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PURIFICATION OF PHYTOTOXIN FROM CULTURE FILTRATES OF *FUSARIUM OXYSPORUM* f.sp. *CICERIS* AND ITS BIOLOGICAL EFFECTS ON CHICKPEA

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Abstract

Fusarium oxysporum f. sp. *ciceris* was grown on liquid minimal medium for the production of culture filtrates. Highest phytotoxic activity was extracted by petroleum spirit while n-hexane could not recover any toxicity from culture filtrates. The fungal bioassay revealed that different solvent extracts yielded 5 different inhibitory zones and for the recovery of maximum toxicity from culture filtrates, extraction with petroleum spirit followed by ethyl acetate should be done. Chloroplast assay revealed 6 different active bands in different solvent extracts. No solvent extracted the major compound extracted by petroleum spirit at Rf value 0.60. Two phytotoxic compounds 1 & 2 were purified from culture filtrates of FOC, which caused wilting and leaf burning of chickpea cuttings. The culture filtrate of FOC inhibited the synthesis of chlorophyll 'a' and 'b' of both chickpea varieties which might be responsible for the yellowing/chlorosis of leaves. The inhibition was more prominent in Aug-424 susceptible as compared to CM 98 resistant cultivars. The culture filtrates of FOC reduced the root length of germinating seeds of chickpea and might also be major factor for effecting the germination of seeds in wilt-infested soils.

Introduction

Fusarium wilt caused by *Fusarium oxysporum* Schlect. Fr. f.sp. c*iceris* (Padwick) Matuo & Sato (FOC) is the most important disease of chickpea, causing 10-50% yield losses in dry areas of Pakistan during the last several years, while in irrigated belts of Punjab, farmers have shifted to other crops due to this disease (Ikramul & Farhat, 1992). The best way to overcome the yield losses due to disease is the use of resistant cultivars. Seven races of *F. oxysporum* f. sp. *ciceris* have been identified based on their interactions with a set of chickpea varieties (Haware & Nene 1982; Jimenez- Diaz *et al*., 1989; Trapero-Casas & Jimenez-Diaz, 1985). Kelly *et al*., (1994) have found two distinct groups (yellowing causing and wilt causing) in FOC by using genetic fingerprinting and random amplified polymorphic DNA techniques.

Pathogenic fungi may often damage their host plants by producing phytotoxins, which cause various symptoms including necrosis, chlorosis, wilting, water soaking and eventually death of plants (Scheffer, 1983). In several host-plant interactions phytotoxins have been reported as pathogenecity or virulence factor (Khan *et al.,* 1998, Jin & Hartman 1996, Yoder, 1980). The phytotoxins, which have been categorized as virulence or pathogenecity factor could be used in identifying resistant cells in tissue culture or in screening and breeding for disease resistance (Song *et al*., 1994; Bajwa *et al.,* 2000; Vidhyasekaran *et al.,* 1990).

The present studies deals with the purification of phytotoxins of *F. oxysporum* f. sp. *ciceris* so as to utilize them in chickpea screening program and also in identifying resistant cells in tissue culture.

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Materials and Methods

Fungal isolates

A virulent, wilt causing isolate (2012) of *Fusarium oxysporum* f. sp. *ciceris* (Khan *et al*., 2002) was used in this study.

Production of culture filtrates

Liquid Minimal medium (MM) as described by Hanif & Ikram (1998) was prepared. The medium was distributed in 100 ml aliquots in one liter roux bottles and after autoclaving at 121°C for 15 min and cooling, inoculated with two 2mm disc of 7 days old culture of FOC isolate. Roux bottles were incubated at 25°C in dark without shaking and culture filtrates were harvested 14 days by filtering through muslin cloth and phytotoxicity was measured by cut seedling method as described by Huang & Hartman (1998).

Standardization of solvent for maximum extraction of phytotoxicity

PH of the culture filtrates was adjusted to 3.0 with 2 M HCl. Culture filtrates (100 ml) were extracted three times into half the volume of n-hexane, toluene, chloroform, diethyl ether, petroleum spirit (60-80^{0}C) and ethyl acetate solvents separately. The organic phases were dried over anhydrous sodium sulphate (Na_2SO_4), filtered through Whatman filter paper no.1 and then evaporated to dryness at 30^{o}C using a vacuum evaporator (Büchi Rotavapor, model R110). The residues were dissolved in 2 ml ethanol and their toxicity was measured as follows.

a. Cut seedling method: The organic extracts (50 µl, 100 µl, 150 µl and 200 µl) dissolved in 5.0 ml distilled water and their phytotoxicity was tested in three replicates by cut seedling method (Huang & Hartman, 1998) against two weeks old cuttings of Aug424.

b. Fungal bioassay: The activity of the FOC metabolites was also measured by the bioautography of the developed TLC plates (0.5 mm thick self coated silica gel 60 GF_{254} plates developed in solvent system n-hexane: ethyl acetate, 8:2) against test fungus *Cladosporium cucumerinum* as described by Sibtain *et al.,* (2002).

c. Chloroplast assay: It was done by the method described by Arpad Ambrus *et al.,* (1981) with slight modifications. Fresh leaves (15 gm) of chickpea (var. Aug-424) were ground in a pestle and mortar by adding 3.0 ml of glycerin. After complete grinding, 10 ml of distilled water was added and mixed well. The material was then filtered through muslin cloth. The filtrate (10 ml) obtained was mixed with 14 ml of 0.04%DCPIP (2, 6-dichlorophenol Indophenol) solution in borax buffer. The mixture was sprayed on the developed TLC plates and reaction was observed after 3-4 minutes.

Purification of phytotoxin

The culture filtrates from 2.0 litre minimal medium was produced and its toxicity was first extracted in petroleum spirit and then in ethyl acetate as described earlier. The

two phases were mixed, dried over anhydrous sodium sulphate and then concentrated on rotavapour and finally dissolved in 2.0 ml ethanol. The concentrated organic phase was subjected to column chromatography and eluted with 200 ml of the solvents viz., n-hexane, benzene, toluene, chloroform, diethyl ether, ethyl acetate, acetonitrile, acetone and methanol. The phytotoxicity of the following fractions was determined by cut seedling method as described earlier. Phytotoxins were identified and purified by thin layer chromatography of the phytotoxic fractions directed by cut seedling assay of different bands which appeared on the TLC plates after developing them in chloroform: methanol (9:1), benzene: acetone: acetic acid (35:5:1) and n-hexane: ethyl acetate (8:2) solvent systems.

Effects of phytotoxins on chlorophyll synthesis

Chickpea plants of varieties Aug-424 (susceptible) and CM 98 (resistant) were grown in dark for 12 days. The cuttings from etiolated chickpea plants were removed and immersed in fresh culture filtrates of FOC for 48 hrs at 28^{o}C in dark while diluted minimal medium was used in control treatments. Chickpea cuttings were removed from culture filtrates/medium, their stems were washed with distilled water and dipped in distilled water and then incubated at 25^{o}C under fluorescent lights (3310 lux), for 72 hrs. Chlorophyll 'a' & 'b' in leaves of treated and control chickpea cuttings were determined by the official method of AOAC analysis (Anon., 1990) in three replicates.

Effects of culture filtrates on root length of germinating chickpea seeds

Three layers of blotting papers were placed in sterilized Petri plates and soaked with 3.0 ml of FOC culture filtrates at 1.0 N (normal culture filtrates) and 0.5 N (50% diluted culture filtrates), while sterilized, diluted minimal medium was used in control treatments. Germinating seeds of chickpea (8 seeds/Petri plate) of varieties Aug-424 (susceptible) & CM 98 (resistant) were placed in the plates and incubated at 25^{o}C in dark. Three replicates were used for each treatment. After 4 days the increase in length of roots were measured and calculated the percentage decrease in roots length by FOC culture filtrates.

Results and Discussion

Fungus

Based on the symptoms produced by the isolates i.e. yellowing and wilt two main groups have been recognized (Khan *et al.*, 2002; Kelly *et al.*, 1994). Occurrence of wilt causing FOC isolates have been reported in Pakistan, while yellowing causing isolates are not reported in Thal area of Pakistan where 90% chickpea crop is sown (Khan *et al*., 2002). Yellowing causing race 0 isolates are mainly prevalent in Spain and Mediterranean region only and these race 0 isolates have recently adapted to infest the local Kabuli varieties of chickpeas (Jimenez Diaz *et al*., 1990). In contrast, nearly all desi chickpea cultivars (Native of Indian sub continent) are resistant to race 0 isolates (Jimenez-Diaz *et al.,* 1989; Jimenez-Diaz *et al.,* 1992). That's why the wilt causing isolate was used in this study.

Production of culture filtrates

Minimal medium was used for the production of phytotoxins because it has been reported to produce maximum phytotoxic activity (Khan *et al*., 2001). The culture filtrate was toxic and caused wilting to chickpea cuttings of Aug-424.

Standardization of solvent for maximum extraction of phytotoxicity

a. Cut seedling method: Highest phytotoxic activity was extracted by petroleum spirit while n-hexane could not recover any toxicity from culture filtrates (Fig. 1). Ethyl acetate diethyl ether and chloroform also extracted sufficient toxicity while toluene recovered very less toxicity. The results showed that the best solvent for toxin extraction from culture filtrates of FOC is p. spirit and also confirm that major toxic activity is due to less polar compounds. The toxic compounds recovered by intermediate polar solvent i.e. ethyl acetate might be different as extracted by the less polar solvents.

b. Fungal Bioassay: The fungal bioassay revealed that different solvent extracts produced five different inhibitory zones (Fig. 2). The chloroform extract produced two inhibition zones at Rf values 0.082 & 0.31, diethyl ether produced four antifungal zones at Rf values 0.0, 0.082 & 0.21, while ethyl acetate phase produced four inhibitory zones at Rf values 0.0, 0.082, 0.21 & 0.32 and p. spirit exhibited three inhibitory spots at Rf values 0.21, 0.32 & 0.52. These antifungal compounds might have or might not have the phytotoxic properties. Petroleum spirit extracted the active compound at Rf values 0.52 while it did not extract the compounds at Rf values 0.0 and 0.082. It became clear that for extraction of maximum toxicity, the culture filtrates must be first extracted with p. spirit to recover less polar active compounds and then with ethyl acetate to recover the remaining intermediately polar toxic compounds. Although ethyl acetate extracted wide range of active compounds with varying polarities (Alam *et al*., 1997: Kim *et al*., 2000) but it could not extract the less polar toxic compound at Rf value 0.52.

c. Chloroplast Assay: Chloroplast assay revealed weak reaction with phytotoxic metabolites of FOC. The reaction produced light pink color on green background (Fig. 3) in spite of blue color as reported in case of herbicide and pesticides (Arpad Ambrus *et al*., 1981). It might be due to the weak phytotoxic activity of FOC metabolites as compared to herbicides/ pesticides. Six different active bands were observed in different solvent extracts mentioned in Table 1. The petroleum spirit phase showed tailing due to higher amount of less polar compounds (Fig. 3), however it showed two active spots, one at Rf '0.60' and other at 0.080. No solvent could recover the major compound extracted by petroleum spirit at Rf value 0.60.

Most of the bioactive compounds detected by fungal assay were different as detected by chloroplast assay. However the compounds at Rf values 0.0, 0.082 and 0.52 as detected by the fungal assay might be the similar compounds at Rf values 0.0, 0.80 and 0.60 as detected by chloroplast assay. Moreover two non-polar toxic compounds at Rf values 0.96 and 0.85 were only detected in chloroplast assay were not having the antifungal properties.

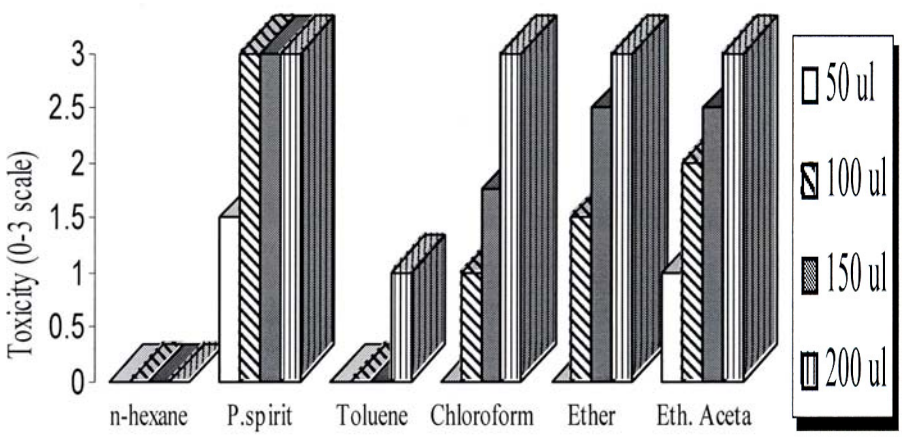

Fig. 1. Toxicity of various solvents extracts for the standardization of solvent for maximum recovery of phytotoxins from culture filtrates of *Fusarium oxysporum* f. sp. *ciceris* (where 0 = no reaction, 1= yellowing/burning of leaves, 2= drooping of leaves and 3= wilting or complete killing of cuttings.

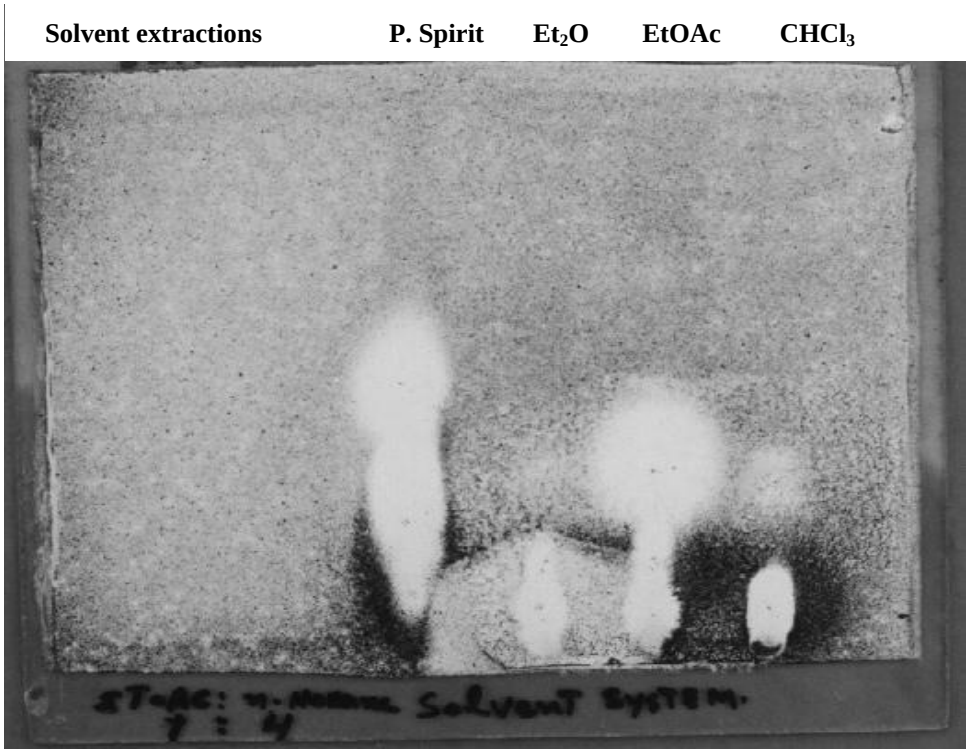

Fig. 2. Antifungal zones exhibited by the solvent extracts from culture filtrates of FOC

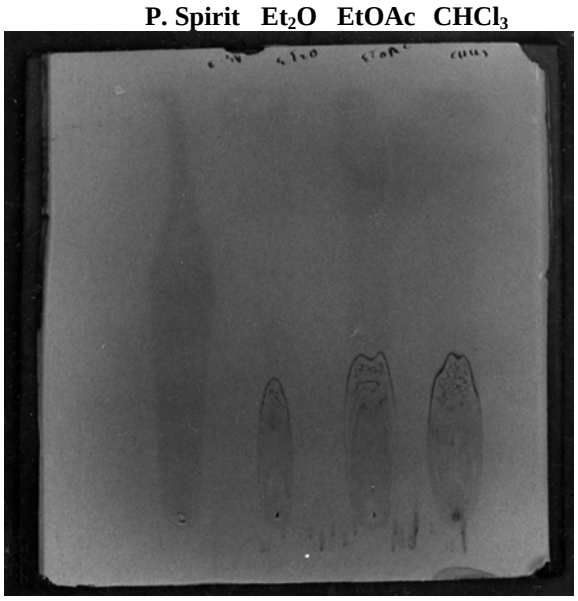

Fig. 3. Chloroplast assay showing phytotoxic bands by different solvent extracts from culture filtrates of FOC.

Table 1. Rf values of active bands recovered by various solvents by chloroplast assay.

Band no.	Ethyl acetate	Diethyl ether	Chloroform	P. spirit
1.	0.96	0.96	0.96	----
2.	0.85	----	----	----
3.	----	----	----	0.62
4.	0.46	0.46	0.46	----
5.	----	0.072	----	0.072
6.	0.0	0.0	----	----

Table 2. Toxicity of various fractions obtained from column chromatography of p. spirit/ ethyl acetate extracts against chickpea cuttings.

S. No.	Solvent	Toxicity
1.	n-Hexane	—
2.	Benzene	—
3.	Toluene	—
4.	Petroleum spirit (60-80^{o}C)	+++
5.	Chloroform	+
6.	Diethyl ether	+
7.	Ethyl acetate	+
8.	Acetonitrile	—
9.	Acetone	—
10.	Methanol	—

+++ = Highly toxic, + = Less toxic reaction, — = Non toxic

Fig. 4. Phytotoxins '1' & '2' produced leaves burning and wilt symptoms to chickpea cuttings

Purification of phytotoxins: The petroleum spirit fraction obtained from column chromatography again showed highest toxicity as compared to other fractions. The fractions of chloroform, diethyl ether and ethyl acetate were also found fairly toxic (Table 2). Other fractions were non-toxic and produced no symptoms on chickpea cuttings even after 5 days incubation. So petroleum spirit fraction was further subjected to thin layer chromatography, which led to the purification of two phytotoxic compounds. Both the compounds produced wilting and leaf burning to chickpea cuttings (Fig. 4). The Rf values of the compounds 1 and 2 were 0.42/0.69 respectively in solvent system n-hexane: ethyl acetate (8:2). The compound 1 produces blue fluorescent under far ultra violet light, while compound '2' was not fluorescent under far and near UV light, it was visualized by placing the developed TLC plate in iodine vapors.

The UV spectrum of compound '1' revealed λmax_{Etoac} values at 254, 274 & a shoulder at 280 nm while compound '2' showed λmax_{Etoac} values at 206, 246 & 261 nm.

Effects of phytotoxins on chlorophyll synthesis: The culture filtrate of FOC inhibited the synthesis of chlorophyll 'a' and 'b' of both chickpea varieties (Table. 3). The inhibition was more prominent in Aug-424 (susceptible) as compared to CM 98 (resistant). Inhibition (34%) of total chlorophyll was produced in Aug-424 while 22% inhibition was produced in CM 98. Phytotoxins have been reported to produce various symptoms including wilting, yellowing, chlorosis etc., (Wheeler, 1975). The pohytotoxins having properties of chlorophyll degradation or inhibition of chlorophyll synthesis can produce these symptoms. The effect of a natural phytotoxin tentoxin to chloroplast has been well established (Andre, 1997).

Table 3. Effect of phytotoxins of FOC on the synthesis of chlorophyll of chickpea.

S. No.	Type of Chlorophyll	Aug-424 Treated	Aug-424 Control	% age Inhibition	CM 98 Treated	CM 98 Control	% age Inhibition
1.	Chlorophyll "a"	3.968	5.89	33	4.689	6.07	22
2.	Chlorophyll "b"	0.96	1.63	41	1.43	2.01	29
3.	Total Chlorophyll	4.93	7.52	34.45	6.3	8.08	22

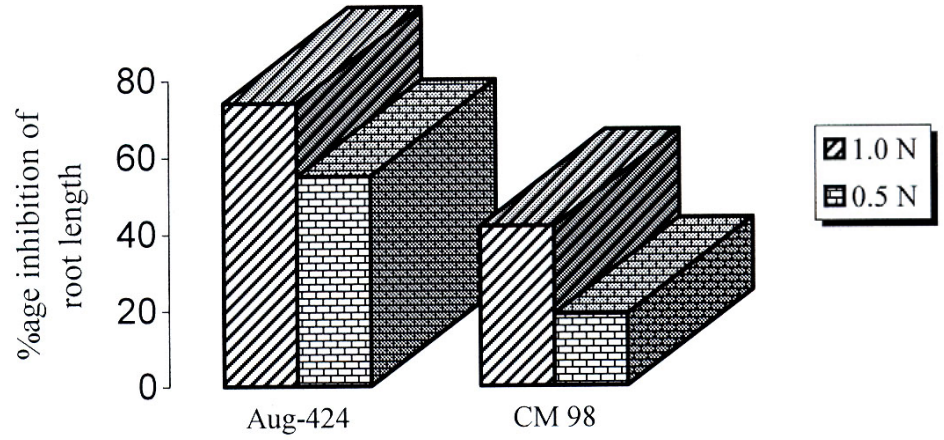

Fig. 5. Percentage reduction of root length of germinating seeds of two chickpea varieties by the phytotoxins of FOC as compare to control.

Effects of culture filtrates on root length of germinating chickpea seeds

The culture filtrates of FOC at both conc. (1.0 N & 0.5 N) reduced the root length of germinating seeds of both varieties when compared to their control. Inhibition (74%) was observed in Aug-424 at 1.0 N concentration as compared to CM 98 where 42% inhibition was observed at same concentration (Fig. 5). The result showed that phytotoxins of FOC do affect the growth of roots and might also be effecting the germination of seeds, as in wilt-infested soil where germination rate is poor as compared to the normal soil. Deoxynivalenol, T-2 toxin, fumonisin B_1, and nivalinol phytotoxins produced by *Fusarium* sp., suppressed the seed germination (Zonno & Vurro, 1999) and the phytotoxins produced by the plant pathogenic fungus *Bipolaris cynodontis* cynA, (cochlioquinone) inhibited the root growth of gramineous plants, such as Italian ryegrass and rice plants, at varying potencies (Lim *et al*., 1998).

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