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Characterization and Identification of Novel Finger Millet (*Eleusine corocana*) Phytoalexins from the Leaves Infected with *Pyricularia grisea*

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Abstract: The phytoalexins are antimicrobial, low molecular weight compounds (secondary metabolites) and considered to be involved in defense reactions against pathogens. The present investigation was undertaken to study the phytoalexins in relation to blast pathogen infection in finger millet leaves in order to understand the nature of resistance to blast in the host. The phytoalexins from blast (*Pyricularia grisea*) infected leaves of finger millet (*Eleusine corocana*) were studied by extracting and subjecting to sephadex LH-20 column chromatography purification and characterization by continuous UV-spectra, EI-mass and IR spectrophotometer. Four phytoalexins isolated from blast infected leaves of finger millet of which two components with an R_f value of 0.22 and 0.28 were identified as oryzalexin A and B, respectively. Two other components with an R_f value of 0.35 and 0.61 are novel phytoalexins produced by finger millet in response to blast infection and needs to be studied further for nomenclature.

Key words: Finger millet, blast, phytoalexin

INTRODUCTION

Finger millet (*Eleusine corocana*) is a staple food crop in drought prone areas of the world. It provides an excellent dietary source of methionine and is productive in a wide range of agro-ecological environments. Finger millet is a hardy crop generally free from severe attack by pests and diseases[1]. But, in recent years, blast (*Pyricularia grisea*) is becoming increasingly severe especially on high yielding varieties. Blast is the most serious disease that affects all aerial parts of the plants at all stages from seedlings to grain formation stage. Plants that shown to define themselves against disease causing agents by activating different defense reactions like the formation of antimicrobial compounds such as phytoalexines, phenolics and hydrolic enzymes[2]. Phytoalexines are low molecular weight antimicrobial compounds that are synthesized and accumulated in plants cells after exposure to plant pathogenic fungi or stress[3,4]. Several phytoalexins are isolated and characterized in rice and *Pyricularia grisea* interactions. Oryzalexins A-D were isolated from foliar parts of rice plants infected with *Pyricularia grisea*[1-4]. Momilactones A and B are also produced in UV-irradiated rice leaves or blast infected rice leaves pretreated with Wl-28325 (2,2-dichloro-3,3-dimethyl cyclopropane carboxylic acid)[1]. Oryzalexin E, F, S and sakuranetin are the novel phytoalexin reported in UV-irradiated and also in blast infected rice leaves[5-10]. The aim of this study was to describe the isolation, characterization and antifungal activities of novel phytoalexins from finger millet leaves infected with *Pyricularia grisea*.

MATERIALS AND METHODS

The experiments were conducted at project co-ordinating unit on small millets and Department of Biochemistry, GKVK, University of Agriculture Sciences, Bangalore during 2000-2002. The leaves of GPU28 finger millet variety infected with *Pyricularia grisea* were collected in the field as the source for phytoalexin compounds. Blast infected finger millet lesions (10 g) are boiled in 120 mL of 70% methanol for 3 min and then ground. The homogenate was centrifuged at 7500 rpm for 15 min. The supernatant was concentrated in vacuum and then applied to a sephadex LH-20 column chromatography (1.5x50 cm) eluted with methanol. In a fraction collector, fraction of 1.5 mL each were collected and monitored

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for the presence of phytoalexins by A225 in UV-VIS spectrophotometer. The eluted fractions were tested for antifungal activity on spore germination of *Pyricularia grisea*. The antifungal fraction were pooled and concentrated in vaccum and purified by preparative TLC silica gel 60F 254, 0.5 mm, (20x20 cm) with benzenethyl acetate (10:1,V/v) as solvent antifungal activity was observed at R_f values of 0.22, 0.35 and 0.61. Each active phytoalexin band was scraped off, extracted with methanol, concentrated in vacuum and further purified on preparative TLC in a similar manner. These phytoalexin compounds were subjected to spectral characters.

The spores of *Pyricularia grisea* were obtained by transferring 5 mm mycelium to conical flask containing sterilized moist finger millet seeds under aseptic condition and incubated at 28±1°C for 15 days, with intermittent shaking. The mycelia growth on seeds was transferred to petridishes containing moist blotting paper and the spores were harvested after 48 h. The spores were suspended in sterilized distilled water and diluted to 15-25 spores in a microscopic field. Each active band on TLC was scrapped off and extracted with methanol. This methanol phase was evaporated and then volume was made to one ml with sterilized distilled water. This was tested on spore germination of *Pyricularia grisea* in cavity slide at different dilutions. After incubation for 8 h at 28±1°C, the present spore germination was measured under microscope.

Statistical analysis: The analysis and interpretation of data was done using Fisher's method of one way Analysis of Variance technique with three replication. The level of significance used in f and t-test was p=0.01 probability level.

RESULTS AND DISCUSSION

Phytoalexines from the blast-infected leaves of finger millet were isolated according to the procedure that was employed in isolating rice phytoalexines schematically[4] (Fig. 1). Sephadex LH-20 column chromatography and Thin Layer Chromatography (TLC) achieved the separation and partial purification of phytoalexines.

The phytoalexin bands with R_f values of 0.22, 0.28, 0.35 and 0.61 on TLC were characterized by continuous UV spectra of all the four-phytoalexin compounds showed a major peak shoulder between 205 to 225 nm.

The phtoalexins compound with R_f value 0.22 and 0.35 showed maximum absorption peak at 208 nm (Fig. 2 and 4). The compound with R_f value of 0.28 and 0.61 showed maximum absorption peak at 209 and 213 nm,

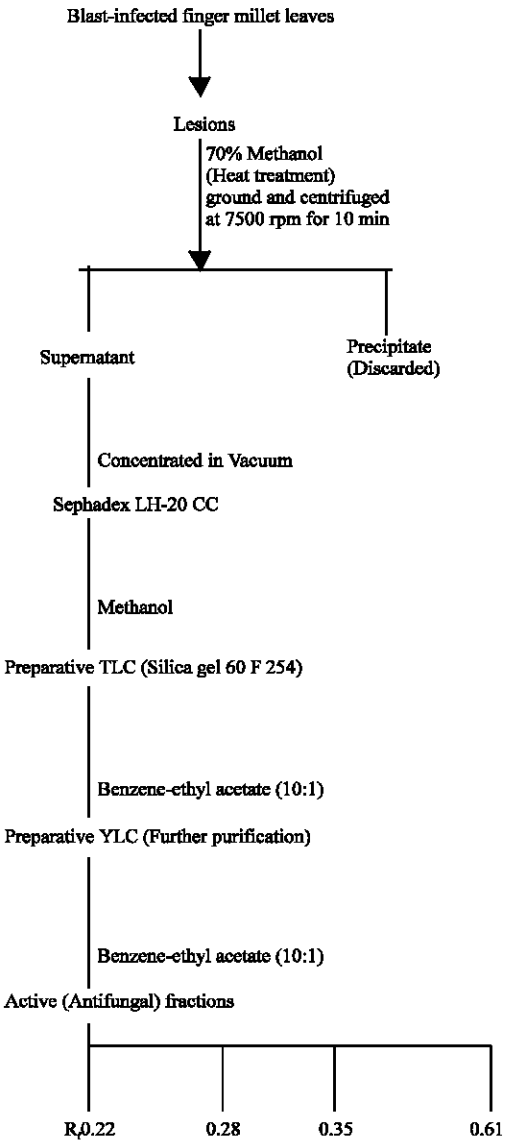

Fig. 1: Isolation procedure for phytoalexins from blast-infected finger millet leaves[4]

respectively (Fig. 3 and 5). The minor peak at 255 nm was observed in the phytoalexin compounds with an R_f value 0.22 and 0.61 (Fig. 2 and 5).

The EI-Mass spectra of all the phytoalexin compounds showed multiple peaks with small molecular mass samples indicate many molecules co-purifying with each other or multiple ionized products. The 300.6, 413.1 and 437.1 mas ionic peaks seem to be common in all the compounds except 437.1 which was absent in compounds in present study showed small peaks at 302 in lesser abundance (Fig. 2).

The IR spectra of phytoalexin compounds with R_f values of 0.22, 0.28, 0.35 and 0.61 showed absorption

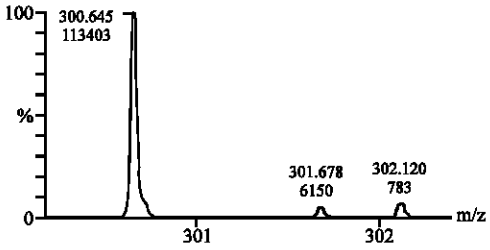

Fig. 2: El-Mass spectra of phytoalexin compound 1 (R_f 0.22)

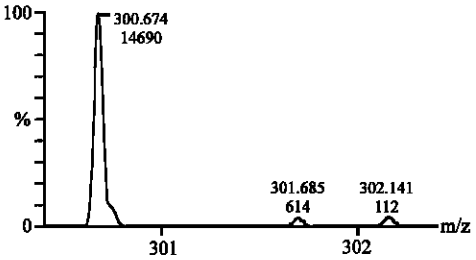

Fig. 3: El-Mass spectra of phytoalexin compound 2 (R_f 0.28)

Table 1: Titer determination of sephadex LH-20 and TLC purified phytoalexin from blast-infected leaves of GPU-28 based on inhibition of spore germination of *Pyricularia grisea*

	Phytoalexin (active) bands with R_f values			
Treatments	0.22	0.28	0.35	0.61
Control	100.00	100.00	100.00	100.00
	(10.03)	(10.03)	(10.03)	(10.03)
1:0	3.64	2.61	4.20	4.91
	(2.03)	(1.76)	(2.17)	(2.32)
1:2	20.42	10.34	11.85	21.49
	(4.57)	(3.29)	(3.50)	(4.68)
1:6	51.29	25.49	31.84	64.86
	(7.20)	(5.09)	(5.69)	(8.08)
1:10	86.47	40.83	62.61	77.21
	(9.33)	(6.43)	(7.94)	(8.81)
1:14	95.69	57.37	81.80	98.06
	(9.81)	(7.61)	(9.07)	(9.93)
1:18	99.40	80.37	92.09	99.92
	(9.99)	(8.99)	(9.62)	(10.02)
1:22	-	94.72	98.87	-
		(9.76)	(9.97)	
1:26	-	99.48	-	-
		(10.00)		
SEM±	0.066	0.091	0.111	0.132
CD at 1%	0.331	0.418	0.557	0.661

* Figures in the parenthesis are square root transformed values

peaks attributed to hydroxyl functions at 3350, 3400, 3400 and 3400 cm^{-1}, respectively. The IR spectra of above compounds showed absorption peaks of α, β-unsaturated carbonyl function at 1685, 1690, 1630 and 1632 cm^{-1}, respectively. The compound with R_f value of 0.22 and 0.35 showed other additional absorption peak at 1620 and 2325 cm^{-1}, respectively (Fig. 2-5).

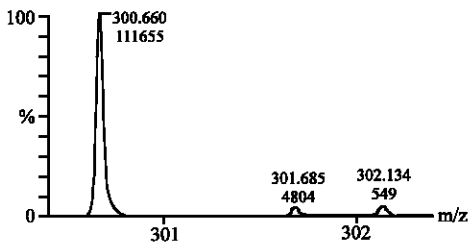

Fig. 4: El-Mass spectra of phytoalexin compound 3 (Rf 0.35)

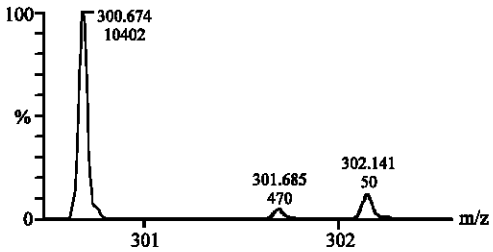

Fig. 5: El-Mass spectra of phytoalexin compound 4 (Rf 0.61)

The UV, EI-Mass spectra along with TLC R_f values showed many similarities with the rice phytoalexins that is oryalexines[4]. Out of four phytoalexin compounds isolated from blast-infected leaves of finger millet variety GPU-28 in the present study, the compound with an R_f value 0.22 and 0.28 could be identified as oryzalexin A and B, respectively. The other two compounds with an R_f value of 0.35 and 0.61 needs to be studied further for nomenclature.

The antifungal activity of the phytoalexin compounds were estimated by using the spore germination method and described in material and methods. The phytoalexin compound with an R_f value of 0.22, 0.28, 0.35 and 0.61 showed significant reduction in per cent spore germination of *Pyricularia grisea* up to dilutions of 1:10, 1:18, 1:14 and 1:10, respectively (Table 1).

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