CHAPTER 4
FUNGAL TOXIN

CHAPTER IV : FUNGAL TOXIN

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FUNGAL TOXIN

INTRODUCTION

Plant pathogenic organisms chiefly fungi and bacteria during their course of infection causes derrangements and degradation of cell wall of their hosts components. The cell wall degrading enzymes like macerozyme, hemicellulase, pectinase and cellulase are produced by almost all pathogenic and non-pathogenic fungi and bacteria (Bateman and Basham, 1976). Breakdown of host pectic polymers by these enzymes leads to the loss of tissue coherence, leading to the separation of cells and water logging of infected tissues, a process known as maceration (Wood, 1967). Apart from these macerating enzymes, certain pathogens produce poisonous substances commonly known as toxins. There are varieties of toxins which have been classified as follows:

i) Phytotoxin, ii) Vivotoxin and iii) Pathotoxin

The fungal metabolites which are directly or indirectly responsible for disease symptoms in higher plants, are normally called phytotoxins (Wheeler and Luke, 1963). This term could include substances that are produced as a consequence of interaction between the pathogens and

their hosts (Harborne, 1983) eg. Lycomarasmin of F. oxysporum sp. lycopersici and alternaric acid of Alternaria alternata (Brain et al 1952). The term 'Vivotoxins' (Dimond and Waggoner, 1953a) is confined to toxins which are indeed formed as a result of the infection of living plant cells by the pathogen e.g. fusaric acid of Fusarium spp (Lakshminarayanan and Subramanian, 1955) and marticins of F. solani (Kern et al, 1970). The term pathotoxin was proposed by Wheeler and Luke (1963). Wheeler (1975) used the term 'host selective toxin' for compounds which exhibit selectivity, rather than specificity, because, they are also active against resistant plants at higher concentration, e.g. Vivotoxin of Helminthosporium victoriae (Meehan and Murphy, 1947; Luke and Wheeler, 1955), T-toxin of Helminthosporium maydis (Hooker, et al, 1970) etc. The genus Fusarium is known to produce several toxins, viz., fusaric acid, lycomarasmin, moniliformin, naphthazarin etc. (Gaumann, 1951, 52; Kern et al, 1967).

Review of Fusarium Toxins

Many of the toxins have been isolated from the pure cultures of Fusarium spp. and chemical and physical properties of some of toxins are known.

Low molecular weight toxins:

i) Napthazarin toxin : Kern and Naef Roth, (1967) identified several phytotoxic compounds from cultures of Fusarium solanii containing common napthazarin structures e.g.marticin, isomarticin, fusarubin, jaramicin, narjaramicin and novarubin. These toxins cause a root and stem rot of peas and Martiella plants. They inhibit seed germination and root growth. (Kern et al., 1970). These substances are detoxified by certain metalions (Cu, Al, Fe), amino acid (glutamic acid) and organic acid (succinic acid) (Kern, 1972). The degree of detoxification varies with the toxin and the compound added. The metal ions chelate the toxin molecules and probably interfere with their transport through vessels and protoplasmic membranes (Kern, 1972).

ii) Lycomarasmin : The first toxic substance to be isolated in pure form from the culture filterate of Fusarium oxysporum f.sp. lycopersici was lycomarasmin (Gaumann, 1951). Tomato plant infected with this pathogen wilted within 7 days of infection. The concentration of lycomarasmin in the pure culture of this pathogen was no higher than 10 mg l^{-1} by 10 days which reaches upto 300 mg l^{-1} after 40 days. As this

toxin has not been detected in the infected plants, it is not considered as a Vivotoxin (Pegg, 1981; Harborne, 1983).

Since lycomarasmin is a peptide, containing aspartic acid, Woolley (1946) suggested that it may be an inhibitory structural analogue of the growth factor strepogenin. He found that the wilting action by lycomarasmin on excised tomato leaves as well as growth inhibition of Lacto bacillus casei could be reversed with strepogenin preparations.

iii) Fusaric acid : Gaumann et al (1952) first described fusaric acid (FA) (5-n-butly picolinic acid) as a wilt toxin of F. oxysporum f.sp. lycopersici and Gibberella fujikuroi. Unlike lycomarasmin, it has been detected in diseased plants (Lakshminarayanan and Subramanian, 1955).

About 200 mg l-1 fusaric acid is produced by F. oxysporum f.sp. lycopersici after 20 days in static cultures or 6 days in shake cultures (Pegg, 1981). Dehydrofusaric acid (with a double bond at the end of the side chain) and other related substances also frequently occur in F. oxysporum (Kern, 1972).

Lakshminarayanan and Subramanian (1955) as well as

Kalyanasundaram and Venkata Ram (1956) detected 17 ug of FA g^{-1} fresh weight of cotton tissue, 2 to 3 weeks after inoculation with F. oxysporum f.sp. vasinfectum. Similarly FA has been detected in various other crops like banana (F. oxysporum f.sp. cubens; Page, 1959); Flax (F. oxysporum f.sp. lini; Davis, 1969), Peas (F. oxysporum f.sp. Pisi; Kern, 1972) etc.

As both lycomarasmin and FA contain nitrogen attached to the carboxyl group (Fig. 4-1), they are able to chelate heavy metals such as Fe+++ and Cu++. The toxicity of these compound depend indeed on their chelating ability like the well known chelating agent versune (EDTA). (Braun and Pringle, 1967).

iv) Moniliformin : This was discovered by Cole et al., (1973) while screening for toxigenic products of F. moniliforme from damaged corn seedlings naturally infected with southern leaf blight. Later Soringer et al. (1974) isolated a moniliformin from Gibberella fujikuroi (perfect stage of F. moniliforme). It contains potassium instead of sodium in its molecule. Later on its toxic effect was also found (Hayes and Hood, 1974), but how the toxicity is brought about is still not known (Hood and Szchech, 1983).

Macromolecular toxins :

A number of macromolecular toxins have been described from cultures of Fusarium spp. some of which are polysaccharides. Pectolysin is an enzyme protein produced by various f.sp. of F. oxysporum (Naef-Roth et al., 1961; Kern 1972). It probably acts in the early phases of pathogenesis and facilitates growth of the parasite in the host tissue and also increases the toxicity of FA (Kern, 1972). A wilt-inducting polysaccharide from F. solani was identified by Thomas (1949). As all these compounds are water soluble, they act simply by physical obstruction of sap flow in the vessels (Harborne, 1983).

MATERIAL AND METHODS

1. Proving Koch's postulate:

A. Collection of Fusarium infected tobacco plant and isolation of the pathogen:

In order to study the Koch's postulate of pathogenicity of the causal organism, isolation of fungus was done from the wilt diseased tobacco. Infected leaves and stem portions of Nicotians tabacum var. Anand2 were collected from a farmer's field located near Baroda. The infected root tissue was cut into small pieces and surface sterilized in 0.1% mercuric chloride solution for 2 minutes. Later it was rinsed with sterile distilled water in a series of three to four washes. Two types of procedures were tried to isolate the fungus from plant tissue. In one, the tissue was inoculated on PDA medium at room temperature. Fungal mycelia appeared within a week. In other method the infected stem and root portions were incubated in moist chamber (modified 'Belljar incubation chamber containing entire infected plants' of Kamat, 1953). Petriplates (15 cm diameter) with 2 filter papers and a Z-shaped ½ cm diameter glass rod in each plate were steam sterilized. They served as moist chambers. The plant material was

treated briefly for 10 seconds with 95% ethanol and washed several times with sterile distilled water to eliminate the surface contaminants. Each material was then placed over a Z-shaped glass rod after moistening the filter papers with sterile distilled water. The plates were sealed and incubated in alternative day light and dark at room temperature.

Both the methods gave satisfactory primary culture of fungus. It was further purified using mycelial tips of one week old culture (emerged from the host ex-plants) which was inoculated on fresh PDA slants. The pure culture was maintained by subsequent transfer on PDA slants. Suspension culture was also prepared by inoculating the fungus into modified Richard's solution (Hendrix and Neilson, 1958) Composition of which is given in Table 10.

B. Preparation of fungal suspension and its culture filterate:

The identity of the pathogen was confirmed as F. oxysporum f.sp. nicotianae using the taxonomical criteria of condiospores as given by Snyder and Hansen (1940). Infection of healthy tobacco with this isolate was carried out thereby proving Koch's postulate (Kamat,

1953). About 10 mg fresh weight of mycelia having 100 ml volume was inoculated in 100 ml of modified Richard's medium (Hendrix and Neilson, 1958) in Erlenmeyer flasks thus giving 200 ml final volume. The cultures were incubated stationary at 34 ± 2°C under 16 h photo period. Under these conditions the mycelia attained 1g wet weight after 10 days. The culture containing 4g wet weight of mycelia was centrifuged at 20000g for 10 minutes to pellet the fungal mycelium and spores which were discarded. The supernatant of the centrifugate was used as the culture filtrate. One hundred ml volume of culture filtrate was produced by 1.25 g of fungal mycelium. The fungal culture filtrate was sterilized using a 0.22 µM pore microbiological filter to remove any residual conidiospores. The culture filterate was partitioned against equal volumes of ethylacetate to remove ethylacetate soluble fraction. Partition was continued three more times so as to completely remove ethylacetate soluble fraction. The aqueous phase was pooled and used for subsequent isolation of water soluble toxin.

C. Inoculation of healthy tobacco plants with pure culture of Fusarium and induction of wilt disease symptoms.

To confirm the Koch's postulates i.e., pathogenecity of the isolate (Kamat, 1953) two months old healthy seedlings of N. tabacum var. A2 were artificially inoculated with the fungal suspension by sick soil and liquid culture methods as described in materials and methods section 2.D.

2. Isolation and purification of glycoprotein from the aqueous phase of culture filterate:

A. Isolation of proteins:

The aqueous phase of culture filterate obtained as above was reduced to one fifth of the original volume in a rotary vaccum evaporator. Thus 100 ml of CF was reduced to 20 ml. To this about 3 volumes of (60ml) chilled acetone was added and the mixture was kept on ice for 30 minutes to precipitate proteins. The precipitated proteins were collected by centrifugation at 3000g at 0°C for 20 minutes. The resultant pellet was air dried to remove the traces of acetone and dissolved in 5 ml of DH_2O. Quantitiy of protein was estimated in this solution using Bradford's method (1976). To 5 ml of Bradford's reagent 0.1 ml of the protein sample was added. The absorbance developed after 10 minutes was read at 595 nm and protein was estimated from the

standard curve prepared using bovine serum albumin.

B. Purification of Proteins:

Purification of toxic protein was done using gel filtration technique. Sephadex G-200 followed by Sephadex G100 matrix of CAPharmacia were used for the gel filtration. The detail of procedure can be found in Selvapandiyan (1987). Briefly crude proteins obtained in (A) were dissolved in small quantity of water at 10 mg/ml concentration. Sephadex gels were hydrated and suspended in distilled water. Small quantity about 5mg. protein was loaded on 100 ml gel filtration column. Elution was carried out by water. Individual fractions were checked for their phytotoxic activity on excised tobacco leaf disks bioassay. Toxin containing fractions were pooled and vaccum concentrated into small volume.

C. Qualitative analysis of purified toxic protein:

Disc electrophonesis using SDS-PAGE of Lamelli was used to check the purity and composition of purified toxic protein. Toxin was dissolved in SDS mercaptoethanol sample buffer at 1mg/1 ml concentration. It was denatured by heating in water bath for 3 minutes. The denatured protein was loaded onto 12% SDS polyacramide gel.

Composition of a Polyacrylamide gel:

	Staking gel	Separation gel
Gel percentage	5 %	12 %
40% Acrylamide	1.25ml	3ml
Ø.5M TRIS HCl pH 6.8/1.5Mtris HCl pH 8.3	2.5 ml	2.5ml
10% aqeous sodium dodecyl Sulphate (SDS)	Ø.1 ml	Ø.1ml
Tetramethylenediamine	4ul	4ul
10% Ammonium per sulphate (APS)	125ul	125ul
Distilled water	made to 10ml	10ml

1. For the stacking gel, Ø.5M Tris HCl buffer pH 6.8 was used. Glycerol was not incorporated in the stacking gel and 65ul of 10% APS was used for the polymerization of stacking gel.

2. Preparation of 40% acrylamide stock solution.

   39 gms of acrylamide. +

   1 gm of NN´ methylene bisacrylamide.

   Volume made upto 100 ml using double distilled water.

   Filtered through whatman No. 1 filter paper.

3. Separation buffer (Tris-HCl 1.5M pH 8.8)

   Tris 18.5 gms.

   Concentrated HCl 6.Øml

   Volume made to 100 ml with D/W

   pH adjusted to 8.8

4. Stacking gel buffer (Tris HCl Ø.5M pH 6.8)

   Tris 6 gm +

   HCl 4.Ø gm

   Volume made upto 1ØØ ml

   pH adjusted to 6.8

5. Electrode buffer (Tris-glycine Ø.ØØ25M pH 8.3)

   Tris 3.Ø gm +

   Glycine 14.3 gm

   Ø.Ø1% SDS

   Volume made to 1ØØØ ml

6. Staining solutions.

   Ø.25% coomassie brilliant blue R-25Ø +

   Methanol 3Ø ml +

   Acetic acid glacial 1Ø ml

   Water 6Ø ml

7. Destaining solution

   Methanol 3Ø ml +

   Acetic acid 1Ø ml +

   Glycerol (1Ø%) 1Øml

   Water 5Ø ml

The electrophoretic apparatus was assembled as follows: Clean, grease free, and oven dried glass plates were

fitted onto the electrophoresis chamber using 0.75 mm thick spacers and were clamped. Three sides were sealed using 1% agar. About 4/5 of the gel chamber was filled with 12% polyacrylamide separation gel solution. Distilled water gently overlaid with pasteur pipette on the gel to ensure even polymerization of the gel. Water was removed from the gel and stacking gel solution consisting of 5% acrylamide was poured. A teflon comb was inserted in the stacking gel to obtain wells for loading protein samples.

After polymerization of the stacking gel the comb was removed and the apparatus was put in a lower chamber after removing basal spacer. The lower chamber was then filled with electrode buffer.

The 1 ml protein sample, containing equal amounts of 1% SDS, 5% mercaptoethonol, 0.5M Tris HCl pH 6.8 and 10% glycerol was heated in a boiling water bath for 5 min to denature protein. After cooling, various volume ranging from 2 to 20µl solutions, were loaded in the individual wells. A small volume of bromophenol blue was added as the tracking dye. Electrophoresis was carried out at a constant voltage of 65 volts which increased to 110 volts when the protein entered the separation gel. The

electrophoresis was discontinued when the tracking dye reached the bottom of separation gel.

The gel was stained for proteins for 3 hours with the coommassie brilliant blue staining solution . Excess stain was removed with frequent changes of destaining solution.

Glycoprotein staining by per-iodic acid Schiff's reaction:

1. The electrophoresed proteins were fixed by immersing the gel in 12.5% aqueous trichloroacetic acid for 20 minutes.

2. The gel was then removed and washed thoroughly with double distilled water.

3. The gel was then placed in 1% periodic acid solution made in 3% acetic acid for 90 minutes.

4. Periodic acid from the gel was washed thoroughly by running tap water and gel was placed in the Schiff's reagent for 2 hours in the dark. Glycoprotein containing band turn purple.

5. Excess stain removed by 3 washings (10 min each) with 0.5% sodium metabisulphite.

3. Biological studies of fungal cultural filtrate and fungal toxin:

Several experiments described here were aimed at studying i) differential toxicity of fungal culture filterate towards Tobacco varieties which in nature are varying in their susceptibility of F. oxysporum f.sp. tobacii and ii) to identify the host specificity of novel protein isolated from the culture filterate.

A. Use of culture filterate in ranking tobacco varieties for wilt disease susceptibility:

Aqueous phase of culture filterate was used to rank tobacco varieties for susceptibility-tolerance to Fusarium oxysporum. For this, whole plants and their corresponding leaf disks were inoculated with dilutions of culture filterate.

i) Ranking varieties using whole plants:

Roots of about 3 month old tobacco plants of varieties A2, GT4 KM, A119, A3, A23, GT5, GT3430 were surface sterilized with 0.1% aqueous $HgCl_2$ solution followed by extensive washing with sterile water. Plants were maintained in the hydroponic solution of 30% v/v solution of culture filtrate at 25 ± 2°C, 80% humidity

and 12h photoperiod in growth rooms. Control plants were maintained in sterile Richard's solution, but in otherwise identical conditions. Observations on morphological and anatomical changes were carried out at a regular intervals. The inhibitory effect of the pathogen on the intact plants was expressed as the wilt index calculated according to Ebells (1967) after 10 days of inoculation.

ii) Ranking varieties using leaf disks:

Fully expanded leaves of varieties A2, GT4, KM, A119, A3, A23, GT5, GT3430 were removed and sterilized with 0.1mg/ml aqueous mercuric Chloride followed by four washes with sterile distilled water. Leaf disks of 0.9 cm diameter were cut using sterile cork borer. They were then transferred aseptically in sterile multiwell dishes at a rate of two disks per well. To each well 2 ml of sterile basal MS medium (Murashige and Skoog, 1962) containing increasing concentrations of sterile fungus culture filtrate (12.5, 25, 28 (=LD 50) and 37.5% v/v) was added. Control leaf disks received similar concentrations of uninoculated Richard's medium (= uninoculated fungal culture medium) in the basal M&S medium.

The leaf disks were cultured under 16 h of 3000 lux light at 25 ± 2°C temperature. Chlorophyll content of leaf disks (Arnon, 1949) was determined in each treatment after 72 h.

iii) Estimation of total chlorophyll content from the leaf disks:

Chlorophyll was extracted from leaf disks with 10 ml of 80% aqueous acetone containing a pinch of $CaCO_3$ to prevent phytin formation. After centrifuging the extract at 2000 rpm for 5 min, the O.D. of supernatent was estimated at 643 and 663 nm in a spectrophotometer. Chlorophyll value was calculated from the values of O.D. using the following formula of Arnon (1949).

Total Chlorophyll (mg l^{-1})=17.3 A663 + 7.18 A643.

Where A663=O.D. at 663 nm A643=O.D. at 643 nm

iv) Loss of electrolyte from tobacco leaf:

This was done by inoculating leaf disk as described above in (b) and measuring conductivity of leached electrolyte in the incubation medium after 72 h.

B. Use of Fusaric acid in ranking tobacco varieties for susceptibility/tolerance to wilt:

Purified sigma grade Fusaric acid was also evaluated at different concentrations of 1,10,50 and 100 ppm for the evaluation of toxic properties on the cultured leaf disks of Azadarichta indica, Mangifera indica, Solanum xanthocarpum and Nicotiana tabacum. Selection of these genera were done as they are not hosts for this pathogen. So if at all fusaric acid were a fungal host specific toxin, then the cultured leaf disks of these plants would not show any toxic effect.

C. Differential toxicity of the purified fungal toxin on varieties of tobacco in leaf disk bioassay:

Different dilutions ranging from 10 to 100 ppm of fungal toxic protein were made and fully expanded leaf disks of 3-4 month old field grown tobacco plants of various varieties were inoculated in a multiwell dish in triplicates. Leaf disks were incubated as described in 1(b). Chlorophyll content of these were measured after 72 h of incubation.

RESULTS

1. Confirmation of Koch's postulate:

When healthy tobacco plants were infected with fungal suspension, disease symptoms appeared after 30 days of inoculation in case of sick soil method (Fig.2A). In the liquid culture method the symptoms were visible within 20 days of incubation (Fig.2B). The disease appeared with characteristic wilt symptoms. Initially vein clearing was mild and yellowing and wilting was prominent in the lowermost leaves. The cortical region of the roots was macerated and eventually sloughed off . In the meantime the entire plant wilted. The decaying roots gave characteristic rotting smell. The pathogen was reisolated by the moist incubation method in the petriplate and was found identical with the original isolate of *F. oxysporum.*

2. Isolation and purification of glycoprotein from the aqueous phase of culture filterate:

a) Quantitative analysis of fungal protein:

The crude culture filtrate obtained from 5, 10 and 20 days old fungus was filter sterilized and crude protein was isolated from aqueous phase as described in

FIG. 2 Tobacco inoculation with F. oxysporum

A. "sick soil" method, left = control, right = treated.

B. "liquid culture" method, left = control, right = treated


A
B

Materials and Method section 2a.

The crude proteins were estimated from 5 day old CF 0.5 mg in 100 ml of culture filterate. The protein content in the 20 day old CF was estimated as 0.9 mg. and no further increase in the protein content was noticed in the CF of beyond 20 days of incubation.

b) Qualitative analysis of Protein: Purified protein sample was electrophoresed on denaturing system of 12% SDS polyacrylamide gel. The toxic protein separated as a single band. The resolution was found better at 12% gel composition. The relative mobility of the protein was calculated as 0.16 with reference to tracking dye. The electrophoresed protein was fixed in 12.5% TCA and were subsequently stained for its carbohydrade fraction using PAS reaction as positive.

3. Biological studies of culture filterate and fungal toxin:

Ranking of Tobacco varieties based on their order of susceptibility to the CF inoculation in leaf disk bio-assay (LD 50) and liquid culture method -

a) Ranking of varieties using whole plants in liquid culture method:

Tobacco plants inoculated with 25% or above v/v of CF, showed wilt symptoms after 10 days of incubation. The wilt index reached 100% at 50% of CF.

Even low volumes of CF (28% v/v) was able to induce several disease symptoms on tobacco plants that appeared during the actual pathological condition. They were yellowing, wilting and epinasty of leaves, formation of new roots and softening of root cortical tissue.

At 28% v/v concentration of CF, A2 variety showed maximum wilt index i.e. 68%. More than 50% plants of this variety were killed. Therefore 28% v/v concentration was judged as LD 50 dose. Thus A2 variety showed maximum susceptibility to wilt and therefore received 8th rank. Whereas var. GT5 and GT3430, in which the wilt index value was '0' at 28% CF were found to be most resistant and therefore received 1st rank. (Table 11).

The order of susceptibility based on wilt index among the 8 varieties screened was as follows:

Most sensitive ----------> Resistant

A2	> GT4	> KM	> A119	> A3	> A23	> GT5	> GT3430.
1	2	3	4	5	6	7	8

b) Ranking of varieties based on Leaf-disk bio-assay:

Toxic properties of the CF of F. oxysporum was also observed in the leaf-disk bio-assay as early as 24 hours after incubation of leaf-disks in the medium containing CF. Toxicity or the inhibitory effect on the CF was expressed in terms of chlorophyll degradation from cultured leaf disks.

When screened at LD 50 (28% v/v) conc. of CF, variety GT3430 showed very little chlorophyll degradation and A2 var showed maximum chlorophyll degradation i.e. 55.5%. Ranking of varieties was therefore possible based on this simple leaf disk bio-assay. Thus the most susceptible variety A2 received the 1st rank and the most resistant variety received the 8th rank.
The order of ranking based on leaf-disk bio-assay was as follows:

A2 > GT4 > KM > A119 > A3 > A23 > GT5 > GT3430
(Most susceptible) (Most resistant)

Control plants in liquid culture method remained healthy throughout the experiment.

TABLE NO. 11.

RANKING OF VARIETIES BY LEAF DISK BIOASSAY VS % WILT INDEX

Sl.No.	Varieties	X (Loss in chlorophyll as % of control)	Y (Wilt Index)	Rank		D= R1-R2	D
				R1	R2		
1	A2	55.5	68	1	1	0	0
2	GT4	37.1	40	2	2	0	0
3	KM	34.0	38	3	3	0	0
4	A23	22.6	28	4	5	-1	1
5	A119	20.0	25	5	4	1	1
6	A3	14.0	10	6	6	0	0
7	GT5	2.0	0	7	7	0	0
8	GT3430	1.8	0	8	8	0	0

$ED^2=2$

Sperman's Rank Correlation:

Therefore Sperman's Rank correlation for the ranking of varieties by culture filterate at whole plant level and ranking with the leaf-disk bio-assay was significant and positive.

Similarly control leaf disks in leaf-disk bio-assay method showed no chlorophyll degradation even after 24 h of incubation in sterile M&S medium with various concentrations of Richard's solution.

The Ranking of varieties made using of leaf-disk bio-assay correlated to their ranking made using the conventional liquid culture method with CF. (Table 11). Sperman Rank correlation for the ranking of varieties between these two methods was found to be positive and significant (Fig .3).

Spermans Rank Correlation ;

$$R_k = 1 - \frac{6 \sum D^2}{n(n^2-1)}$$

Where R_k = Coefficient of rank correlation

n = number of paired observations

$\sum$ = notation meaning "The sum of"

d = difference between the ranks for each pair of observations

$$= 1 - \frac{6 \times 2}{8(8^2-1)}$$

$$= 1 - \frac{12}{504}$$

$$= 1 - 0.023$$

$R_k = 0.977$

P = >98%

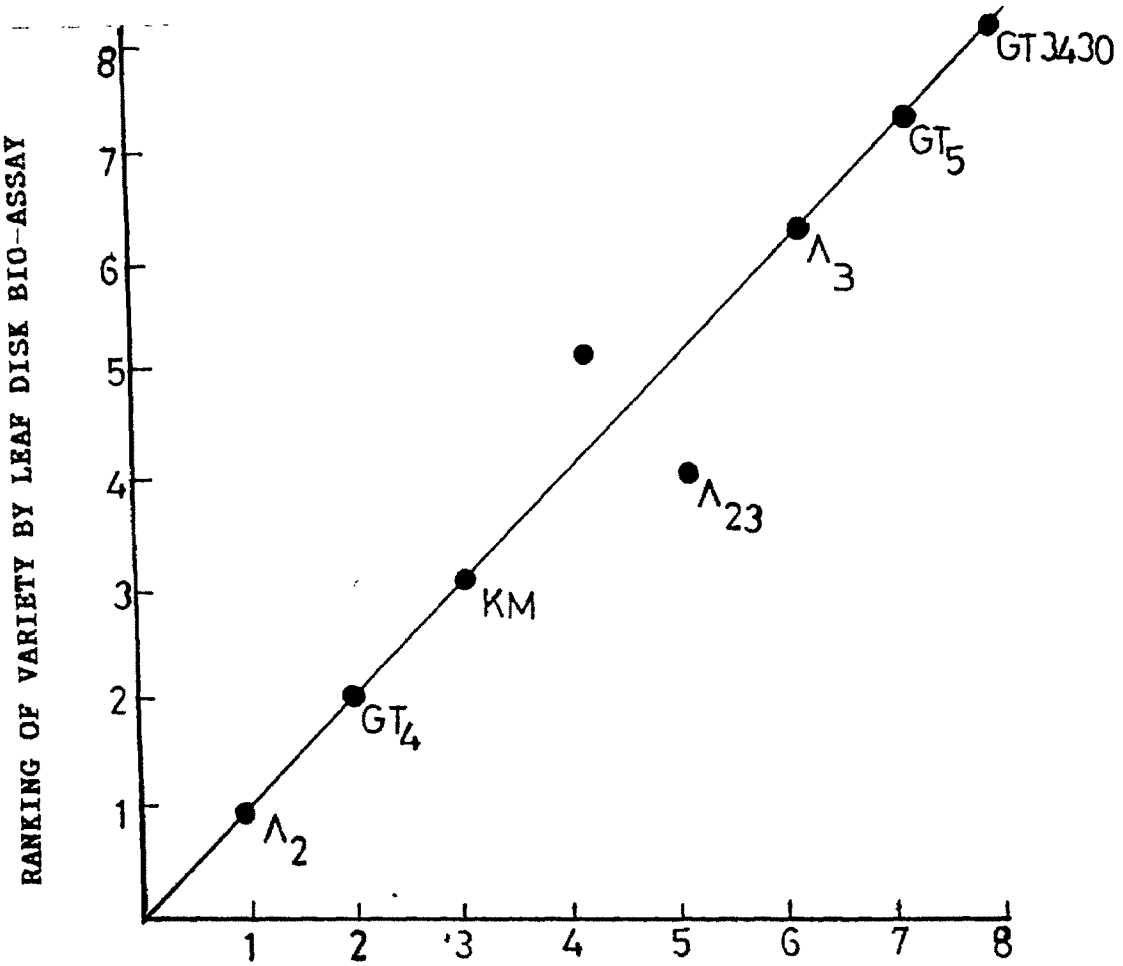

FIG 3- RANK CORRELATION BETWEEN THE VARAITAL RANKING DONE BY LEAF DISK BIOASSAY VS RANKING OF VARIETIES DONE BY % WILT INDEX.

Further, these two methods of ranking of varieties was also, similar to that of ranking done by direct fungal mycelial infection method (Selvapandiyan, 1989).

Differential Toxicity of CF on tobacco varieties:

Leaf disks of all eight varieties were treated with culture filterate concentrations ranging from 12.5% v/v to 37.5% v/v (Table 12) in leaf disk bioassay.

Concentrations of CF as low as 12.5% v/v could bring about considerable damage in chlorophyll content of leaf-disks of most susceptible varieties A2, GT4 and KM i.e. 30%, 25% and 24% respectively. And in most resistant varieties of GT5, GT3430 the chlorophyll degradation value was found to be negligible.
Further increase in CF concentration to 37.5% v/v induced greater loss in chlorophyll content (64.8%) in A2 variety when compared to the most resistant varieties of GT5 and GT3430 i.e. 12.5%.

Thus fungal culture filtrate showed differential toxicity to the in vitro cultured leaf-disks of all these varieties (FIG. 4).

TABLE NO. 12.

DIFFERENTIAL TOXICITY OF CF ON TOBACCO VARIETIES AS JUDGED IN LEAF DISK BIOASSAY. LEAF DISKS WERE INCUBATED IN MEDIUM WITH AND WITHOUT VARIOUS CONC. OF CF FOR 72 h. AND CHLOROPHYLL WAS DETERMINED IN THEM AFTER 72 h.

Sl.No.	Var.	Chlorophyll in control leaf disks (mg/gm fresh weight)	Conc. of CF v/v	Chlorophyll loss as % of control
1	A2	1.037	0.0	0.0
			12.5	30.0
			25.0	34.5
			28 (=LD 50)	55.5
			37.5	64.8
2	GT4	1.102	0.0	0.0
			12.5	25.0
			25.0	35.3
			28 (=LD 50)	37.1
			37.5	39.6
3	KM	1.102	0.0	0.0
			12.5	24.0
			25.0	33.2
			28 (=LD 50)	34.0
			37.5	37.1
4	A119	1.173	0.0	0.0
			12.5	18.0
			25.0	20.0

			28 (=LD 50)	22.6
			37.5	29.0
5	A3	0.6486	0.0	0.0
			12.5	18.0
			25.0	18.0
			28 (=LD 50)	20.0
			37.5	26.8
6	A23	0.7856	0.0	0.0
			12.5	11.0
			25.0	14.0
			28 (=LD 50)	14.0
			37.5	23.3
7	GT5	0.4540	0.0	0.0
			12.5	0.0
			25.0	1.8
			28 (=LD 50)	2.0
			37.5	12.6
8	GT3430	0.5513	0.0	0.0
			12.5	0.0
			25.0	1.9
			28 (=LD 50)	1.8
			37.5	12.5

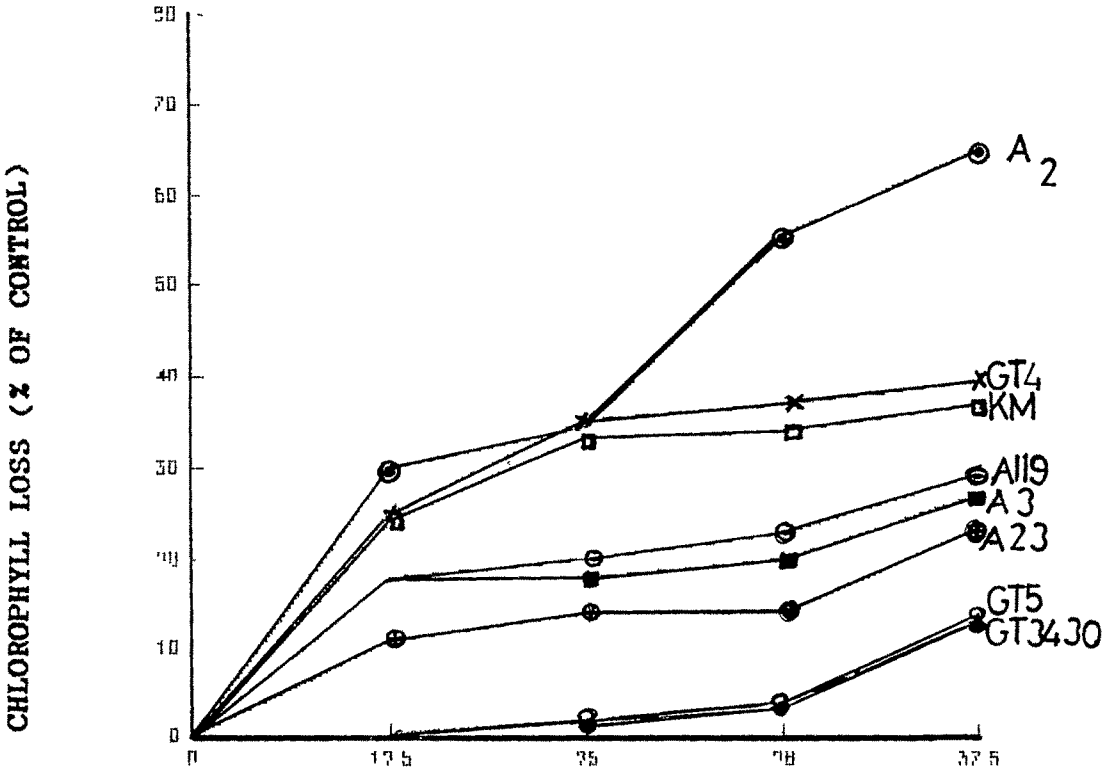

FIG 4. DIFFERENTIAL TOXICITY OF CF ON THE LEAF DISKS OF TOBACCO VARIETIES.

4. Effect of Fusaric acid on the leaf-disks of different plants:

'Sigma grade FA was tested for wilting on different plants like Mango, Solanum sp, Neem and Tobacco. Different dilutions of fusaric acid between 10-100 ppm were made with sterile MS medium.

The order of these plants with respect to chlorophyll degradation was as follows: (Table 13)

Highest degradation -----> Least degradation

Solanum > Neem > Tobacco > Mango

Thus fusaric acid showed very little toxic effect on Tobacco leaf-disks when compared to Solanum and Neem

.

Thus fusaric acid could neither caused chlorophyll degradation to the extent caused by the CF nor it showed any host specificity towards the Tobacco plant which is a natural host to this pathogen.

Effect of fungal Toxic protein (FTP) on leaf-disks of Tobacco:

The effect of FTP at different concentrations of 10, 50 and 100 ppm were tested on in vitro cultured leaf-disks

of Tobacco var A2. The results of leaf-disk bio-assay showed that 100 ppm conc. of toxic protein brought about more than 50% of chlorophyll degradation i.e. 54.21%, and therefore judged as LD 50 concentration. (Table 14)

TABLE 13.

EFFECT OF FUSARIC ACID ON CULTURE LEAF DISKS OF VARIETIES OF NON HOST PLANTS. TOBACCO IS INCLUDED HERE AS CONTROL AS IT IS THE NATURAL HOST OF THIS PATHOGEN:

Sl.No.	Plant Name	Conc. of Fusaric Acid (in ppm)	Loss in Chlorophyll as % of control
1	Tobacco	Control	0.00
		10	3.08
		50	18.06
		100	26.57
2	Solanum sp.	Control	0.00
		10	37.40
		50	60.28
		100	76.40
3	Mango	Control	0.00
		10	0.00
		50	25.30
		100	39.90
4	Neem	Control	0.00
		10	29.00
		50	31.90
		100	29.00

FIG.5. Effect of fungal glycoprotein on leaf disks of different types of plants like neem, mango, solanum sps and tobacco. Note the highest chlorophyll degradation coupled with electrolyte leachete into the media in the case of tobacco.

TREATED CONTROL

MANGO

SOLANUM

NEEM

TOBACCO

Effect of Fungal Toxic protein (FTP) on leaf-disks of non host plants of F. oxysporum f.sp. nicotianae to confirm host specificity (Fig. 5).

Leaf disks of three different plants like Solanum sp., Neem and Mango along with that of Tobacco when inoculated at LD 50 concentration of (=100 ppm) of toxic protein, showed greatest loss in chlorophyll content in case of Tobacco. (Table 15). The chlorophyll content remained unaltered in case of Neem and Mango even after 24 h incubation at this concentration whereas in case of Solanum sp. the chlorophyll content was reduced to a negligible value (0.6%). The chlorophyll degradation in Tobacco leaf disks found to be 54.28% which was maximum when compared to the other plants, indicating its high degree of susceptibility of tobacco to this proteins and high degree of host specificity of the toxic protein.

TABLE 14.

DOSIMETRY OF FUNGAL PROTEIN ON CHLOROPHYLL DEGRATION OF CULTURED LEAF DISKS OF TOBACCO:

Sl.No.	Conc. of toxic protein (in ppm)	Total chlorophyll content mg/g/f.wt.	% loss of Chlorophyll
1	Control	1.4	0.00
2	10	0.75	46.72
3	50	0.72	48.57
4	100	0.64	54.28

TABLE 15.

EFFECT OF FGP (FUNGAL GLYCO PROTEIN) ON LEAF DISKS OF PLANTS THAT ARE NOT HOST TO FUSARIUM OXYSPORUM f.sp. NICOTIANAE. DATA ON TOBACCO INCLUDED HERE FOR COMPARISON TO SHOW EFFECT OF FGP ON LEAF DISK OF NATURAL HOST.

Sl.No.	Plant Species	Total chlorophyll content mg/g/f.wt.		% of loss in Chlorophyll
		Control	treated with LD 50 (=100 ppm) toxic protein	
1	Tobacco	1.4	0.64	54.28
2	Solanum	0.593	0.590	0.60
3	Neem	1.23	1.23	0.00
4	Mango	1.14	1.14	0.00

DISCUSSION

Selvapandiyan (1987) screened about six tobacco varieties to Fusarium wilt using the pure culture of F. oxysporum f.sp. tobacii as well as its crude CF. In the present study we have screened total 8 commercially important bidi tobacco varieties to Fusarium wilt. Besides crude CF, we also used the aqueous phase of the CF of F. oxysporium. The aqeous phase was obtained by partition chromatography of crude CF with ethylacetate. This was done to remove organic toxic principles like the fusaric acid, dihydrofusaric acid etc. from the crude CF as used by Selvapandian (1987). The aqueous phase identified var A2 and GT4 as the most susceptible amongst all other varieties. The varieties GT5 and 3430 were found to be most resistant and var. A23 and A114 were found to be moderately susceptible. Thus tobacco varieties showed identical susceptibility/tolerance to the aqeous phase of CF as that shown when subjected to the actual fungal infection or its crude CF.

Varietal screening was extended in leaf disk bioassay using LD50 (28% v/v) of aqueous phase of CF. The test indicated that the varieties that were susceptible to Fusarium in the whole plant assay were also susceptible

in the leaf-disk bio-assay of aqueous phase of crude CF and vice versa. Further the result of Speerman rank correlation for the ranking the varieties with these two methods was highly significant and positive.

Earlier, Buiatti et al (1985) observed a correlation between the ranking of the varieties of carnation to the actual pathogen F. oxysporum f. sp. dianthi and the tolerance of their in vitro cultures to the culture filtrate, but stastistical validity was lacking. Further, no information was given of the effect of culture filtrate on whole plants of different varieties of carnation.

To our knowledge, this is the first report on the use of leaf disk bioassay in screening tobacco varieties for susceptibility to the wilt pathogen F. oxysporum f.sp. nicotianae using its aqueous phase of culture filtrate. Although the number of varieties screened is small, the method of leaf-disk bio-assay to screen varieties shows a potential due to the rapidity of assessment and ease of manipulation. Impetus to discover host specific glycoprotein from the CF came from our observation in which the aqeous phase of CF was able to mimic the actual pathogen and the crude CF in bringing about the characteristic wilt symptoms on the intact plant.

The purification was made easier by testing individual fraction of gel filtration chromatography of aqueous phase of CF in leaf disk bioassay. The fractions were also tested for their absorbance at 280 nm which gives presence of proteins. The fraction showing highest toxicity in leaf disk bioassay also showed highest absorbance at 280 nm prompting as that the toxin might be proteinaceous. Therefore the fractions showing highest, toxicity were pooled and treated with acetone to precipitate the proteins. The precipitated protein was redisolved in water and was tested for its toxicity in leaf disk bioassay. As little as 10 ppm of protein was able to exert strong chlorophyll degradation in the cultured leaf disks of tobacco. It had however no effect on cultured leaf disks of plants like Neem, Mango & Solanum which are not known to be host of this pathogen. At this juncture it would be worth while to remember that when Selvapandian (1987) tested the effect of Fusaric acid on intact plants, he found that .it caused only vein clearing and wilting of leaves. But did not cause many other characteristic wilt symptoms such as necrotic and interveinal chlorosis, inhibition, of formation of new roots yellowing and wilting of leaves and softening of root cortical tissues. In

contrast the aqueous phase of culture filtrate was able to mimic the actual pathogen in bringing the wilt symptoms. Further fusaric acid was toxic to the leaf disks of Neem, Solanum and Mango unlike the crude protein that we have isolated.

There is also report by Pegg (1981) that FA pH 8.7 did not show any reaction on the host since it was denatured in alkaline condition. Literature (Gaumann, 1957; Kuo and Scheffer, 1964) too indicates that FA is not directly involved in causing symptoms in diseased plants. Hence, FA injury is not at all considered as a true symptom (Gaumann, 1957). Gaumann et al, (1952) further pointed out that FA never acts alone on the host but always in association with other products of fungal and host metabolism.

In our studies, Fusaric acid when tested at different concentrations (1-100 ppm) on the leaf disks of different plants, showed greater chlorophyll degradation in the leaf disks of solanum spp. than in Tobacco. In tobacco even higher concentrations of FA such as 100 ppm could cause very low levels of chlorophyll degradation i.e. 26% when compared to the toxic protein which caused 54.28% of chlorophyll degradation at similar

concentrations. Hence, all the evidences indicate that FA is not responsible for the true wilting and that it could have no more than a secondary role in disease development. Further, it is not host specific.

Many of the plant pathogens grown in vitro secrete their toxic product (= toxin) in the culture media. In cases where the nature of toxin secreted by a pathogen is not known it is customary to use the crude CF as selection agent or for the varietal screening (Selvapandiyan, 1987) or for the progeny analysis as in our case. In such experiments caution should be taken in interpreting results. Because the CF may contain some residual elements of the original fungus culture medium which may be inhibitory for the growth of the plant cells. Thus the observed inhibitory effect of CF may not always be due to the presence of presumptive fungal toxin(s) but also due to the elemental composition of residual fungal medium not suitable for plant cell or organ growth. We have maintained proper control in the material and methods, where control leaf disks were inoculated with MS medium containing uninoculated Richard's medium (Hendrix and Neilson, 1958). Therefore the observed inhibitory effect of CF of F. oxysporum is definitely due to toxin secreted by F. oxysporum in the medium.

These type of controls are not used by some previous workers in selection or screening experiments for disease resistance using in vitro cultures (Behnke, 1980a,b; Hartman; 1984 b, etc.)

In many cases the mechanism responsible for toxin-induced changes in plants is not known. The mode of action of toxin is considered as one of the most challenging and perhaps most fruitful aspects of toxin research (Misaghi, 1982). However, there are reports regarding the consequences due to the pathogen action on the host; permeability (Gaumann, 1957; Wheeler and Black, 1963; D'Alton and Etherton, 1984); irreversible damage to the chloroplast (Koruge, 1978); changes in the rate of CO_2 uptake (Duniway and Stayer, 1971); alterations in photosythetic rate (Steele et al, 1976, 1978) increase in rate of respiration (Collins and Scheffer, 1958).

Experiments conducted by the present study too gave similar results when aqueous phase of CF as well as purified toxic protein were tested on the leaf disks of N. tabaccum. The damage occured to the photosynthetic pigments of leaf disks was correlated with a decrease in the growth of the leaf disks, pH change of the culture medium and electrolyte leakage into the ambient

solution. Hence, a linear relationship was established between toxin concentration and the various uncoupling activities in the plant metabolism. In all the cases, maximum damage of the leaf disks occured above 37.5% of CF.

Necrosis and interveinal chlorosis are some of the symptoms found in tobacco leaves infected with F. oxysporum. Hence, leaf-disk bio-assay was taken as parameter for testing the effect of CF (aqueous).

The isolated fungal toxic protein was not dissolved in any organic polar or non polar solvents. It was dissolved in water. It was found to be heat stable since the autoclaved CF had the identical inhibitory properties.

It was resolved into a single band on the SDS polyacrylamide gel. and was positive for its carbohydrate fraction using PAS reaction. Hence we confirm that the principle fungal toxin present in the aqueous phase of CF as a glycoprotein. Such water soluble toxins so far has not been reported in case of F. oxysporum f. sp. nicotianae. Therefore we consider it as novel compound with high toxicity capable of eliciting many host specificity symptoms that the fungus

is capable to do.

With biologically active substances such as phytotoxin, successful extraction of a particular compound usually requires the development of a suitable bio-assay. In the present report purified fungal protein was bio-assayed by the leaf disk showing degradation of chlorophyll pigments. Concentrations as low as 10 ppm of fungal protein alone could be able to bring about chlorophyll degradation which is comparable to LD 50 dose of crude culture filtrate.