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NECROSIS INDUCING HERBICIDAL PROTEIN FROM COWPEA PATHOGENIC ISOLATE FUSARIUM *OXYSPORUM* AND ITS HERBICIDAL ACTIVITY AGAINST COMMON WEED *PEPOROMIA WIGHTIANA*

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ABSTRACT
Necrosis inducing herbicidal protein with a molecular weight of 66KDa was isolated from cowpea pathogenic isolate of *Fusarium oxysporum* 07 strain and its phytotoxic activity was evaluated against common weed *Peporomia wightiana*. The protein was isolated from methanol extract of culture filtrate of *F. oxysporum* grown in modified Fries media and partially purified by silica gel chromatography and the fractions obtained were tested for their herbicidal activity against *Peporomia wightiana* adopting detached leaf necrosis assay. The protein induced necrotic lesions as fungal conidia after 24 hours on detached leaves. The effect of temperature on phytotoxic activity of the toxin was studied. The protein retained more than 90% of its activity after 30 minutes incubation at 40, 50, 60, 70 and 80^{o}C. The impact on seedling emergence was also studied with paddy, wheat, black gram and horse gram. Distinct reduction in seedling emergence was not observed in any tested seeds. Antibacterial activity of the protein was evaluated against *Eschercia coli*, *Pseudomonas aeuroginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The protein was inhibitory to *E. coli* and *K. pneumoniae* and inactive against *Staphylococcus aureus* and *Pseudomonas aeuroginosa*.

KEYWORDS: Necrosis, phytotoxic activity, *F. oxysporum,* antibacterial activity etc.

INTRODUCTION

Weeds have been problem for farmers ever since agriculture began. A weed is a plant that is considered to be a nuisance, and normally applied to unwanted plants in human-made settings such as gardens, lawnes or agricultural areas. More specifically, the term is often used to describe native or nonnative plants that grow and reproduce aggressively. Weeds may be unwanted for a number of reasons: they might be unsightly, or crowd out or restrict light to more desirable plants or use limited nutrients from the soil they can harbor and spread plant pathogens that infect and degrade the quality of crop or horticultures (Khanh *et al*.,2007) Some weeds are a nuisance because they have thorns or prickles, some have chemicals that cause skin irritation or are hazardous if eaten, or have parts that come off and attach to fur or clothes (Marcello Pennacchio *et al*., 2005) Even synthetic herbicides are used to control, the indiscriminate use of synthetic herbicides causes various effects such as harmful to host plants, affects soil fertility and beneficial microorganisms and ground water etc. Considering the increasing awareness of herbicide resistant and the restriction of the usage, novel compounds from microorganism may provide new chemistries for weeds that may otherwise be difficult to control (E.g. parasitic weeds). The use of microbially derived compounds in biological control of weeds may represent a promising alternative to the use of chemical herbicide. A bioherbicide is a herbicide that is based on a living organism, or their metabolites such as fungi and bacteria. Now being extensively used to control various parts of the world to control economic important weeds. The toxic metabolites of *Colletotrichum gramnincola, colletotrichum gloeosporioides, Ascochyta caulina, Alternaria alternata* and *Fusarium* sp have been known to cause herbicidal activity against wide range of economc important weeds (Abbas *et al*., 2002). In the present study, necrosis inducing protein was isolated from culture filtrate of cowpea pathogenic isolate of *Fusarium oxysporum* 07 strain and evaluated the activity against common weed *Peporomia wightiana.*

MATERIALS AND METHODS

Fungal strain

F.oxysporum 07 strain was isolated from necrotic spot in naturally infected leaves of cowpea grown in research plot, at Sathyabama University,Chennai,Tamil Nadu,India and the isolate was maintained on Potato agar slants as monosporic culture

Extraction and purification of Necrosis inducing protein

For necrosis inducing protein production,500 ml of modified Fries media (sucrose 10g, casein hydrolysates 2g, sodium nitrate 1.5g, dipotassium hydrogen ortho phosphate 1g,potassium chloride 0.5g, Magnesium sulfatate 0.5g. Ferrous sulfate 0.01g, distilled water 1L., pH 6.8) was prepared and sterilized by autoclaving. 0.1 ml of spore suspension derived from 10 days old PDA slant culture of *F.oxysporum* was inculated and the inoculated flasks were kept at 28 ^{o}C on a rotatory shaker (Scigene) at 150rpm for 21 days. (Figure 1b). Growth of *Fusarium oxysporum* 07 strain in Modified Fries media). After 11 days of growth, the broth was filtered through three layers of cheesecloth and the collected filtrate was extracted with methanol (1:5 ratio). Solvent extracts were then evaporated using a flash evaporator, the final residues collected in screw cap vials. The residues was weighted and subjected to thin layer chromatography. Analytical silica gel plates (0.26mm) thick were used and developed

in butanol: acetic acid: distilled water (9:5:1). Band on TLC plates were observed after spraying with ninhydrin reagent which reveals presence of protein. The protein was further partially purified using G_{60} silica gel (0.063 to 0.20mm) column chromatrography with a solvent Chloroform; methanol; ethylacetate (18:1:1 ratio of 100:1 w/v). Fractions were collected and analyzed using thin layer chromatography as described earlier. Similar fractions were mixed, lyophilized and used for further studies. The quantification of protein was determined by Lowry's method with BSA standard.

FIGURE 1. Necrotic lesions produced by F.oxysporum conidia on Peporomia *wightiana*

FIGURE 2. Necrotic lesions in *Peperomia wightiana* by the necrosis inducing protein extracted from*Fusarium oxysporum* 07 strain

Herbicidal activity assay

Initially leaf necrosis assay was carried out with *F. oxysporum* fungal conidia. The expanded leaves of *Peperomia wightiana*; were detached from plant and cut into 6-9cm^2, surface sterilized with ethanol and washed with sterile distilled water to remove ethanol from surface. The cut pieces were inoculated with 10^8 spores/ml of *F. oxysporum* 07 strain fungal condia by wounding them with sterile needle on the surface of the leaf and transferred to Petri plate containing moistened cotton ball and filter paper. Later plates were incubated at 25˚c for one week (Amusa, 2005). The leaf bioassay with protein was performed as descried earlier with 1.0mg/ml final concentration of lyophilized protein. A daily observation was made for the development of necrotic lecisions on the protein inoculated leaves. The protein was re extracted from necrotic lesion that developed on the tested weed. After one week, 30gms of necrotic leaves was collected, chopped and treated over night with 50 ml of methanol and chloroform at room temperature. Extracts were filtered through four layers of cheese cloth. Residues were added to 50ml of methanol and chloroform for further extraction for four hrs and once again filtered through four layers of cheese cloth as well. Solvents were removed by using flash evaporator and water fractions were partitioned with methanol. Water fraction was discarded and methanol was evaporated to dryness in vacuum. Residues were then collected into sterile 10ml screw cap vials and partially purified by column chromatography as described earlier and the fraction obtained was identified TLC and bio leaf assay were done as explained earlier.

Effect of temperature on herbicidal activity

The effect of temperature on herbicidal activity of herbicidal protein was studied. The protein with final concentration of 100mg/ml was dissolved in 5ml of Tris HCL buffer in a 10ml of test tube was heated at 40˚C, 50˚C, 60˚C, 70˚C and 80˚C for one hour. After the heat treatment phytotoxic assay was performed using leaf bio assay as discussed earlier.

Effect of necrosis inducing protein on seedlings emergence

The impact of the protein on seedlings emergence of four economic important cereals was also carried out Seeds of paddy, wheat, black gram and horse gram were dipped in protein (final concentration 100mg/ml) for 30 minutes, and the treated seeds were transferred to petridish containing moistened filter paper on cotton ball at temperature at 25C for 48 hours. Seedling emergence was recorded.

Antibacterial activity

Antibacterial activity of the protein against human pathogenic bacteria viz *Escherichia coli, Pseudomonas aeuroginosa, Klebsilla pneuomoniae, Staphylococcus aureus* was also studied. All the clinical isolates were obtained from Meenakshi clinical laboratory, Chennai and the isolates were maintained on trypticase soy agar slants.18 hour's trypticase soy broth culture of respective clinical isolates was swabbed with sterilized cotton swabs on Muller Hinton agar plates. Wells were made using 8mm sterile cork poder. Wells were filled with 100ul of protein (final concentration 100mg/ml). Plates were incubated at 37^{o}C for 24 hours and observed for zone of inhibition.

RESULT AND DISCUSSION

The principle responsible for the herbicidal activity of the culture filtrate was readily extracted into organic solvents such as methanol at pH 4 to 5. The methanol soluble extract was concentrated and partially purified by silica gel G_{60} column and the pooled fractions were lyophilized and stored at -20^{o}C, used for bioassay. The partially purified protein showed phytotoxic effect on the tested weed *Peporomia wightiana*. First symptom appear within 24 hours as weak chlorotic marking which subsequently developed into well defined chlorotic spots which forms deep brown lesions. The diameter of necrotic area was 7.0mm^2(Table 1 & Figure 2a} the fungal conidia also caused same phytotoxic effect and the diameter of necrotic lesions 7.2mm^2 (Figure 2).

TABLE 1: Surface necrotic area of respective treatments

S.No.	Treatment	Diameter of the necrotic area (mm2)
1	*F. oxysporum* 07 strain conidia	7.2±0.0b
2	Necrosis inducing protein	7.0 ±0.1 b
3	after re extraction from necrotic lesion	6.8±0.1 b

* Mean ± S.E

In column, the mean followed by same letter is not statistically significant (P>0.05) by DMRT.

TABLE 2. Effect of necrosis inducing protein on seedling emergence %

S.No.	Tested seeds	Seedlings emergence	
		Control	Treatment
1	*Oryza sativa (*Paddy)	100.0	91.07
2	*Triticum. aestivum*(Wheat)	100.0	93.7
3	*Vigna mungo* (Black gram)	100.0	89.57
4	*Macrotyloma uniflorum* (Horse gram)	100.0	77.14 b

In column, the mean followed by alphabet is statistically significant (P>0.05) by DMRT

TABLE 3: Antibacterial activity of herbicidal protein on pathogenic protein

S.No.	Tested Bacteria	Zone of inhibition (mm)
1	*E.coli,*	12.1±0.3
2	*Pseudomonas aeuroginosa*	0.0
3	*Klebsiella pneumonia*	10±0.2
4	*Staphylococcus aureus*	0.0

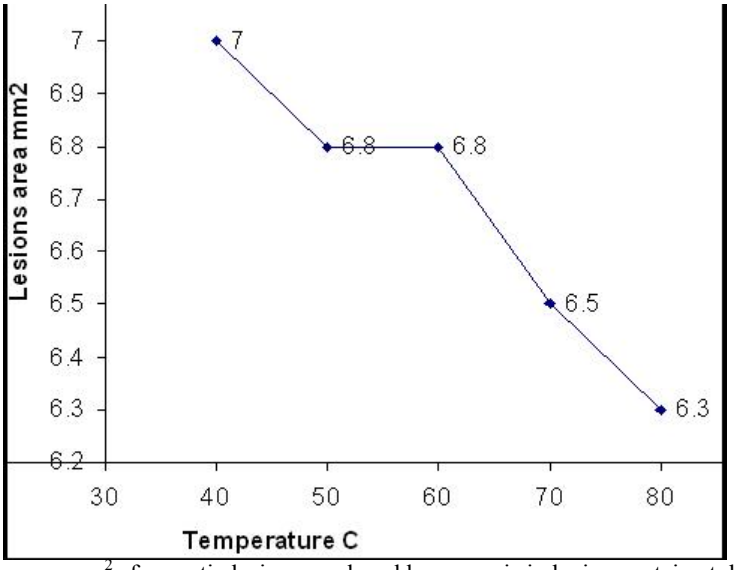


FIGURE 3. Surface area mm ^{2} of necrotic lesions produced by necrosis inducing protein at different temperature

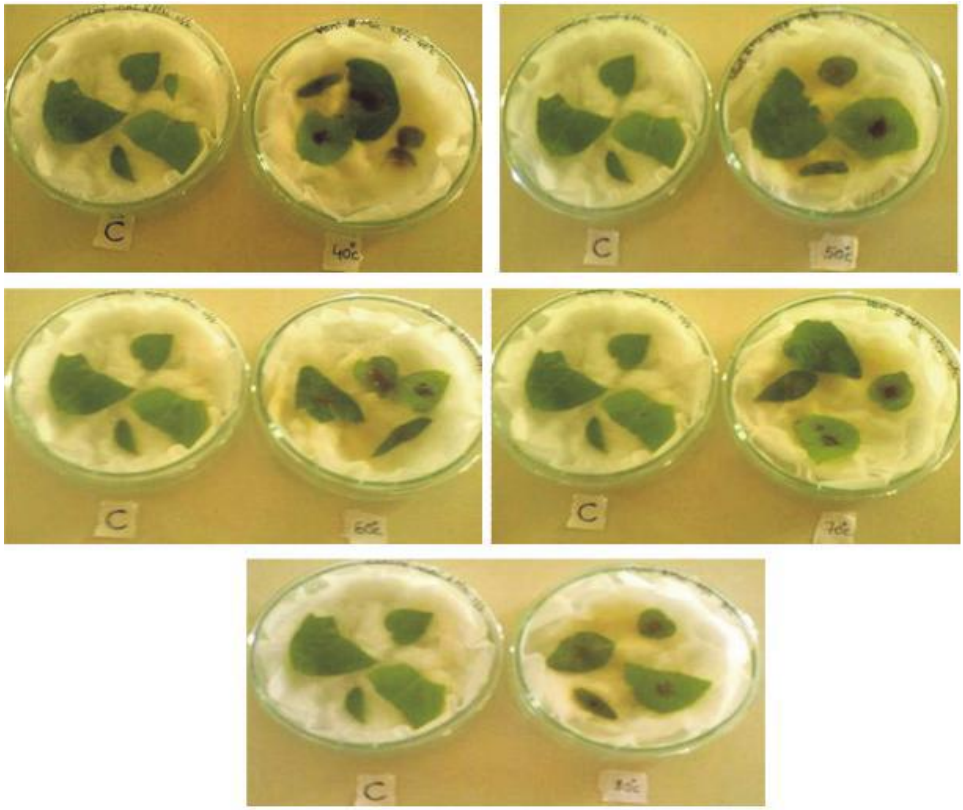


FIGURE 4. Efect of temperature on phytotoxic activity of herbicidal protein

A .Oryza sativa B) *Vigna mungo*, C) *Macrotyloma uniflorum, Triticum aestivum*

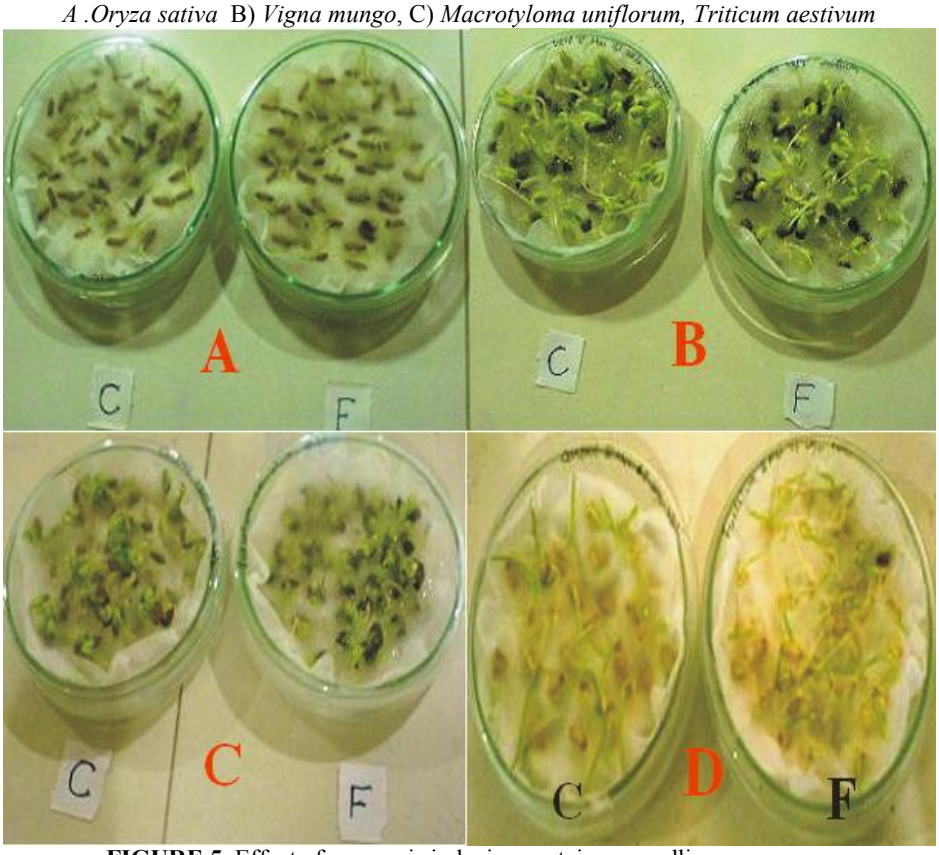
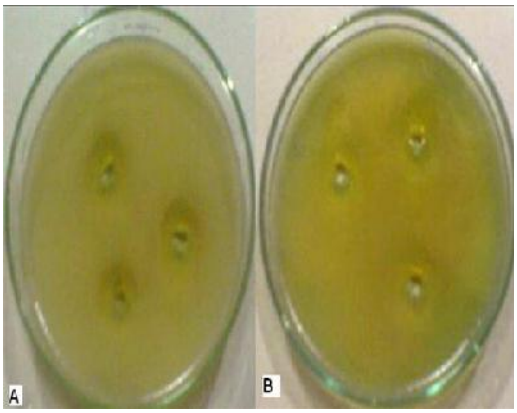


FIGURE 5. Effect of necrosis inducing protein on seedling emergence

The protein extracted from infected leaves also showed the same phytotoxic effect on fresh *Peporomia wightiana* leaves which shows the virulence of the protein after re-extraction and the surface area of necrotic lesions is 6.8mm^2.Effect of temperature on herbicidal activity reveals no distinct effect on was observed after heating the protein at 40, 50, 60, 70 and 80^{o}C for 30 minutes The toxin heated at respective temperature induced necrotic lesions with surface area of 7.0, 6.8, 6.8, 6.5 and 6.3 mm^2 (Figure 3.) respectively which clearly reveals the herbicidal protein could withstand at high temperature and retained its herbicidal effect. Effect of herbicidal protein on seedling emergence of four important cereals reveals that maximum emergence of wheat (93.7%) followed by paddy (91.07%), 89.57 and 77.14 % seedling emergence was recorded in black gram and horse gram (Table 2 & Figure 4). Antibacterial activity showed the protein inhibited the growth of *E. coli*, *and Klebsiella pneumonia.* The zone of inhibition was 12.1mm and 10.0mm respectively (Table 3 & Fig. 5). No effect was observed in *Pseudomonas aeuroginosa* and *Staphylococcus aureus*.

The herbicidal activity of fungi and their metabolites on various weeds have been reported by many workers Zhang and Watson (1999) isolated phytotoxin produced by *Exserohilum monoceras*, a potential bioherbicide for control of *echinochloa sp*. Chanudattan and Rao (1981) isolated Bostrycin and 4-deoxybostricin from culture filtrate of *Alternaria eichokorniae* known to cause phytotoxic effect on water hyacinth. Phytotoxin produced by *Collectotrichum dematium* produces viable necrotic lesions on various weeds (Yoshida *et al*., 1999). Physiological and ultrastutural effect of "fumonisin" a phytotoxin produced by *Fusarium monoliforme* was studied by Abbas *et al*. (2002) In the present study a phytotoxic protein was isolated from methanol extract of culture filtrate of *F. oxysporium* 07 strain cultivated in unique liquid modified fries media . The protein was extracted and partially purified from the culture filtrate by column chromatography. The protein was lyophilized and lyophilized protein was evaluated for its herbicidal activity against a weed *Peporomia wightiana*. *Peporomia wightiana* is a tropical perennial having thick transparent stems and glossy leaves. They are mostly natives of tropical America, they leaves are smooth and fleshy and may be heart shaped and lance shaped. These plants are main wild in nature and grow as weed in shaded regions and agricultural lands, majority of them are found at Maharashtra; Chennai, vellore and tanjore in Tamilnadu; samallcot and rajamundry in Andhra Pradesh, India. The partially purified protein obtained from *F. oxysporum* 07 strain showed distinct herbicidal activity on *Peporomia wightiana*. The protein caused well defined necrotic lesions with diameter of 7.0 mm^2. The protein caused same phytotoxic effect after rextraction of infected leaves which shows the protein could retain the phytotoxic effect. Similar effect was reported by quayyum *et al*. (2002) in necrosis inducing host specific protein toxin (SGP) from spore germination fluid of *Alternaria panax*.



A) – *Escherichia coli*
B) **–** *Klebsiella pneumoniae*
FIGURE 6.Antibacterial activity of culture filtrate extracts of *Fusarium sp* using well diffusion assay

The thermo stability of the toxin reveals the toxin could withstand high temperature (up to 80^{o}C) and retained the herbicidal activity upto 80 C and the diameter of surface area of necrotic lesions was found to be similar as at 30C. (Fig. 4, 4a.) Effect of Necrosis inducing protein on seedling emergence of paddy, wheat, Black gram, Horse gram, reveals no distinct effect on all the tested seeds except horse gram. 77.14 % seedling emergence was regarded (Table 2 & Fig 5).The activity of the toxin against pathogenic bacteria reveals the protein has antibacterial effect, among the four pathogenic bacteria tested *E. coli and Klebsiella pneumoniae* were susceptible to herbicidal protein. Inhibitor effect was not observed in *Pseudomonas aeuroginosa*, and *Staphylococcus aureus*. The antibacterial activity of Bostrycin has been previously isolated from *Bostryconema alpestre cesati* by noda *et al*. (1968)' *Nigrospora oryzae petch* by Furuya and shirsaka (1969), and *Arthrinium phaeospermum (*Vaneijk, 1965) the testing herbicidal protein did not cause any significant effect on seedling emergence of all the tested seeds. The mimicking of pathogenic necrotic symptoms produced by herbicidal protein isolated from *F. oxysporum* on *Peporomia wightiana* suggests a herbicidal role for the protein in *Peporomia wightiana* necrotic lesions. Characterization of this herbicidal protein, mass production, formulation and herbicidal activity on other economic weeds (Invitro and field trial) will be carried out in future study.

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