CHAPTER 4

SOME PHYSIOLOGICAL AND BIOCHEMICAL STUDIES:.. ..CF .. .. ..

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SOME PHYSIOLOGICAL AND BIOCHEMICAL STUDIES: THE EFFECTS OF F. OXYSPORUM CF ON TOBACCO PLANTS AND IN VITRO CULTURED TISSUES

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SOME PHYSIOLOGICAL AND BIOCHEMICAL STUDIES : THE EFFECTS OF CF OF F. OXYSPORUM ON TOBACCO PLANTS AND IN VITRO CULTURED TISSUES

4-A INTRODUCTION

Derangements in the structures of the plant cell walls and degradation of their components are the common features of infection by pathogenic agents. These changes are brought about by cell wall degrading enzymes 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, causes separation of cells and waterlogging of infected tissues, a process known as maceration (Wood, 1967). Apart from this, the pathogens also produce poisonous substances known as toxins.

Various toxins have been classified as :

i) phytotoxin, ii) Vivotoxin and iii) pathotoxin.

Various terms have been used for different toxins produced by pathogens. 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 produced in pure culture, which may not necessarily be formed as a consequence of an interaction between the fungus and a higher plant (Harborne, 1983) e.g., Lycomarasmin, and alternaric acid (Brian, 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 fungi, e.g., fusaric acid (Lakshminarayanan and Subramanian, 1955) and marticins (Kern et al., 1970). 'Pathotoxin', proposed by Wheeler and Luke (1963), describe a 'host specific toxin' (Pringle and Scheffer, 1964). 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 (Meehan and Murphy, 1947; Luke and Wheeler, 1955), T-toxin (Hooker, et al., 1970) etc., Fusarium oxysporum is known to produce several toxins, viz., fusaric acid, lycomarasmin, moniliformin, naphthazarin etc.

4-B REVIEW ON FUSARIUM TOXINS

Many of the toxins were isolated from cultures of Fusarium spp. and many of their chemical and physical properties are known.

4-B.1 Low molecular weight toxins :

i) Naphthazarin toxins : Kern and Naef-Roth, (1967) identified several phytotoxic compounds, viz. marticin, isomarticin, fusarubin, jaramicin, narjaramicin and novarubin, with a common naphthazarin structure produced by F. solani, causing a root and stem rot of peas and Martiella plants.

These toxins inhibit seed germination and root growth by inhibiting the respective enzyme reactions (Kern et al., 1970). These substances are detoxified by certain metal ions (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 CF of a pathogen was lycomarasmin (Gäumann, 1951), produced by F. oxysporum f. sp. lycopersici the causal agent of tomato wilt disease. A tomato plant is wilted within 7 days of fungal infection, but the concentration of lycomarasmin in CF at a corresponding time is no higher than

10 mg l^{-1}, whereas it reaches upto 300 mg l^{-1} after 40 days. As this has not been detected in the diseased plants, it is not considered as a vivotoxin (Pegg, 1981; Harborne, 1983).

Since lycomarasmin is a peptide, containing aspartic acid (Fig.4-1), Wooley (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 Lactobacillus casei could be reversed with strepogenin preparations.

iii) Fusaric acid : Gäumann et al. (1952) first described fusaric acid (FA) (5-n-butyl picolinic acid; Fig. 4-1) as a wilt toxin from F. oxysporum f. sp. lycopersici and Gibberella fujikuroi. But unlike lycomarasmin, it has been detected in diseased plants (Lakshminarayanan and Subramanian, 1955).

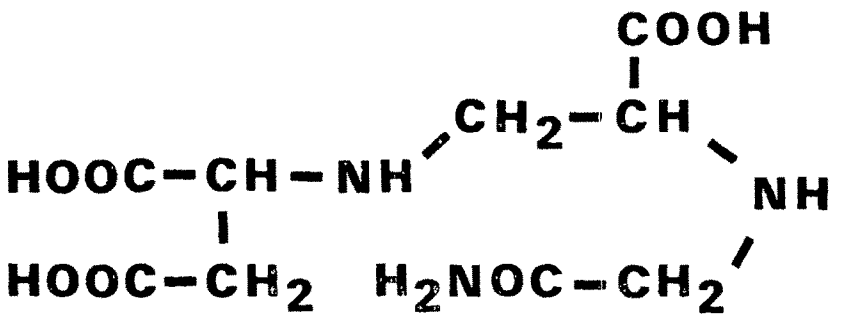
About 200 mg l^{-1} fusaric acid is produced by F. oxysporum f. sp. lycopersici after 20 days in static cultures or 6 days in shake cultures (Pegg, 1981). Dehydrofusaric acid (With a double bond at the end of the side chain) and other related substances also frequently occur in F. oxysporum (Kern, 1972).

Lakshminarayanan and Subramanian (1955) as well as Kalyanasundaram and Venkata Ram (1956) detected 17 µg 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

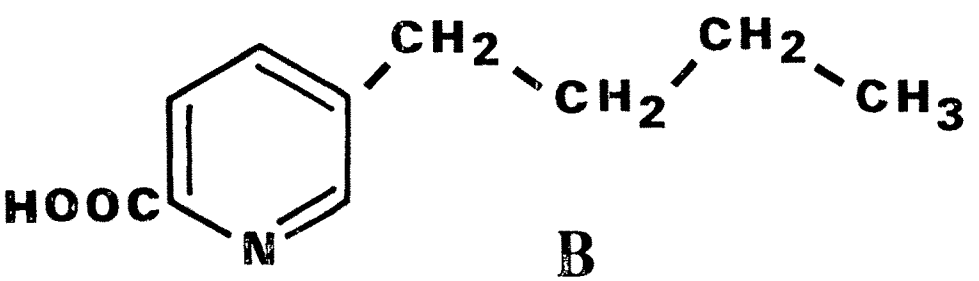
Fig.4-1 Low molecular weight toxins of *Fusarium* :

A) Lycomarasmin
(N-acetyl aspartic acid)

B) Fusaric acid
(5-butylpicolinic acid)


COOH
CH2
CH
HOOC
CH
NH
NH
HOOC
CH2
H2NOC
CH2

A


CH2
CH2
CH2
CH3
HOOC
N

B

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 α carbon to the carboxyl group (Fig.4-1), they are able to chelate heavy metals such as Fe^{+++} and Cu^{++}. The toxicity of these compounds depend indeed on their chelating ability, since the well known chelating agent versune (EDTA) produces effects which resemble those produced by lycomarasmin and FA in tomato (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 Springer et al. (1974) isolated a moniliformin that contained potassium instead of sodium in its molecule, from Gibberella fujikuroi (perfect stage of F. moniliforme). Later on its toxic effect was also found (Hayes and Hood, 1974), but how the toxicity is brought about is still not known (Hood amd Szczech, 1983).

4-B.2 Macromolecular toxins :

A number of macromolecular toxins have been described from Fusarium, some of which are polysaccharides. Pectolysin is an enzyme protein produced by various f. sp. of F. oxysporum (Naef-Roth et al., 1961; cf. 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-inducing 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).

4-C OBJECTIVE OF THE PRESENT STUDY

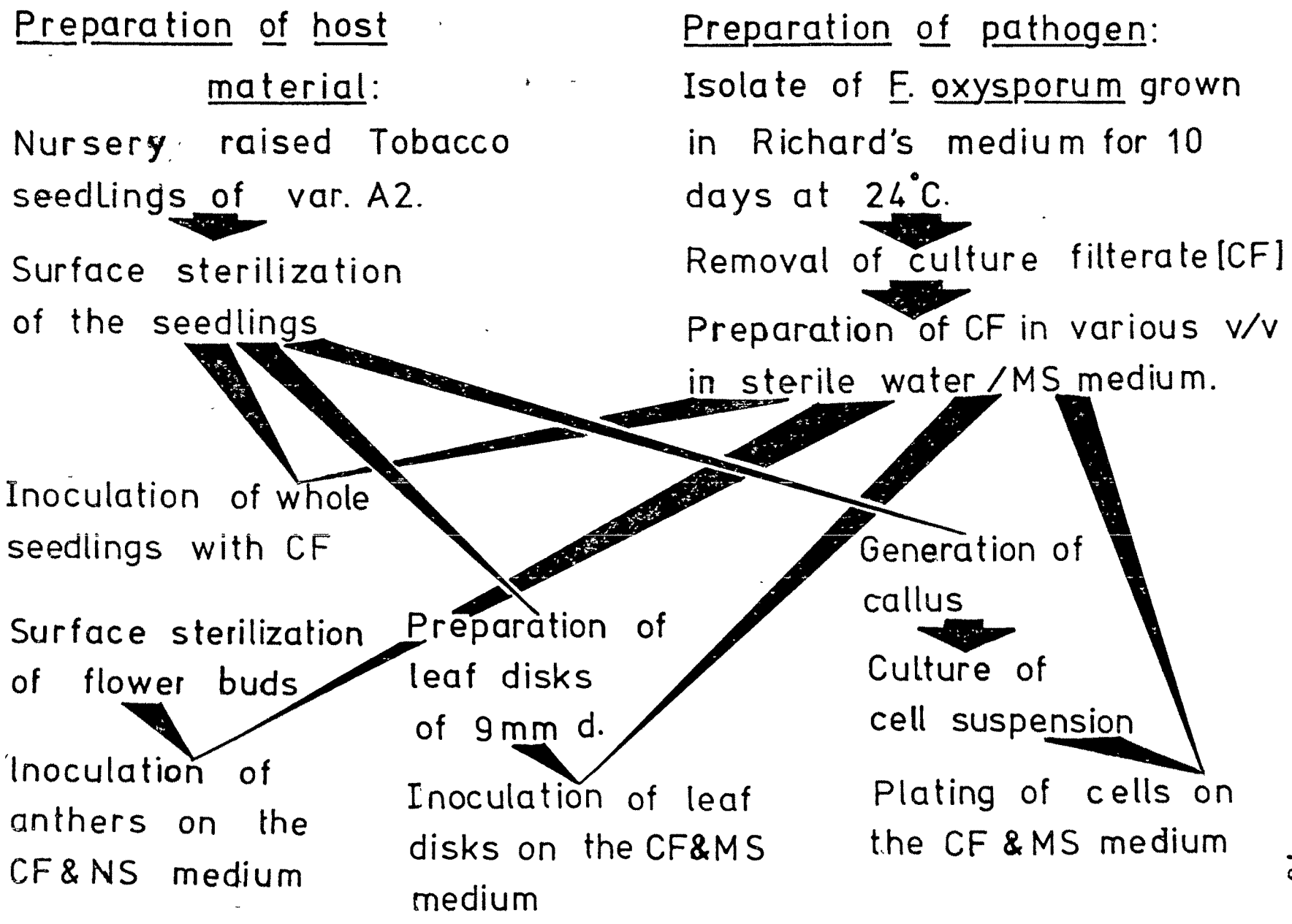
Our experimental reports in the previous chapter showed that CF alone can mimic all the wilt symptoms on tobacco plants like the fungal mycelium, indicating the presence of some toxin in CF that affects plant metabolism. The present study aimed to find the effects of CF on different in vitro and in vivo cultures of tobacco and to attempt to characterize the toxic metabolite(s) present in the CF.

4-D MATERIALS AND METHODS

4-D.1 Inhibitory effects of CF of F. oxysporum on the growth of tobacco plants, and in vitro grown anthers, leaf disks and cell suspension culture (Fig.4-2) :

i) Preparation of CF and media containing CF: The isolate F. oxysporum f. sp. nicotianae was grown in the modified Richard's (MR) medium (Hendrix and Nielson, 1958). The mycelial mat and the conidiospores were removed by filtering through a nylon mesh. The CF was again filtered twice through Whatman No.1 filter paper and finally filter sterilized with 0.22 μ microbiological filter. It was then

EXPERIMENTAL PROTOCOL 2



mixed with molten 0.8% agar based MS medium to obtain various volume to volume concentrations: 12.5, 25, 37.5 and 50%. No CF was added to control medium. The CF was mixed with MS medium in such a way that the strength of MS medium remained constant. The MS medium was supplemented with 0.1 μM IAA, 2 μM Kn and 2% sucrose.

In carrying out studies on the effect of fungal CF on in vitro grown plant tissues, a proper control is necessary in which the effect of various strength of fungal culture medium (MR medium) is studied on in vitro grown plant tissues. We prepared MS medium that contained increasing amount of either MR medium or the CF as shown in Table 4-1 and used this as either the control or treatment respectively.

ii) Inoculation of host tissues on media containing CF: Roots of about three months old tobacco plants were surface sterilized with detergent water three times. Plants were treated with CF by immersing their entire root portions in the MS-CF media.

To study the effect of CF on the androgenesis, anthers from 0.9-1.1 cm long young flower buds were excised and inoculated on the NS medium supplemented with 15% coconut milk and 1% activated charcoal along with different concentrations of CF.

Table 4-1 : MS-MR-CF media combinations

S.No.	MS medium %	MR medium %	CF %	Total volume %
1	50	50	0	100
2	50	37.5	12.5	100
3	50	25	25	100
4	50	12.5	37.5	100
5	50	0	50	100

% = v/v

MS = MS basal + 0.1 μM IAA + 2 μM Kn + 2% sucrose

MR = Modified Richard's solution

Final pH was adjusted to 5.8 in each case

Leaf disks (0.9 cm diam.) cut from the surface sterilized young leaves of three months old plants, were inoculated on various MS-CF media.

The action of CF was also observed on the isolated cells grown in vitro. The callus obtained from the inoculated leaf disks (on MS+2% sucrose + 0.1 μM IAA + 2-10 μM Kn) was subcultured every 2 wks in the same medium for 3-4 times. The friable callus was transferred to liquid media of same chemical composition minus agar. The suspension culture obtained after 2 sub-cultures in same liquid medium was filtered through various sizes of mesh to obtain single cells or groups of 3-6 cells. They were mixed with molten (40 to 50°C) agar based MS-CF medium upto 60% (v/v) of CF was used in the MS medium and spread on petriplates.

The plants/cultures except anthers were incubated at 24±1°C with 18 hr photoperiod. The inoculated anthers were incubated under dark in the culture room. The inhibitory effect of CF on intact plants was expressed as the wilt index (see section 3-B.5).

Leaf disks were grown in the presence of CF for 10 days. The inhibitory effects of CF on the in vitro grown leaf disk was expressed using three different parameters: a. decrease in dry weight, b. decrease in chlorophyll content and c. increase of the conductivity of the culture medium.

Chlorophyll was extracted with 10 ml of 80% of 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 determined at 643 and 663 nm in a spectrophotometer. Chlorophyll value was calculated from O.D. using the following formula of Arnon (1949).

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

Loss of electrolyte from tobacco leaf disks inoculated in MS-CF was studied as a measure of permeability change under infected condition (Samaddar and Scheffer, 1968; Dube, 1973). Tobacco leaf disks (0.9 cm diam.) from surface sterlized leaves were inoculated in liquid MS medium combined with different doses of CF: o, 12.5, 25, 37.5 and 50%. MS medium was supplemented with 0.1 µM IAA and 2 µM Kn and 2% sucrose. The inoculated leaf disks were kept on a gyratory shaker. After 3 days of incubation the leaf disks were removed and rinsed twice in double distilled water. The disks were blotted and 100 mg portions were placed in 20 ml sterile double distilled water. Conductance measurements of the bathing solutions were taken after a period of 12 hr using Toshniwal conductivity Bridge-type CL01/01A.

The specific conductivity readings were expressed in µ mho. The temperature of the bathing solutions was maintained at 25 ± 1°C during the conductivity. The specific conductivity was calculated using following formulae.

$$\text{a. Cell constant} = \frac{\text{Sp. conductivity of N/10 KCl} \times 10^6}{\text{Range factor} \times \text{Dial readings}}$$

$$\text{b. Sp. conductivity of bathing solution} = \text{Dial reading} \times \text{Range factor} \times \text{Cell constant}$$

The anthers from the culture were periodically harvested and squashed in 1% acatocarmine and the androgenetic microspore development was confirmed. After 4 to 5 weeks of incubation per cent productive anther was calculated according to method of Dunwell (personal communication) as mentioned below.

$$\text{Percent productive anther} = \frac{\text{Number of anthers which produced plants}}{\text{Total number of anthers inoculated}} \times 100$$

In cell plating after 12 days of incubation, numerous 10-15 celled colonies were observed. Their number was counted under an inverted microscope to calculate the plating efficiency using the following formula (Bhatt et al., 1986).

$$\text{\% plating efficiency} = \frac{\text{Number of colonies per petridish}}{\text{Number of plating units seeded in the dish}} \times 100$$

4-D.2 Isolation of Phytotoxin from the CF :

i) Isolation of CF : Ten days old culture of F. oxysporum was filtered through Whatmen No.1 filter paper to remove fungal mat. The CF was further ~~classified~~ clarified by passing through 0.22 µ size microbiological filter to remove contaminating condiospores.

ii) Partition chromatography : The classified fungal CF was partitioned against equal volume of ethyl acetate to remove ethyl acetate soluble fractions, viz. fusaric acid dihydrofusaric acid etc., Partitioning was done three times and aqueous phase separated in the separating funnel. The aqueous phase was reduced to one fifth of the original volume in a rotary vacuum evaporator. The aqueous phase did not show presence of fusaric acid on paper chromatography performed as suggested by Lakshminarayanan and Subramanian, (1955).

iii) Gel chromatography : One/three ml of the concentrated aqueous phase was loaded on 1.8 X 18 cm Sephadex G100 column. The column was eluted with distilled water. Fractions of 3 ml were collected and were assayed for the presence of toxin using the leaf-disk-assay (chlorophyll assay; see section 4-D.1). The UV spectrum of those fractions showing phytotoxicity was determined on a. Shimadzu OPI-4/UV-240 (Japan) spectrophotometer. A typical spectrum is shown in Fig.4-8. Fractions showing high phytotoxicity were pooled together and were rechromatographed on Sephadex G25 (1.8 X 23 cm). Three separate peaks exhibiting phytotoxic compounds were visible (Fig.4-10).

4-D.3 Effect of toxins on wilting :

i) Effect of purified toxin on wilting : The fractions (obtained after gel filtration) that contained high levels

of phytotoxicity were oven dried at 60°C for 48 hr and the white powder obtained was used for inducing wilt at different concentrations: 0, 0.01, 0.1 .....1000 ppm.

ii) FA test on wilting : To see the effect of FA in developing wilt symptoms on tobacco plants, FA ('Sigma' grade) was mixed with sterile distilled water in different concentrations: 0.01, 0.1 and 10 mM. As it dissolves slowly in water, it was shaken well to ensure complete dissolution. Control was maintained in which FA was not added. Three months old tobacco plants were inoculated in these solutions of FA and incubated in the culture and wilt index was determined after 3 days of inoculation.

4-D.4 Effect of increase in carbon source in the medium for the fungal toxicity :

The normal concentration of sucrose in the MR fungal culture medium is 0.5%. To find out the optimum level of carbon source in the MR medium for maximum synthesis of phytotoxin, different levels of sucrose 0.5, 1.5.....4.5% were added to MR media. The CF was filtered from 10 days old cultures and gel filteration was carried out as mentioned in section (4-C.2). They were tested for absorption spectrum and toxicity using the leaf-disk bioassay method.

The nature of the culture of F. oxysporum f. sp. nicotianae growing at different levels of sucrose to the media was observed and recorded separately.

4-E RESULTS

4-E.1 Effects of crude CF of F. oxysporum on whole plant and on various types of in vitro grown tissues :

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

The CF at concentration of $\leq$ 25% was stimulatory to growth of excised leaf disks as well as to plated cells. Concentrations of CF above this level was inhibitory. No growth occurred in leaf disks incubated with 50% CF. Similarly when the concentration of CF was raised beyond 50%, the plating efficiency of cells reached zero. The cultured anthers were much more sensitive than other tissues to the presence of CF. The CF showed inhibition of androgenesis right from the lower concentration (15%) and complete inhibition was recorded at 37.5% of CF in the medium (Fig.4-3).

In all cases, the cultures grown in control (without CF) showed typical sigmoid pattern of growth during incubation. But the cultures at complete lethal level turned brown and dead.

Necrosis and interveinal chlorosis are some of the symptoms found in tobacco leaves infected with F. oxysporum. Hence the effect of CF on the destruction of chlorophyll was studied by estimating the chlorophyll contents of in vitro cultured leaf disks with CF.

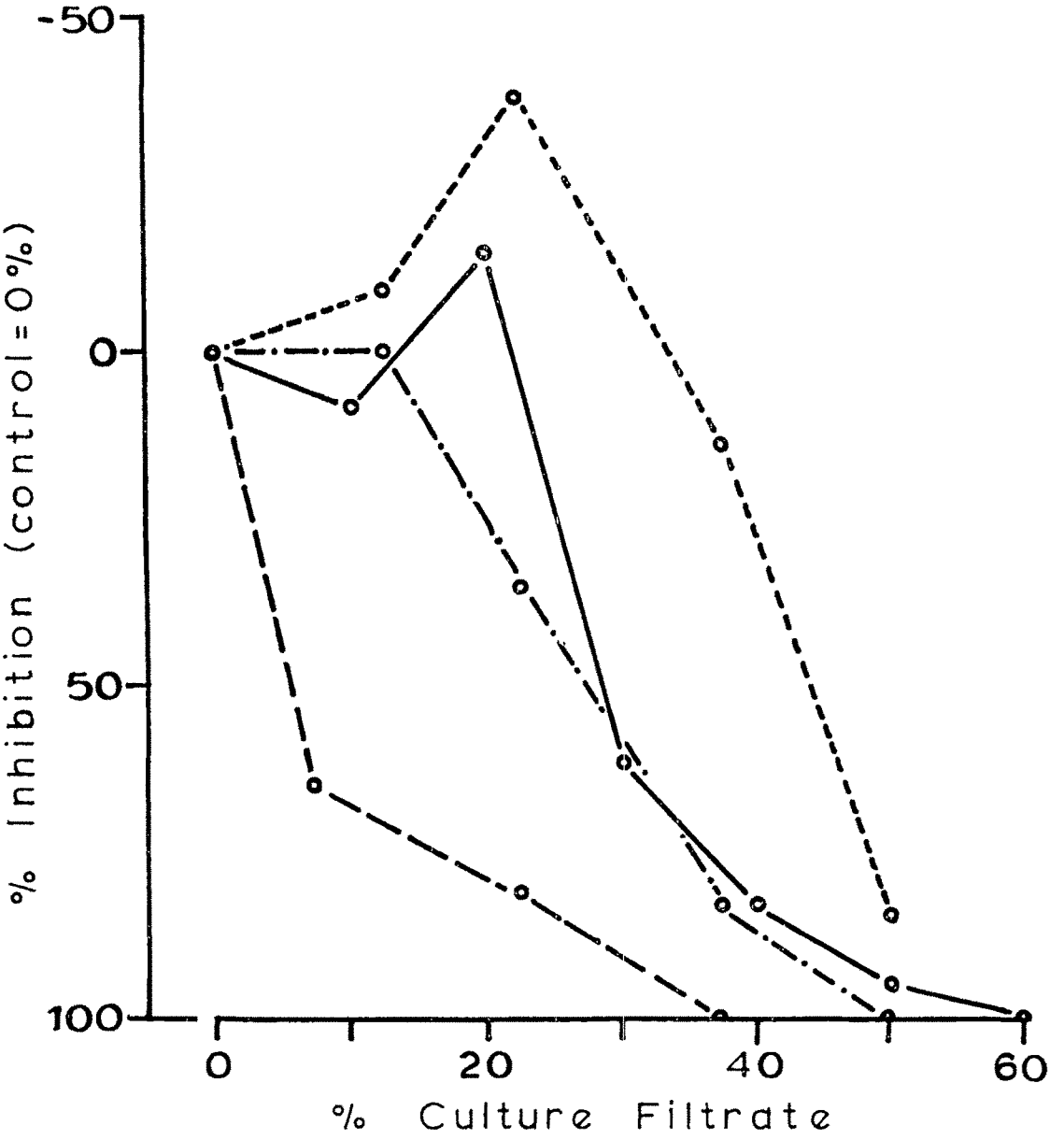
Fig.4-3 Growth inhibition of seedlings, excised leaf disks, anthers and cells of tobacco due to CF of *F. oxysporum* :

0—·—·—··0 whole plant

0--------0 leaf disks

0———— —0 anthers

0————————0 cells


-50
0
50
100
% Inhibition (control=0%)
0
20
40
60
% Culture Filtrate

The chlorophyll content was found almost constant in the control leaf disks throughout the experiment (Fig.4-4). The pigment loss was noticed right from the beginning in the disks inoculated with CF at 25% and above. At 12.5% CF there was very little inhibition in the content of chlorophyll in the early hours (between 6 and 12 hr) and its toxic effect on the pigment content was well pronounced only after 24 hr. Leaf disks treated from 12.5 to 37.5% showed more or less similar amount of pigment loss (from 1.5 to 1 mg g^{-1} fresh wt), at the 30th hr. At this period 50% CF reduced the chlorophyll level upto 0.75 mg (50%). Changes in dry weight increase, chlorophyll content of the leaf disks, ~~changes in~~ pH of the culture medium and electrolyte leachate of the bathing medium, were compared.

The leaf disks were inoculated with different concentrations of CF and incubated for 24 hr. The chlorophyll content, decrease in growth of the disks and pH change of the medium were recorded (Fig.4-5). To measure the electrolyte leakage, leaf disks were kept in distilled water for 6 hr. on a gyratory shaker and the specific conductivity of the bathing solution was measured.

The chlorophyll content remained unaltered at 12.5% level of CF, but a further increase in CF brought about a gradual decline in the total chlorophyll content. The pH value of the control medium showed a slight drop from the initial pH,

Fig. 4-4 Effect of CF levels on chlorophyll-contents of tobacco leaf disks at different hours of incubation.

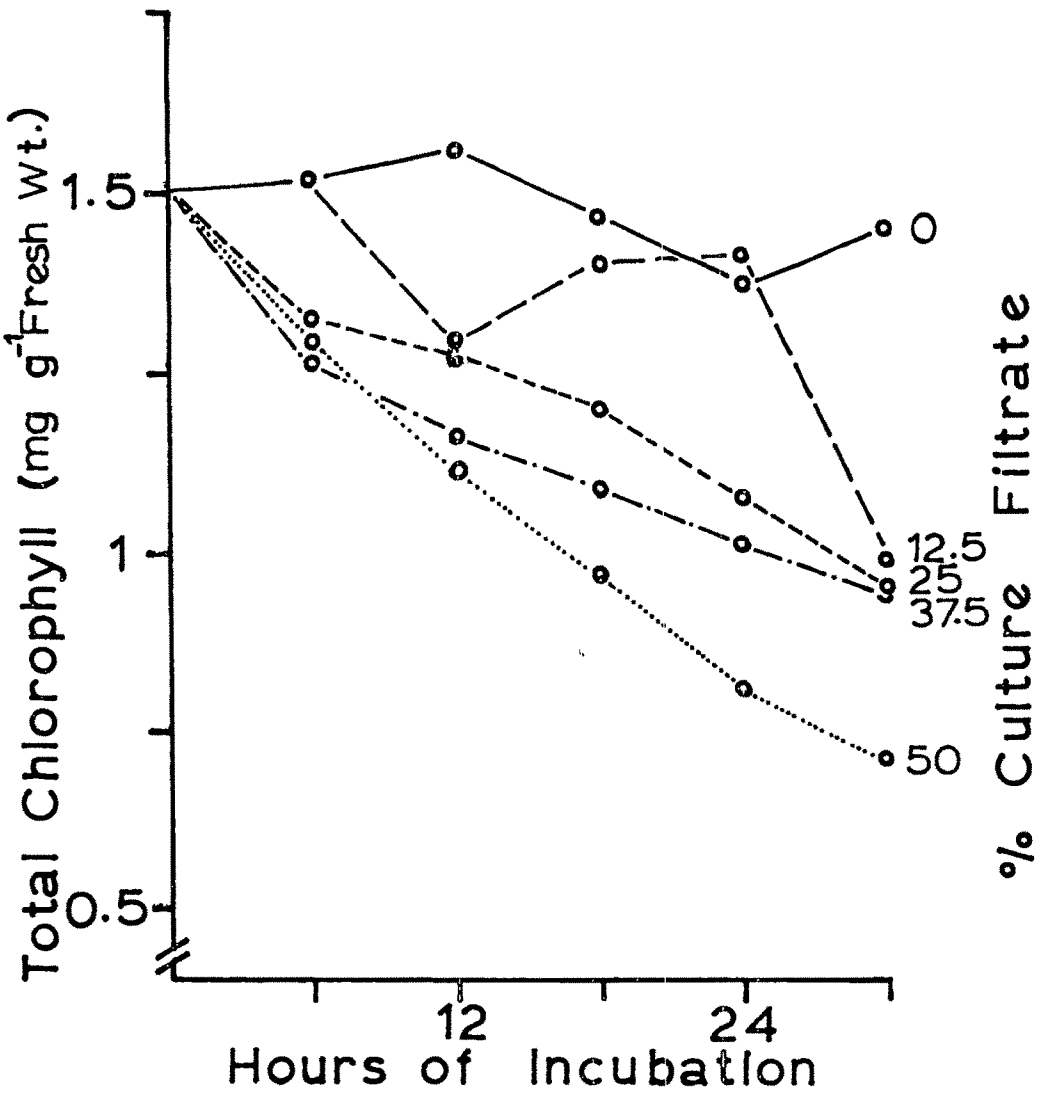

Total Chlorophyll (mg g⁻¹Fresh Wt.)
1.5
1
0.5
12
24
Hours of Incubation
0
12.5
25
37.5
50
% Culture Filtrate

Fig. 4-5 Effect of CF levels on dry weight of leaf disks, chlorophyll pigments, pH change of the culture medium and the electrolyte leachete in the bathing solution (after 24 hr of incubation).

Dry Weight of Leaf Disk (mg)
0
5
10
15
20
Total Chlorophyll (mg g⁻¹Fresh Wt.)
1.5
1
0.5
% Culture Filtrate
0
12.5
25
37.5
50
pH of Medium
7
6.6
6.2
5.8
5.4
Sp. Conductance (μ mhos)
40
30
20
10
0

however, with addition of CF to the medium, pH value also increased. There was a parallel increase in the specific conductivity of the culture medium, indicating a gradual increase in leakage of electrolytes. The CF has not very significant effect on dry weight accumulation of leaf disk upto 37.5%.

4-E.2 Toxins isolated :

The preceding section indicated that some toxic compound(s) is present in the fungal CF that has a growth inhibitory property. A preliminary experiment was done to reveal the size, stability and solubility of toxic principle of the CF. The crude CF was partitioned against ethyl acetate. When the ethyl acetate soluble fractions as well as the water soluble fractions were individually tested on the tobacco plant, we found that aqueous phase was highly wilt inducing indicating the water soluble property of the toxic principle. When the water soluble fraction was autoclaved, the inhibitory property was not lost. However, when the aqueous phase was dialysed against water it lost its growth inhibitory property (Fig.4-6).

4-E.3 FA on wilting :

'Sigma' grade FA was tested for wilting on bidi tobacco. Various dilutions between 0.01 to 10 mM were prepared with sterile tap water and seedlings were inoculated with those solutions. After 5 days of incubation noticeable toxic effect

Fig.4-6 Wilt inducing activity of different fractions of CF on tobacco plants :

FCF = filter sterilized CF

ACF = autoclaved CF

DFCF = dialyzed phase of FCF

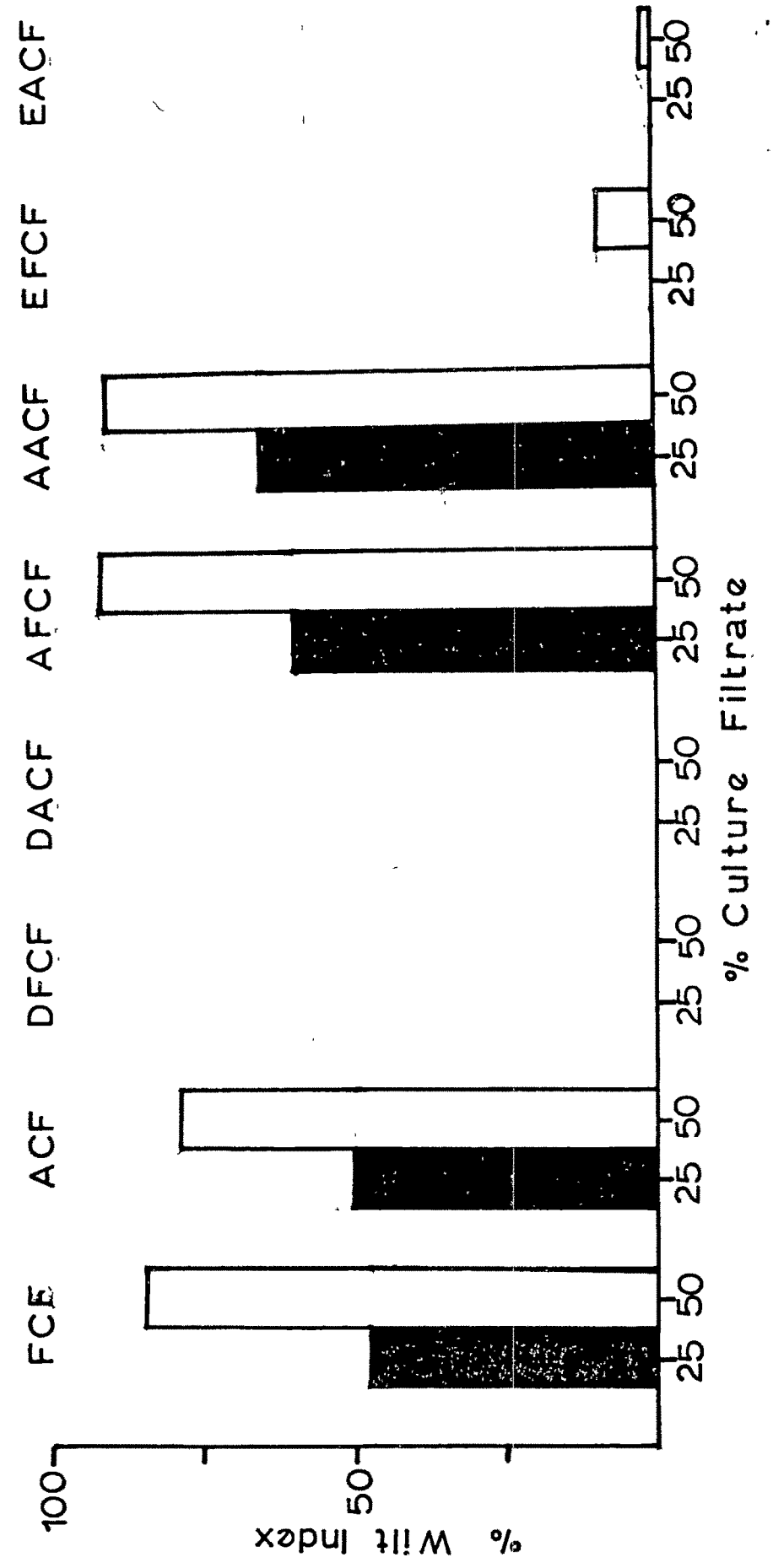
DACF = dialyzed phase of ACF

AFCF = aqueous phase of FCF

AACF = aqueous phase of ACF

EFCF = ethyl acetate phase of FCF

EACF = ethyl acetate phase of ACF


% Wilt Index
100
50
FCE
ACF
DFCF
DACF
AFCF
AACF
EFCF
EACF
25 50
% Culture Filtrate

was found only above 1 mM of FA, and at 100 mM FA, wilt index was 100% (Fig.4-7). However, in all these plants, the leaves showed only vein clearing and wilting. Unlike the effect of CF or the aqueous phase of CF, it did not show any necrotic or chlorotic lesions.

4-E.4 Gel-filtration of crude aqueous phase:

Attempts were also made to purify the phytotoxin(s) present in the aqueous phase of CF. Four ml of 5X concentrated aqueous phase of CF was chromatographed on a Sephadex G100 (1.8 X 18 cm) bed and the column was eluted with distilled water. Fractions of 3 ml were collected. Individual fractions were tested for the presence of phytotoxin in the leaf-disk-assay described in section 4-C.1. Toxicity was calculated in terms of per cent decrease of chlorophyll pigments. Maximum toxicity (100%) was present in the 11th fraction (Fig.4-8). The fractions 10 and 12 showed 80 and 56% toxicity respectively. Fractions 11 to 14 were scanned in the UV-visible range (190 to 700 nm) using Schimadzu spectrophotometer. Two absorption peaks were visible in the UV region of spectrum at 220 nm and 296 nm (Fig.4-9). Only the peak obtained at A296 was showing phytotoxicity. Further fractions 11 to 14, that showed decrease in their toxicity, were positively correlated with the decrease in the absorption at 296 nm. Pooled fractions from 11 to 14 were rechromatographed on Sephadex G25 to increase the resolution. A column (1.8 X 23 cm) of smaller bead sized Sephadex (G25) was prepared. The sample

Fig. 4-7 Fusaric acid and wilt induction on tobacco

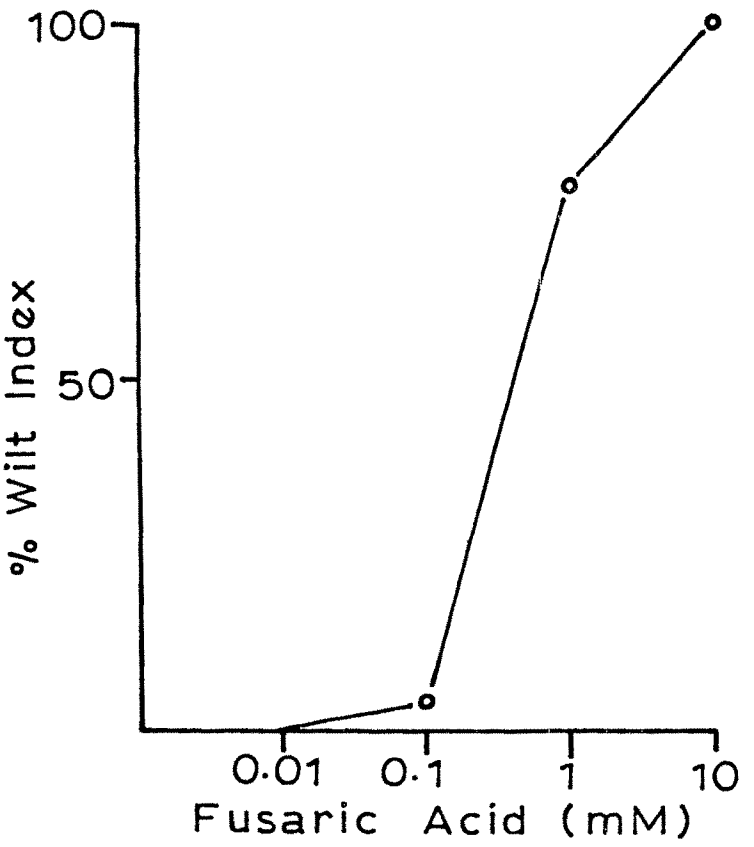

100
50
% Wilt Index
0·01
0·1
1
10
Fusaric Acid (mM)

Fig. 4-8 Fractions obtained from Sephadex G 100 by passing concentrated aqueous CF - absorption and bioassay (chlorophyll estimation).

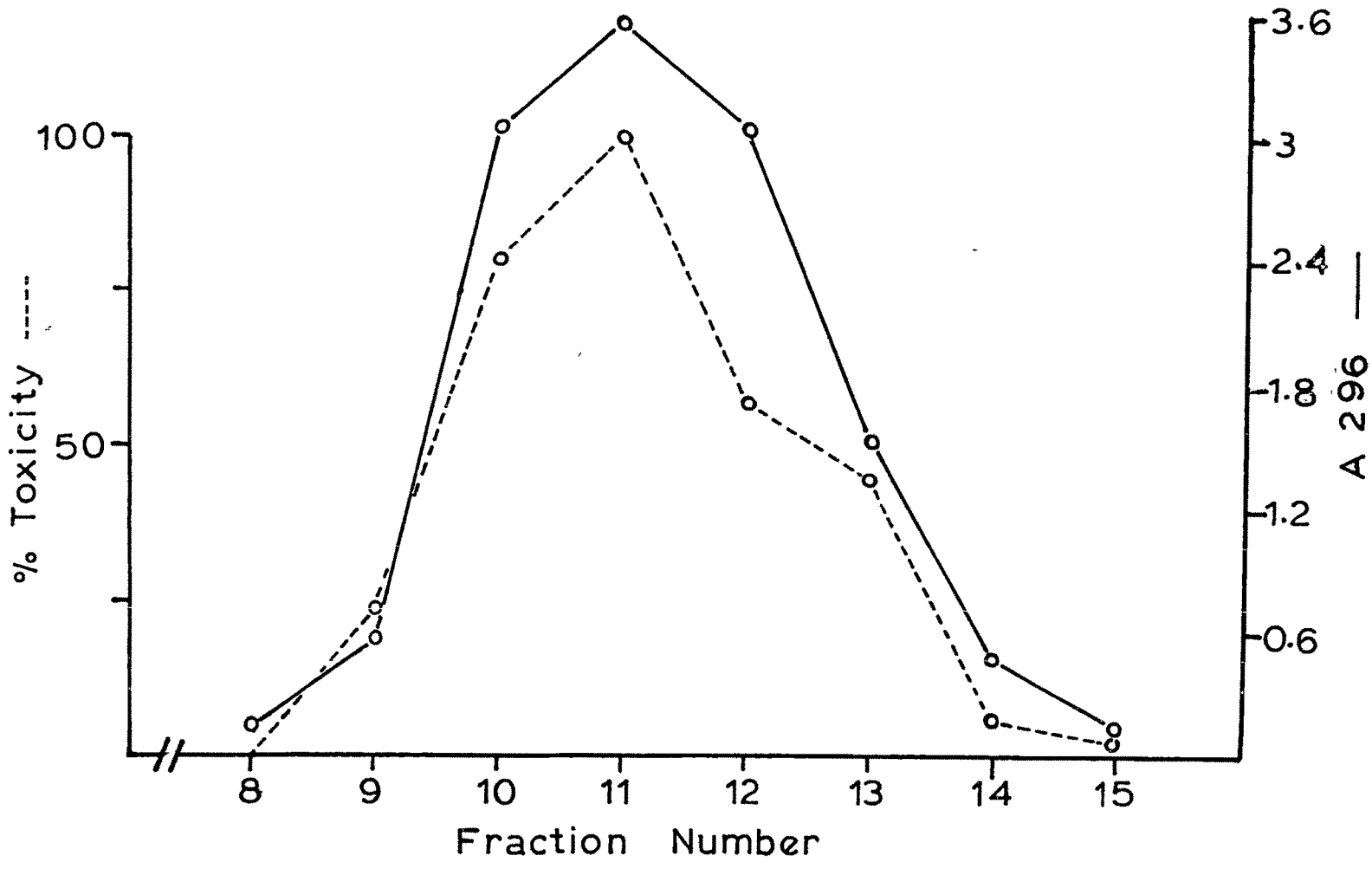

100
50
% Toxicity ----
3.6
3
2.4
1.8
1.2
0.6
A 296 —
8
9
10
11
12
13
14
15
Fraction Number

Fig. 4-9 Spectrophotometric scanning for absorption of the column eluted culture fractions (Nos. 11 to 14).

1522-01
320.0
100.0
896
14
13
12
11
DATE 09-86
2.000
SAMPLE 11 to 14
4
4

volume was reduced to 1 ml and 3 ml fractions were collected. Fractions were tested for absorbance at 296°A and toxicity. The Presence of 3 different substances was indicated by absorption (Fig. 4-10) peaks of fractions 7 and 8, 10 and 11 and 14 and 15. When tested in the leaf disk bioassay, they showed 81, 86 and 80% toxicity respectively as indicated by chlorophyll degradation (Table 4-2).

4-E.5 Cultural parameters affecting toxin production by F. oxysporum:

The CF collected from F. oxysporum grown on MR medium supplemented with various concentrations of sucrose was filtered and filter sterilized. The aqueous phase obtained after fractionation of CF with ethyl acetate was concentrated and gel filtration was carried out through Sephadex G25. The fractions were tested for absorbance (Fig.4-11) as well as toxicity on seedlings (Wilt index: Fig.4-12). While culturing the fungus on media containing different levels of sucrose, the characteristics of the fungal culture and sporulation types were also observed and recorded in Table 4-3. Fresh weight of the mycelium increased with an increase in concentration of sucrose. Aerial mycelium was also found maximum at the highest level of sucrose (4.5%). The white colour of the mycelium gradually changed to pinkish with an increase in sugar level. There was a change in colour of CF too. It was colourless at the lowest level of sucrose and gradually intensified in

Fig. 4-10 UV absorption of fractions obtained from Sephadex G25 by passing concentrated aqueous CF.

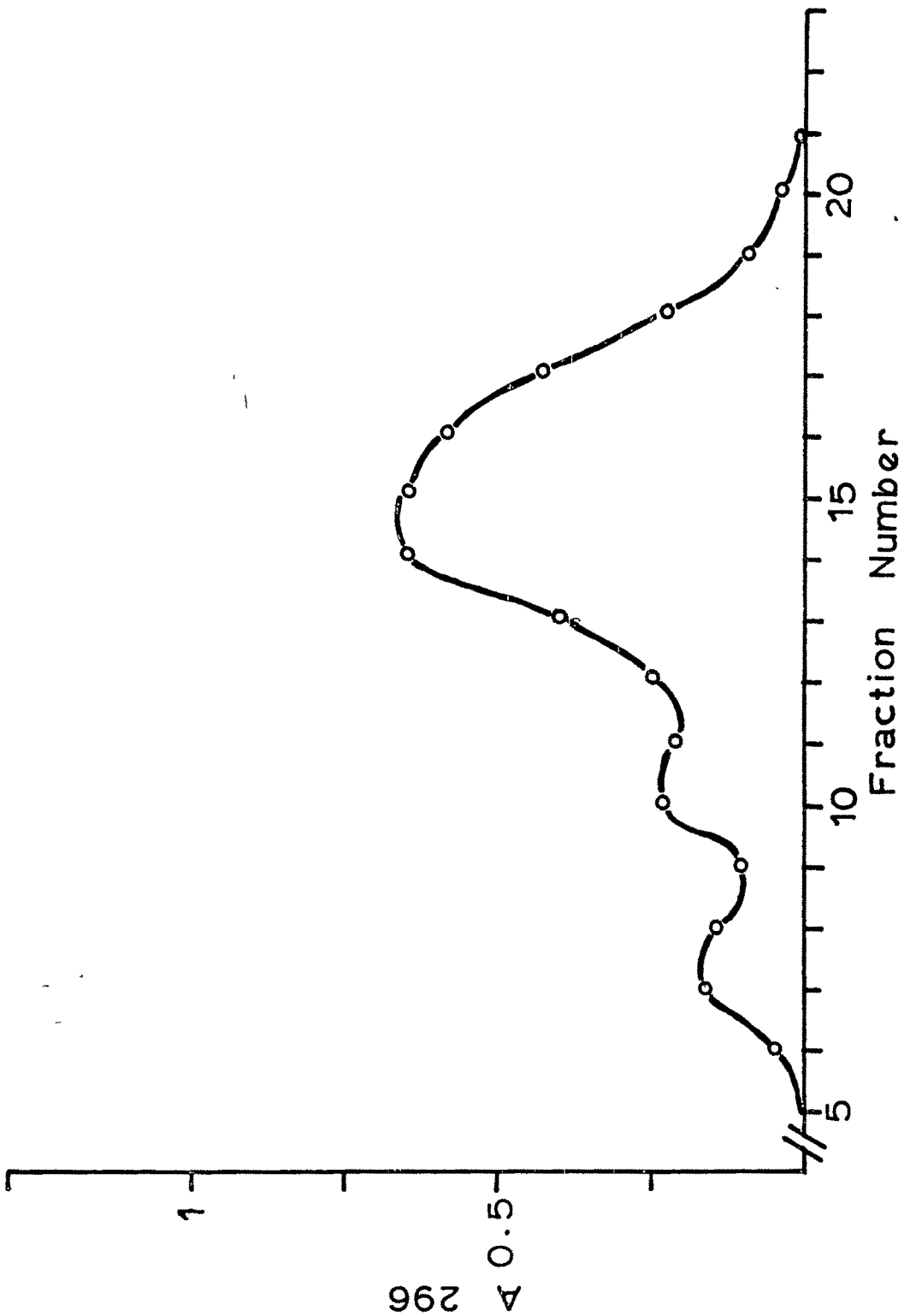

A 296
0.5
1
5
10
15
20
Fraction Number

Table 4-2 : Fractions obtained from Sephadex G25, their absorption and toxicity

Fraction Nos.	*Absorption at 296 nm	% Toxicity
7 & 8	0.15	81
10 & 11	0.22	86
14 & 15	0.65	80

* Mean of 3 replicates

Fig. 4-11 Effect of sucrose conc. in culture medium on the synthesis of fungal toxin(s) (data represents the UV absorptions of fractions (No.14 & 15) obtained after gel filtration).

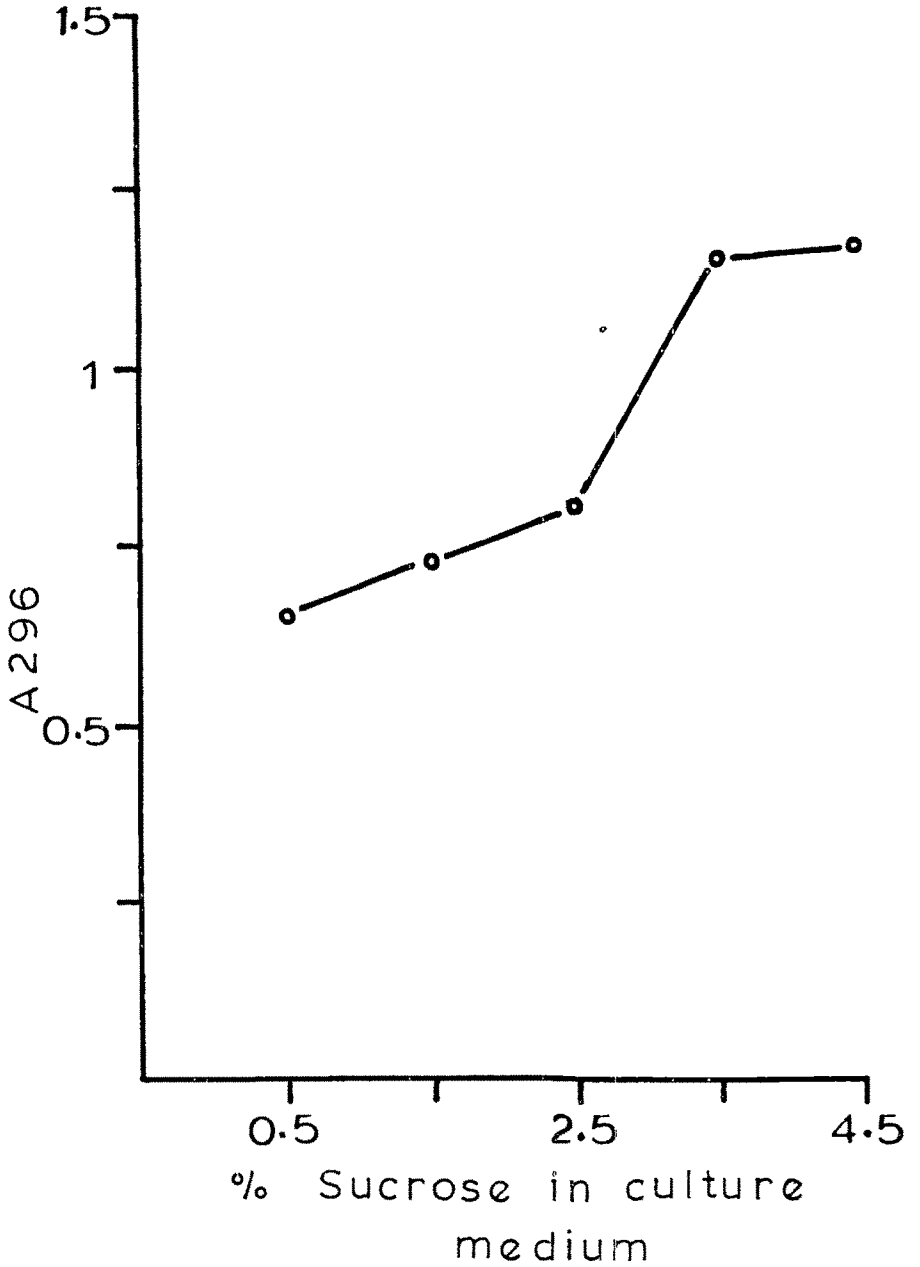

1.5
1
0.5
A 296
0.5
2.5
4.5
% Sucrose in culture medium

Fig. 4-12 CF from various sucrose Conc. media and its wilt induction on tobacco plants:

1 = 0.5% sucrose

2 = 1.5% "

3 = 2.5% "

4 = 3.5% "

5 = 4.5% "

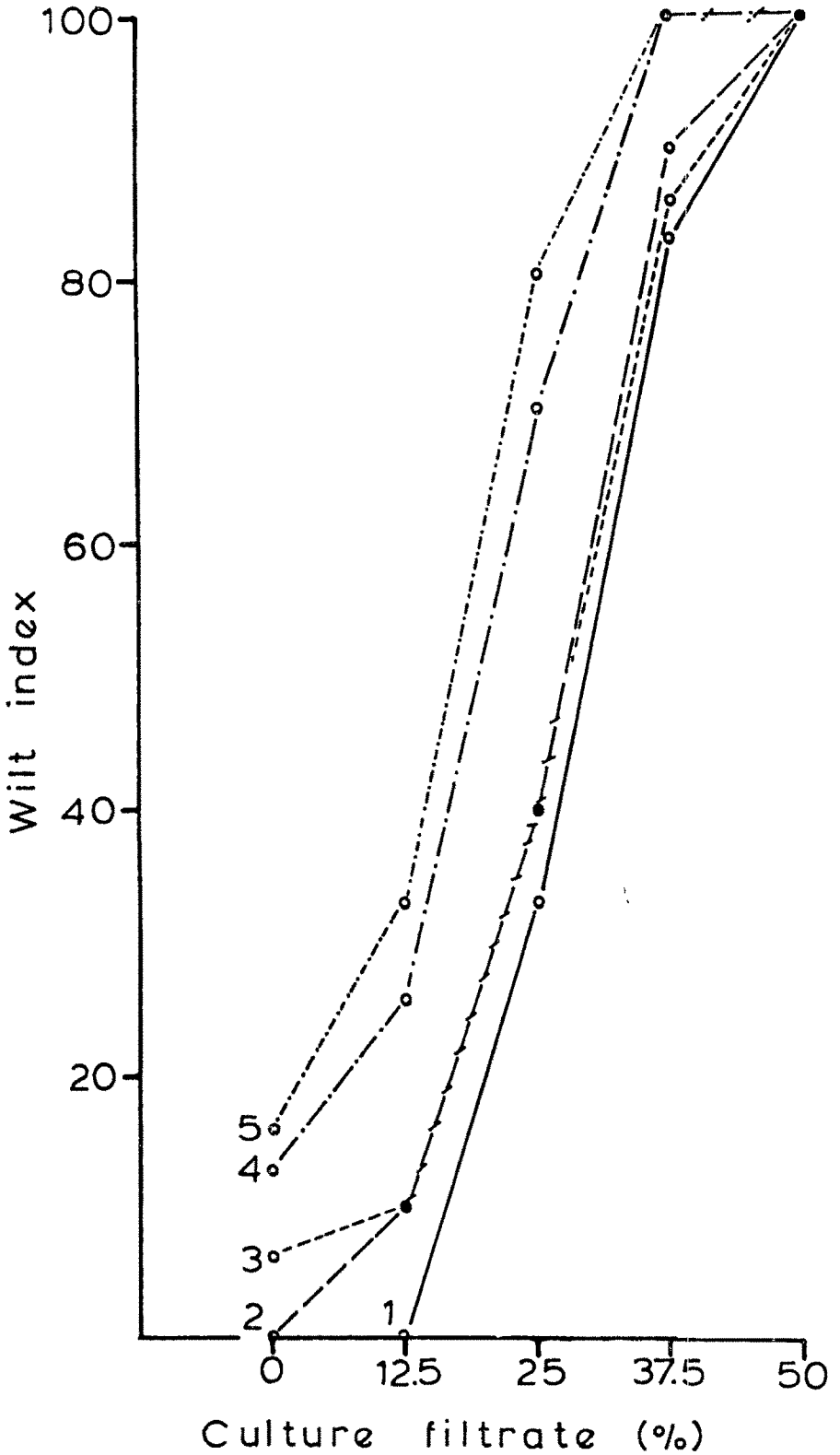

100
80
60
40
20
Wilt index
5
4
3
2
1
0
12.5
25
37.5
50
Culture filtrate (%)

Table 4-3 : Morphological characters of Fusarium culture on various sucrose level media.

S.No.	Sucrose conc. (%)	Fresh weight of culture (mg)	Aerial mycelium	Pigmentation		Sporulation *		
				Mycelium	Medium	A	B	C
1	0.5	60	+++	white	colourless	+++++	++	++++
2	1.5	73	+++	"	pale vinanceous	++++	+	++
3	2.5	85	++++	dirty white	light brown	++	++	++
4	3.5	85	+++++	white in pinkish tinge	deep purplish	++	++	+
5	4.5	86	+++++	"	"	+	+++	+

+++++	Very good	*	A	Macroconidiospores
++++	good		B	Microconidiospores
+++	moderate		C	chlamydospores
++	fair			
+	scanty			

colour from blue to deep purple. Macro-conidiospores and chlamydospores were maximum at the lowest level of the sucrose concentration and their occurrence decreased as the sucrose concentration increased. The situation with microconidiospores was more or less the reverse.

The wilt index (Fig.4-11) also increased gradually when the level of sucrose increased to higher concentration. At 0.5% sucrose level, complete wilting of the plants was observed at 50%.CF. A similar level of wilting was observed with only 37.5% CF from media containing either 3.5 or 4.5% sucrose. The level of toxicity of the CF thus increased with an increase in amount of carbon source in MR medium.

The absorption peaks of various fractions of CF from different concentrations of sucrose also increased at 296 nm as shown in Fig.4-12. This would indicate, that increasing the level of carbon source increases the level of phytotoxin/s. Moreover 2 new substances were also resolved from CF of higher sucrose (3.5%) media.

4-E.6 Purification of phytotoxin and its effect on wilting:

The fractions (14 and 15) that showed maximum absorption at 296 nm were oven dried (at 60°C for 12 hr) and the white powder obtained was collected. The average concentration of the substance present in the fractions 14 and 15 was 6,650 and 6,225 ppm respectively. This was diluted to different

levels with sterile tap water in the range of 0, 0.01, 0.1 ..... 1000 ppm. In these solutions pH was adjusted to 5.8. Young tobacco plants were inoculated with these solutions as mentioned earlier and the wilt index was calculated.

In another study, leaf disks were inoculated on agar based MS (MS + 0.1 µM IAA and 2 µM Kn+ 2% sucrose) media containing various levels of the purified phytotoxin as mentioned above. After 10 days of inoculation the fresh weight change of the leaf disk was calculated and recorded in Fig.4-13.

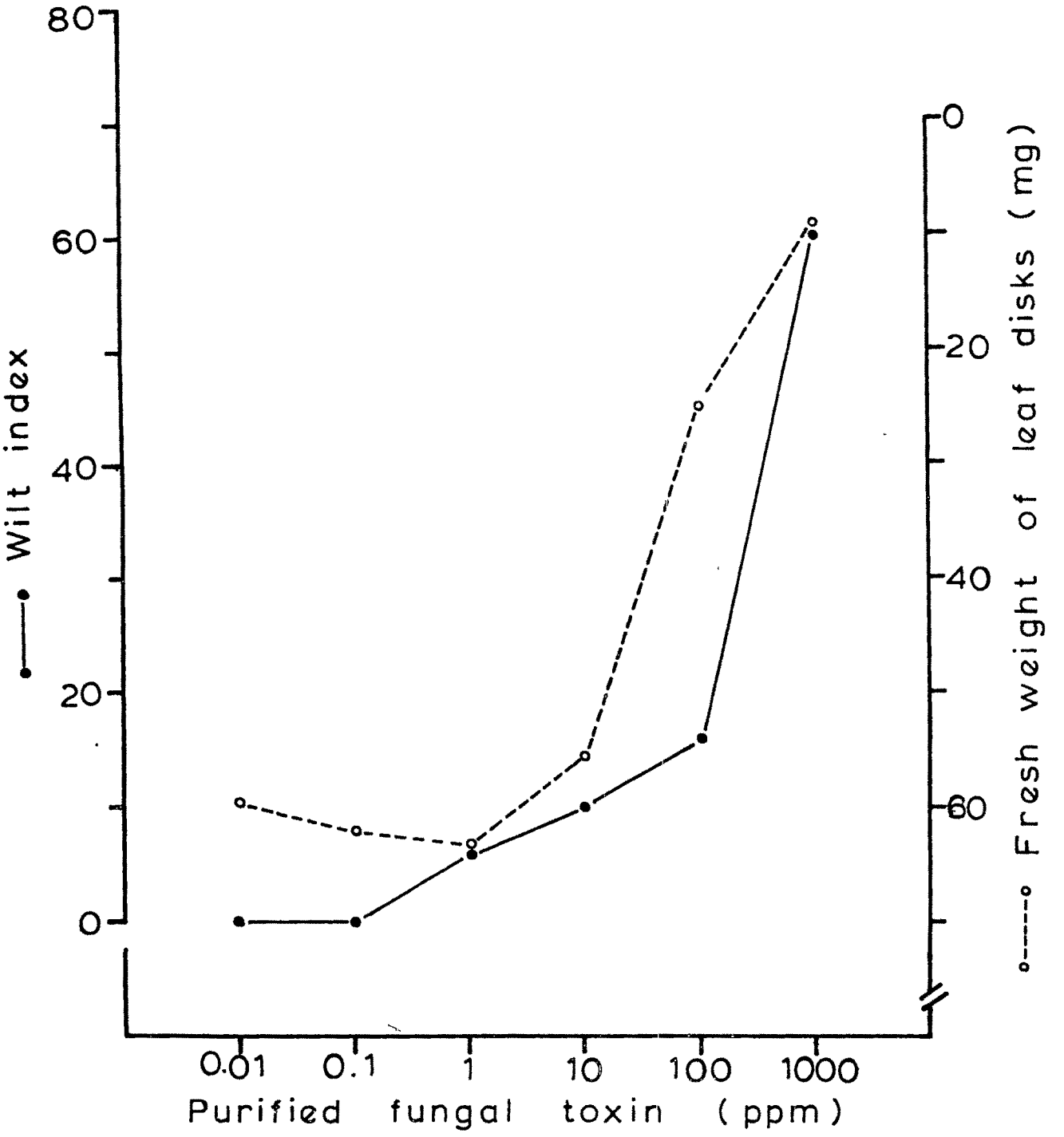
No wilt symptoms occurred on the plants at 0, 0.01 and 0.1 ppm of the fungal toxin. At 1, 10 and 100 ppm. the wilt index was 6.7, 10 and 16.7% respectively. The symptoms were found most prominent at 1000 ppm of the phytotoxin. It caused all the wilt symptoms including, vein-clearing, chlorosis and necrosis. These results were also found significantly correlated with the fresh weight decrease of the leaf disks.

4-E.7 Further characterization of the purified toxin :

i) Melting point : The melting point of the compound ranged 108°C as measured with the Gallenkamp Micro [illegible] Melting Point, MF359, England.

ii) Ninhydrin reaction : A 2 ml of 1000 ppm solution of the purified compound prepared in distilled water was mixed with 2 ml of buffered ninhydrin reagent and heated in a boiling water bath for 15 min. While heating, the solution turned

Fig.4-13 Effect of purified Fusarium toxin on the growth of excised leaf disks and seedlings of tobacco.


80
60
40
20
0
Wilt index
0
20
40
60
Fresh weight of leaf disks (mg)
0.01
0.1
1
10
100
1000
Purified fungal toxin (ppm)

purple in colour, indicating that the compound was ninhydrin positive.

iii) Stability : When the compound was left for 3-4 weeks after purification and then tested for its toxicity, it did not produce show any disease symptoms on whole plants as it did with freshly purified compound (mentioned in previous section). The freshly purified dry powder was very hygroscopic in nature and was able to bring about wilting of tobacco plants. When this hygroscopic compound, was redried in oven and later left for aeration, it lost its hygroscopic character. When 1000 ppm of this compound was inoculated to the tobacco plants, no wilt symptoms were observed.

iv) Solubility : This compound was not soluble in any of the polar and non-polar organic solvents, i.e., chloroform, ethyl acetate, dimethyl sulfoxide, methanol, carbon tetrachloride, or petroleum ether.

4-F DISCUSSION

The dose response curve of CF on the growth of whole plants, leaf disks, and cells of suspension culture were fairly identical. Addition of small amount of CF (10-20%) to culture/ incubation medium stimulated growth, and at higher concentrations, it showed growth inhibition. However, the CF inhibited plantlet formation from cultured anthers at a much lower concentration. Thus, the inhibitory action of CF is more or less similar to various types of differentiated tissues.

Many of the plant pathogens grown in vitro secrete their toxic product (=toxin) in the culture media. In cases where the nature of the toxin secreted by a pathogen is not known, it is customary to use the crude CF to select the resistant plant cell clones or callus. In such experiments caution should be exercised in interpreting results because the CF may contain some residual elements of the original fungus culture medium which may be inhibitory for growth of plant cells. Thus the observed inhibitory effect of CF may not always be only 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 growth. We have mentioned proper controls as mentioned in materials and methods (4-C.1). Therefore, the observed inhibitory effect of 50% CF of F. orysporum is definitely due to toxin secreted by F. oxysporum in the medium. These type of controls were not used by any previous worker selecting for disease resistance using in vitro cultures (Behnke, 1980b; Hartman, 1984b, 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 (Gäumann 1958; 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 Satyer, 1971); alterations in photosynthetic rate (Steele et al., 1976, 1978) increase in rate of respiration (Collins and Scheffer, 1958).

Experiments conducted in the present study too gave similar results when CF of F. oxysporum was tested on the leaf disks of N. tabacum. The damage occurred to the photosynthetic pigments of leaf disks was correlated with a decrease in the growth of leaf disks, pH change of the culture medium and the 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 tissues inoculated was confirmed above 37.5% of CF.

Selman and Pegg (1957) showed that dry weights of leaf, stem and root of 8 weeks old tomato after inoculation with Verticillium albo-atrum, decreased by 72, 70 and 65% respectively. But stunting of the whole plant, often in the absence of wilting, was a major symptom of the disease. Similarly Barash et al. (1981) reported that Phoma tracheiphila, the causal organism of mal-secco disease in citrus secretes a low molecular weight phytotoxin during growth on a synthetic medium. The toxin is capable of inciting interveinal chlorosis and necrosis in lemon leaves. It also induces electrolyte leakage from lemon leaves and carrot disks and causes uncoupling of electron transport in lettuce chloroplasts.

Using standard microelectrode technique, D'Alton and Etherton (1984) measured the effects of fusaric acid (FA) on the membrane potential of tomato incipient root hair cells. They found that FA has both direct and indirect effects on the plasma membrane. The direct effect is indicated by a rapid, FA-induced depolarization, possibly due to change in membrane permeability. The indirect effects are (a) a transient hyperpolarization, possibly reflecting a FA-mediated influx of protons followed by their electrogenic extrusion and (b) a slow depolarization, possibly caused by reduced ATP levels inhibiting electrogenic extrusion of H^+.

Various phytotoxins have been identified from F. oxysporum CF with respect to different host. Results presented in this study indicate that in addition to FA, F. oxysporum f. sp. nicotianae secretes in culture a low molecular weight phytotoxin which is capable of inducing chlorosis, necrosis and wilting in tobacco plants.

Using different fractions of CF to treat whole plants of tobacco, it was found that the toxin is a heat stable dialisable (hence, low molecular weight) and water soluble compound(s). It was also found that this compound alone can cause all the wilt symptoms: necrosis, chlorosis and wilting of individual leaves in the beginning and entire plant later.

Fusaric acid (5-n-butyl pyridine-2-carboxylic acid) was detected in the ethyl acetate extract of CF and by chromatography and bioassay on the tobacco plants. Tobacco plants when incubated in standard 'Sigma' grade FA solution at 1 mM or above show signs of wilting and the plants were completely wilted at 10 mM of FA. When the ethyl acetate fraction of filter sterilised CF was tested for wilt on tobacco plants, it showed only 9% wilt index. Comparing those results, the concentration of FA present in the CF could be deduced as 0.11 mM.

There is also report by Pegg (1981) that FA solution adjusted to pH 8.7 did not show any reaction on the host since it was denatured in alkaline condition. Literature (Gäumann, 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 (Gäumann, 1957). Moreover its action is not specific to plants which serve as hosts of various Fusarium spp. (Gäumann et al., 1952). Gäumann (1957) further pointed out that FA never acts alone on the host but always in association with other products of fungal and host metabolism. The effect of absorbed FA on the 7 flax varieties demonstrated that the wilt resistant varieties were not more FA-resistant then were the wilt-susceptible varieties.

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.

In contrast, our present work indicates that in comparison to FA, much more potent toxin is/are present in the aqueous phase. Such water soluble toxins so far has not been reported in case of F. oxysporum f.sp. micotianae. Therefore, we consider them as novel compounds with high toxicity, capable of eliciting all disease symptoms that the fungus is capable to do. These novel compounds have UV absorption at 296 nm. Further their synthesis in the culture medium can be enhanced by increasing carbon source of the culture medium. We propose that they are involved in the development of disease symptoms by F. oxysporum since the regenerated plants which are resistant to these toxins are also resistant to pathogenic attack.

The aqueous phase obtained after the separation of CF with the ethyl acetate, was further purified in the chromatography of Sephadex G25 column. More than three substances with different molucular weights were separated out in different times through the column. All the substances were absorbed maximum at A296 and were equally responsible for causing wilt symptoms. The secretions of these phytotoxins in the CF was enhanced when the carbon source (sucrose) of the medium was increased upto 3.5%.