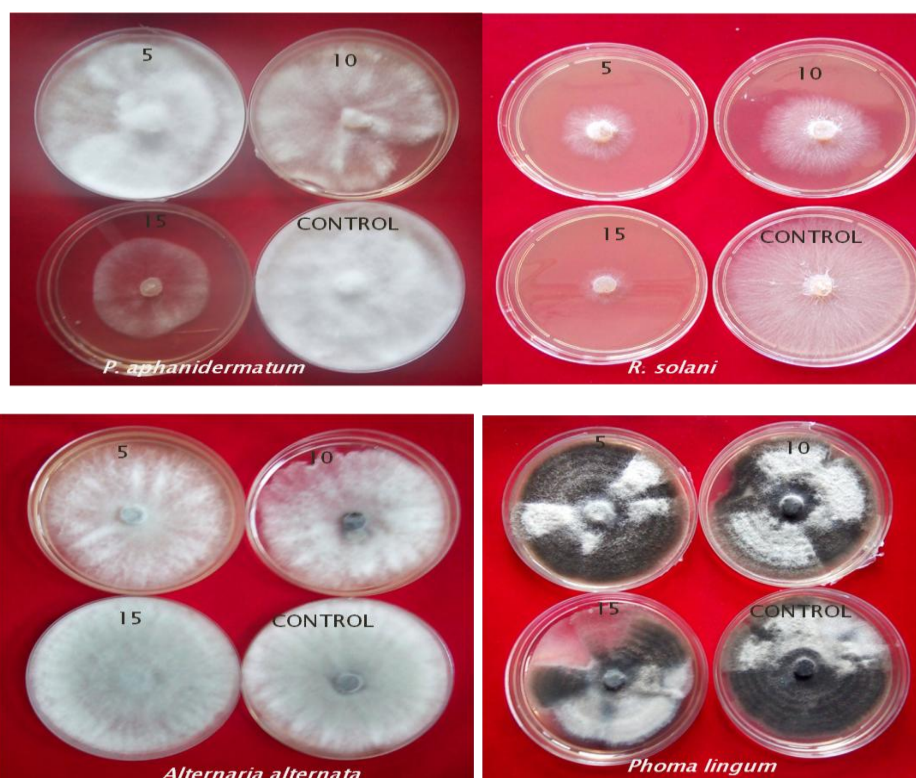
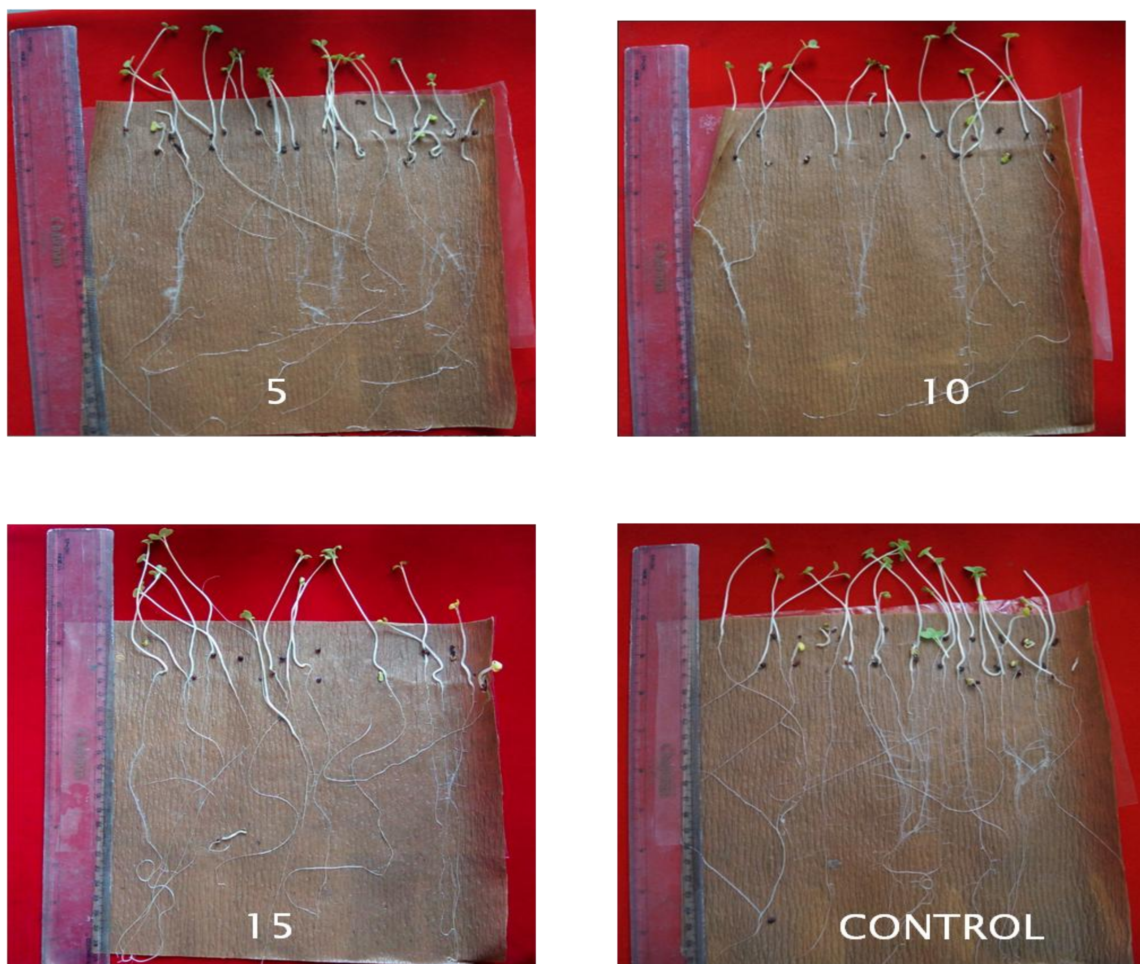


Fig.2 Effect of metabolites at 500 ppm on the growth of mustard seeds



5, 10 and 15 stands for *R. oryzae* isolate number

It's not new that the *R. oryzae* produce a plethora of bioactive secondary metabolites drawing many a curious researcher to the tidal swamps. In the present study we also tried to identify the compounds responsible for the bioactivity against major pathogens. Some of the major peaks are having more abundance ratio as listed in Table 3. Interestingly several compounds containing benzene like Benzene, 1,2-dimethoxy-4-(2-propenyl) commonly known as methyl eugenol. Hence from the GC/MS profile, the antibiotic activity of the isolate may be due to the presence of benzene and thiol group based compounds. Methyl-eugenol and eugenol extracted form *Piper diverticum* were tested individually at concentrations of 0.25 to 2.5 mg/mL against *F. solani* f. sp. *piperis*, and it expressed the strong antifungal index, from 18.0% to 100.0% (da Silva *et al.,* 2014). Eugenol and methyl eugenol are phenylpropanoids and are known to possess antimicrobial activity, induction of oxidative stress characterized by elevated levels of free radicals and an impaired antioxidant defence system. It may be a possible mechanism of cell death (Khan *et al.,* 2011). In additions to antifungal activtity we have also reported for the first time that the existence of Methyl Euginol in *Rhizopus oryzae* (Faisal and Lakshman, 2016).

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