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Partial Characterization and Leaf Bioassay of Toxins of *Fusarium udum* Butler

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ABSTRACT: ***Wilt caused by Fusarium udum Butler, is the major constraint to pigeonpea production worldwide, which can cause yield losses up to 100% in susceptible cultivars, These toxic metabolites also known is one of the weapons used by pathogen inducing disease condition in susceptible host plants. Many pathogens are known to produce toxins both in vitro and in vivo and these toxins have been implicated in the symptom development on the host tissues. In the present research, we have isolated and partially characterized the Fusarium udum toxin. The efficacy of the toxins were further tested using detached leaf bioassay. The LCMS analysis of crude and methanolic fraction revealed more than 20 peaks among which seven known mycotoxins were detected on the basis of m/z ratio. The analysis of mycotoxin in present investigation revealed FusrenonX (FX), T2toxin (T2), HT2toxin (HT2), Zearalenon (ZON), Tricothecen-T-2-Diacetoxyscipenol (DAS), .3-Acetyldeoxy-nivalenol (ADON). Detached leaf bioassay results exhibit that necrotic symptoms was increased upon increasing the concentration of the toxin in both crude and menthanolic fraction of the toxins appeared at all the concentration but the severe necrotic lesion was noticed in methanolic fraction of toxins. The maximum lesion of 5.13 mm^2 was observed in 200 µl of methanolic fraction of toxin. The identified phytotoxins may prove to be useful tool for screening pigeonpea at earlier stages.***

Keywords: Pigeonpea, fusarium wilt, toxin, leaf bioassay

INTRODUCTION

Fusarium wilt (*Fusarium udum* Butler) is an important soil-borne disease of pigeon pea, which causes serious yield losses in susceptible cultivars. The disease can occur at any stage of plant development from the young seedling to the pod filling stage (Mesapogu et al., 2012). In India alone, the loss due to this disease is estimated to be US $71m (Kannaiyan *et al.*, 1984, Reddy *et al.*, 1993). The fungus enters the host vascular system at root tips through wounds or invasion by nematodes, leading to progressive chlorosis of leaves, branches, wilting and collapse of the root system (Butler, 1906, Jain and Reddy, 1995). It is rapidly spreading to newer areas in India and other countries (Singh *et al.*, 1988).

Fusarium udum is responsible for 15.5-47% crop losses. The percentage of disease incidence varies from 5.3 to 22.6%. In Africa the estimated losses from wilt are over US $5m annually (Kannaiyan *et al.*, 1984). Breeding for wilt resistance is likely to remain the most wide-spread control measures as well as the core item in the integrated control strategies.

Fungi of the genus *Fusarium* are known to produce a variety of biologically active substances in their culture filtrate reported to be toxic to callus, cotyledons, germinating seeds, and plants of many vegetable species. Culture filtrates from isolates of *Fusarium udum* causing wilt of Pigeon pea were reported to be toxic to callus, germinating seeds, and plants (Singh and Husain, 1970; Nusrath, 1979; Reddy and Raju, 1996). Variability in response of soybean calli and cotyledons to culture filtrate among fungal isolates and soybean cultivars was also observed (Lim et al., 1990).

Pathogenic fungi often damage their host (plants) tissues by producing toxic metabolites, which induced various symptoms such as necrosis, chlorosis, wilting, water soaking and eventually the death of plants. These toxic metabolites also known is one of the weapons used by pathogen inducing disease condition in susceptible host plants. Many pathogens are known to produce toxins both *in vitro* and *in vivo* and these toxins have been implicated in the symptom development on the host tissues. Many of these phytotoxic metabolites have also been extracted from diseased plant tissues. Based on the reactions of host crops to the toxic metabolites of respective hosts, methods of rapid screening of germplasm for resistance to plant diseases have been developed. Their application has successfully resulted in resistant lines in some tropical crops like cowpea, cassava, maize,

yam, and soybean. Nowadays, these evaluation techniques are becoming an important complement to classical breeding methods. The knowledge of the inactivation of microbial toxins has led to the use of microbial enzymes to inactivate phytotoxins thereby reducing incidence and severity of disease induced by microbial toxins. Considering the increasing awareness of herbicide resistance, and the restriction of the use of chemical pesticides in agriculture against plant pathogens, novel compounds from microorganisms also provided new environmentally friendly bio-herbicides for the control of parasitic weeds that are normally difficult to control. A variety of *Fusarium* fungi, which are common soil fungi, produce a number of different mycotoxins of the class of trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol (DON) and nivalenol) and some other toxins (zearalenone and fumonisins). The *Fusarium* are probably the most prevalent toxin-producing fungi are capable of producing to a variable degree two or more of these and other toxins (Eriksen and Alexander, 1998; Eriksen, 1998).

The toxins released by *Fusarium udum* causes serious yield and economic loss. So, the main aim of our study was to isolate and partially characterize the toxins released by *Fusairum udum* and and analyzing the effect of the toxins using host leaf biassay.

MATERIALS AND METHODS

In vitro Production of Phytotoxin

The pathogenic *Fusarium udum* was isolated from the infected root of Pigeon pea and its monoculture was maintained on Potato Dextrose Agar (PDA). ***F. udum*** isolate was grown on Potato dextrose broth. The cultural filtrate was collected after 21 days of incubation at 26^0C. The culture filtrate was treated with an equal volume of methanol and precipitates overnight at 4^0C. Precipitates were removed by filtering through Whatman No 1 filter paper. Methanol was evaporated in vaccum at 50^0C and the aqueous fraction was extracted using a separating funnel. The solvent and water fractions were evaporated to dryness under vaccum separately. The residues were dissolved in 10ml of bidistilled water and stored in glass vials until further use.

Total Soluble Proteins and Carbohydrate Estimation

The total Protein content was estimated by Lowry *et al.* (1951) methods. The Carbohydrates were estimated by DNS method.

Mass spectroscopy:

The LC-MS analysis crude toxin and methanolic extract sample was carried out.

Effect of Phytotoxins on Host Plant by Leaf Bioassay

The leaf of host plant (Pigeon pea) was detached from plants. Sets of glass slides were prepared by inserting both ends of the slide into filter paper (Whatman No. 3) so that a 4 cm mid-portion was exposed. The tip and basal portion of a detached leaf were fixed by inserting them between the slide and filter paper and by placing a small cotton ball on the basal end of the detached leaf (to keep the leaf from drying). The prepared leaf set was placed into a Petri dish. The filter paper and cotton ball were then moistened with distilled water. The detached leaf was wounded with a glass capillary tube and a droplet of test solution containing 100 µl, 200 µl. The plates were incubated at 28°C in the dark. After 48 h incubation, lesions were observed.

RESULTS AND DISCUSSION

The auxenic culture of *Fusarium udum* were cultured in Potato Dextrose Broth for the production of toxins. The filterate was separated using methanol and the methanolic fraction along with the crude toxins was used for the further study. DNS method for carbohydrate content of toxic metabolite indicating that methanolic fraction of PDB had 1245µg/ml of carbohydrate while in the crude toxin the concentration was 280 µg/ml. The Lowry's method for the estimation of protein content of toxic metabolite indicating that methanolic fraction had 4050 µg/ml while PDB crude toxin showed the concentration of about 1325 µg/ml. (Table 1)

The toxic metabolites of *Fusarium udum* present some similarities to other reported toxins such as *Rhizoctonia solani* toxin and Ceratocystis ulmi toxin, which is composed of

carbohydrate and protein. (Karthikeyan et al. 2007). Song et al. (1993) purified the phytotoxins using Sephadex G-25 and DEAE- cellulose. They found 17 kilo Dalton protein band that cause necrosis in detached leaf. The phytotoxins of cultrate filtrate of *Septoria glycines* contain large polysaccharide in uronic acid.

The LCMS analysis of crude and methanolic fraction revealed more than 20 peaks among which seven known mycotoxins were detected on the basis of m/z ratio (Fig 1 and 2). The analysis of mycotoxin in present investigation revealed FusrenonX (FX), T2toxin (T2), HT2toxin (HT2), Zearalenon (ZON), Tricothecen-T-2- Diacetoxyscipenol (DAS), .3-Acetyldeoxy-nivalenol (ADON) (Table 2).

Since Fusarium are capable to producing wide range of toxins, the LCMS determination of mycotoxins having an advantage of other technique that include its general apply capibility to a broad range of compound high sensitivity and outstanding selectivity. Biselli et al.(2005) detected 18 mycotoxin including, Fusrenon (FX), T-2toxin (T2), HT2toxin (HT2), Zearalenon(ZON), 3-Acetyldeoxy-nivalenol(ADON), Tricothecen-T-2, Diacetoxyscipenol(DAS). Several researchers have reported the Fusarium mycotoxins like fumonisins, A- and B-trichothecenes, and zearalenon (ZON), T-2 and HT-2 toxin. T-2 toxin is one of a group of trichothecene mycotoxins produced by various species of Fusaria. A related compound, HT-2 toxin, is thought to be produced by the deacetylation of T-2 toxin by microflora (Biselli and Hummert, 2005; Busman et al., 2011). T-2 toxin is a potent inhibitor of DNA, RNA and protein synthesis, and shows immunomodulatory and cytotoxic effects both in vivo and in vitro. The toxicities of T-2 and HT-2 toxins are quite similar and caused severe damange. DON is the most prevalent mycotoxin, but it is orders of magnitudes less toxic compared with many other trichothecenes (Biselli and Hummert, 2005; Biselli et al., 2005).

The results of detached leaf bioassay are depicted in Table 3 and Fig. 3. Two concentration of the toxins, 100 µl and 200 µl, were used in order to analyze the effect of toxin on host plant leaves. Detached leaf bioassay results exhibit that necrotic symptoms was increased upon increasing the concentration of the toxin in both crude and menthanolic fraction of the toxins appeared at all the concentration but the severe necrotic lesion was

noticed in methanolic fraction of toxins (Table 3). The maximum lesion of 5.13 mm^2 was observed in 200 µl of methanolic fraction of toxin.

Several researchers observed the significant differences in sensitivity to the culture filtrate. Karthikeyan et al (2007) observed that the toxin produced brown necrotic symptoms was composed of carbohydrates and proteins. The pathological significances of phytotoxins are assayed by evolution of the correlation between toxins production and diseases occurrence by pathogen and sensitivity to the toxins and susceptibility of the plant to diseases (Chen and Swart 2002).

Calli bioassay was also done for testing phytotoxic effect of culture filtrate the carbohydrate and protein content of the phytotoxins play major role in diseases development in plant as well as in animals. On detached leaflets, the symptom appeared at 500ppm and above concentration and the severe necrotic lesion (>10mm) was noticed above 5000ppm concentration. The crude toxin produced the brown necrosis symptom at 5000ppm in detached coconut leaves. (Karthikeyan et al 2007).

CONCLUSIONS

Fusarium udum causes one of the major devastating disease and cause severe yield loss in pigeonpea. Hence, early diagnosis of these diseases is the thrust area of research to overcome the heavy loss. The objectives of the investigation include, the knowledge of collection of material, standar. The mass spectroscopy of the crude toxin and methanolic extract of PDB and CZB was done. FusrenonX (FX), T2toxin (T2), HT2toxin (HT2), Zearalenon (ZON), Tricothecen-T-2- Diacetoxyscipenol (DAS), .3-Acetyldeoxy-nivalenol (ADON) mycotoxins was detected using LCMS method. The detached leaf bioassay was done on the detached leaflets of host plant (Pigeon pea). It was observed that the methanolic extracts of at 200 µl were produced more necrotic lesions. In the partial characterization of phytotoxins, the amount of carbohydrate and protein were estimated and it was found that the methanolic fraction of PDB showed higher concentration as compare to PDB crude toxin. The identified phytotoxins may be useful as tool for screening pigeonpea plants for resistance and susceptibility.

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SN	**SAMPLE**	**Carbohydrate content (µg/ml)**	**Protein content (µg/ml)**
1.	PDB crude toxin	280	1325
2.	PDB methanolic fraction	1245	4050

Table 1: Concentration of total carbohydrate and protein in *Fusarium udum* toxin

Sl. No.	**PHYTOTOXINS**	**m/z**
1.	HT-2 toxins	424
2.	Tricothecen-T-2	466.57
3.	Zearalenon(ZON)	321
4.	3-Acetyldeoxy-nivalenol(ADON)	337
5.	Fusarenon X(FX)	353
6.	Diacetoxyscipenol(DAS)	366.4
7.	T-2-toxin	488

Table 2: Identification of phytotoxins in methanolic fraction of *Fusarium udum* culture

Toxin concentration	**Average leaf lesion area (in mm^2)**
100 µl	
1.Potato dextrose broth crude toxin	1.90
2. Potato dextrose broth methanolic fraction	2.45
200 µl	
1.Potato dextrose broth crude toxin	4.21
2. Potato dextrose broth methanolic fraction	5.13

Table 3: Toxins concentration and average leaf lesion area

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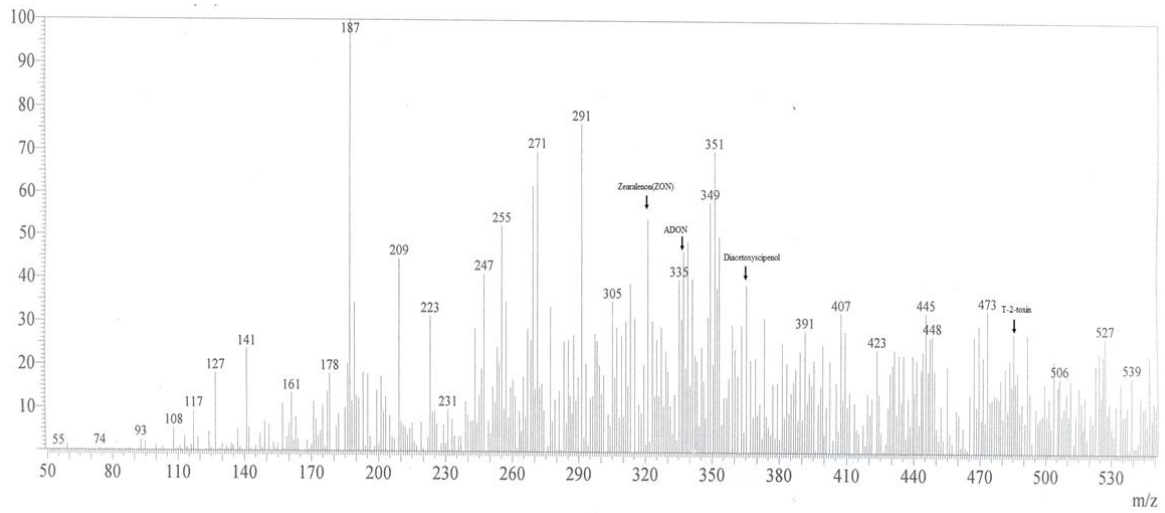

Fig 1- Characterization of toxin by LCMS Method (PDB Crude fraction)

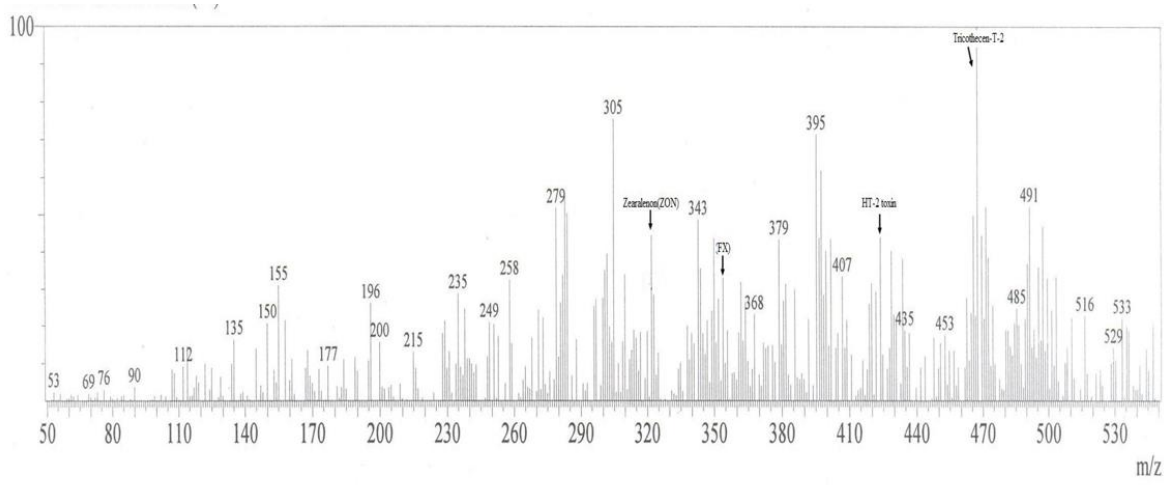

Fig 2- Characterization of toxin by LCMS Method (PDB methanolic fraction)

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Fig 3: Detached leaf bioassay of crude toxin and methanolic fraction of toxin at different concentration {A. PDB crude toxin (100µl) B. PDB methanolic fraction (100µl) C. PDB crude toxin (200µl) D. PDB methanolic fraction (200µl)}

