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Effect of *Cyperus rotundus* rhizome extract on *Fusarium udum*

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ABSTRACT : The effect of aqueous and methanol, petroleum ether, chloroform and ethyl acetate extracts of rhizomes of a tropical weed *Cyperus rotundus* L. was seen on the spore germination of *Fusarium udum*. Various parameters, namely, percent germination, germ tube length and mode of germination were affected. Increase in percent germination, germ tube length and lateral germination was observed at increasing dilution of aqueous as well as methanol, petroleum ether and chloroform extracts. Characteristic bulging of spores was observed at higher concentration. Ethyl acetate extract exhibited inhibitory effect on spore germination at 1000 µg ml^{-1}. Fractionation of ethyl acetate extract on silica gel column resulted in eluents rich in flavones and terpenes. Rhizome-meal-amended potato dextrose agar medium induced sector formation and profuse sporulation as compared to potato dextrose agar medium where sectors were not formed. It is suggested that the chemical(s) responsible for the morphogenetic changes in this fungus may be present in petroleum ether/chloroform fraction(s) of rhizomes.

Key words : *Cyperus rotundus,* rhizome, *Fusarium udum*

Fusarium udum Bulter is a soilborne fungus which survives in tissues of diseased plants that it has colonised as a parasite (Subramanian, 1955). It causes the most destructive disease commonly known as fusarial wilt in pigeonpea (*Cajanus cajan* (L) Millsp.). Plant disease control, since the beginning of the present century, was based on the use of synthetic fungicides that included elemental sulphur, Bordeaux mixture and mercurial compounds (Nene and Thapliyal, 1979). However, the indiscriminate use of synthetic fungicides has caused potential threat to human health, increase in pathogen resistance/mutation and are often costly and not available to common farmers in developing countries. In the quest of solution to this problem, alternate methods, particularly biological control, are being adopted in disease control.

Numerous reports on the use of crude plant extracts (Dixit *et al.,* 1976; Krishna Reddy, 1987; Prithiviraj *et al.,* 1996) and their active principles (Fawcett *et al.,* 1969; Hoffmann, 1975; Preston, 1977; Goyal and Gupta, 1987; Oswald *et al.,* 1987; Bandara *et al.,* 1988; Nair *et al.,* 1990; Singh *et al.,* 1990; 1991, 1995) against several micro-organisms *in-vitro* and *in-vivo* are now available in the literature.

In a previous study from this laboratory, *Cyperus rotundus* rhizome meal and extracts induced sexual reproduction in three basidiomycetes fungi (Singh *et al.,* 1996). The present experiment was conducted to see if this plant does have similar effect on *Fusarium udum*, a soilborne fungus, as there is ample probability of its interaction with rhizomes of *C. rotundus* present in the tropical soils.

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MATERIALS AND METHODS

Fungus

Fusarium udum was isolated from wilted pigeonpea plants and purified by single spore isolation and maintained on Potato Dextrose Agar (PDA) (peeled potato 250 g, dextrose 20 g, agar 15 g and distilled water 1l) medium.

Preparation of aqueous extract of *Cyperus rotundus*

Cyperus rotundus rhizomes were collected from the field and washed thoroughly in tap water, blotted-dry and stored at -20°C. For the preparation of aqueous extract, rhizomes were crushed with equal amounts (w/v) of distilled water in a grinder. The resulting homogenate was passed through several folds of muslin cloth. The filtered extract was then centrifuged at 1300 x g for 15 min and the supernatant used for experiments. Different concentrations, namely, 1:1, 1:3, 1:5 and 1:10 (extract : distilled water) were prepared and

sterilized at 121°C for 10 minutes.

Preparation of aqueous extract after soaking the meal for 24 h in water

The washed rhizomes were crushed in a grinder with equal amounts (w/v) of water and left for 24 hours in an incubator (25 ±2°C). The meal was once again crushed after 24 h and extracted through a muslin cloth. The extract was centrifuged as above. Different dilutions were prepared in the ratios of 1:1, 1:3, 1:5 and 1:10 (extract : distilled water). The various dilutions of extract were sterilized as described previously.

Preparation of methanolic extract of rhizomes and its fractionation

The rhizomes were dried at room temperature and crushed to a fine power. The powder (3 kg) was extracted in Soxhlet apparatus for 7 days with methanol. Later, the extract was dried at reduced pressure to remove residual methanol and moisture. A thick chocolate brown paste constituted the extract. The extract was mixed with equal volume of water, filtered and fractionated successively with petroleum ether, chloroform and ethyl acetate in a liquid-liquid apparatus. The resulting extracts were dried under reduced pressure. The required amount of the extract was initially dissolved in methanol and further diluted to a required concentration (100, 500, 1000 and 5000 $\mu g\ ml^{-1}$) in distilled water. The solutions were sterilised at 121°C for 10 min. before use.

The resulting methanol extract was mixed with equal amounts of water and filtered to remove extraneous matter and extracted successively with petroleum ether, chloroform and ethyl acetate in that order. Each of the extracts was dried under reduced pressure for use in the study. Thin layer chromatography profiles of all the extracts were seen with precoated TLC plates (60 F_{254}' 0.2 mm thickness, E. Merck, Germany) with different solvent system and developed either in iodine chamber or LB reagent.

The extracts, namely, ethyl acetate (4 g) and chloroform (20 g) were subjected to further fractionation by column chromatography. The extract was dried completely and redissolved in methanol and adsorbed onto silica gel (601-2 mesh). This was then padded over a column of silica gel approximately four times the volume of adsorbed silica gel. The column was eluted starting with petroleum ether, benzene, chloroform and methanol and their combination in the increasing order of polarity. The eluents were evoporated off the solvent and checked on TLC.

Effect of fresh aqueous extract, 24 h. stored, methanol, petroleum ether, chloroform and ethyl acetate extracts of *Cyperus rotundus* on spore germination of *Fusarium udum*

0.1 ml of the test solutions of each dilution was kept on grease-free glass slides and approximately 200-300 macroconidia from freshly growing cultures of *F. udum* were mixed in these solutions with the help of an inoculation needle under aseptic condition. The slides were then kept in moist chambers at 25±2°C for different periods (2,3 or 4 h). At the end of desired period, the slides were removed from the moist chamber and immediately fixed in lactophenol-cotton blue. The slides were observed under light microscope for percent germination, type of germination, namely, unipolar, bipolar or lateral, germ tube dimensions and spore size and the data were subjected to standard statistical analysis. The experiments were conducted in five replications.

Effect of *Cyperus rotundus* rhizome-meal-amended PDA on growth of *Fusarium udum*

Fresh rhizomes of *C. rotundus* were crushed to a fine meal. PDA was amended with the meal at different concentrations, viz., 5,10 and 15% (w/v). Such medium was sterilized at 121°C for 10 min. Twenty milliliter of the medium was poured in sterile petri plates (9 cm dia). Four millimetre disc from freshly growing colonies (7-10 days old) was inoculated in the centre of each plate. All the plates were incubated at 25±2°C for 10 days. Observations on radial growth were taken every 24 h. PDA without meal served as the control. Each treatment had five replications. A similar set was maintained for observation on the development of sectors and conidium production. After 10 days of incubation, the number of sectors was observed. To determine the conidial numbers, 4 mm^2 discs were cut from the colony by a cork borer and transferred into culture tubes containing 10 ml of distilled water. A drop of detergent (Teepol) was added to make a uniform suspension of spores. The tubes were shaken in a vortex mixure and the concentrations were measured with haemocytometer. The chlamydospores were also counted by taking 4 mm mycelial disc from the plate on to the slides and stained with cotton-blue in lactophenol and finally covered with cover glass for observation.

RESULTS

The effect of freshly prepared aqueous extract from *C. rotundus* rhizomes, mixed in different ratios in water, on spore germination, percent germination and germination types of conidia of *F. udum* has been presented in Table 1. The percent germination increased

with increase in dilution of the extract and was maximum at extract dilution of 1:10 (extract : water). Maximum spores (60-70.6%) showed 100% unipolar germination in water (control) while following treatment with extract unipolar and bipolar germinations were discerned. The maximum number of bipolar germ tubes was observed at highest dilution of the extract. In bipolar germination, one germ tube was shorter than the other one even in some spores of the control. A similar trend with a significant increase in the length of germ tubes was observed with increasing dilution of the extract. The formation of lateral germ tubes was not observed in the control. In the diluted extracts, a single germ tube from the middle cell of the spore was invariably observed when the incubation period was prolonged. Two way ANOVA showed significance ($P<0.001$) among the variables. Intervariable correlation matrix (Table 2) reveals that different variables are correlated differentially among themselves.

The effect of methanolic extract from *C. rotundus* rhizomes, in different concentrations with distilled water, on spore dimension, percent germination and germination types in *F. udum* was also observed (Table 3). Spore dimension increased in different concentrations of extract. The percent germination increased in the extracts containing 100, 500, 1000 and 2000 μg ml^{-1} but decreased at highest concentration (5000 μg ml^{-1}). There was 100% unipolar germination in the control while, in the treatments, bipolar germination was invariably seen. With bipolar germination one germ tube (Pole A) was always considerably longer than the other one (Pole B). The germ tube dimension gradually increased with increasing concentrations of the extract. Formation of lateral germ tubes was not observed in the control, while in different concentrations of extract, a single germ tube emanating laterally from the middle cell of the spore was observed after prolonged incubation periods. The lateral germ tubes were very small in

Table 1. The effect of different dilutions of fresh *Cyperus rotundus* rhizome extract and water on spore germination of *Fusarium udum* macroconidia

Treatment (Extract: water)	Period of treatment (h)	Spore dimension (μm)	Unipolar germination			Bipolar germination			Lateral germination	
			Germination (%)	Germination (%)	Germ tube dimension (μm)	Germination (%)	Germ tube dimension (μm) A	Germ tube dimension (μm) B	Germination (%)	Germ tube dimension (μm)
1:1	2	8.6 × 2.7	13.4	88.5	17.0 × 0.8	10.95	13.7 × 0.8	7.6 × 0.8	0.0	0.0 × 0.0
	3	8.4 × 2.6	13.7	62.7	19.1 × 1.3	22.20	13.7 × 1.3	8.0 × 1.3	14.4	9.2 × 1.37
	4	9.8 × 3.2	23.8	66.1	16.8 × 2.4	18.70	14.9 × 2.4	9.3 × 2.4	31.1	8.3 × 2.43
1:3	2	9.5 × 2.5	26.5	77.9	12.0 × 0.7	21.72	10.8 × 0.7	6.5 × 0.7	0.0	0.0 × 0.0
	3	8.4 × 2.5	27.1	62.0	20.9 × 1.5	20.68	17.1 × 1.5	10.0 × 1.5	17.2	9.7 × 1.5
	4	10.0 × 2.5	76.6	55.9	58.6 × 1.7	38.86	47.2 × 1.7	25.5 × 1.7	4.8	22.0 × 1.7
1:5	2	8.7 × 2.5	19.9	83.6	17.1 × 0.6	16.07	14.5 × 0.6	7.3 × 0.6	0.0	0.0 × 0.0
	3	8.6 × 2.5	20.3	44.4	21.3 × 1.0	31.71	17.6 × 1.0	8.4 × 1.0	23.8	8.3 × 1.0
	4	9.1 × 2.5	94.4	63.2	77.2 × 1.3	25.50	54.0 × 1.3	30.1 × 1.3	11.6	26.7 × 1.3
1:10	2	8.1 × 2.5	23.0	79.5	14.3 × 0.6	20.00	11.8 × 0.6	7.5 × 0.6	0.0	0.0 × 0.0
	3	8.2 × 2.5	22.6	45.7	38.0 × 1.0	30.90	17.8 × 1.0	10.0 × 1.0	23.5	8.5 × 1.0
	4	8.9 × 2.5	97.3	59.9	75.9 × 1.2	28.56	56.9 × 1.2	32.2 × 1.2	14.4	24.2 × 1.2
Control	2	8.3 × 2.5	22.5	65.4	19.0 × 0.9	34.19	20.7 × 0.9	10.8 × 0.9	0.0	0.0 × 0.0
	3	8.4 × 2.5	37.0	71.0	50.8 × 0.9	28.75	36.5 × 0.9	12.4 × 0.9	0.0	0.0 × 0.0
	4	8.3 × 2.5	71.5	60.8	28.2 × 0.7	4.10	18.2 × 0.7	10.2 × 0.7	0.0	0.0 × 0.0

Table 2. Intervariable correlation matrix for fresh rhizome extract

Variables	Treatment	Germination (%)	Unipolar germination (%)	Germ tube length	Bipolar germination (%)	Lateral germination (%)
Treatment	1.000000					
Percent germination	.862300*	1.00000				
Unipolar germination(%)	.912226*	.583299	1.000000			
Germ tube length	.307972	.715461	-.102218	1.000000		
Bipolar germination(%)	-.931284*	-.626049	-.985097*	-3.94509E-003	1.000000	
Lateral germination	-.852034*	-.898749*	-.678799	-.410309	.645982	1.000000

*Significant

comparison to the unipolar and bipolar germ tubes. Bulging of conidial cells was observed at higher concentrations (2000 and 5000 µg ml^{-1}). Similar bulging was evident in 1:1 aqueous extract also. The correlation matrix in Table 4 shows that the variables correlated differentially among themselves. Similar effects were observed also with petroleum ether and chloroform extracts (results not shown). Extract of meal of rhizomes soaked for 24 h also affected the spore germinaton significantly ($p < 0.01$) as did fresh extract (Table 5). Surprisingly, ethyl acetate fraction was inhibitory at low concentration (1000 µg ml^{-1}). There was significant inhibitory effect ($p < 0.001$) of ethyl acetate extract on spore germination.

Table 6 shows the influence of rhizome meal amended PDA on growth of *F. udum*. Higher concentration inhibited the mycelial growth. The colonies of *F. udum* inoculated on *C. rotundus* meal (5%) + PDA showed sector formation from one to several in each plate (average 1.7 sector per plate), whereas there was no sectored growth on PDA. Sectored and nonsectored growth showed significant difference in sporulation. A high number of spores was observed on non-sectored growth (1.884×10^6 conidia / 4 mm mycelial disc of PDA + meal) or on colonies grown on PDA (0.88×10^6 conidia / 4 mm mycelial disc of PDA) as compared to sectored growth (0.24×10^6 conidia / 4 mm mycelial disc of PDA + meal). Chlamydospores were significantly more in colonies grown on meal + PDA than in colonies grown on PDA alone.

The fractionation of ethyl acetate extract yielded fractions rich in terpenoids and tested positive for sinode test indicating the presence of flavonoides.

DISCUSSION

Very little work on the biological effect of *C.*

Table 3. Effect of methanolic extract of *Cyperus rotundus* rhizomes on spore germination of *Fusarium udum*

Treatment (µg ml^{-1})	Period of treatment (h)	Spore dimension (µm)	Unipolar germination			Bipolar germination			Lateral germination	
			Germination (%)	Germination (%)	Germ tube dimension (µm)	Germination (%)	Germ tube dimension (µm) A	Germ tube dimension (µm) B	Germination (%)	Germ tube dimension (µm)
100	2	8.6 × 2.5	24.7	96.0	16.5 × 1.4	3.9	14.0 × 1.4	9.4 × 1.4	0.0	0.0 × 0.0
	3	8.4 × 2.5	38.4	97.0	21.5 × 1.3	2.8	17.5 × 1.3	9.0 × 1.3	0.0	0.0 × 0.0
	4	9.8 × 2.5	41.1	94.7	20.3 × 1.3	3.4	16.6 × 1.3	10.9 × 1.3	1.5	6.2 × 1.3
500	2	9.5 × 2.5	47.5	97.8	20.0 × 1.4	2.1	13.9 × 1.4	9.0 × 1.4	0.0	0.0 × 0.0
	3	8.4 × 2.5	52.8	94.1	20.6 × 1.3	4.0	15.7 × 1.3	7.9 × 1.3	1.7	12.7 × 1.3
	4	10.0 × 2.5	56.7	90.8	27.0 × 1.3	7.5	18.1 × 1.3	9.5 × 1.3	1.6	7.7 × 1.3
1000	2	8.7 × 2.5	53.6	98.6	20.1 × 1.5	1.8	16.2 × 1.5	10.0 × 1.5	0.0	0.0 × 0.0
	3	8.6 × 2.5	57.6	93.8	21.7 × 1.2	4.5	18.0 × 1.2	10.5 × 1.2	1.5	10.3 × 1.2
	4	9.1 × 2.5	63.2	95.1	23.0 × 1.3	4.4	18.6 × 1.3	10.6 × 1.3	1.4	8.1 × 1.3
2000	2	8.9 × 2.5	59.9	99.1	19.1 × 1.2	0.7	17.6 × 1.2	11.1 × 1.2	0.0	0.0 × 0.0
	3	8.1 × 2.5	62.6	98.3	21.5 × 1.3	1.6	20.2 × 1.3	11.1 × 1.3	0.0	0.0 × 0.0
	4	9.3 × 2.5	91.2	86.6	38.7 × 1.4	10.2	31.3 × 1.4	14.3 × 1.4	2.0	17.1 × 1.4
5000	4	9.0 × 2.5	85.2	86.0	39.2 × 2.0	1.4	30.2 × 2.0	14.7 × 2.0	1.4	10.5 × 2.0
Control	2	8.3 × 2.5	18.4	91.4	20.1 × 1.2	8.5	14.6 × 1.2	9.0 × 1.2	1.7	12.7 × 1.3
	3	8.4 × 2.5	21.3	99.6	17.4 × 1.2	0.0	0.0 × 0.0	0.0 × 0.0	0.0	0.0 × 0.0
	4	8.3 × 2.5	69.0	100.0	24.6 × 1.2	0.0	0.0 × 0.0	0.0 × 0.0	0.0	0.0 × 0.0

Table 4. Intervariable correlation matrix for the effect of methanolic extract of *Cyperus rotundus* on spore germination of *Fusarium udum*

Variables	Treatment	Germination (%)	Unipolar germination (%)	Germ tube length	Bipolar germination (%)	Lateral germination (%)
Treatment	1.000000					
Percent germination	.913717*	1.000000				
Unipolar germination(%)	-.941243*	-.776823*	1.000000			
Germ tube length	.990800*	.892469*	-.970693*	1.000000		
Bipolar germination(%)	-.808132*	-.525472	.836990*	-.789425*	1.000000	
Lateral germination	.721044	.697192	-.814994*	.758118*	-.555824	1.000000

* = Significant

Table 5. Effect of different ratios of *Cyperus rotundus* rhizome extract (after 24 h dipping) and water on spore germination of *Fusarium udum*

Treatment	Period of treatment (h)	Spore dimension (µm)	Unipolar germination			Bipolar germination			Lateral germination	
			Germination (%)	Germination (%)	Germ tube dimension (µm)	Germination (%)	Germ tube dimension (µm) A	Germ tube dimension (µm) B	Germination (%)	Germ tube dimension (µm)
1:1	4	9.8 × 2.5	26.6	87.4	26.1 × 2.0	9.06	28.2 × 2.0	13.2 × 2.0	3.3	8.7 × 2.0
1:3	4	10.0 × 2.5	28.1	86.6	20.6 × 2.3	9.46	20.3 × 1.5	- × 1.5	6.5	8.3 × 2.3
1:5	4	9.1 × 2.5	36.6	88.1	23.0 × 1.5	9.43	18.5 × 1.5	11.6 × 1.5	2.3	9.2 × 1.0
1:10	4	8.9 × 2.5	46.8	83.6	24.8 × 1.5	8.03	19.4 × 1.0	13.1 × 1.0	8.1	10.8 × 1.5
Control	4	8.3 × 2.5	71.5	98.6	27.1 × 0.7	1.36	6.0 × 0.2	1.4 × 0.2	0.0	0.0 × 0.0

Table 6. Radial growth (mm) of *Fusarium udum* on *Cyperus rotundus* rhizome meal-amended potato dextrose agar medium

Treatment (meal %)	Radial growth (mm) in hrs					
	48	72	96	120	144	160
5	15.6	23	30.3	36	44.6	48.6
10	19	26	31.6	37	43.3	50.3
15	13	18.6	24.6	29.3	35.3	40
Control	34	49	59.3	70	81	82
Mean	20.41	29.16	36.5	43.08	51.16	55.83
CD at 5%	4.19	6.20	5.48	6.99	5.42	4.37

rotundus rhizomes has been carried out in the past. Masood *et al.* (1985) reported that extracts of tubers inhibited larval hatching in *Meloidogyne incognita*. In the present study, the aqueous, methanol, petroleum ether and chloroform extracts stimulated the germination and germ tube elongation in *F. udum* conidia. This differential effect of germination stimulation by extract at lower concentrations and inhibitory effect of meal mixed PDA and vice-versa may be attributed to hormone-like or paradoxical action of the chemicals present in *C. rotundus* rhizomes as observed for some other chemicals (Wainwright, 1994).

The reason for the inhibitory effect of ethyl acetate fraction as evident from the results is, presumably, because of the presence of chemical(s) detrimental to fungal growth. The TLC profile of the ethyl acetate extract was strikingly different from the other two fractions, namely, petroleum ether and chloroform. Many chemical constituents have been isolated from rhizomes of *C. rotundus*. Korchiro *et al.* (1991) established chemotypes in *C. rotundus* based on the occurrence of seven different sesqueterpenes. New flavonol-glycoside was isolated from mature tubers of *C. rotundus* (Singh *et al.*, 1986). Further, an insect juvenile hormone III was isolated from rhizomes (York *et al.*, 1988). However, there is no detailed study of the effect of rhizomes of *C. rotundus* on fungi. The results of the present study indicate that rhizomes present in the soil may influence the thallus of *F. udum*, a soil-borne fungus, as rhizome meal increases chlamydospore formation, sector formation (an indication of genetic segregation) and also affect the germination of conidia of *F. udum*. This may be used in the study of sporogenesis and sexual stage formation as seen in some fungi (Singh *et al.*, 1996). Part of the chemical constitution of rhizomes may be utilized for disease control as ethyl acetate fraction is inhibitory to spore germination. Isolation of active principles from different extracts is in progress which will be reported later. This, to our knowledge, is the first report of the effect of *C. rotundus* on *F. udum*.

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