# CHAPTER 3

# PATHOLOGICAL STUDIES

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# 3

## PATHOLOGICAL STUDIES

### 3-A INTRODUCTION

India is third in world tobacco production, the first and second being China and United States respectively (Anonymous, 1983a). The cultivated area under tobacco in India is around 4 to 4.5 hundred thousand hectares producing annually about 40/50 million kilograms of cured tobacco. The crop generates an annual excise revenue of about Rs.8.50 millions from national market and 120 millions from international market and ranks second among excisable commodities next to mineral oils (Anonymous, 1984). Among the tobacco growing states of India viz., Gujarat, Andhra Pradesh, Karnataka, Tamil Nadu, West Bengal, Orissa, Bihar, Uttar Pradesh and Maharashtra, Gujarat ranks first from the productivity view point (Anonymous, 1983b).

In Gujarat state bidi tobacco (N. tabacum) is cultivated in about 99,400 ha. It suffers from several biotic and abiotic factors which affect the successful cultivation of tobacco both quantitatively as well as qualitatively. The principle diseases attacking young plants of bidi tobacco are damping-off (Pythium aphanidermatum (Edson) Fitzpatrick.), Fusarium wilt (Fusarium oxysporum (Schlecht) f. sp. nicotianae J. Johnson.), black shank (Phytophthora parasitica f. sp. nicotianae (Breda de Haan) Tucker.), brown spot (Alternaria alternata (Fries) Keissler.), anthracnose (Colletotrichum dematium (Pers. ex Fr.) Grove), leaf curl mosaic and root-knot nematodes (Meloidogyne incognita (Trenb) Chitwood and M. javanica (Treub) Chitwood.) (Patel, 1960; Lucas, 1965, 1975; Akehurst, 1981). Among these, the most disastrous disease of bidi tobacco is 'chitri' disease (Prasad et al. 1957). Due to considerable loss caused by this disease, it has become a menace to tobacco cultivation in this area.

Chitri a complex of wilt and root-rot (Fig.3-1) is a local name given to the disease in which groups of plants are affected as patches which extend irregularly but slowly. Usually the disease appears in Oct. and Nov. months. It has been observed that the disease normally reappears in the same portion of the field year after year. However, epidemic occurrences are rare. It is considered that the disease complex involves F. oxysporum f. sp. nicotianae, and

Fig.3-1 Fusarium wilt :

A) Yellowing and wilting of leaves on one side of the plant.

B) Wilting of entire plant (right).


**Fusarium wilt**

F. Solani f. sp. nicotianae (Prasad and Patel, 1952). However, wilting of the plant is due to F. oxysporum (Akehurst, 1981).

The experiments described in the present chapter were designed to study the pathological features of causal organism F. oxysporum f. sp. nicotianae . Cellular selection for disease resistance was attempted. Six varieties of bidi tobacco commonly cultivated in Gujarat were used to select out a variety which is susceptible to the wilt fungus.

In order to select wilt resistant tobacco plants the culture filtrate (CF) of the fungus must be used as the selective agent. Uniform infection of plant cells in vitro by the pathogen is very difficult to achieve. Even if the infection could be achieved, the growth of selected resistant plant cells might be retarded or prevented as a consequence of the growing fungus depleting the nutrients from the culture medium. The in vitro strategy of cellular selection might be useful if the pathogen secrets a specific toxin or the crude CF of the pathogen could mimic most if not all, the disease symptoms. Several workers have examined the effect of crude CF on plants and results are inconsistent (Nene and Kannaiyan, 1982). Therefore, we examined in detail the effect of CF on different varieties of tobacco. Information gained in this experiment became the basis of our cellular selection approach discussed in chapter 5.

## 3-B MATERIALS AND METHODS

### 3-B.1 Collection of infected tobacco material and isolation of the pathogen :

In order to study pathogenicity of the causal organism, isolation of the fungus was done from the diseased portions of tobacco. Infected leaves and stem portions of Nicotiana tabacum var. Anand 2 were collected from a cultivated plot around Baroda. The infected tissue was cut into small bits and surface sterilized in 1:1000 mercuric chloride solution for 2 minutes. Later it was rinsed with sterile distilled water in a series of three to four washes. One or two bits were transferred onto potato-sucrose-agar (PSA) slants and incubated at room temperature.

### 3-B.2 Moist chamber incubation :

The infected stem and root portions also were incubated in a moist chamber (modified to 'Belljar incubation of entire infected plants' of Kamat, 1953). Petriplates (15 cm diameter) with 2 filter papers and a Z-shaped 1/2 cm diameter glass rod in each plate were steam sterilized. They served as moist chambers. The plant material was surface cleaned with 95% ethanol and washed in 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.

To get pure cultures of the pathogen, mycelial tips from one week old fungal culture (emerged from the host explants) were taken and inoculated on fresh PSA slants. The pure culture was maintained by subsequent transfer on PSA slants. Suspension culture was also prepared by inoculating the fungus in modified Richard's solution (MR).

3-B.3 Morphological studies on pathogen :

To confirm the exact identity of the pathogen, morphological studies of the mycelium and conidiospores were done by measuring the length and breadth of the spores under high power magnification using ocular micrometer. Camera lucida drawings of the fungal mycelium and spores were also prepared.

3-B.4 Preparation of fungal inoculum for liquid culture :

The fungal mat was collected by filtering 10 day old suspension culture through Whatman filter paper. The mat was washed three times with sterile water. It was then weighed and homogenized in a known volume of sterile distilled water in a blender. Finally the homogenate was diluted with sterile distilled water to make the required per cent solution (w/v) for inoculation.

3-B.5 Pathological studies (Fig.3-2) :

Seeds of 6 varieties of N. tabacum, viz. Anand 2 (A2), Anand 119 (A119), GT4, 3430, Anand 3 (A3) and Anand 23 (A23) used in the present investigation were obtained from Bidi Tobacco Research Station, Gujarat Agricultural University, Anand. They were germinated in garden soil in the greenhouse. One month old seedlings were removed aseptically from the soil, surface sterilized briefly (1 minute with 95% ethanol and then with sterile tap water).

Screening of these seedlings to evaluate their relative tolerance to F. oxysporum was accomplished by 2 techniques: 'sick soil' technique of Kamat (1953) and 'liquid culture' technique of Roberts and Kraft (1971) with slight modifications.

i) Sick soil method : To prepare the fungus for inoculation by sick soil method, it was grown on Richard's solution (Hendrix & Nielson, 1958) in 250 ml Erlenmeyer flasks. After 10 days the culture was centrifuged for 10 minutes and the supernatant discarded. Fungal concentrate weighing 10% was mixed with 90% of the steam sterilized composit soil (garden soil:sand:farm yard manure, 7:2:1). Required amount of stock of sick soil was diluted with the sterile soil to generate various concentrations of sick soil which was transferred into earthernware pots. Surface cleaned seedlings were repotted in the 'wilt sick soil' (after 24 hr of its

# EXPERIMENTAL PROTOCOL 1

**Preparation of host material:**

Nursery raised Tobacco seedlings of var. A2
A119
GT4
3430
A3
A23

**Preparation of Pathogen:**

Isolate of *F. oxysporum* grown in modified Richard's Solution for 10 days at 24°C.

↓

Removal of fungal mat

↓

Preparation of fungal mat/CF in various concentrations in sterile water

↓

Inoculation of host with pathogen/CF

↓

Incubation of exp. material at 25°C at 85% R.H.

↓

Rating of following disease Symptoms

1. Decrease in formation of new roots
2. Yellowing of leaves
3. Bad odour & softening of roots
4. Wilting of entire plants
5. Anatomical changes

preparation). The incubation period of plant commenced on the day of inoculation. Inoculated pots were incubated in the glasshouse with alternative day light and dark at 24±2°C.

ii) Liquid culture method : Various concentrations of fungal suspension prepared as described in section A-4 was added to 250 ml volume sterile Erlenmeyer flasks. Surface sterilized tobacco plants of different varieties were positioned in these flasks in such a way that their roots remained fully immersed in the fungal suspension (Fig.3-4). Control plants were subjected to sterile distilled water instead of fungal suspension. The neck of the flasks were closed by sterile cotton 'bungs' which provided support to the aerial shoots as well as prevented entry of contaminants in the flasks. The experimental set was incubated at normal room temperature (28±2°C).

Observations on morphological and anatomical changes (Fig.3-2) were carried out at regular intervals. The inhibitory effect of the pathogen on intact plants was expressed as the wilt index (% wilt score). It was calculated according to Ebells (1967). Each plant was given a rating of 0-5 according to per cent leaves wilted as follows.

0 = No wilt symptom

1 = Fewer than 25% leaves wilted

2 = 25-50% leaves wilted

3 = 50-75% leaves wilted

4 = 75-100% of leaves wilted but the apical shoot tip still green bearing turgid leaf primordia

5 = Dead plant

Sum of wilt score of individual plants in each treatment was done to calculate wilt index (% wilt score) as follows:

$$= \frac{\text{Sum of wilt scores of all plant in the treatment}}{5 \times \text{Number of plants per treatment}}$$

3-B.6 Light microscopy :

The lowermost internode (i.e., the internode immediately above the soil level) was excised from the infested plant and was cut into 0.3 cm thick sections for fixation in FAA fluid (6% formalin, 25% acetic acid and 50% ethanol in water) as recommended by Johnston and Booth (1983). After 25 hr in fixative they were dehydrated by passing through alcohol:xylene series and ultimately embedded in paraffin wax (Merck (52-54°C M.P.) or Tissue prep (61°C M.P.)). Paraffin blocks were trimmed and transverse and longitudinal sections were cut at 6-10 µm thickness by AO. Spencer's 820 rotary microtome. The sections thus obtained were stained with 0.05% toluidine blue in 50 mµ sodium phosphate buffer (pH 6.8) for 75 sec and then rinsed in water as suggested by Harling and Taylor (1984). Stained sections were dehydrated through alcohol:xylol series and finally mounted in Euperol or DPX.

3-B.7 Effect of crude culture filtrate of F. oxysporum on Tobacco :

CF from 10 day old culture of F. oxysporum was sterilized by passing through 0.22 µ microbiological filter. It was mixed with sterile water to achieve various strength of per cent solution (v/v). In control, autoclaved Richard's medium was mixed with equal volume of sterile distilled water. Plants of each tobacco variety were incubated in the various concentration of CF in the manner described in section 3-B.5.ii. The following parameters of toxicity were recorded on weekly intervals: i) inhibition of the root formation, ii) yellowing and wilting of lower leaves, iii) vein clearing, iv) softening of the root cortical portions and v) wilting of entire plants.

The dose response curve of CF on variety A2 indicated 30% concentration of CF as that concentration which caused 50% wilt index (=abbreviated as GI50*). All other varieties of tobacco, were screened to only this single GI50 concentration of the CF. The variety which exhibited lowest value of wilt index is considered as the most resistant and the one with greatest value is considered as the most susceptible.

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* GI = Growth inhibition

3-B.8 Test for significance of correlation :

To confirm the mutual expression of similar varietal difference in susceptibility on the host by the fungus as well as its CF, the significance of correlation was calculated. It was calculated between the two variables x and y, where x is the wilt score due to fungal inoculation on 6 varieties of tobacco and y is the wilt score due to CF on the same varieties. The method of calculation followed by Cavalli-S forga and Bodmer (1971, cf. Redei, 1982).

i) Principle and method of calculation : Two variables are said to be correlated, if an increase in the average of the other, or vice-versa. Two variables cannot be considered in light of dependence and independence and still there is a relation of some sort to estimate what is the relation and to what extent the variables vary together of influence on another. The coefficient of correlation 'r' is calculated by the formula:

$$r = \frac{n(\Sigma xy) - \Sigma(x)\,\Sigma(y)}{\sqrt{n(\Sigma x^2)-(\Sigma x)^2} \times \sqrt{n(\Sigma y^2)-(\Sigma y)^2}}$$

Hence, the test of significance is calculated by 't'

$$= r\sqrt{\frac{N-2}{\sqrt{1-r^2}}}$$

The computed 't' value was compared with the tabular 't' value (from Table 3 of Hays, 1981) with $(n_1+n_2-4)$ degree of freedom. The correlation would be highly significant only

if the computed 't' value is greater than the tabular 't' value. The graphical representation of the correlation was also made taking x variables in x axis and y in y axis to see whether the correlation is positive or negative.

## 3-C RESULTS

### 3-C.1 Identification of the Fungal Isolate :

The pathogen was isolated by inoculating the diseased root and stem portions of N. tabacum var. A2 on PSA medium. Fungus appeared on the incubated infected host material in the humidity chamber. Morphological observations were made from 10 days old culture. The camera lucida structures of the spores are presented in Fig. 3-3.

All the morphological characters and descriptions of the isolate were closely in accordance with the descriptions given by Snyder and Hansen (1940), Gilman (1967), Booth (1971) and Bessey (1979). They also agree with the type culture received from Dr. G.J. Patel, Director, Bidi Tobacco Research Station, Gujarat Agricultural University, Anand, India. Hence the isolate has been confirmed as Fusarium oxysporum (Schlecht) f. sp. nicotianae J. Johnson.

### 3-C.2 Confirmation of Koch's Postulates :

To confirm the Koch's postulates i.e., pathogenicity of the isolate (Kamat, 1953) two months old healthy seedlings of

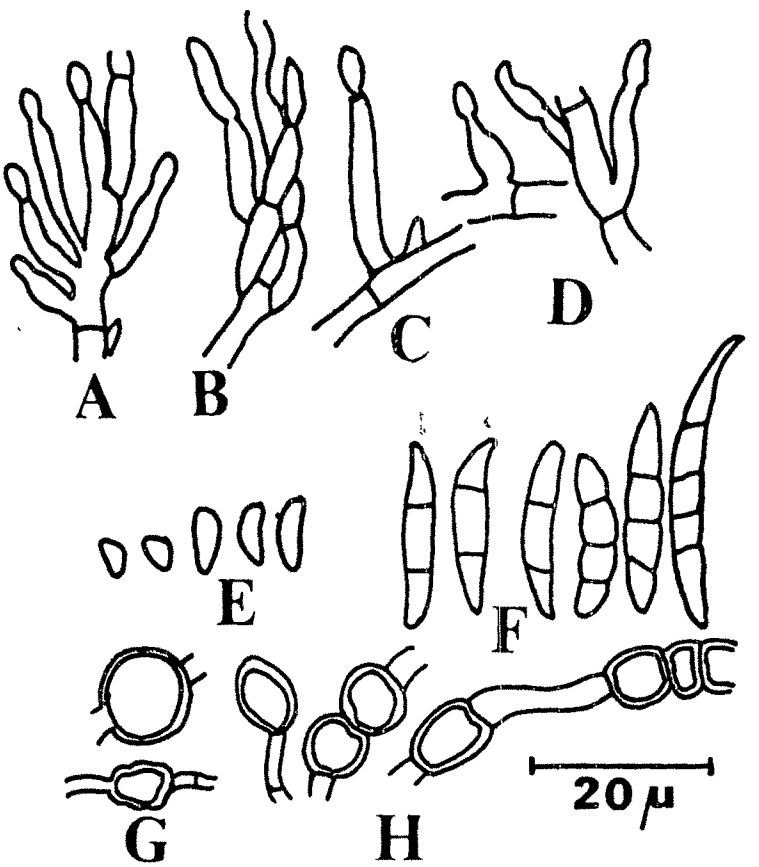
Fig.3-3 Camera-lucid drawings of *Fusarium oxysporum* Schlechtendahl f. sp. *nicotianae* (J. Johnson) Snyder & Hansen :

A - D Fungal hyphae with phialides giving out conidiospores

E microconidiospores

F macroconidiospores

G - H chlamydospores


A
B
C
D
E
F
G
H
20 µ

N. tabacum var. A2 were artificially inoculated with the fungal suspension by sick soil and liquid culture methods as described in materials and methods. In the sick soil study disease symptoms appeared after 30 days of incubation (Fig.3-4). In the liquid culture method the symptoms were visible within 20 days of incubation (Fig.3-4). The disease appeared with characteristic wilt symptoms. Initially vein clearing was mild, and yellowing and wilting was prominent in the lower-most leaves. The cortical region of the roots was macerated and eventually sloughed off (Fig.3-5). In the mean time the entire plant wilted. The decaying roots gave a characteristic rotting smell.

The pathogen was reisolated by the 'moist incubation' method in the petriplate and was found to be identical with the original isolate of F. oxysporum.

3-C.3 Fusarium-inoculum potential :

The influence of inoculum potential of F. oxysporum on N. tabacum var. A2 was studied by varying the concentration of the fungus in the liquid culture method. The fungal concentration varied from 0.04% to 40% w/v. About one month old seedlings were inoculated in each concentrations. The characters observed during incubation were i) formation of new roots, ii) yellowing of the lower leaves, iii) vein clearing, iv) bad odour and softening of the root region,

Fig. 3-4 Tobacco inoculation with *F. oxysporum* :

A 'sick soil' method; left=control; right = treated.

B 'liquid culture' method; left = control; right = treated.

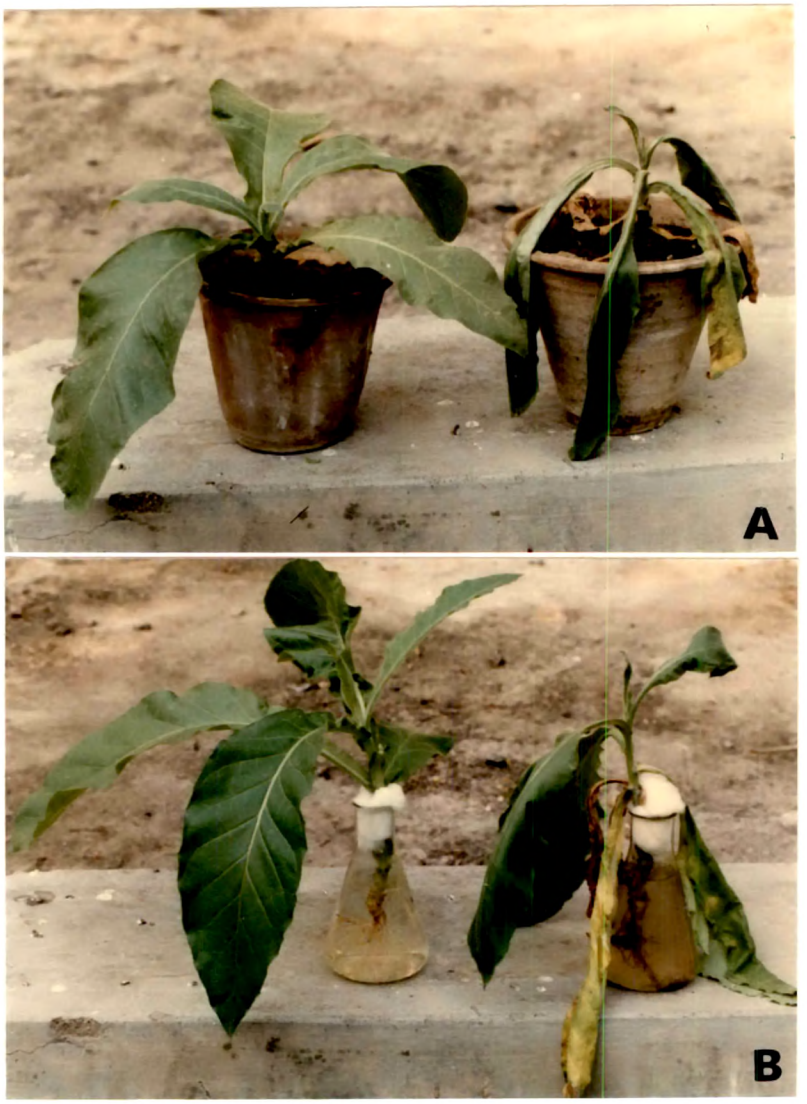


Fig. 3-5 Wilt symptoms on tobacco :

A) Yellowing and wilting of leaves.

B) Browning and rotting of roots; arrow points the sloughing off of the cortical regions.


A
B

and v) wilting of the entire plant (Table 3-1).

Formation of new roots was observed in all the control plants as well as in those in which lower levels of the inoculum density (viz. 0.04 and 0.4%) were used. New root formation did not occur at higher inoculum densities. Yellowing and wilting of the lower leaves, vein clearing and bad smell around the root region were noticed in those plants treated with higher levels of inoculum. Wilting of the entire plant was observed after 20 days in all treated plants in 4% and 40% inoculum levels. Thus we deduced 4% w/v level of inoculum as the optimal level for the pathogenicity test.

3-C.4 Anatomical study of diseased tobacco plant :

In a cross section, the stem of a 15 day old fungal infested plant at the lowermost node showed destruction of xylem vessels and surrounding tissues. The majority of fungal hyphae were seen occupying the vessel lumen (Fig. 3-6 A, B). The cytoplasm of paratracheal parenchyma cells became dense and was intensively stained with toluidine blue thereby indicating a great accumulation of polysaccharides in them. The cortical parenchymatous tissues generally got rotted and were eventually sloughed off from the roots, exposing dark brown central stele. Conidiospores were often visible in the stelar region (Fig. 3-6 C). In the terminal stage of infection, even the stelar region became destroyed and the lowermost node of the stem became hollow

Table 3-1 : Inoculum density and influence of the pathogen *F. oxysporum* on the host *N. tabacum* var. A2.

| Symptoms observed | Fungal inoculum density % (w/v) | *Number of plants responded on day... | | | |
|---|---|---|---|---|---|
| | | 5 | 10 | 15 | 20 |
| Formation of new roots | 0.0 | 6 | 6 | 6 | 6 |
| | 0.04 | 6 | 6 | 6 | 6 |
| | 0.4 | 3 | 4 | 4 | 4 |
| | 4.0 | 0 | 0 | 0 | 0 |
| | 40.0 | 0 | 0 | 0 | 0 |
| Yellowing and wilting of lower leaves | 0.0 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 1 |
| | 0.4 | 0 | 0 | 0 | 2 |
| | 4.0 | 0 | 1 | 3 | 6 |
| | 40.0 | 1 | 3 | 3 | 6 |
| Vein clearing | 0.0 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.4 | 0 | 0 | 1 | 2 |
| | 4.0 | 0 | 2 | 6 | 6 |
| | 40.0 | 0 | 4 | 6 | 6 |
| Bad odour and softening of the root regions | 0.0 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.4 | 0 | 0 | 0 | 0 |
| | 4.0 | 1 | 3 | 5 | 6 |
| | 40.0 | 1 | 4 | 6 | 6 |
| Wilting of the entire plant | 0.0 | 0 | 0 | 0 | 0 |
| | 0.04 | 0 | 0 | 0 | 0 |
| | 0.4 | 0 | 0 | 0 | 1 |
| | 4.0 | 0 | 0 | 1 | 6 |
| | 40.0 | 0 | 0 | 2 | 6 |

* Number of plants treated in each inoculum level = 6

Fig. 3-6 Anatomical changes in the wilted plants due to fungus and CF inoculation.

A - C Part of stem sections of N. tabacum var. A2 showing presence of fungal mycelium and spores.

D A portion of T.S. of stem showing changes due to fungal CF.

g gel

m fungal mycelium

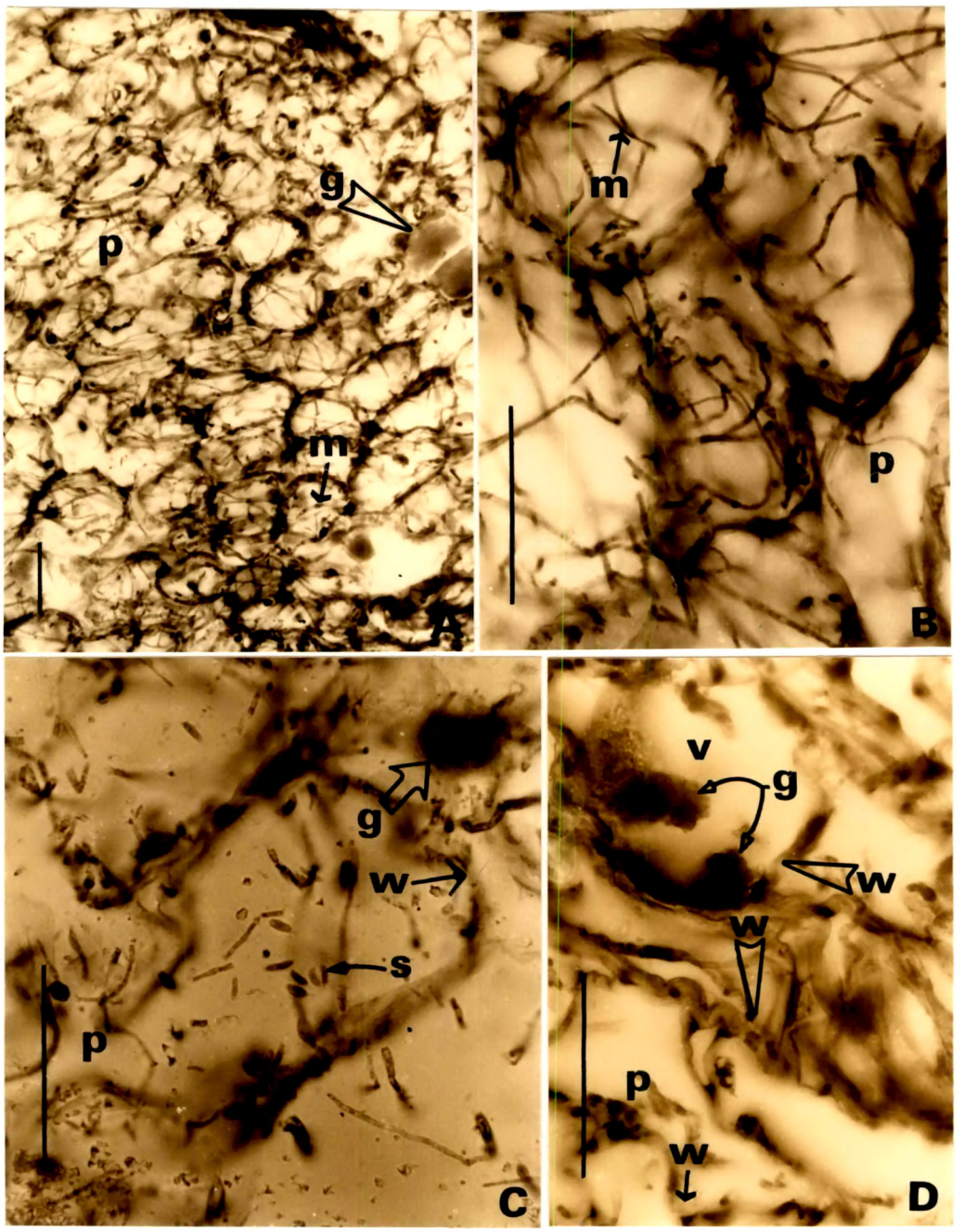
P prenchymatic cells

S conidiospores

v vessel

w destruction of cell wall

In all bar represents 10 μ.


g
p
m
A
m
p
B
g
w
s
p
C
v
g
w
w
p
w
D

by the formation of lytic cavities. No such anatomical changes were observed in the control plants.

Similar type of tissue maceration and wilting were observed in the plants inoculated with the filter sterilized CF (Fig. 3-6 D). Gelation, xylem degeneration and parenchymatous destruction were observed as noticed in mycelium infection.

3-C.5 Screening of tobacco varieties for susceptibility to the pathogen and its culture filtrate :

Nicotiana tabacum var A2, A119, A3, A23, GT4 and 3430 were treated with the fungus and its CF to determine differences in the susceptibility of varieties to the pathogen and/or its CF. Ranking of varieties was based on their relative tolerance to the pathogen and to its CF. Finally ranks of each variety obtained by these two different methods were compared using coefficient of correlation (Redei, 1982).

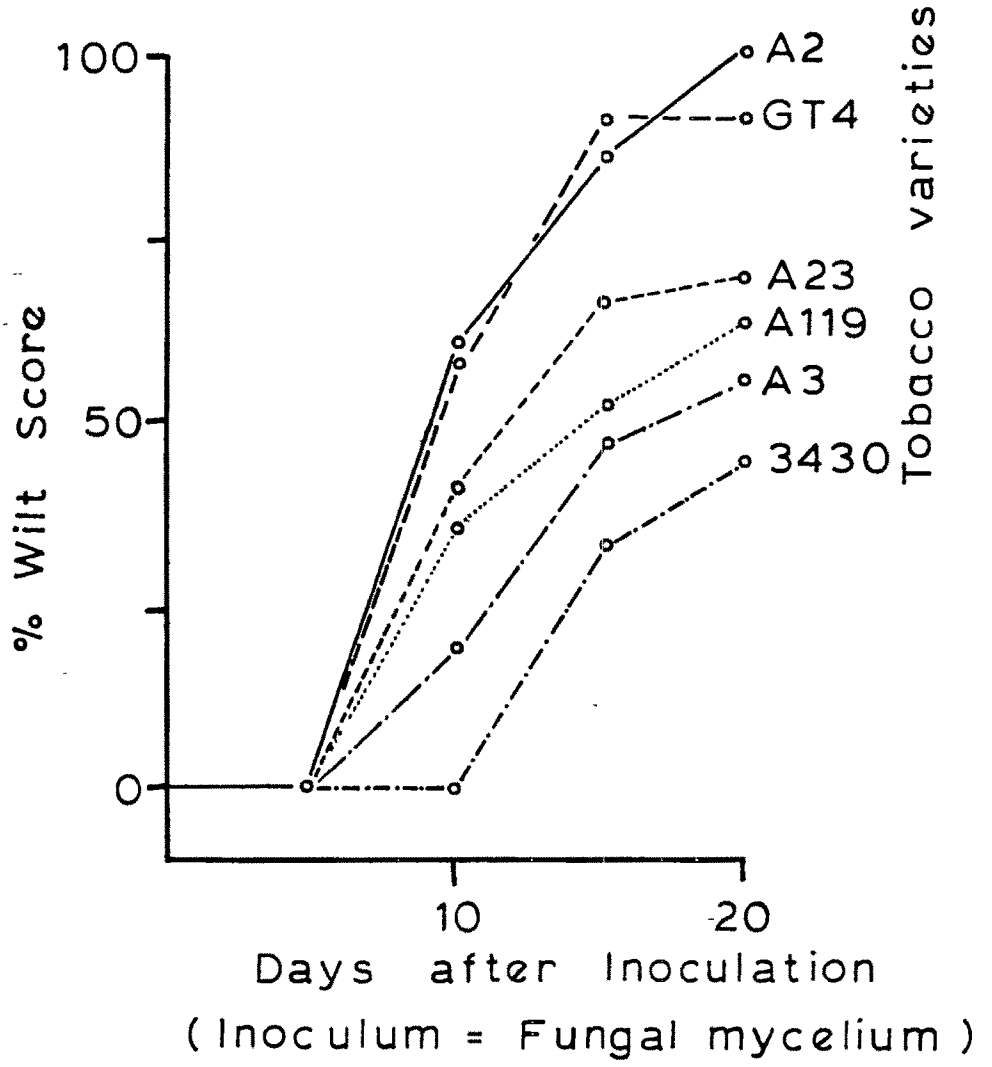
Ranking of varieties is based on their relative tolerance to the pathogen. All six varieties of tobacco were inoculated by 0.4% w/v fungal mycelium as mentioned under materials and methods. At least 6 plants for control and 9 plants for treatments were used. Symptoms were recorded at every 5 day interval after inoculation (Table 3-2, Fig. 3-7). New root formation was observed in all control plants and in most of plants of varieties 3430, GT4, A2 and A23. After about 15 days of incubation, yellowing of the lower leaves, vein clearing, bad

Table 3-2 : Response of tobacco varieties to fungal inoculation.

| Symptoms observed | Tobacco variety | *Number of plants responded on day | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 5 | | 10 | | 15 | | 20 | |
| | | + C | T | C | T | C | T | C | T |
| Formation of new roots | A2 | 5 | 2 | 6 | 2 | 6 | 2 | 6 | 2 |
| | A119 | 6 | 4 | 6 | 4 | 6 | 4 | 6 | 4 |
| | 3430 | 5 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| | GT4 | 4 | 5 | 6 | 5 | 6 | 5 | 6 | 5 |
| | A3 | 5 | 5 | 6 | 5 | 6 | 5 | 6 | 5 |
| | A23 | 6 | 4 | 6 | 4 | 6 | 4 | 6 | 4 |
| Yellowing and wilting of lower leaves | A2 | 0 | 0 | 0 | 8 | 1 | 9 | 1 | 9 |
| | A119 | 0 | 0 | 0 | 4 | 1 | 5 | 1 | 7 |
| | 3430 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 5 |
| | GT4 | 0 | 0 | 0 | 7 | 1 | 9 | 1 | 9 |
| | A3 | 0 | 0 | 0 | 2 | 0 | 5 | 0 | 6 |
| | A23 | 0 | 0 | 0 | 5 | 1 | 7 | 0 | 7 |
| Vein clearing | A2 | 0 | 0 | 0 | 6 | 0 | 8 | 0 | 9 |
| | A119 | 0 | 0 | 0 | 3 | 0 | 5 | 0 | 6 |
| | 3430 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 4 |
| | GT4 | 0 | 0 | 0 | 5 | 0 | 8 | 0 | 8 |
| | A3 | 0 | 0 | 0 | 2 | 0 | 4 | 0 | 5 |
| | A23 | 0 | 0 | 0 | 4 | 0 | 6 | 0 | 6 |
| Bad odour and softening of root cortical regions | A2 | 0 | 0 | 0 | 8 | 0 | 9 | 0 | 9 |
| | A119 | 0 | 0 | 0 | 4 | 0 | 5 | 0 | 6 |
| | 3430 | 0 | 0 | 0 | - | 0 | 3 | 0 | 4 |
| | GT4 | 0 | 0 | 0 | 9 | 0 | 9 | 0 | 9 |
| | A3 | 0 | 0 | 0 | 3 | 0 | 4 | 0 | 5 |
| | A23 | 0 | 0 | 0 | 6 | 0 | 7 | 0 | 7 |
| Wilting of entire plant | A2 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 9 |
| | A119 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 4 |
| | 3430 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 3 |
| | GT4 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 7 |
| | A3 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 4 |
| | A23 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 5 |

* Number of plants treated in control of each variety = 6
Number of plants treated in treatment of each variety = 9

+ C : Control
T : Treatment




( Inoculum = Fungal mycelium )

odour and softening of the root cortical portions were observed in the varieties A2, A119, GT4 and in some plants of A3 and A23. These symptoms were severe in variety A2. Leaves of this variety showed vein clearing in all the plants. Root cortical regions were sloughed off easily in the affected plants. Finally all plants of this variety were wilted in about 20 days of incubation. Seven out of nine and five out of nine were wilted in varieties GT4 and A23 respectively. Only few plants showed wilt symptoms in other varieties. Control plants of all varieties were healthy throughout the experiment.

The order of susceptibility of the six varieties of tobacco to the fungal infection is as follows.

A2 > GT4 > A23 > A119 > A3 > 3430

All six varieties treated with the GI50* concentration of the CF did not produce prominent vein clearing as that by direct fungal infection. However, many other symptoms of fungal infection viz., inhibition of formation of new roots, yellowing and wilting of leaves, softening of root cortical tissue could be induced by the CF (Table 3-3, Fig.3-5, 3-8). Further appearance of these symptoms by the CF was much earlier by at least 10 days than by the direct fungal infection. The

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* Detailed procedure to calculate GI50 concentration is given in materials and methods.

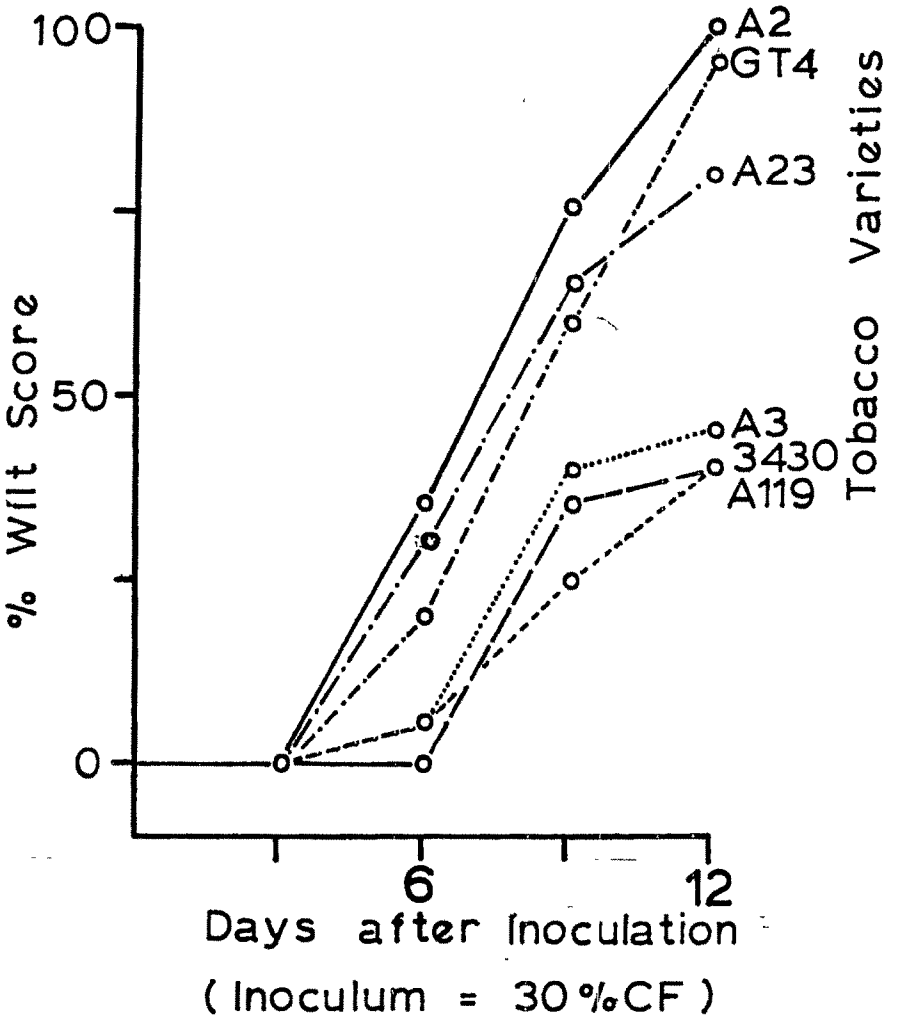
Table 3-3 : Response of tobacco varieties to GI50 concentration ( 30% ) of the CF

| Characters observed | Tobacco varieties | *Number of plants responded on day | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 3 | | 6 | | 9 | | 12 | |
| | | C | T | C | T | C | T | C | T |
| Formation of new roots | A2 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 |
| | A119 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 |
| | 3430 | 0 | 0 | 0 | 2 | 3 | 2 | 3 | 2 |
| | GT4 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 3 |
| | A3 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 3 |
| | A23 | 0 | 0 | 0 | 2 | 3 | 2 | 3 | 2 |
| Yellowing and wilting of lower leaves | A2 | 0 | 0 | 0 | 2 | 0 | 3 | 0 | 5 |
| | A119 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 5 |
| | 3430 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 4 |
| | GT4 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| | A3 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| | A23 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 4 |
| Vein clearing | A2 | 0 | 0 | 0 | 2 | 0 | 4 | 0 | 5 |
| | A119 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 4 |
| | 3430 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| | GT4 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| | A3 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 3 |
| | A23 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 4 |
| Softening of root cortical portions | A2 | 0 | 0 | 0 | 2 | 0 | 4 | 0 | 5 |
| | A119 | 0 | 0 | 0 | 2 | 0 | 4 | 0 | 5 |
| | 3430 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 5 |
| | GT4 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| | A3 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 2 |
| | A23 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 4 |
| Wilting of entire plant | A2 | 0 | 0 | 0 | 1 | 0 | 4 | 0 | 5 |
| | A119 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 5 |
| | 3430 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| | GT4 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| | A3 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| | A23 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 4 |

* Number of plants kept in control of each variety = 3
Number of plants kept in treatment of each variety = 5

C : Control,

T : Treatment


100
50
0
% Wilt Score
A2
GT4
A23
A3
3430
A119
Tobacco Varieties
6
12
Days after Inoculation
( Inoculum = 30 %CF )

order of ranking of varieties based on their relative susceptibility to the CF is as follow.

A2 > GT4 > A23 > A3 > A119 = 3430

Most susceptible — Least susceptible

Correlation between the two screening procedures : The wilt scores recorded in six varieties of tobacco after inoculation with either the fungus or its CF were analysed statistically to compare the correlation of variability. The results are entered in Table 3-4 and the further calculation is as follows.

$$\text{Coefficient of correlation } r = \frac{n(\Sigma xy) - \Sigma(x)\,\Sigma(y)}{\sqrt{n(\Sigma x^2) - (\Sigma x)^2} \times \sqrt{n(\Sigma y^2) - (\Sigma y)^2}}$$

$$\text{Test of significance } t = \frac{r\sqrt{N-2}}{\sqrt{1-r^2}}$$

$$= 6.78$$

In this study the tabular 't' value obtained from Table 3 of Hays (1981) with $(n_1+n_2-4=8)$ 8 degrees of freedom is 3.055 for the 0.005 level.

$t.005 = 3.055$ level

$t = 6.78$

$t = t.005$

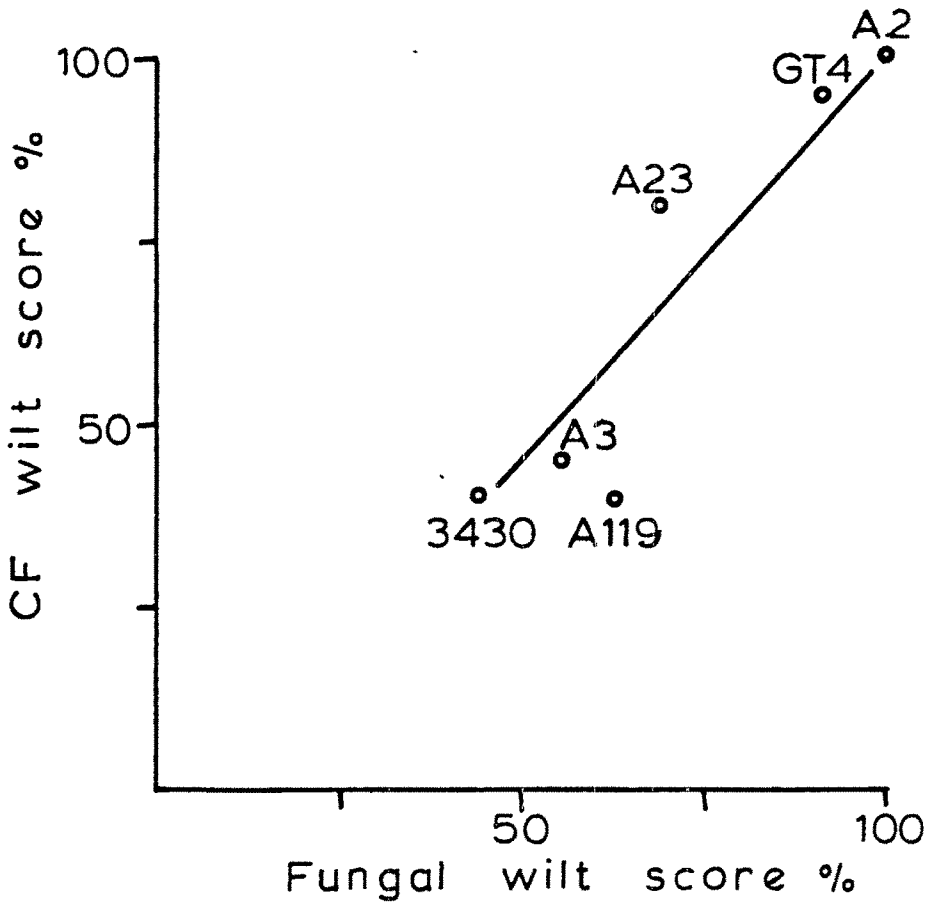
Hence, the correlation is highly significant.

Table 3-4 : Computation of x and y variables for comparisons of paired observations

| Sr. No. | Varieties | Wilt score X | Wilt score Y | $X^2$ | $Y^2$ | XY |
|---|---|---|---|---|---|---|
| 1 | 3430 | 44.0 | 40 | 1971.36 | 1600 | 1776 |
| 2 | A3 | 55.6 | 45 | 3091.36 | 2025 | 2502 |
| 3 | A119 | 63.9 | 41 | 4083.21 | 1681 | 2619.9 |
| 4 | A23 | 69.4 | 80.5 | 4816.36 | 6400.25 | 5586.7 |
| 5 | GT4 | 91.7 | 95 | 8408.89 | 9025 | 8711.5 |
| 6 | A2 | 100 | 100 | 10000 | 10000 | 10000 |
| | Total | 425 | 401.5 | 32371.18 | 30811.25 | 31196.1 |
| | Mean | 70.83 | 66.91 | | | |

x = wilt score due to fungal mycelium inoculation

y = wilt score due to CF inoculation


A2
GT4
A23
A3
3430
A119
100
50
CF wilt score %
50
100
Fungal wilt score %

The line graph method of correlation drawn in Fig.3-9, taking x variables in x axis and y variables in y axis showed that the correlation is positive.

## 3-D DISCUSSION

Wilt disease typically appears during October and November months in Gujarat state. The disease is found in distinct patches of various sizes in the field. Isolated diseased plants may be found throughout the field. One of the first discriminating symptoms is the drooping of the tips and leaves of the plant due to loss of turgor. Yellowing of the leaves and maceration of the root cortical region are observed in later stages.

The fungus isolated from the infested material inoculated on PSA medium as well as in 'moist chamber' was identified as F. oxysporum f. sp. nicotianae. Neither F. solani nor any other species were identified. The classification by Snyder and Hansen, (1940) has been used for the determination of the species as F. oxysporum.

Fusarium oxysporum f. sp. nicotianae has been identified as the causal agent of the disease by Butler (1918), Prasad and Patil (1952) and Prasad et al. (1957).

The present study demonstrated that all the symptoms reported in the 'chitri' complex of tobacco could be induced by F. oxysporum alone. Root cortex and pith maceration simultaneously occurred with plugging of xylem vessels.

Prasad et al. (1957) reported that the fungal hyphae initially are confined to the xylem vessels and can be observed in other tissues only when the disease has practically destroyed the plant. In contrast, wilt studies of other plants show different results, (Pennypacker and Nielson, 1972, and Harling and Taylor, 1985). Hyperplasia in response due to invasion by Verticillium albo-atrum has been reported in the vascular cambium of hop by Talboys (1958) and in the pith cells of tomato by Selman and Buckley, (1959). F. oxysporum f. sp. dianthi was found simultaneously in the xylem vessels and other parenchymatous tissue of carnations (Harling and Taylor, 1985). In the present study, I found that the fungal hypae plug the xylem vessels and simultaneously attack the root cortex and pith.

Simultaneous infection of the host with nematodes and F. oxysporum was not attempted. Therefore, the results can not be compared with those by Jenkins (1948); Mathrani et al. (1951); Caperton et al. (1986); Griffin (1986). In my system, the seedlings were grown aseptically in soil and infected with the fungal pathogen. In spite of the fact that there were no nematodes causing preliminary injury to host tissues, the host was infected. This indicates that F. oxysporum is the primary pathogen. This is further supported by studies on the effect of CF on the growth of different host systems (Chapter IV - showing that the fungus can secrete toxic substances to digest the host tissue for its entry and establishment in the host).

Complete structural derangements observed in the light microscopy of the Fusarium wilted tobacco tissue resemble the wilt as mentioned in carnation (Pennypacker and Nelson, 1972; Harling and Taylor, 1985), in tomato (Langcake and Drysdale, 1975), and in Chrysanthemum (Robb et al., 1975, 1978; Emberger and Nelson, 1981; Stuchling and Nelson, 1981).

We have made comparison of two methods commonly used to study the pathogenicity of fungi as well as to evaluate the relative susceptibility of varieties to the pathogen. In the soil inoculation method, complete wilt symptoms were noticed only after four weeks. In the liquid culture method symptoms were brought about as early as 20 days. Further, instead of using fungal inoculum, when a CF was used (30% CF), wilting occurred even earlier, 11 days after incubation. Rating of varieties by these two different methods, i.e., fungal inoculum and CF is identical and significant. Although the sample size in terms of number of varieties screened is small in our study, we feel that our method of using CF to screen varieties shows a great potential due to its rapidity and ease of manipulation.

Nene and Kannaiyan (1982) found that the CF of Fusarium were not toxic to pigeonpea seedlings even when the entire contents of suspension culture of the fungus was used as inoculum. In our study, CF alone can mimic the action of fungal hyphae and spores in bringing about all the wilt symptoms. Moreover, the use of CF shows the wilt symptoms much earlier

than the fungus itself. The toxic effects of the fungal CF on tobacco seedlings further indicate that there are materials toxic to plants elaborated by this fungus in culture solutions. This supports similar findings of Wolf and Wolf (1948) and Trione, (1960).

In the present study six local (Gujarat) varieties of bidi tobacco have been screened to Fusarium wilt for the first time. There is no previous mention of varietal screening of Indian tobacco upto 1983 (Raja Rao and Suryanarayana, 1986). I found that all the varieties showed different levels of susceptibility to F. oxysporum. N. tabacum var. A2 and GT4 were the most susceptible among all other varieties. The varieties A119 and 3430 showed less susceptibility. Var. A3 and A23 were moderately susceptible. The results obtained by the fungus inoculation procedure were identical with the CF inoculation method. Those two variables were tested for correlation and found to be significantly correlated.