A STUDY OF THE WILT DISEASE OF PHASEOLUS SPP. CAUSED BY FUSARIUM OXYSPORUM

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by

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ABSTRACT

Different varieties of beans were tested for their reaction to infection by _Fusarium oxysporum_ and it was found that all tested varieties were susceptible.

When inoculated through roots, plants developed disease symptoms 7 to 9 days later and died within the next 3 weeks. The most common visible symptoms of the disease were, wilting, yellowing, vein clearing and necrosis of the leaves and a general dwarfing of the plant. Although the pathogen entered the plant as early as 3 days after inoculation, its spread up the stem was delayed and the foliar symptoms appeared in advance of the pathogen. The petiole and the lamina were seldom or never invaded by the fungus.

The vessels were not sufficiently blocked by the fungus before symptom expression to cause obstruction to water flow. However, the fungus grew profusely and invaded most of the vessels later in the development of the disease but it seldom invaded cells other than the vessels. Vascular discoloration was a common feature of the disease but it was found only in vessels occupied by the fungus and in close proximity to it.

The symptomless leaves of the infected plant respired at a higher rate than those of the healthy

plant. The rate of water loss of the infected plant was less than that of healthy plant but the loss exceeded the intake, just before or the time of symptom expression externally.

Cell free filtrates obtained from cultures of the fungus on liquid CCA medium was found to be toxic even when diluted to 50 times and induced disease symptoms in cuttings but toxicity was lost after 48 hours dialysis. The water against which the filtrate was dialysed became toxic and caused wilting in cuttings. The fungus was found to grow well in the sap from healthy plants when supplemented with glucose, and the cell free filtrate from this culture was toxic to cuttings.

Fusaric acid was detected in culture filtrates and infected plants, but the quantity found in an infected plant was not sufficient enough to cause disease symptoms in the plant. Evidence obtained suggests that bean wilt is caused by one or more low molecular weight substances and fusaric acid may be one of them.

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INTRODUCTION

Fusarial wilt of beans caused by *Fusarium oxysporum* was first reported in California (W.C.Snyder, personal communication). More recent reports of this disease comes from Egypt (Yousuff, 1961).

The most common symptoms of this disease are wilting and yellowing of the leaves accompanied by vein clearing and laminar necrosis. These symptoms appear first on the primary leaves and then spread upwards to other leaves in an acropetal succession. With the appearance of symptoms, the plant is stunted and eventually dies. Most of these symptoms appear in advance of the pathogen and even in an advanced stage of infection the pathogen remains confined to the vessels. How the pathogen brings about these changes is not clear. One hypothesis suggests that these symptoms are the results of obstruction in the vessels to water flow caused by the physical presence of the fungus or substances produced in response to infection. Another hypothesis suggests that the pathogen produces toxins which are carried by the transpiration stream to the leaves. In the leaves it affects the cell permeability resulting in an excess water loss.

In this work an attempt has been made to find the relation of infection to the production of symptoms and

the mechanisms involved in disease development.

The first part of this work is concerned with the relation of infection to the production of external symptoms and with anatomical changes inside the host. This was done mainly by anatomical studies of infected plant material taken from different regions of the plant at different stages of infection.

The second part deals with a study of the physiology of infected plants, in particular, rate of transpiration, the ratio of water loss to water intake, the rate of conduction, occlusion of vessels and its effect on conduction, the stomatal behaviour of the infected plant, the effect of humidity and temperature on symptom expression and such other details.

The next part is mainly concerned with the effect of culture filtrates; on transpiration, respiration and conduction of cuttings.

The last part deals with the identification of fusaric acid in culture filtrate and in diseased plants, and the role of fusaric acid in the production of disease symptoms.

REVIEW OF LITERATURE

This review deals with the literature on the more prominent symptoms of diseases caused by vascular wilt parasites and with the mechanisms involved in their production.

1. Wilting

There is still considerable disagreement on the cause of wilting in vascular wilt diseases. Two main hypothesis have been proposed to account for wilting. According to the first, conduction of fluids by the xylem is impeded by occlusions which obstruct the flow in the vascular elements. The second suggests that metabolites produced in the plant alter the permeability of the leaf cells and consequently, the water relations of the whole plant. These hypotheses will be dealt with separately.

a) Occlusion hypothesis

Smith (1899) was one of the first to work on this aspect of vascular wilt diseases. He recorded a considerable reduction in the rate of flow of liquid in the vessels of infected cotton, watermelon and cowpea plants and concluded from histological evidence that it was caused by mechanical plugging of the water conducting

elements by gums and tyloses and by fungal hyphae in the lumens of vessels. But, Ashby (1913), and Brandes (1919) studying *Fusarium* wilt of bananas have doubted that the amount of mycelium in infected vessels is sufficient to account for wilting. The findings of Ludwig (1952), Waggoner and Dimond (1954), and Threlfall (1957) support this view. Ludwig (1952) measured the reduction in the rate of water flow in stems of *Fusarium* infected tomato plants and found that the number of vessels blocked by mycelium could not fully account for the differences between diseased and healthy stems. He found that cutting up to 83 per cent of the vascular system of the stem region of a tomato plant did not cause the plant to wilt. Dimond and Waggoner (1954) constructed a scale model of a xylem vessel with the same Reynolds number as the host and showed that the reduction in flow due to the physical presence of mycelium in the lumens of vessels did not account fully for the large decrease inflow in some wilt diseases.

There are many reports of tyloses and gums in diseased plants; in Verticillium wilt of potato (Pethybridge, 1916; Van der Meer, 1926), tomato (Derbyshire, 1956), hops (Talboys, 1958), in *Fusarium* wilt of banana (Wardlaw, 1930), watermelon (Wilson, 1936),

sweet potato (McClure, 1950), tobacco (Powers, 1954), banana (Beckman _et al_., 1962). In oak wilt caused by _Ceratocystis fagacearum_, Struckmeyer _et al_., (1954) considered that there were sufficient tyloses in most of the vessels to account for wilting. Zentmeyer (1942) reported a similar condition in Dutch elm diseases caused by _Ceratostomella ulmi_ and Beckman _et al_., (1953) observed an 85 per cent drop in the movement of radio-active rubidium in the vascular tissues of infected oak during a period of 3 or 4 days before the appearance of the first permanent leaf symptoms; this was attributed to plugging of vessels by gums and tyloses. Continued development of tyloses and gums caused further plugging and this caused permanent wilting. A direct correlation between plugging by tyloses and gums and wilt symptoms has also been found in oak wilt by Parmeter _et al_., (1956) and by Schoeneweiss (195[illegible]).

Dimond and Waggoner (1953) found that the rate of movement of radiophosphate was reduced by 96 to 98 per cent in the vessels of _Fusarium_ infected tomato plants and posed the hypothesis that disease symptoms result from an impediment to water flow caused by high molecular weight substances in the vessels. This hypothesis was supported by the fact that wilting tomato leaves re-covered when the affected vascular tissue was removed

and the leaves were placed in water. Scheffer and Walker (1953) also found reduced flow of water in stems and petioles of *Fusarium* infected tomato plants compared with healthy tomato plants. The movement of radiophosphate in diseased stems was 0.0045 cm./sec. as against 0.25 cm./sec. in healthy stems under the same conditions. Scheffer and Walker (1953) induced plugging of vessels and wilting of healthy stems by placing cut ends of tomato shoots in dilute solutions of various polysaccharides. Threlfall (1957) and Scheffer *et al.*, (1956) observed a decrease in transpiration in *Verticillium* infected plants and they attributed it to an increase in resistance to water flow in the vessels. Wood (1961) and Blackhurst and Wood (1963) induced wilting by immersing the cut ends of healthy stem in very dilute solutions of pectic substances. Chamberlain and McAlister (1954) found a similar resistance to water flow in soybean wilt caused by *Cephalosporium gregatum*.

Ludwig (1952) in his studies on the physiology of *Fusarium* wilt of tomato plants found increased resistance to water flow in diseased plants and that a hyaline substance visible only in fresh sections and possibly causing vascular occlusion is present in vessels of infected plant. He suggested this as the cause of wilting. This was

supported later by Gothoskar *et al*., (1954), Winstead and Walker (1954), Waggoner and Dimond (1955) and Pierson *et al*., (1955) who suggest that macerating enzymes liberate fragments of pectic substances into the lumen of vessels where they increase the viscosity of vascular fluids or accumulate to form occluding plugs. Beckman and Halmos (1962) found that in *Fusarium* infected banana roots gels accumulate in the region of infection. This gel contains pectic materials and appears to come from the swelling of primary walls and perforation plates of vessels. They suggest that the pathogen produces a metabolite, possibly a chelating agent, that increase the hydration of native pectin to form gels. Paquin and Coulombe (1962) found that a virulent strain of *Fusarium oxysporum* f. *lycopersici* produced more pectic enzymes *in vitro* than did an avirulent strain. Leal and Villanueva (1962) working with 40 strains of *Verticillium* spp. found that pectic enzymes were produced only by the virulent strains. These observations demonstrate the good correlation between the ability of pathogens to produce cell wall degrading enzymes and cause symptoms to develop in infected plants. But Mann (1962) and McDonnel (1962) both working with mutants of *Fusarium oxysporum* f. *lycopersici* found no relation between the pathogenicity

of a strain and its ability to produce pectic enzymes. Chambers and Corden (1963) point out that plugs of pectic material are rarely formed in Fusarium infected tomato plants and imply that they are not responsible for wilting.

b) Toxin hypothesis

According to this, toxic metabolites produced by the pathogen are translocated to the leaf where they reduce or increase the permeability of leaf cells and so cause wilting.

Young and Beumot (1921), Limford (1931) and Fisher (1935) were some of the early proponents of the toxin hypothesis. In other diseases, Thatcher (1942) showed that permeability to water and to solutes increases in cells of tissues infected with obligate or facultative parasites.

Gaumann and Christ (1960) isolated eleven toxins from culture filtrates of various fungi which altered the permeability of protoplasts of Rhoeo discolor. Of these toxins only fusaric acid qualifies for consideration because it is the only toxin known to be produced in vivo (Lakshminarayanan and Subramanian, 1955).

Gottlieb (1943 detected a toxin in the tracheal fluid of Fusarium infected tomato plants. By using special techniques he collected it anaerobically and found that

it was stable at 100°C and caused a reversible wilting of cuttings and increased cell permeability.

Kern and Kluepfel (1956) using isotope methods demonstrated fusaric acid-like compounds in *Fusarium* infected tomato plants. Lakshaminarayanan and Subramanian (1955), and Kalyanasundaram and Venkata Ram (1956) detected fusaric acid in cotton plants infected with *Fusarium oxysporum* f. *vasinfectum*. Nishimura (1957c) and Page (1959) detected fusaric acid in the *Fusarium* wilts of watermelon and banana.

Though *in vivo* production of fusaric acid has been demonstrated and it is known that it affects cell permeability in very low concentrations, the way in which it affects transpiration of infected plants is not well understood. Factors such as concentration of available toxin, pH, temperature and ability to chelate, all seem to play a major role in the development of wilt symptoms.

Bachmann (1956) treated tomato cuttings with fusaric acid and found an unfavourable disturbance of the transpiration-absorption balance. She also found that the permeability of epidermal cells of *Rhoeo discolor* to water doubled with fusaric acid at 1 x 10 M; this effect decreased when higher concentrations were used.

Gaumann (1957) and Kern *et al*., (1957) point out

that fusaric acid is a weak acid and that it dissociates as the pH rises from 4,3 to 8.7. At a low pH, it causes injury to the stem and at a high pH it moves upwards in the transpiration stream to cause leaf injury. Bachmann (1956) studying the effect of pH on water permeability of cells of Rhoeo discolor and spirogyra sp. found pH to be important only at concentrations of fusaric acid greater than 1 x 10^{-5}M.

Considerable importance has been given to the metal chelating properties of fusaric acid by Kalyanasundaram and Saraswathi Devi (1955) and Kalyanasundaram and Subba Rao (1957) who found at temperatures above 37.5°C that there is an increase in detectable fusaric acid in infected plants because of the preferential chelation of iron by cystine which is formed at this temperature. At low temperatures and in the absence of cystine, the iron chelates with fusaric acid so that the quantity of detectable fusaric acid in infected plant is less.

Severe plasmolysis of leaf cells, disintegration of plastids, appearance of brown occluding materials at vein endings and changes in permeability are now recognized as typical symptoms of wilt diseases. They all suggest the activity of some metabolites that act in advance of the pathogen.

2. Yellowing, Vein Clearing and Necrosis

Dawson (1922) suggested that a toxin was responsible for yellowing in *Verticillium* wilt of Michaelmas daisies and Gilman (1916) suggested that an increasing water deficit as the cause of yellowing in cabbage infected with *Fusarium oxysporum* f. *conglutinans*. Pegg and Selman (1959) stimulated yellowing and wilting of leaves by supplying detached leaves with indole-3 acetic acid at 1-5 p.p.m.

Vein clearing has been reported in only a few cases. Foster (1946) noticed a 'netted' appearance and a clearing of veinlets in *Fusarium* infected tomato leaves, 24 hours after inoculation when observed under fluorescent light. Kalyanasundaram (1955) found a similar condition in leaves of *Fusarium* infected cotton plants and Raade (1958) reported vein clearing in *Verticillium* wilt of garden stock. Toxic metabolites of the pathogen are reported to be responsible for development of Necrosis. Talboys (1958) attributed necrosis and desiccation of leaves in *Verticillium* wilt of hops to the action of toxic metabolites.

In none of these diseases was the fungus present in leaf lamina. This implies that the changes are caused by some metabolites produced in infected plants.

3. Epinasty and Initiation of Adventitious Roots

Petiolar epinasty is characteristic of several vascular wilt diseases and has been attributed to the production of ethylene, (Dimond and Waggoner, 1953). Sufficient ethylene is produced by *Fusarium* in culture and also in diseased plants to cause epinasty (Dimond and Waggoner, 1955). Fergus and Wharton (1957) noted similar symptoms in oak wilt but found that *Ceratocystis fagacearum* does not produce ethylene *in vitro*; they did however detect another growth substance. IAA and many synthetic growth regulators cause epinasty and abnormal adventitious rooting when applied to healthy plants (Zimmerman and Wilcoxin, 1935; Zimmermann and Hitchcock, 1941). Selman and Pegg (1959) detected significant amounts of IAA in *Verticillium* infected tomato plants and in cultures of the parasite and suggest that accumulation of IAA in diseased plant might be responsible for these symptoms. Sequeira and Kelman (1962) also found a significant increase in the level of IAA in tobacco and banana plants infected with *Pseudomonas solanacearium*. But, Threlfall (1959) suggests that shortage of water inside the stem could be responsible for yellowing and epinasty.

4. Stunting

In most vascular wilts infected plants are much stunted with reduced root systems and with shorter internodes to give compact dwarfed plants. Stunting was found to be an invariable symptom of *Verticillium* infected tomato plants by Sinha (1964), and Selman and Pegg (1957 and 1958) interpreted the reduction in leaf area and stunting of the *Verticillium* infected tomato plants as the effect of IAA. According to Selman and Pegg, small quantities of IAA and other toxic substances produced by the fungus inside the vessels are carried by the transpiration stream to all parts of the plant. In the leaf it affects the permeability of cells to water. Interference with the metabolism of leaves thus caused reduces the photosynthetic efficiency and the supply of elaborated materials to the younger leaves and so reduce their size. As the stem has a relatively high content of natural IAA it does not respond as readily as the leaf to IAA produced by the parasite. But eventually it interferes with the growth of the cells of the meristematic tissues of the stem also resulting in the reduction in the length of the internodes. Sinha (1964),noticed a 35 per cent reduction in stem height in *Verticillium* infected tomato.

III. MATERIALS AND METHODS

A. Growing of plants

Seeds of different varieties of beans used in this study were obtained from the following sources.

	Variety	Source
1.	Prince.	Sutton and Sons, Reading.
2.	Canadian Wonder.	" " " "
3.	Comptesse De Chambord	" " " "
4.	Masterpiece	" " " "
5.	Brown Dutch	" " " "
6.	Top Crop	" " " "
7.	Mont De Or.	" " " "
8.	Bounteous	" " " "
9.	Bremier	" " " "
10.	Black Prince	" " " "
11.	Tender and True	" " " "
12.	Suttons Best of Al .	" " " "
13.	Giant Painted Lady	" " " "
14.	Scarlet	" " " "
15.	Carters Merton	Carters Ltd., England.
16.	Carters Granda	" " "
17.	Carters Lightning	" " "
18.	Carters Pershore Favourite	" " "
19.	Crusader.	" " "

	Variety	Source
20.	White Monarch	Carters Ltd., England.
21.	Red Mexican	Bean Research Laboratory, College of Agriculture, Idaho.
22.	High Grade	" " " "

For most of the experiments, plants were grown in the roof Greenhouse at Imperial College, London. The greenhouse was heated during winter months and the temperature rarely went below 65°F. (18°C). Additional light was provided by banks of 80.w. 'white' fluorescent tubes suspended approximately 1 ft. above the tops of plants giving a light intensity of about 800 f.c. For varietal resistance tests and also for a few other preliminary experiments, plants were grown in John Innes potting compost supplemented with John Innes base fertilizer (2 parts hoof and horn, 2 parts superphosphate and 1 part sulphate of potash) and ground chalk added at the rate of 5 lb. and 1 lb. per cubic yard respectively. Seedlings for these experiments were raised in groups of 5 per 6" clay pots and were transferred into 4" clay pots after inoculation; one plant per pot. These plants were watered daily with tap water.

For most other experiments plants were grown in Horticultural Vermiculite No.3 (obtained from Pan Britannia

Industries Ltd., England) and watered every 3 days with Long Ashton nutrient solution (Hewitt, 1952), but omitting aluminium, gallium, cobalt and nickel salts. The ferric citrate component of the nutrient was replaced by iron-ethylene-diaminotetra-acetic acid complex (Fe-EDTA) supplying iron at 0.15 milli equivalent per litre of nutrient solution. Seedlings were raised in groups of 3 in 5" plastic pots and after inoculation they were planted separately in 4" plastic pots. (Preliminary experiments showed that plants grew well in vermiculite when supplied with a suitable quantity of nutrient solution).

Cuttings were grown in bottles (160 ml. capacity) containing nutrient solution. For most experiments with cuttings, they were kept in the constant-temperature cabinet (growth cabinet) at $22^{o}C \pm 1^{o}C$ and provided with additional light by six 80 w. fluorescent tubes giving a light intensity of about 400 f.c. The bottles were wrapped with black polythene to cut off light from the roots.

B. Culture of fungus

1. Origin of culture

The isolate of *Fusarium* came originally from wilted French bean plants growing in the Evesham area.

It was used to infect bean plants in which it caused a typical, rapidly developing vascular wilt. It was reisolated from a diseased plant and pure cultures were established from single spores. The reisolated specimen was identified by Dr. Booth of the Commonwealth Mycological Institute as Fusarium oxysporum.

2. Media

The following media were used for growing fungus. Unless otherwise stated, all the chemicals used for media were of analytical reagent grade.

a. Sucrose-casamino acids (SCA)

Sucrose	-	15.0 g
KH_2PO_4	-	1.0 g
Mg SO_4, $7H_2O$	-	0.5 g
Casamino acids (Difco)	-	4.6 g
Trace element soln.	-	1 ml.
Water	-	to 1 litre.

The trace element solution was made up as shown below. (One ml. of this was added to a litre of the medium).

Trace element	Salt used	Amount in mg./100 ml.	p.p.m. trace element in stock soln.	p.p.m. trace element in final soln.
Iron	$FeSO_4,7H_2O$	249	501	0.5
Copper	$CuSO_4,5H_2O$	40	100	0.1
Zinc	$ZnSO_4,7H_2O$	44	100	0.1
Manganese	$MnSO_4,4H_2O$	41	101	0.1
Molybdenum	$Na_2MaO_4,2H_2O$	51	202	0.2
Water		100 ml.		

Agar was added at 20 g per litre when a solid medium was required and steamed for 1 hour.

The medium was sterilized by autoclaving for 15 minutes at 120°C.

b. Sap-glucose medium

Glucose	0.1 g
Plant sap (exudate)	95 ml.
Water	5 ml.

Glucose was dissolved in water and sterilized by autoclaving for 15 minutes at 120°C. The solution was then added to plant sap sterilized by micropore filtration.

3. Culture vessels

Cultures for preparing inoculum were grown in 250 ml. medicine bottles containing 40 ml. of liquid or solid medium

and incubated on the flat side.

For reisolation of fungus from plant material and for obtaining monospore culture, petri dishes containing 20 ml. of solid SCA medium were used.

4. Maintenance of stock culture

After establishing a monospore culture, the fungus was grown on slopes of sucrose casamino acid (SCA) medium in McCartney bottles. After incubation at 25°C for 10 days a few culture bottles were stored in at - 20°C and the others were kept at room temperature (20°-22°C) under sterile liquid paraffin. Occasionally, subcultures were made from the stock cultures. For experiments on growth of fungus in plant sap Erlenmeyer flasks of 150 ml. capacity were used.

5. Inoculation and incubation of cultures

Solid media in medicine bottles were inoculated by drawing a bit of sporulating mycelium over the surface of the agar. Liquid medium in medicine bottles and plant exudate medium in Erlenmeyer flasks were inoculated with 1 ml. spore suspension made by washing a 8-10 day old culture on solid SCA medium with 10 ml. of sterile water. Agar slopes in McCartney bottles were inoculated with a small bit of sporulating mycelium on the agar with a sterile inoculating needle.

All cultures were incubated at $25^{o}C \pm .5^{o}C$.

C. Preparation of inoculum

For root inoculation, inocula were prepared from 8-10 day old cultures growing on SCA liquid medium. The mycelial mats were collected on muslin and washed several times in running water to remove all traces of medium. They were then homogenized in a Waring type Blender for 10-15 seconds at maximum speed with water added at the rate of 40 ml. per mycelial mat of one bottle.

Spore suspension was obtained by shaking 8-10 day old cultures on solid SCA medium with 10 ml. of water. The spores in suspension after filtration through 3 layers of muslin were washed thrice by centrifugation and then resuspended in water. The concentration of spore suspension was adjusted after haemocytometer slide counts of spore density.

D. Inoculation of plants

1. Root inoculation

Plants were root inoculated by dipping the washed root system in a mycelial suspension. For most experiments some roots were deliberately damaged by removing their tips with scissors, before dipping in the mycelial suspension. In the controls roots were dipped in water instead of mycelial suspension.

For plants growing in nutrient solution, a dense live spore suspension containing 1,000,000 - 1,500,000 spores per ml. was used instead of mycelial suspension. Controls were dipped in heat inactivated spore suspensions.(By heating suspension at different temperatures for varying length of time and then incubating them on plates of solid SCA, it was found that all spores were killed when heated for 10 minutes at 70°C and this did not cause them to clump.)

2. Shoot inoculation

Cuttings were inoculated with spore suspensions, adapting the technique used by Keyworth (1950). Generally, 16-18 day cuttings at 4 leaf stage (2 primary and 2 trifoliate leaves) were used. They were cut at ground level with a wet razor blade. After removing a few mm. of the stem under water, shoots were placed in a spore suspension. For the controls heat inactivated spore suspension was used. The cuttings in spore suspension were kept in the growth cabinets. After six hours, the cuttings were removed from the spore suspension, their ends were trimmed again and then washed well under tap water before transferring into bottles or vials containing nutrient solution.

E. Dry weight of plant organs

The plant materials were cut into small pieces and dried in a ventilated oven at 100°C for 24 hours.

F. Surface sterilization of plant materials

Plant tissues for reisolation of fungus were surface sterilized by immersion in sodium hypochlorite solution (5 per cent active chlorine) for five minutes followed by 3 or 4 washings with sterile water. Before immersing in the sodium hypochlorite solution, the cut ends of the material were sealed by smearing with melted paraffin wax.

For experiments requiring an undamaged root system, the seeds were surface sterilized by dipping in sodium hypochlorite solution containing 3% active chlorine for five minutes followed by 3 or 4 washings in sterile water and then sown in sterile containers.

G. Studies with culture filtrates

1. Collection and sterilization of culture filtrate

Culture filtrate was obtained from 8-10 day old liquid culture by straining the latter through 2 layers of muslin. The filtrate was then centrifuged at 10,000 r.p.m. for 10 minutes. The cell free filtrate thus obtained was either used immediately or stored in McCartney bottles at - 20°C. When sterile culture filtrate was

needed, it was sterilized by micropore filtration using 'Oxoid' membrane filters approximately 120 u thick with pores 0.5 to 1.0 micron diameter.

2. Dialysis of culture filtrate

Visking tubing of 8/32" diameter (obtained from H.M.C. Scientific Instrument Centre, England) were used for dialysing culture filtrate. After soaking in water for a few minutes, the tubing was cut in length of 15 cm. They were then tied tightly at one end and filled with cell free culture filtrate (5 ml. per tube). After filling, the free end was also tied well and then placed in glass distilled water in a beaker and kept in a refrigerator. The water in the beaker was changed 4 times within 24 hours.

For certain experiments, the water into which the substance was dialysed was used. To do this, the Visking tubings containing culture filtrate were placed in known volume of glass distilled water and the latter was not changed during dialysis.

The solutions were sterilized by micropore filtration after dialysis.

H. Examination of plants

1. Evaluation of symptoms

The nodes and leaves of plants were numbered from below upwards; the first node being the one bearing the primary leaves. The primary leaves were numbered separately as 1 and 2 and the trifoliate leaves were numbered as 3, 4, et. upwards for evaluation of morphological symptoms.

The severity of infection of intact plants and cuttings as expressed by wilting or yellowing of leaves was assessed by using a disease index based on the following arbitrary scale used by Sinha (1964).

0	-	healthy or no symptom
1	-	25% wilting and/or yellowing
2	-	50% " " "
3	-	75% " " "
4	-	complete wilting and/or yellowing of leaf.

2. Anatomical studies

Anatomical studies of the plant material were made by taking free hand and microtome sections. When plant materials were not used immediately, they were fixed and preserved in Formalin-Aceto-Alcohol by the method of McLean and Ivimey-Cook (1952).

a. Free hand sectioning

Free hand sections of fresh plant materials were stained in various ways for particular studies, the details are given later.

b. Microtome sectioning

For a more critical study of the growth of fungus in the plant and also of the response of host tissues to infection, microtome sections were taken with a Cambridge Rocking Microtome. The plant materials, for this purpose, were fixed and preserved in buffered gluteraldehyde fixative, (adapted from that of Sabatini, Bensch and Barrnett, 1963, but with some modifications).

The fixative was prepared by mixing 25 per cent gluteraldehyde in water with phosphate buffer (0.1 M. K_2HPO_4) and the PH was adjusted to 6.8. To prevent any fall in the PH of the gluteraldehyde solution on storage, a small quantity of barium carbonate was added (0.1 per cent w/v) to the stock solution. The precipitate formed was removed by centrifugation.

Fresh plant materials as disks 5 mm. thick were fixed and washed thrice for 15 minutes each time in buffer solution (pH. 6.8-?), before dehydrating. The dehydration was done slowly in water/alcohol mixture, with two final washings in absolute alcohol for 12 hours each before clearing in chloroform. Infiltration

was done in several steps and after 24 hours the materials were embedded in paraffin (MP. 52°C).

Sections were cut in thickness varying from 10 to 24 u and they were variously stained and mounted, adopting mainly the techniques described by Johansen (1955).

Safranin-fast green staining.

After removing wax in xylol, sections were brought down to 70 per cent alcohol and stained in safranin for 12 hours. Excess stain was removed in running water and the sections differentiated in 95 per cent alcohol containing a trace of picric acid. The action of picric acid was stopped by washing in 95 per cent alcohol containing a trace of ammonium hydroxide followed by another wash in absolute alcohol for 10 seconds. Sections were then stained in fast green for 15 minutes and cleared in a mixture of 50 parts clove oil, 25 parts absolute alcohol and 25 parts xylol. This was followed by 2 washings in xylol before mounting in Euparol.

Xylem elements were stained red and other tissues green when sections were dyed as mentioned above.

Composition of stains used

Safranin stain

Safranin O	1.0 g
Methyl cellosolve	50 ml.
95% Alcohol	25 ml.
Distilled Water	25 ml.
Sodium acetate	1.0 g
Formalin	2 ml.

Fast Green stain

Fast Green	1.0 g
Methyl cellosolve	50 ml.
Absolute Alcohol	50 ml.

Staining with Pianese stain and acid alcohol

This stain was used mainly for detecting fungal hyphae in microtome sections.

After washing for 2 hours in xylol, sections were brought down to 50 per cent alcohol and then stained in pianese stain for 30 minutes. Excess stain was removed in running water and sections then differentiated with acid alcohol for 10-15 seconds. After rinsing in absolute alcohol, the slides were cleared in clove oil and mounted in Canada Balsam.

The vessels were stained deep green and the hyphae pink.

Composition of Pianese stain and acid alcohol

Pianese stain (obtained from Edward Gurrs Ltd., London.)

Malachite green	0.1 g
Acid fuchsin	0.1 g
Martius yellow	0.05 g
Alcohol	50 ml.
Water	150 ml.

Acid Alcohol

70% Alcohol	100 ml.
HCI (conc.)	3 ml.

I. Studies in transpiration

1. For studying transpiration root inoculated plants were grown in plastic pots (4") with lid and sealed base. The lid was pierced to take a two holed rubber bung, through one hole of which passed a glass tubing. The other hole held the plant stem. The bung was split longitudinaly along the side to insert the plant stem without damaging the root. The pot was filled to $\frac{3}{4}$" of the upper rim with vermiculite. When nutrient solution was needed, it was poured through the glass tubing. (Plate I).

2. Transpiration of cuttings

Cuttings were grown in 40 ml. vials closed with rubber bungs having central holes through which the stems of the cuttings were inserted, a known volume of

Plate 1

H- Healthy plant growing in vermiculite in plastic pot with lid and sealed base.

nutrient in the test solution was placed in each vial.

3. Transpiration of detached leaves

Detached leaves were kept in 30 ml. vials containing known volumes of ½ strength nutrient solution. They were closed with aluminium foil caps with a hole in the centre for inserting the petiole.

For studies on transpiration, cuttings and leaves were placed in the growth cabinet.

4. Measurement of leaf area

The leaf area was measured by a weighing method. The leaf was traced on to thin paper of known weight per unit area. After tracing, the paper was cut along the line and then weighed. From this weight, the area of the leaf was estimated.

J. Studies in respiration

Respiration of plant tissues was measured as the rate of oxygen uptake of leaf tissues, in a Warburg apparatus.

Fifteen leaf disks were used in each flask containing 3 ml. of phosphate buffer solution (0.1 M solution of K_2HPO_4). The pH of the buffer solution was adjusted to 6.4, unless otherwise stated. The leaf disks were cut with a cork borer 10 mm. diameter. The central well in each flask contained 0.2 ml. of 10 percent KOH solution to absorb CO_2 evolved as the tissues respired. Each flask

was wrapped in aluminium foil to exclude light. In most of the experiments manometer readings were taken for 3 hours at 30 minute intervals. The temperature of the water in the bath was maintained at 25°C for all experiments. At the end of the experiments, the disks were removed and dried in an oven at 70°C for 48 hours and cooled over $CaCl_2$ in desiccator before taking the dry weights. From the dry weights the respiration rate was calculated as ul/oxygen uptake/mg. dry weight of leaf tissue.

When the respiration of leaf tissues in plant sap was studied, the sap from healthy or infected plant replaced the buffer solution in the flask.

K. Collection of tracheal sap

Tracheal sap of plants was collected from plants 24-26 days old. Sap from infected plant was collected 6-8 days after inoculation.

The shoots of plants were cut off with a wet razor blade, one inch above ground level. A glass tubing 12" x ¼" was attached to the freshly cut plant stump with rubber tubing. The free end of the glass tubing was loosely plugged with sterile cotton wool. The tubing was kept vertical with a support. The tracheal sap that rose in the glass tubing was collected after 24 hours. The sap was either used immediately or stored

in McCartney bottles at $-20^{o}C$, after sterilization by micropore filtration.

L. Preparation of plant extract

Juice of plant was extracted by using a Roller Mill. A known weight of fresh plant material was washed in distilled water and then squeezed through the rollers of the Roller Mill. A known volume of glass distilled water was used to wash the rollers after squeezing and the combined juice and water was filtered through several layers of muslin. The filtrate was then centrifuged 6-8 times at 3500 r.p.m. for 10 minutes each. The supernatant was either used immediately or stored in 10 oz. bottles at $-20^{o}C$.

In certain experiments, the plant materials were blended in a Waring type blender with known volumes of 80 per cent ethanol. The supernatant was decanted and after centrifuging at 3500 r.p.m. for 15 minutes at $2^{o}C$, it was immediately or stored in 10 oz bottles at $-20^{o}C$.

M. Studies with fusaric acid

1. Source of fusaric acid

Pure, crystalline fusaric acid was kindly supplied by Professor H. Kern of Zürich.

2. Extraction of fusaric acid

The techniques for the extraction of fusaric acid from culture filtrate and also from plant extracts were adapted from Page (1959) with some modifications.

a. From culture filtrate

The pH of the cell free culture filtrate was adjusted to 4.0 with N/10 HCI. The acidified filtrate was extracted in a separatory funnel with an equal volume of ethyl acetate. The ethyl acetate extract was then dried in a rotary evaporator at 40°C and the residue was taken up in 95 per cent ethanol.

b. From plant extract

The method of preparing plant extract is described earlier (Section L.). The pH of the plant extract was adjusted to 4.0 with N/10 HCI. The acidified extract was then extracted 10 times in a separatory funnel with chloroform using a tenth of the volume of juice at each extraction. The combined chloroform extract was then dried in rotary evaporator at 30°C. The dried residue was then taken up in 95 per cent ethanol.

c. Identification of fusaric acid

Fusaric acid was identified by paper chromatography. The residue obtained from the culture filtrate and also from plant extract were spotted separately and also with

pure fusaric acid at the origin of the paper (Whatman No.1), with an "Algla" syringe. All samples were complexed with copper. After spotting the required quantity of each solution, the papers were dried by air blown from a hair dryer. They were then fitted to the shandon frame and developed by an ascending technique (Smith, 1960) using n-Butanol/acetic acid/water (120/30/50) as solvent, for 6 hours at 22°C. After developing, the papers were dried in the fume cupboard andthen sprayed with rubeanic acid in acetone (w/v). The fusaric acid-copper complex appeared as bluish spots when the papers were dried.

N. Studies with Indole-3-acetic acid (IAA)

Identification of IAA in diseased plants

Plant extract for these studies was obtained as described earlier. The technique used for the identification of IAA was adapted from that of Smith (1960). To remove chlorophyll and other pigments, the extract was washed in a separatory funnel with petroleum ether in a fume cupboard. After 2 or 3 washings, the ethanolic phase was made alkaline with a few drops of ammonia and dried in a rotary evaporator at 40°C. After addition of a drop of ammonia, the ethanol was blown off in an air stream and the residue was dissolved in a few drops of

water using a whirlimixer and the solution was spotted at the origin of Whatman No.1 chromatogram paper. Authentic indole-3-acetic acid (obtained from L.Light and Co.,) in 50 per cent aqueous acetone was spotted next to the extract to serve as markers. The dried chromatogram papers were developed for 10 hours at 22°C. by an ascending method using isopropanol/ammonia/water (200/10/20) as solvent. After developing, the papers were dried in the fume cupboard and then dipped in a mixture of 1 vol. 10 per cent dimethylaminobenzalclchyde (m/u) in concentrated HCI to 4 vol. acetone in a dip tray. The papers were again dried in the fume cupboard and the IAA spots appeared as pinkish spots.

IV. EXPERIMENTAL WORK

A. Morphological and anatomical studies

1. Relation of infection to the production of external symptoms

The external symptoms of the disease were studied mostly by visual observations in two preliminary experiments. The variety 'Prince' was chosen for this purpose.

Experiment I.

Five 18 day old plants at the 4 leaf stage were root inoculated by dipping in mycelial suspensions, after a few roots had been damaged by cutting off their tips in the expectation that root injury would facilitate infection. After inoculation, they were planted in John Innes potting compost and allowed to grow in the greenhouse. Five other plants were kept as controls after their roots had been dipped in water instead of in mycelial suspension.

After inoculation, plants were examined daily for external symptoms. Wilting symptoms appeared in the primary leaves of all inoculated plants 7 to 9 days after inoculation and the symptoms became very severe after 14 days. (Plate 2.)

Plate 2   IN.8.D. Inoculated plant - 8 Days after inoculation

In.14.D.   "   " -14 Days after inoculation

C -   Control

In most plants, the yellowing which followed wilting appeared 9 days after inoculation. Both wilting and yellowing were assessed on an arbitrary scale of 0-4 as mentioned under 'Materials and Methods'. Only leaves measuring 1 cm. or more across the lamina were considered.

Another external symptom in infected plant was vein clearing. This was very prominent in the primary leaves as yellowing became intense. (Plate 3). Vein clearing was assessed as slight, moderate or extensive (Table 1).

Table 1 Wilting, yellowing, vein clearing and necrosis in healthy and infected plants

Disease Symptoms	Days after inoculation					
	7	8	9	10	11	12
Wilting	0.25	0.25	0.5	1	1.25	2
Yellowing	0	0	0.25	0.5	0.5	1
Vein clearing	0	0	0	S	S.M.	M.E.
Necrosis	0	0	0	0	S	S

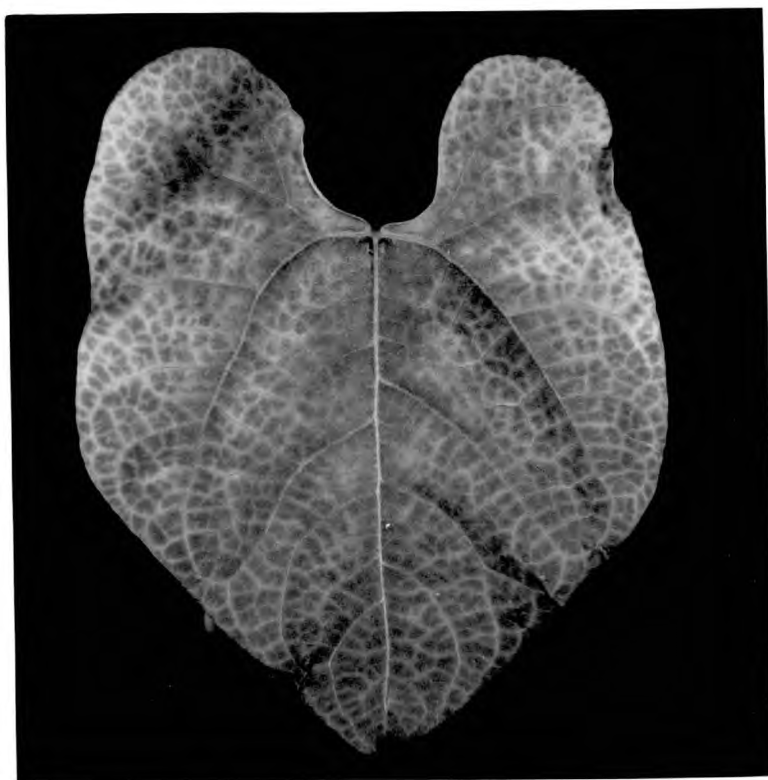
S - Slight

M - Medium

E - Extensive

Average of 5 replicates

Petiolar epinasty was seen in the primary leaves of three plants but it disappeared as severe wilting developed.



Primary leaf of an inoculated plant showing pronounced Vein Clearing.

During this experiment it was observed that diseased plants were considerably shorter than control plants. The following experiment investigated the extent of this dwarfing.

Experiment 2

Nine 18-day old plants were root incculated with mycelial suspension and 9 were kept as controls. All plants were planted in John Innes potting compost and kept in the greenhouse. The heights of 3 inoculated and 3 control plants taken at random, were measured 6, 9, 12 and 15 days after inoculation. From these readings, the percentage reduction in heights was calculated. There was no significant effect until 6 days after inoculation. Thereafter, the rate of growth of the infected plant was markedly reduced. At the end of the experiment, the percentage reduction in stem height was about 25. (Table 2).

Table 2 Effect of infection on plant height.

Days after inoculation	Heights (cm.) control	inoculated	Percentage reduction in height
6	25.3	24.3	4
9	29.3	25.2	14
12	32.5	35.9	20
15	34.8	26.1	25

Average of 3 replicates

The dry weights of samples of three plants were taken, 6 and 12 days after inoculation. The results (Table 3) show that there is considerable reduction in dry matter production by the plant. This was not only due to failure of the leaves to expand as found by Selman and Pegg (1957, and Threlfall (1957), but also due to a reduction in leaf number. The rate of leaf formation became less in infected plants, soon after symptoms appeared.

Table 3 Effect of infection on dry weight of plant organs.

Days after inoculation	Treatment	Dry Wt. (mg.)			Percentage reduction in dry wt.		
		Plant	Root	Stem	Plant	Root	Stem
6	control	170	45	125			
	inoculated	155	40	115	8.9	12	8.4
12	control	321	75	235			
	inoculated	165	55	175	49	37	26

Average of 3 replicates

2. Varietal resistance tests

Twenty-two varieties of beans were examined for their reactions to infection by *Fusarium oxysporum*. Only wilting symptoms were considered in assessing the effect of infection because they were an invariable symptom of this disease.

Five 18 day old plants of each variety were inoculated by dipping their roots in a mycelial suspension. Five other plants of the same variety, age and size were kept as controls; their roots were dipped in water instead of in a mycelial suspension. A few secondary roots of all plants were damaged deliberately by cutting off their tips. After inoculation, the plants were planted separately in John Innes potting compost and placed in the greenhouse.

In most varieties wilting developed 6 to 15 days after inoculation. The results are summarised in Table 4.

Table 4 Reactions of different varieties of beans to infection. (Assessed on the basis of wilting).

	Variety	Days after inoculation					
		3	6	9	12	15	18
1.	Red Mexican	0	1	2.5	3.2	3.75	4
2.	Suttons Best of all	0	0.5	1.5	2.5	3	3.75
3.	Giant Painted Lady	0	0.5	1.25	2.5	3.2	3.6
4.	Masterpiece	0	0.25	0.75	1.75	2	3
5.	Carters Lightning	0	0.25	1.25	2	2.25	3.25
6.	Comptesse De Chambord	0	0.25	0.75	1.75	2.25	3.5
7.	Tender and True	0	0.25	1	1.75	2.5	3.2
8.	Carters Merton	0	0	0.5	1.25	2.5	3.5
9.	Black Prince	0	0	0.25	1.5	2.5	3.25
10.	Crusader	0	0	0.75	2	2.9	3.5
11.	Prince	0	0	0.5	2.25	3.5	3.75
12.	Canadian Wonder	0	0	0.25	1.75	2.5	3.5
13.	Pershore Favourite	0	0	0.5	1.5	2.75	3.5
14.	White Monarch	0	0	0.75	1.75	2.75	3.75
15.	Carters Granda	0	0	0.25	1.5	2.5	3.25
16.	Scarlet	0	0	0.25	0.5	1.75	2.75
17.	High Grade	0	0	0.25	0.75	2.75	3.75
18.	Brown Dutch	0	0	0.25	1.25	2.5	3.25
19.	Mont De Or	0	0	0.3	1.5	2	2.5
20.	Top Crop	0	0	0	0.75	1.75	2.75
21.	Bounteous	0	0	0	0.5	1.75	2.25
22.	Premier	0	0	0	0.5	1.25	2

Average of 5 replicates

3. Root injury as a factor in infection

In *Fusarium* wilt of tomato, Keyworth and Dimond, (1952), dound that damage to the root system increases infection. This was also reported in *Verticillium* infection of tomato by Selman and Buckley (1959), and Sinha (1964). Bewley (1922), Derbyshire (1950) and Selman and Pegg (1957) state that undamaged roots of tomato plants can be penetrated by *Verticillium albo-atrum*.

The following experiment was designed to determine the effect of damaging roots in bean plants at the time of inoculation.

Seeds were surface sterilized and allowed to germinate on moist filter paper in sterile petri-dishes kept in a lighted constant temperature cabinet. When the seeds had germinated they were placed on corks at the mouths of beakers (6" x 3") containing nutrient solution. The corks had been pierced in the centre to allow the roots to grow into the nutrient solution. After 15 days, the plants were carefully taken out and the roots were dipped in a dense spore suspension containing 5 x 10^6 spores per ml. In some plants the secondary roots were deliberately damaged by removing their tips. The controls were dipped in a suspension of heat inactivated spores. Following inoculation, the spores were allowed

to incubate on the roots by suspending the roots in a beaker, the inner wall of which was lined with moist blotting paper. After 24 hours, they were immersed again in nutrient solution in beakers and grown in the constant temperature cabinet. (A preliminary experiment showed that spores germinated on the roots in twentyfour hours when incubated at 22°C.)

In all the inoculated plants with damaged roots symptoms developed 7 to 10 days after inoculation, and wilted severely in another ten days, where as in the plants with undamaged roots, symptom appearance was delayed to the 15th day after inoculation. But afterwards, the plants wilted rapidly. None of the control plants showed any sign of wilting. The results are shown in Table 5.

Table 5. Mean wilting indices per leaf in infected plants with damaged and undamaged root system.

Treatment	Days after inoculation 3	6	9	12	15	18
Undamaged	0	0	0	0	.5	2.8
Damaged	[illegible]	0	0.4	2.5	3.2	3.8

Average of 5 replicates.

From this result, it is evident that injury to the roots at the time of inoculation allows symptoms to develop considerably earlier but that injury is not essential for infection and the late development of severe symptoms.

4. Effect of plant age on infection

This experiment was designed to see whether the age of the plant at the time of inoculation had much effect on disease development. This has been reported for *Verticillium* wilt of tomato plants (Nelson, 1950; McLean, 1955; McLeod and Thompson, 1959 and Selman and Pegg, 1957).

Seeds were sown in vermiculite at intervals of 6 days. When the youngest plants were 12 days old, 3 plants from each set were root inoculated with mycelial suspension and 3 plants were kept as controls. As it had been found that root injury leads to earlier symptom development, in this as in all subsequent experiments, some roots were damaged by removing their tips before inoculation. All plants were planted in vermiculite and allowed to grow in the greenhouse until the severity of disease development in each group of plants was assessed 9, 12 and 15 days after inoculation. At the time of first disease assessment, plants of the different age groups had an average of 3, 5, 7 and 10 leaves

each. The wilting indices for each age group are given in Table 6.

Table 6 Effect of age at the time of inoculation on disease development

Age at the time of inoculation (Days)	Days after inoculation		
	9	12	15
12	0.75	2.5	3
18	0.5	2.3	3
24	0.4	2.6	3.2
30	0.25	2.6	3.1

Average of 3 replicates

The result showed that there was not much difference in disease development among plants of different age groups. The difference in wilting indices noticed at first assessment was due to the difference in the number of leaves in each set because wilting symptoms developed in the primary leaves of all replicates between 8 and 9 days after inoculation and when symptoms were assessed on the 9th day, only the primary leaves had wilted irrespective of the number of leaves on each plant. Symptoms in the other leaves developed suddenly; this accounts for the uniformity of the disease indices on the 12th and 15th days.

5. Effect of high humidity on symptom expression

To investigate the effect of high humidity on symptom expression, the following experiment was designed.

Ten 18 day old plants were root inoculated with mycelial suspension and planted in vermiculite in 4" pots. Five of the inoculated plants were kept inside a large polythene chamber on the greenhouse bench and the other five alongside but outside the chamber. The inside of the chamber was kept humid from a tray of water on the floor of the chamber and by periodically spraying the inner walls of the chamber.

The inoculated plants outside the chamber developed disease symptoms 7 to 9 days after inoculation and these became very severe by the 12th day, whereas symptom expression in inoculated plants inside the chamber was delayed until the fifteenth day. But with the expression of the first wilting symptom, disease development was quite rapid and most of the leaves were shed within the next four days. Yellowing was also a prominent symptom but vein clearing was not conspicuous.

Table 7 Wilting of plants under normal and humid conditions

Treatment	Days after inoculation						
	3	6	9	12	15	18	21
Normal conditions	0	0	.6	2.4	3	3.8	3.9
Humid conditions	0	0	0	0	.4	3.2	3.7

Average of 5 replicates

6. a. Relationship between foliar symptoms and infection

It was found in the foregoing experiments that foliar symptoms developed 7 to 9 days after inoculation under greenhouse conditions. The following experiment was designed to determine the extent to which these symptoms were related to the entry of fungas.

Fifteen inoculated and ten control plants were used in this experiment. Plants were inoculated by dipping the roots in mycelial suspension. Control plants had their roots dipped in water instead of in mycelial suspension. After inoculation, all plants were planted in vermiculite and kept in the greenhouse. Starting from the 3rd day and then at regular intervals of 3 days, 3 inoculated and 2 control plants were examined on each occasion to see where fungus was in the plant. This was done by using an isolation technique.

The root, stem and petioles were cut into 1" pieces and these were surface sterilized after sealing both ends with melted wax. Thin disks of tissues were cut from these pieces with a sterile scalpal and plated on solid SCA in petri dishes. They were incubated for 48 hours at 25°C and later examined for hyphae of the parasite. The results are given in Table 8.

Table 8 Isolation of fungus from different regions of the plant at various stages of infection

Days after inoculation	Root	Hypo-cotyle	Coty-ledon-ary node	First node	First inter node	Second node	Second inter node	Third node	Third inter node	Peti-oles 1 & 2	Peti-ole 3	Peti-ole 4
3	+	-	-	-	-	-	-	-	-	-	-	-
6	+	+	+	-	-	-	-	-	-	-	-	-
9	+	+	+	-	-	-	-	-	-	-	-	-
12	+	+	+	+	+	+	+	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	*	+	-

- 51 -

Average of 3 replicates

+ Fungus isolated

- Fungus not isolated

* Petioles shed

In this experiment, the fungus entered the primary root within 3 days of inoculation and in one of the replicates had reached the hypocotyl region. Nine days after inoculation, it was found up to cotyledonary node in all plants. At the time of sampling on the 9th day, primary leaves of all plants had developed pronounced symptoms but none of their petioles contained the fungus. In fact, in only one plant had the fungus reached the 1st node. Twelve days after inoculation, when most of the plants had developed severe wilting symptoms, only two plants had the fungus in the petioles of the primary leaves. Although the other leaves were rather severely wilted by this time none of their petioles contained fungus.

From these results, it appeared that symptoms developed well in advance of the pathogen and that extensive invasion of the host was delayed to a late stage of infection.

6. b. Relationship between foliar symptoms and vascular distribution of fungus

Having found that the fungus may enter the plant as early as 3 days after inoculation, it was thought necessary to investigate the extent to which vessels or other tissues were invaded and its relationship with foliar symptoms.

Eighteen root inoculated and eighteen control plants were used. Plants were inoculated by the usual method, and then grown in the greenhouse. Sampling started 3 days after inoculation and continued until the 18th day at intervals of 3 days. Three each of inoculated and control plants were taken on each occasion. As before, plants were cut into short segments and after surface sterilization bits of tissues from each were plated on SCA to see if fungus was present. The remaining plant material was preserved in FAA for sectioning and also in buffered gluteraldehyde fixative for microtome sectioning.

Free hand sections of materials preserved in FAA were taken with a razor blade after bringing the material to water through alcohol-water mixtures. The sections were washed in lactophenol and stained with cotton blue in lactophenol (McLean and Cook, 1952), while warming over a gentle flame for 30 seconds. After staining, they were again washed twice in pure lactophenol and then mounted in Euparol. The slides were examined microscopically and the percentage of vessels occluded by hyphae are shown in Table 9.

Table 9 Percentages of vessels with hyphae at different regions of the plant

Days after inoculation	Root	Hypocotyle	Cotyledonary node	First node	First internode	Second node	Second internode	Third node	Third internode	Fourth node	Fourth internode	Apex	First and second petiole	Third petiole	Fourth petiole
3	10	4	0	0	0	0	0	0	0	0	0	0	0	0	0
6	11	7	0	0	0	0	0	0	0	0	0	0	0	0	0
9	20	18	13	8	3	0	0	0	0	0	0	0	0	0	0
12	33	34	35	31	17	24	9	15	10	0	0	0	7	0	5
15	54	43	46	38	31	30	16	19	15	10	0	0	*	6	7
18	67	84	83	78	54	50	44	46	36	28	4	0	*	*	7

* Petioles shed

Average of 3 replicates

As there was a possibility of fungus being lost during free hand sectioning, the material was examined by microtome sections. Before dehydrating, material was washed thrice for 15 minutes each time in 0.1M phosphate buffer solution at pH 6.8. Dehydration was done slowly in water-alcohol mixtures and finally in absolute alcohol. After clearing and infiltration, they were embedded in paraffin. Sections were cut in thickness varying from 10 to 24 [illegible] with a cambridge microtome. A few sections were stained in safranin-fastgreen and the rest in acid-pianese stain. After staining and clearing they were mounted in Canada Balsam and examined under the microscope. The results are summarised in Table 10.

Table 10 Percentages of vessels with hyphae at different regions of the plant

Days after inoculation	Root	Hypocotyle	Cotyledonary node	First node	First internode	Second node	Second internode	Third node	Third internode	Fourth node	Fourth internode	Apex	First and second petiole	Third petiole	Fourth petiole
3	7	2	0	0	0	0	0	0	0	0	0	0	0	0	0
6	10	6	0	0	0	0	0	0	0	0	0	0	0	0	0
9	18	17	14	5	0	0	0	0	0	0	0	0	0	0	0
12	20	30	34	31	19	21	4	11	10	0	0	0	8.5	0	3
15	60	42	40	35	31	30	15	15	13	7	0	0	*	5.5	4
18	66	87	80	72	52	48	41	18	32	29	9	0	*	*	7

Average of 3 replicates

* - Petiole shed.

These observations showed that the number of vessels containing hyphae was very similar to the figures given in Table 9. Hyperplasia was found in a few sections, but this is described later.

The results of these studies with free hand and microtome sections can be summarized as follows.

1) At least 80 percent of the vessels are free of fungus when the primary leaves had developed clear wilting symptoms; 8 to 9 days after inoculation.

2) Wilting of leaves started in advance of invasion by the pathogen and petiolar vessels were almost free of pathogen at this stage.

3) The pathogen invaded the petiole only at a late stage of infection; the maximum percentage of petiolar vessels invaded was 7.

4) The fungus was confined to xylem vessels until at a late stage of infection.

These observations posed the question as to whether obstruction in 20 per cent vessels by hyphae could cause wilting, particularly in view of the fact that even in invaded vessels only some 5 per cent had the lumen completely occluded by mycelium. In view of the findings of Ludwig (1952), Waggoner and Dimond (1954), Threlfall (195[illegible]),

and Sinha (1964), it seems unlikely that this amount of occlusion by hyphae can itself be responsible for wilting.

Occlusion of petiolar vessels is more important than similar occlusion in the stem. Dimond and Edgington (1960) have shown that in *Fusarium* infected tomato plants, when 50 per cent of the lumen vessels of petioles was occluded by hyphae, the pressure required to maintain a steady flow of water was at least 500 times more than that required when the vessels of stems are occluded to this extent. In this case, the maximum percentage of petiolar vessels occluded at any time was 7 and that at a late stage of infection.

6. Relationship between vascular distribution of fungus and discoloration of vessel walls

c. Free hand sections of the material used for studies on distribution of fungus were used in these studies. The sections were stained in 0.1 per cent (aqueous) safranin 'O' followed by picro-aniline blue after the method of McLean and Cook (1952). The hyphae appeared bluish; the brown walls of vessels, bluish grey and the unaffected walls, pinkish.

It was found (Figures 1, 2 and 3) that there was a close relationship between the presence of hyphae and vascular discoloration. Discoloration of walls was

RELATIONSHIP BETWEEN VASCULAR DISTRIBUTION OF FUNGUS AND DISCOLORATION OF WALLS.

VESSELS WITH DISCOLORED WALLS

VESSELS CONTAINING FUNGUS.

Fig.1

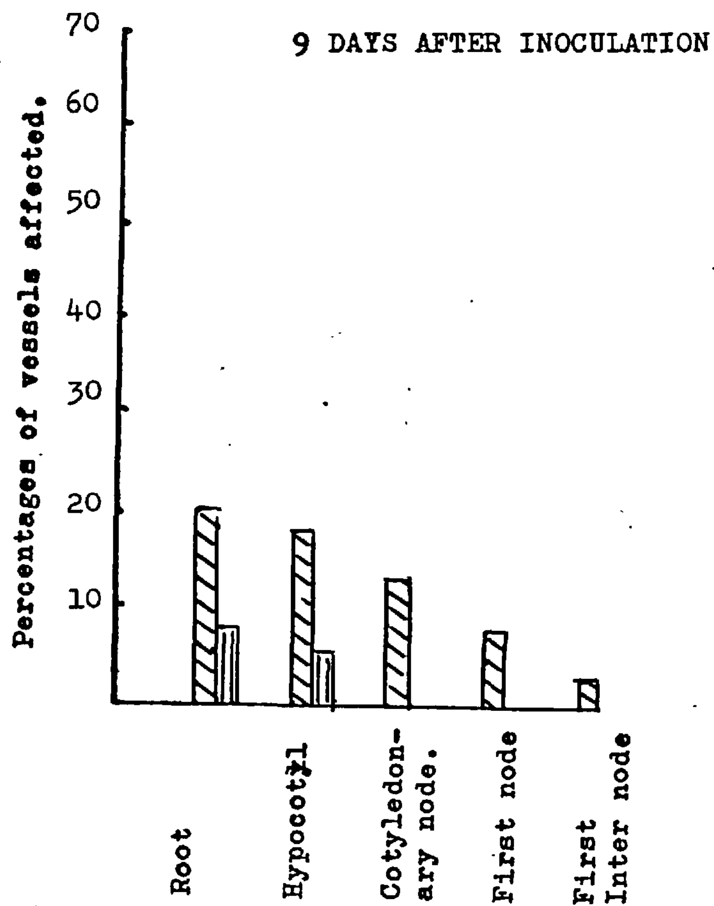


Fig.2.

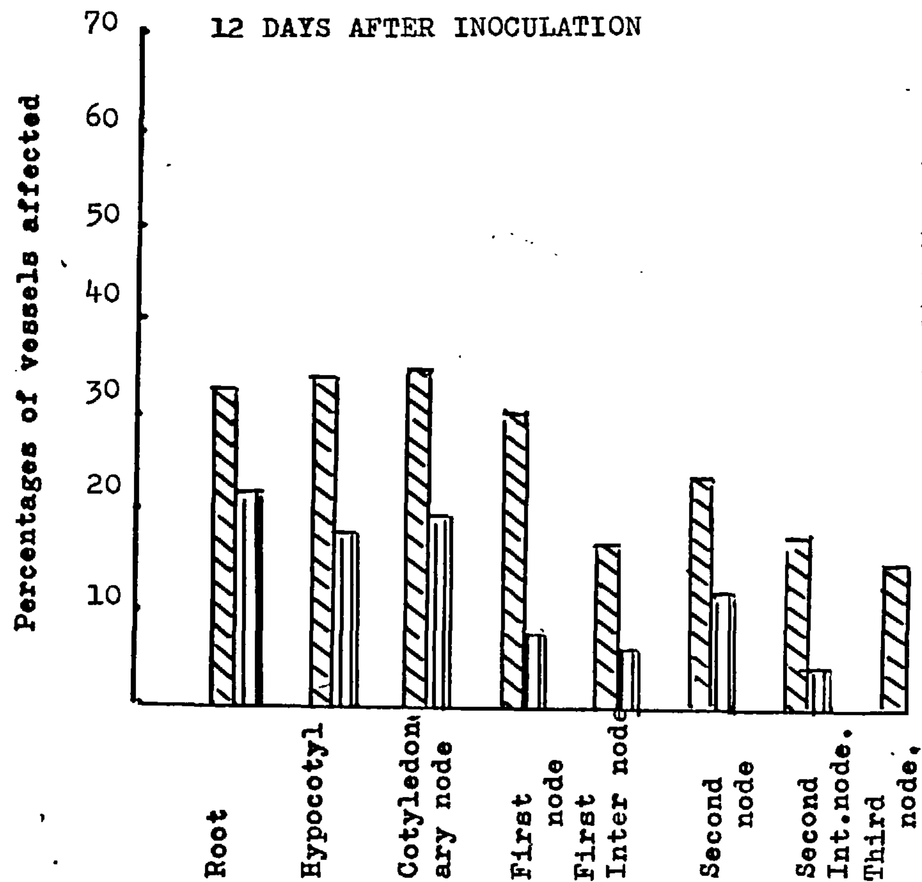
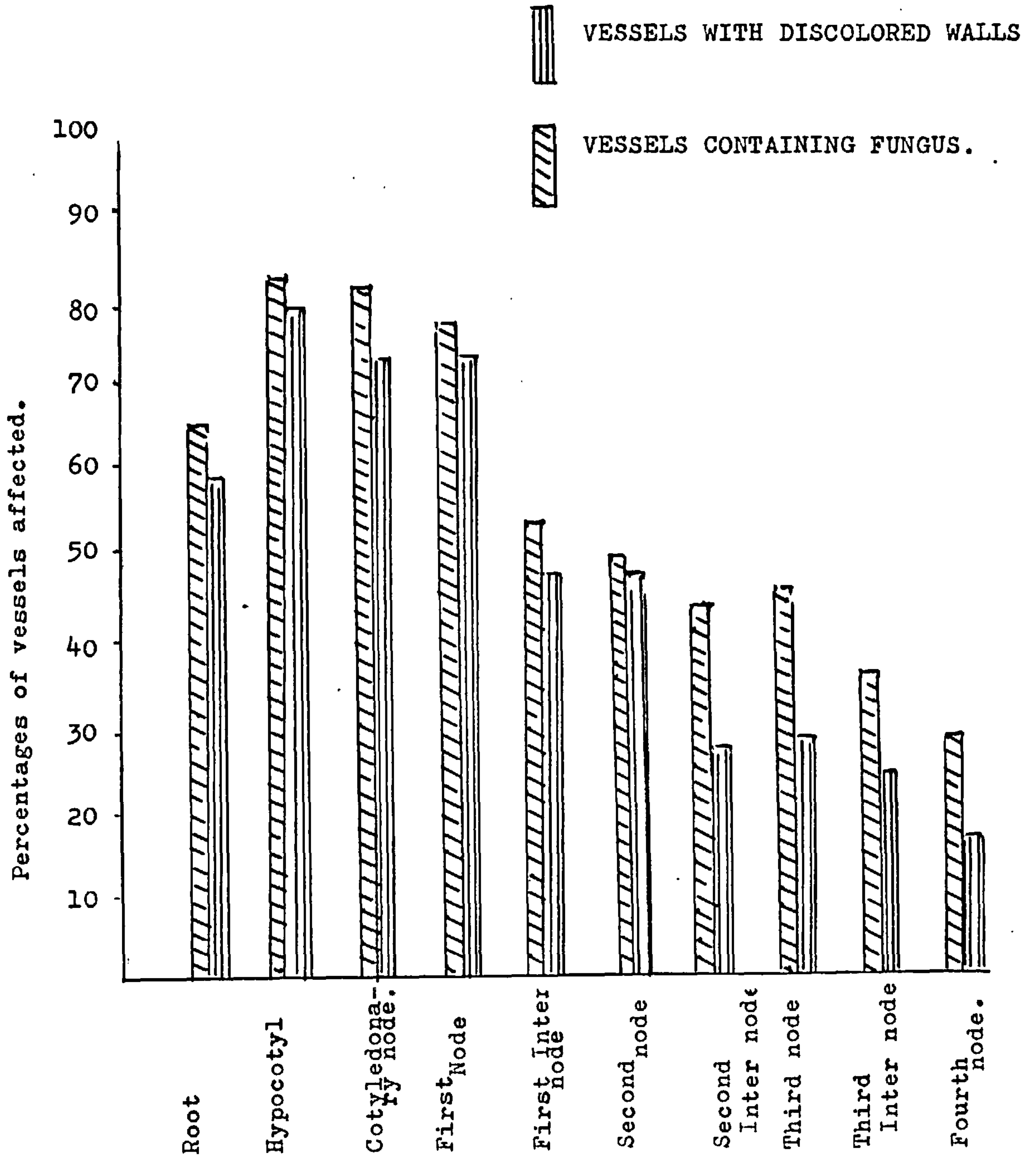


Fig.3

RELATIONSHIP BETWEEN VASCULAR DISTRIBUTION OF FUNGUS AND DISCOLORATION OF WALLS

18 DAYS AFTER INOCULATION.



seen only in vessels occupied by the fungus and it occured after the fungus had become established in the vessel. The first occasion when discoloration of walls was observed, was 9 days after inoculation, when wilting of leaves was already apparent. The percentage of vessels that had discolored walls at this stage was 9. But in material collected 18 days after inoculation, all vessels containing hyphae had discolored walls. The high percentages of vascular discoloration at this stage is not very significant because the infected plant had already wilted, almost completely by this time.

Discoloration of vessel walls is a generally recognised feature of vascular infection. In *Fusarium* infected potato and tomato plants Davis *et al.*, (1953), it to liberation of phenols from conjugated forms by hydrolytic enzymes and oxidation by polyphenol-oxidases are present in healthy stems and these oxidise the phenols and the latter would polymerize to form pigments. Mace (1963), studied the phenols of healthy and *Fusarium* infected banana and pointed out that phenols occur in a free unconjugated form, localised in cytoplasmic masses in parenchyma cells. Following infection, both fungal and host polyphenoloxidases and possibly

oxidation of the phenol and discoloration occurs. The discoloration products then diffuse into xylem vessels.

Whatever be the cause of discoloration of vessel walls, it is evident from the above studies that in Fusarium infected beans, the discoloration occurs only in walls containing hyphae.

6. Relationship between foliar symptoms and other anatomical abnormalities in the infected plant.

d. For these studies, microtome sections of plant materials used in the above experiment and free hand sections of fresh plant materials were used.

d 1. Hyperplasia

Microtome sections of root, stem and petiole taken from different regions of the plant and at various stages of infection were double stained with safranin-fast green, (Jensen, 1962). Cuticle and lignified cell walls appeared red and the other structures green. After staining and clearing, they were mounted in canada balsam. It was found that no abnormal structural changes took place in infected plants until a late stage of infection. However, in samples collected on the 12th day and after, hyperplasia was found in the hypocotyl and first and second internodal regions of infected plants. No such change was seen at any stage in any of

the petioles. In sections of plants from the 15 and 18 day samples, a dark brown substance was seen in the intercellular spaces in the vascular tissues 4). Chambers and Corden (1963) found similar substances in *Fusarium* infected tomato plants and suggested that such substances would disrupt the connections between vessels and adjacent parenchyma cells. In some cases, many of the xylem parenchyma cells were also seen to be fi[illegible] with a black substance.

d. 2. Tyloses

Free hand sections of fresh plant material were used. The plants were grown and inoculated by the usual method. Fifteen inoculated and fifteen control plants were used. They were sampled at 3 day intervals after inoculation and sections were stained with picro-aniline blue. After mounting in 50 per cent glycerine they were examined under the microscope. The results showed that tylose formation was not a prominent feature of this disease, especially at the early stages of infection. In sections of 9 day old infected plants, they were rarely found. However, they were found in fairly large numbers in infected plants after 12 days when the disease had become very severe. They were found in the hypocotyle and internodal regions, but not in any of the petiolar vessels.

Tylose formation is generally considered as a defense mechanism in infected plants. Wardlaw (1930) and Linford (193[illegible]) found in *Fusarium*-infected banana and pea plants a limited distribution of parasites in the resistant varieties because of tyloses. Sinha (1964) found more tyloses in a resistant than in a susceptible variety of tomato when infected by *Verticillium albo-atrum*. Similar observations were made by Rishbeth (1957) for banana, and McClure (1950) for potato. In view of this, the low incidence of tyloses in the early stages of infection of bean plants is what that would be expected because of the high susceptibility to infection.

6. 3. *Gels and gums*

Gels and gums are reported to be of frequent occurence in many vascular wilt diseases. Ludwig (1952) and Pierson *et al.*, (1955) reported gels and gums of pectinaceous orgin in *Fusarium*-infected tomato plants. Scheffer and Walker (1953), Gothoskar *et al.*, (1955) and Waggoner and Dimond (1955) also found gels in *Fusarium* wilt of tomato plants and suggested that macerating and pectic enzymes liberate fragments of pectin into the lumen of vessels where they accumulate to form plugs. Gels of similar nature have been described in *Fusarium*-infected banana roots (Beckman *et al.*, 1962)

and cabbage wilt by _Fusarium_ conglutinans (Melhus _et al_., 1924).

To investigate whether substances such as gels or gums are present in infected bean plants, free hand sections from different regions of the plant in various stages of colonization were stained in an aqueous solution of ruthenium red (1:5000 w/v). After staining and two subsequent washings in water, they were mounted in 50 per cent glycerine for immediate observation. (It was assumed that substances of pectic nature would stain red with ruthenium red).

It was found that there was not much difference between the vessel walls of healthy and infected plants in their reaction to ruthenium red. This result was confirmed by staining sections with hydroxylamine-ferric chloride, adapting the method used by Reeve (1959), as described below.

Free hand sections of root and hypocotyles of healthy and infected plants were placed in alkaline hydroxylamine solution for 3 to 5 minutes. Then an equal volume of a solution of 1 part concentrated hydrochloric acid and 2 parts of 95 per cent alcohol was added. The sections were then washed in 10 per cent ferric chloride in 60 per cent alcohol containing 0.1N.

hydrochloric acid. The sections were then mounted in 50 per cent glycerine.

The results showed that gels and gums were not formed in sufficient amounts to block the vessels. The maximum proportion of vessels showing gels or gums was less than 10 in plant samples after 9 days when they were showing typical wilt symptoms.

7. Disease development in cuttings from root inoculated plants

In earlier experiments, it was found that root inoculated plants developed wilting symptoms 7 to 9 days after inoculation, under greenhouse conditions. The next experiment was designed to see whether shoots would still develop symptoms, if they were removed during this period and grown in nutrient solution. It was thought that if these shoots did develop symptoms, the cause of wilting could be something other than mere occlusion of vessels by hyphae or some other products because it had already been found that the proportion of vessels occluded in infected plants, 9 days after inoculation was less than 20 per cent.

Fifteen inoculated and fifteen control plants (10 day-old) were used in this experiment. Root inoculation was done by the usual method and plants

were grown in vermiculite in the green-house. Six days after inoculation and at 3-day intervals afterwards 3 inoculated and 3 control plants were cut at ground level with a wet razor blade for microscopic examination, cuttings were placed in half-strength Long Ashton nutrient solution in 160 ml. bottles and then kept in lighted cabinets at 22°C. They were examined daily for wilting symptoms. The results are given in Table 11.

Table 11 Wilting indices per leaf of cuttings from root inoculated plants

Time of sampling (Days after inoculation)	Days after sampling										
	1	2	3	4	5	6	7	8	9	10	11
6	0	0	.3	.5	.4	0	0	0	0	0	0
7	0	0	.6	.75	.5	.4	0	0	0	0	0
8	0	0	.8	1.2	1	.75	.25	0	0	0	.4
9	.5	1	1.4	1.5	1.2	.75	.25	0	0	0	.5
10	.75	1	1.4	1.5	1.5	1	.75	.5	.5	.75	1

Average of 3 replicates

Free hand sections of stem segments removed from the bases of cuttings were examined for hyphae, tyloses and occluding substances using stains given in experiment 6.

The results of these findings are given in Table 12.

Table 12 Occlusion of vessels in the bases of cuttings from root inoculated plants.

Time of sampling (Days after inoculation)	Percentages of vessels occluded with:		
	Hyphae	Tyloses	Gels or Gums
6	7	0	3
7	8	0	3
8	11	0	3.5
9	12	3	7
10	18	8	9

Average of 3 replicates

One of the most striking observations made in this experiment was that cuttings taken after 6, 7 and 8 days after sampling developed typical wilt symptoms 2 to 5 days after sampling. Some control plants also showed a little wilting within few hours after sampling, but they all recovered within 24 hours. Nine and ten day cuttings that had already started wilting at the time of sampling continued to wilt for the next few days. But all cuttings except one in the 10th day samples recovered between 5 and 8 days after sampling, though eventually all developed typical disease symptoms again after 7 to 10 days.

It would seem from the results in Table 12 that the wilting in these cuttings could not have been caused by occlusion of vessels by the fungus or its products. An alternative explanation is that some substances that had already been formed in the lower regions of the infected plant when the pathogen had become established might have entered the leaves and caused them to wilt. As the supply of this was cut off when the shoot was severed from the plant, the leaves recovered and it took quite some time for the hyphae in the cuttings to grow and produce sufficient of the substance to make the cutting wilt again.

8. Disease development in cuttings inoculated with spore suspension

This experiment was designed to find whether cuttings inoculated through cut ends with spore suspension would respond to infection in the same way as would root inoculated plants.

Twenty-five cuttings from 15 day old plants were used. Spore suspension was prepared as described under materials and methods in four concentrations: 0.5×10^5; 1×10^5; 5×10^5; 1×10^6. Quantities of 4 ml. were placed in calibrated tubes of 10 ml. capacity. Cuttings were taken at ground level with a wet razor blade and after trimming 5 mm. from the base under water they were placed in the spore suspensions. Three cuttings were used for each strength of spore suspensions. Controls were placed in heat inactivated spore suspension containing 1×10^6 spores per ml. The cuttins were kept in the constant temperature cabinet for 4 to 6 hours until each cutting had taken up 1 ml. of the suspensions. Afterwards the cuttings were removed from the suspensions and 1 cm. was trimmed off their bases

which were then washed under running water before transferring to 160 ml. bottles containing ½ strength nutrient solution. All cuttings were then kept in the constant temperature cabinet until the end of the experiment.

The cuttings were examined daily for disease development and the symptoms were assessed on the basis of wilting, as in earlier experiments.

The results of this experiment are shown in Table 13.

Table 13 Wilting indices per leaf of cuttings inoculated with spore suspensions

Strength of inoculum (spores/ml.)	Days after inoculation									
	1	3	5	7	9	11	13	15	17	19
0.5×10^5	0	0	0	0	0	0	0	0	.25	.5
1×10^5	0	0	0	0	0	0	.5	.8	1.5	1.75
5×10^5	0	0	0	.3	.5	1	2.2	2.6	3	3.5
1×10^6	0	0	.2	.5	1.4	1.9	2.8	3.2	3.8	4

Average of 3 replicates

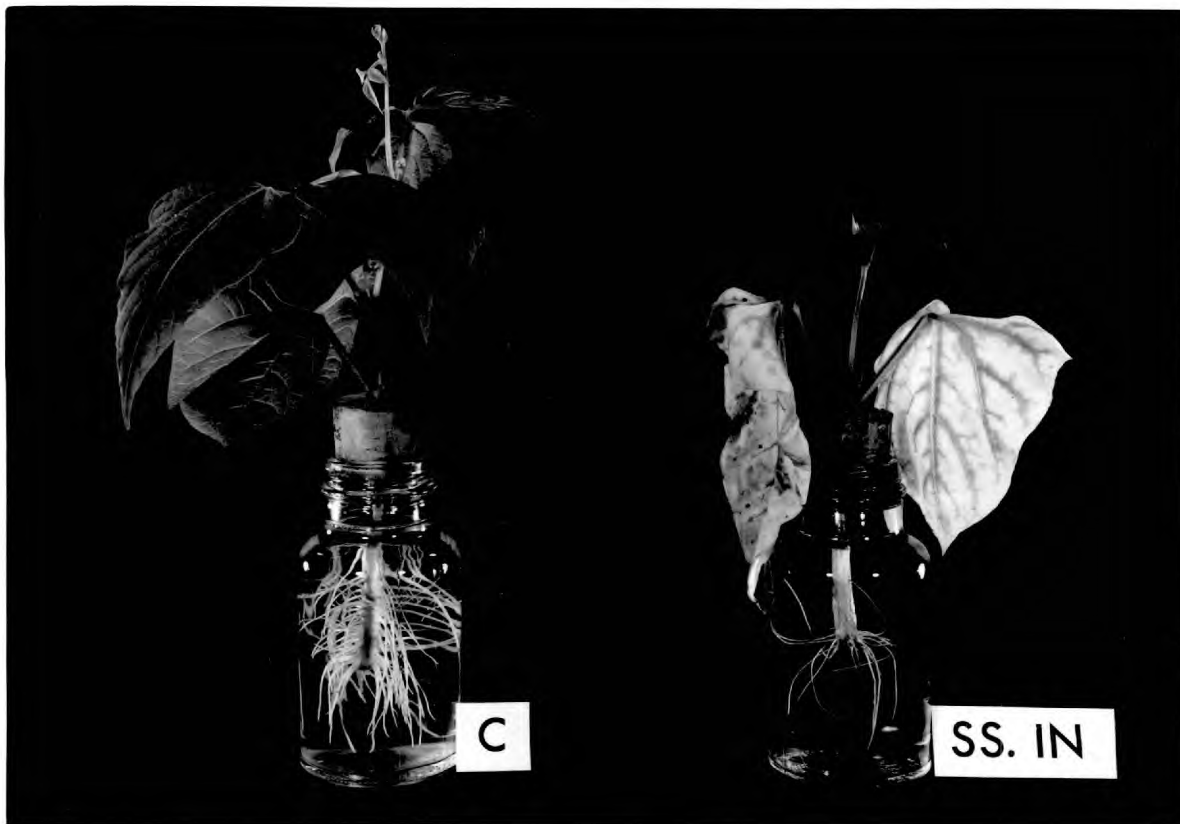


Plate 4

C - Control (Inoculated with heat inactivated spore suspension)

SS.IN - Cutting inoculated with live spore suspension.

All cuttings developed typical wilting symptoms when inoculated with spore suspensions.(Plate 4). The cuttings that had taken up 1 ml. of the suspensions containing 5 x 10^5 developed symptoms with those in root inoculated plants with conspicuous leaf yellowing, vein clearing and pronounced necrosis in the intervenal regions of the leaves. (Plate 4). The root growth was greatly reduced in inoculated cuttings and a striking feature associated with this was a general swelling of the base of the cutting. (Plate 4).

9. Effect of infection on growth of lateral roots

Reduced root growth is characteristic of most vascular wilt diseases. The experiment described below was designed to determine the effect of infection on root growth of bean plants.

Ten, 12 day old plants were cut at ground level and the cuttings were placed in half-strength nutrient solution in 160 ml. bottles and grown in constant temperature cabinet. After 7 days when a sufficient number of roots had been produced, cuttings were carefully taken out and 5 lateral roots of each were measured in length from the point of origin and labelled so that they could be identified later. Five of the cuttings were inoculated by dipping the ends of roots in a

spore suspension (5 x 10^6 spores per ml.). The other five cuttings were kept as controls and their roots were dipped in heat inactivated spore suspension. The tips of 10 roots each, of all cuttings had been removed before inoculation to facilitate infection. After inoculation, the cuttings were placed in 6" x 3" glass beakers, suspended in the centre by means of cork. The beakers were lined inside with moist filter paper. After 24 hours incubation at 22°C, the cuttings were transferred to fresh beakers containing nutrient solution and kept in the constant temperature cabinet. All beakers were wrapped with black paper to keep light from the roots. After 10 days, when typical wilting symptoms had developed, the labelled roots were separated from the stem. Their lengths were measured and the percentage reduction in the length of infected root was determined. (Table 14).

Table 14 Effect of infection on growth of lateral roots

Treatment	Average length of roots (cm.) at time of inoculation	at the end of the exp.	Percentage reduction in length
Control	5.8	16	
Inoculated	6	10.5	77

Average of 5 replicates and 25 roots.

The results showed that there was a reduction of 77 per cent in the length of roots of infected cuttings.

B. PHYSIOLOGICAL STUDIES

a) Respiration

Alteration of respiratory pattern in plants following infection by microorganisms has been reported in a number of diseases; in sweet potato infected by *Rhizopus trilici*, (Weimer and Harter, 1921) and *Ceratostomella fimbriato*, (Akazawa and Uritani, 1956); in rust and mildew diseases of cereals (Shaw and Samburski, 1957); in tobacco plants infected by *Pseudomonas solanacearum* (Maine *et al.*, 1959) and in mildew disease of barley (Bushnell and Allen, 1962). There are also reports of enhanced respiration following infection by *Fusarium oxysporum*. Collins and Scheffer (1958) found that respiration of leaves of tomato plants increased considerably following infection by *F. oxysporum* f. *lycopersici*; Scheffer (1960) noticed that *Fusarium* infected tomato plants had a high respiratory rate and that the increase was associated with symptom development. Similar increases are reported for cabbage (Keitefuss *et al.*, 1960). In tomato plants infected by *F. oxysporum* f. *lycopersici* Kuo and Scheffer (1964), found that the respiration of leaves increased gradually following inoculation and remained relatively high until symptom development

and then fell suddenly.

It is known that increase in the rate of respiration is one of the important metabolic changes occurring in plants following infection by microorganisms. Whether this change has a direct role in inducing symptom development is still unknown. Collins and Scheffer (1958) found that the infection of tomato plants by *Fusarium oxysporum* f. *lycopersici* increases Co_2 production and O_2 uptake by non symptomatic leaves that do not contain fungus. The fact that respiratory changes occur in host tissues away from the region of infection and before significant symptoms develop suggests that some diffusible metabolite is involved. In this connection, Allen and Goddard (1938) working on mildew diseases of wheat concluded that the increase is caused by diffusible substances coming from the parasite.

In view of the above reports, a number of experiments were done to see whether increased respiration is also a feature of bean plants infected with *Fusarium oxysporum*.

1. Effect of Infection on Respiration of Root Inoculated Plants

In earlier experiments it was found that fungus was present in the vessels of the stems of infected plants 72 hours after inoculation. As the presence of the fungus is likely to alter the rate of respiration of the infected region only leaf tissues that are not invaded by the fungus at any time during the course of the disease were used in the experiments described below.

Eighteen-day old plants were inoculated by dipping the roots in a mycelial suspension. The plants to be grown as controls had their roots dipped in water instead. Both types were planted in 4" plastic pots containing vermiculite and watered with Long Ashton nutrient solution as required. As all plants showed transient wilting for the first 24 hours because of the disturbance caused by transplanting, sampling was postponed until 48 hours after inoculation.

By means of a Warburg apparatus, respiration of three replicates of inoculated and control plants were determined daily from the second day after inoculation until first symptom development. Fifteen leaf disks, each 10 mm. diameter and cut from the primary leaves were used in each flask. The leaf disks were floated in 3 ml.

phosphate buffer solution of pH.6.4, contained in the outer vessel of the flask. The central well of the flask contained 0.2 ml. of 10 per cent KOH solution to absorb Co_2 evolved by the leaf tissue. Each flask was wrapped in aluminium foil to exclude light. The temperature of water in the bath was 25°C. Manometer readings were taken at 30 minute intervals for 2½ hours. The leaf disks were then removed and dried for 48 hours in an oven at 70°C. Respiration rate was calculated as ul. oxygen uptake/mg. dry wt. of leaf tissue.

Table 15. Respiration of healthy and root inoculated plants. (O_2 uptake ul./mg. dry wt./hour).

Days after Inoculation	Healthy	Inoculated	Percentage Increase or reduction - inoculated
4	3.6	4.1	+ 13.9
5	3.8	4.5	+ 18.4
6	3.5	4.5	+ 28.5
17	3.6	5	+ 38.8
8	3.5	2.9	- 17.1

Average of 3 replicates

Fig. 4

RESPIRATION OF HEALTHY AND INOCULATED PLANTS

7 DAYS AFTER INOCULATION

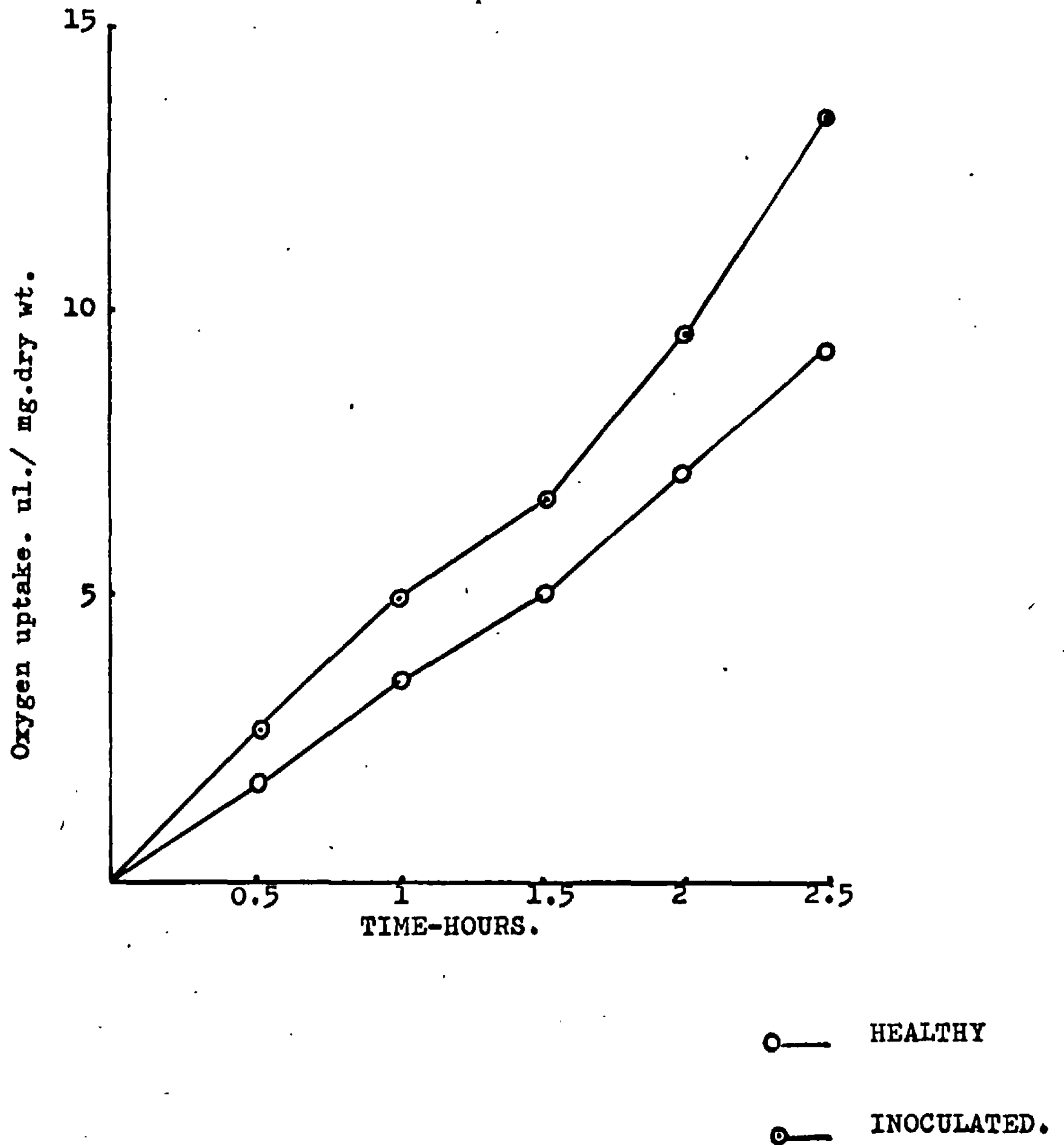
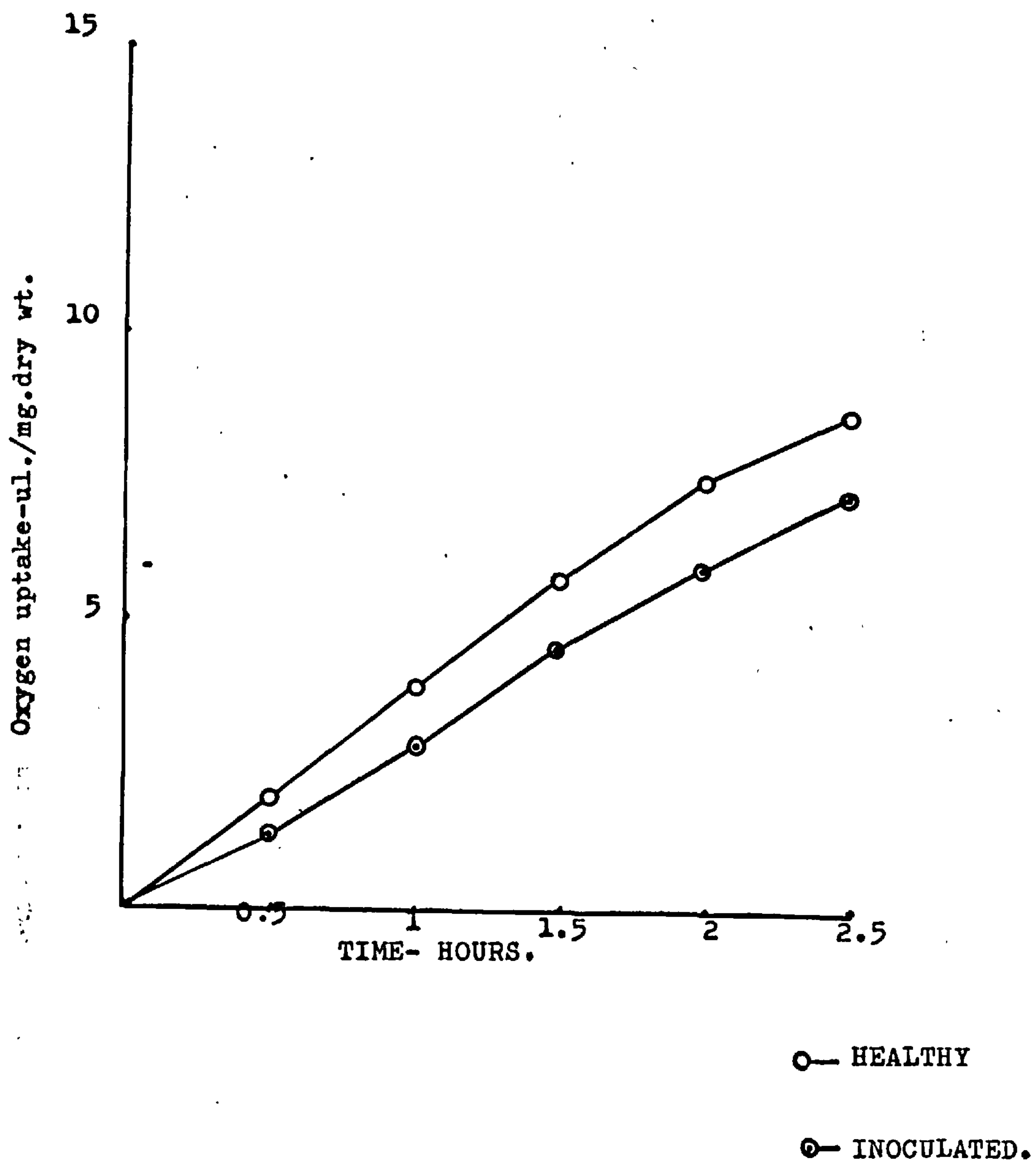


Fig.5

RESPIRATION OF HEALTHY AND INOCULATED PLANTS

8 DAYS AFTER INOCULATION



The results of these experiments (Table 15 and Figure) show that there is a gradual increase in the rate of respiration in the infected plant starting from the fourth day after inoculation. This increase reached a peak 7 days after inoculation and then respiration fell suddenly with the appearance of the first symptoms of wilt in the primary leaves.

2. Effect of Infection on Respiration of Inoculated Cuttings

Bean cuttings, 18-days old and about 15 cm. in height were used in this experiment. They were inoculated with spore suspensions in the way described under Materials and Methods. In earlier experiments it had been found that 18-day old cuttings developed disease symptoms similar to those of root inoculated plants when they had taken up 1 ml. of spore suspension containing 10,000 to 15,000 spores per ml. for 4 to 6 hours under conditions favouring normal transpiration in the glass house. Controls were placed in heat inactivated spore suspension for the same period. After inoculation, cuttings were taken out of the spore suspension and their bases were trimmed under water before putting into bottles containing half strength nutrient solution. All cuttings were kept in an illuminated constant temperature cabinet at $22^{o}C \pm 1^{o}C$.

The procedure adopted for root inoculated plants was followed in measuring respiration rate of cuttings. Sampling started 48 hours after inoculation; all cuttings had recovered from the transient wilting following removal from the plant by that time. Disks of leaves from three infected and three healthy cuttings were used at each sampling. Only disks from symptomless leaves were used. The experiments were done for six days after inoculation; by this time most of the remaining cuttings had developed slight wilting or epinasty. The results are shown in Table 16 and Fig. 4+5

Table 16 Respiration of Healthy and Spore Inoculated Cuttings (O_2 uptake ul./mg. dry wt./hour)

Days after Inoculation	Healthy	Inoculated	Percentage increase or reduction - inoculated
3	3.8	3.9	+ 2.8
4	3.7	4.1	+ 18.1
5	3.7	5.1	+ 37.8
6	3.9	2.5	- 35.8

Average of 3 replicates

Fig.6

RESPIRATION OF HEALTHY AND SBORE INOCULATED CUTTINGS

5 DAYS AFTER INOCULATION.

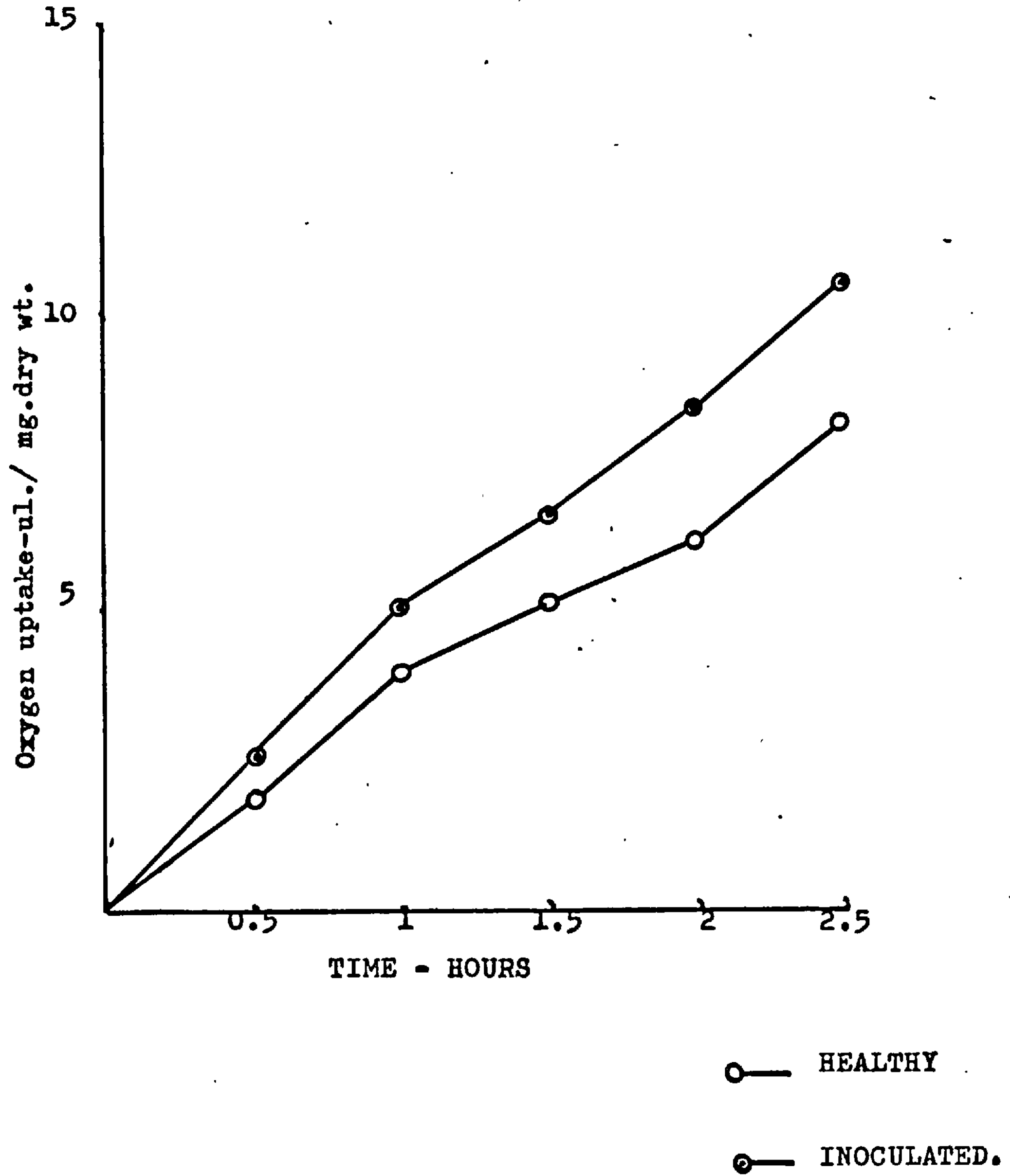
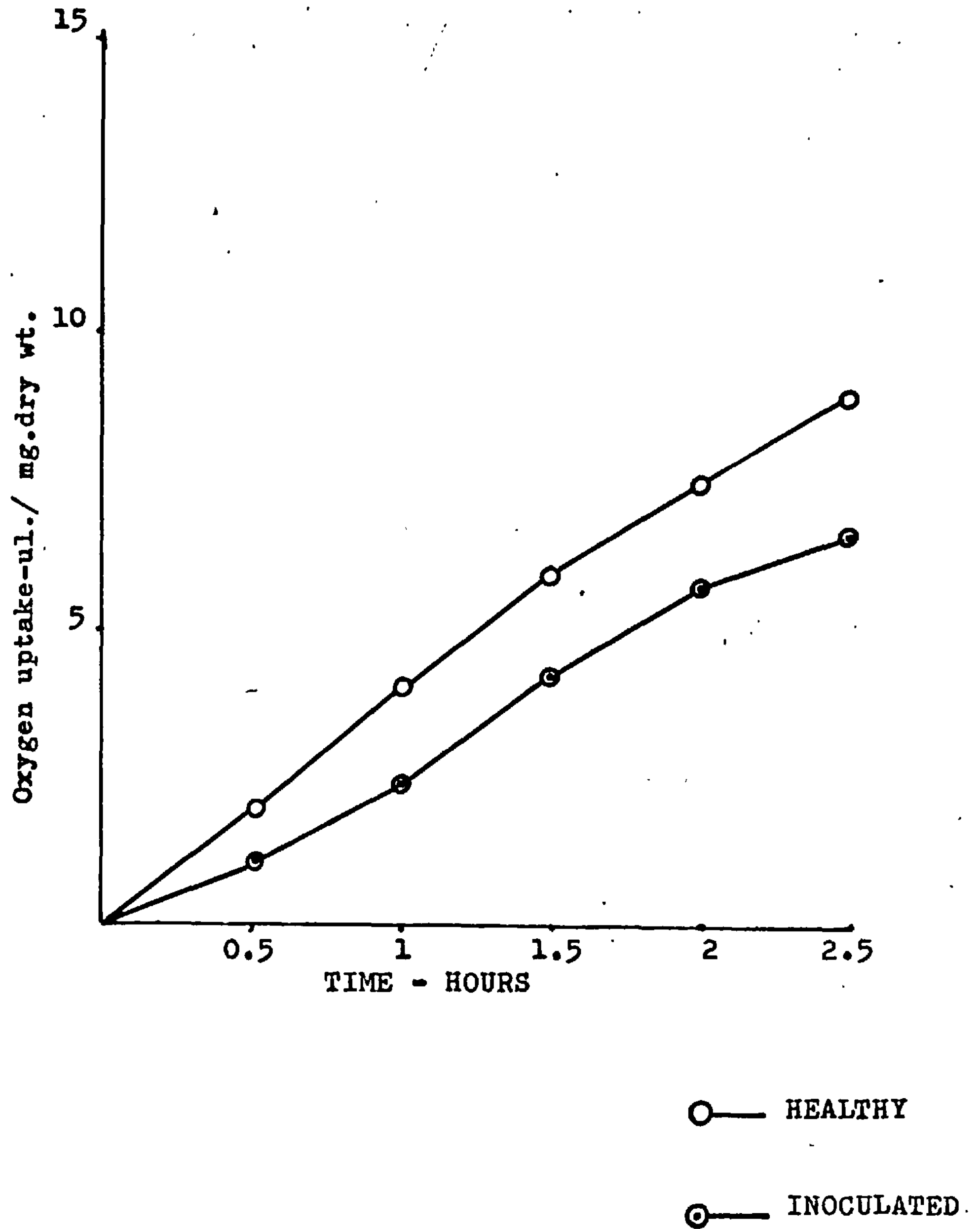


Fig.7

RESPIRATION OF HEALTHY AND SPORE INOCULATED CUTTINGS

6 DAYS AFTER INOCULATION.



The results (Table 16 Figs. 6 & 7) show that the majority of inoculated cuttings respired at about the same rate as the controls for the first 3 days after inoculation. Later, differences in the rate of respiration between controls and inoculated plants became apparent. Although, there was considerable variation among the replicates in the inoculated series, all had a higher rate of respiration than the controls. The variation in the rate of respiration among the replicates of the inoculated plants may have been due to the differences in quantity of the fungus taken up.

3. Respiration of Leaf Disks in Sap From Infected Plants

It is apparent from the foregoing experiments that symptomless leaves of plants respire faster than those of healthy plants before disease symptoms develop. As this happens in tissues some distance from the parts of plants carrying the parasite in root inoculated plants, metabolites produced in the region of infection are carried by the vascular stream to the leaves and produce their effects there. If this is the case, the vascular sap of the infected plant should be toxic to leaf tissues. The following experiment was, therefore, devised to test the toxicity of the sap and the effect of the sap on respiration.

Again with a Warburg apparatus, the respiration rates of healthy leaf tissues in the sap from infected and healthy plants were studied.

The sap was collected as described under Materials and Methods from healthy and infected plants, 6 days after inoculation. Three flasks were used with 3 ml. sap, after adjusting its PH.to 6.4. Control flasks had 3 ml. phosphate buffer solution instead of sap. Fifteen leaf disks from healthy leaf tissues were floated in each flask. The central well of each flaskhad 0.2 ml. of10 percent KOH sol. to absorb the Co_2 evolved as the leaf disks respired. Each flask was wrapped in aluminium foil to exclude light during the experiment.

The experiment was run for 2.5 hours and the manometers were read every 30 minutes. At the end of the experiment, leaf discs from each flask were taken out dried saparately in an oven at 70° C. for 48 hours. The oxygen consumption was calculated as ul/mg.dry wt./ leaf tissue

Fig.8

RESPIRATION OF LEAF DISKS IN THE SAP FROM HEALTHY AND INOCULATED PLANTS.

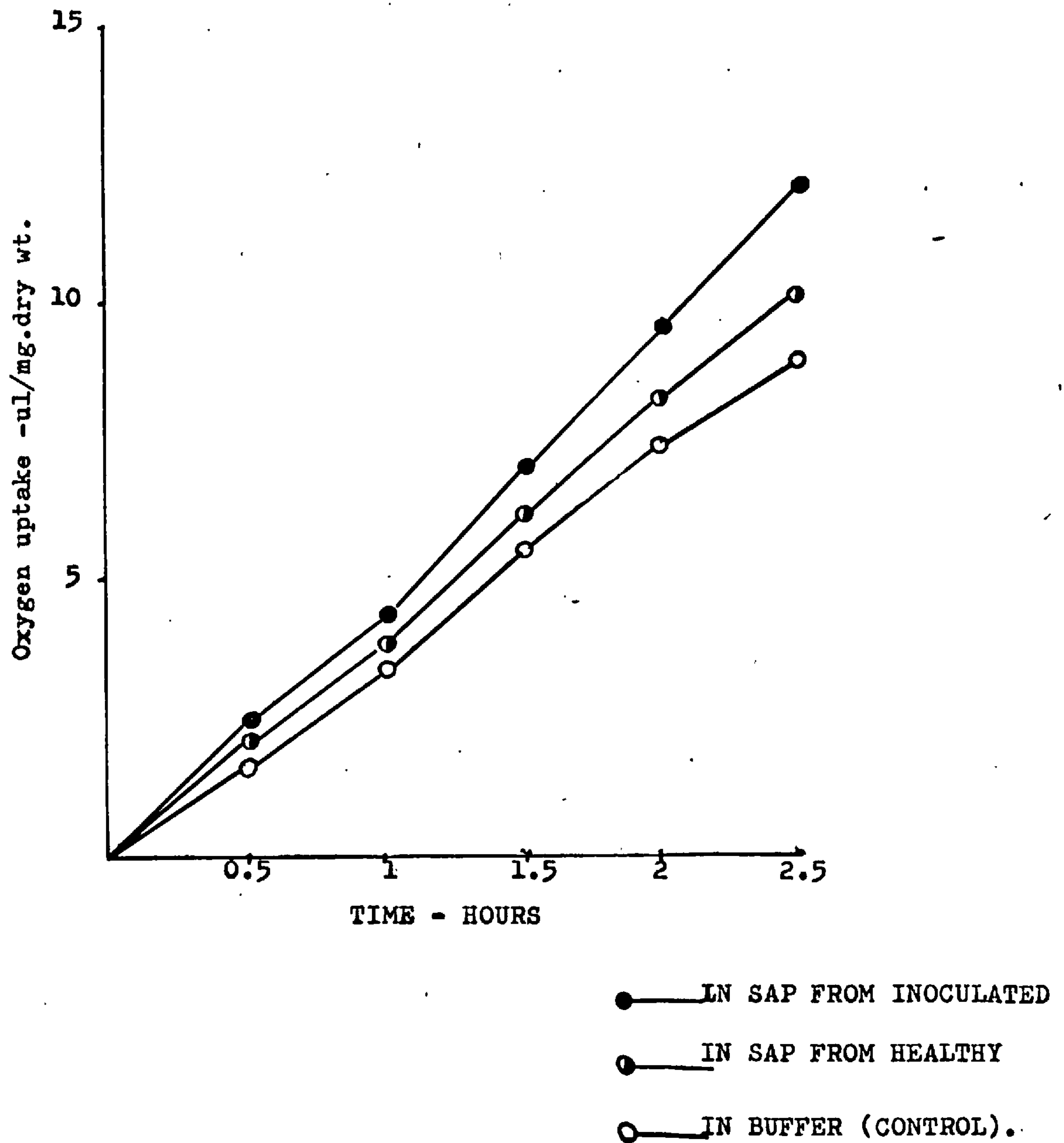


Table 17 Respiration of leaf disks in sap from healthy and inoculated plants

Time (Hours)	Control	% sap from: Healthy	% sap from: Inoculated	Percentage increase in sap from: Healthy	Percentage increase in sap from: Inoculated
0.5	1.75	1.8	2.3	2.8	3.1
1	3.6	3.8	4.2	5.5	16.6
1.5	5.5	6	7.1	9.1	29
2	7.5	8.2	9.7	9.3	29.3
2.5	9.2	10	12.4	8.7	34.7

Average of 3 replicates

The results (Table 17 Figure 8) show that leaf disks in infected and healthy sap respire at a higher rate than those in the buffer solution, and that disks in infected sap respire more than do those in healthy sap. It is probable, but not certain, that these increases in respiration are both attributable to toxic substances present in both healthy and diseased sap as used and that such substances develop in healthy sap after extraction. Gottlieb (1943, 1944) has obtained similar results and circumvonted this difficulty by preparing extracts anaerobically, a precaution not

taken in the current work. Nevertheless, inspite of this deficiency, the results show that sap from infect-ed plants contain substances stimulating respiration additional to those present in sap from healthy plants.

b.) Transpiration and Conduction

B.1. Transpiration of root inoculated plants

This experiment was designed to study the transpiration rate of root inoculated plants. Eighteen-day old plants of uniform size were selected from a large number and were inoculated with mycelial suspension. They were then planted in vermiculite in 4" plastic pots with lids and sealed bases as mentioned under 'Materials and Methods'. (Plate 1). Five inoculated and five control plants were used. Roots of the controls were dipped in water instead of in mycelial suspension. All plants were kept in the growth cabinet for 24 hours before weighing and transferring to the green house bench. Each unit was then weighed daily at 4 p.m. The leaf area was also measured daily. (Preliminary experiments showed that the primary leaves of experimental plants had reached the maximum size when they were 15 to 18 days old). As the primary leaves (1 and 2) had already reached their full size at the time of setting up the experiment, their area was measured only at the end of the experiment. The area of leaves 3, 4 and in some cases 5 were measured by choosing leaves of the same length from the check plants growing besides the experimental plants and tracing their outlines onto paper of known

weight. From this, the daily increase in leaf area of healthy and inoculated plants were determined. The experiment was conducted for 8 days until the first wilt symptoms appear in infected plants. At the end of the experiment the areas of all leaves were determined. The daily transpiration rate of inoculated and control plants are shown in Fig.9 and in Table 18.

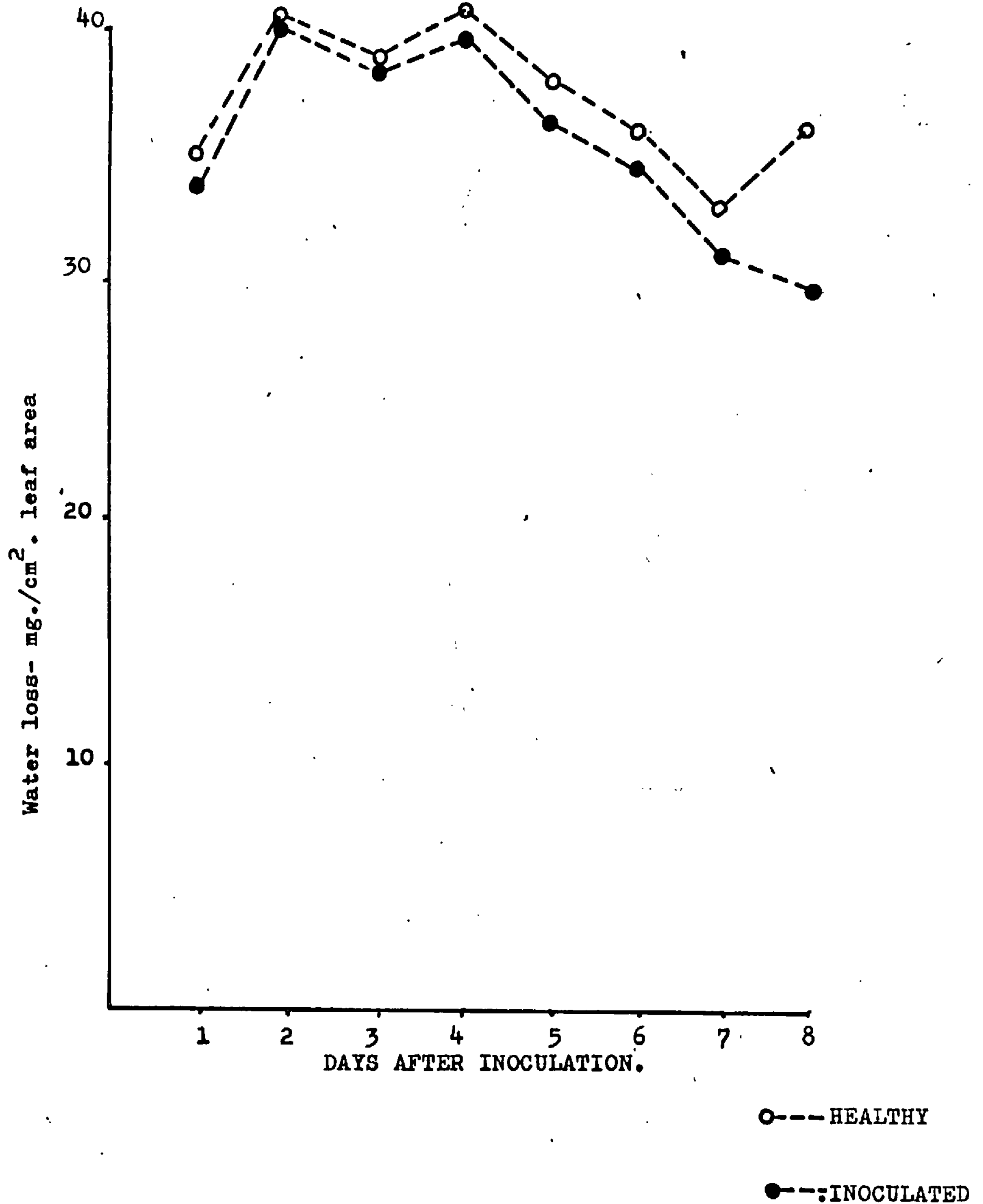
Table 18 Water loss from healthy and inoculated plants

Days after inoculation	Water loss (mg/cm^2 leaf area)		Percentage reduction inoculated
	Healthy	Inoculated	
1	35	34	2.8
2	40	39	2.5
3	39	38	2.56
4	41.5	40	3.6
5	38	36	5.2
6	36	34.5	6.7
7	34	31	8.8
8	36	29.5	18.1

Average of 5 replicates

Fig.9

WATER LOSS FROM HEALTHY AND INOCULATED PLANTS



The results of this show that there was a gradual fall in the rate of transpiration from 4 days after inoculation. With the appearance of wilt symptoms on the 8th day, there was considerable reduction in the rate of water loss, compared with the control.

A pronounced fall and rise in the transpiration rate was seen in both control and inoculated plants during this experiment.

2. Transpiration of root inoculated plants with primary leaves only

In preliminary experiments, it was found that plants with buds removed to leave only primary leaves wilted, about the same time as entire plants when inoculated with mycelial suspension. So, it was thought appropriate to study the rate of transpiration of plants with primary leaves only as this would eliminate the possible errors in estimating the rate of transpiration when a large number of continuously growing leaves were used.

The transpiration rate of 5 inoculated and 5 control plants were studied, their buds had been removed when they were 12 days old. They were root inoculated with mycelial suspension and planted in plastic pots with lid and sealed base as before. After inoculation, all units were placed in the green house. They were weighed

at the time of transferring to the green house and then daily at 4 p.m. After 8 days when the inoculated plants had developed wilt symptoms the areas of all the leaves were measured. The daily rates of water loss per unit area of healthy and infected leaves are presented in Fig. 10 and summarized in Table 19

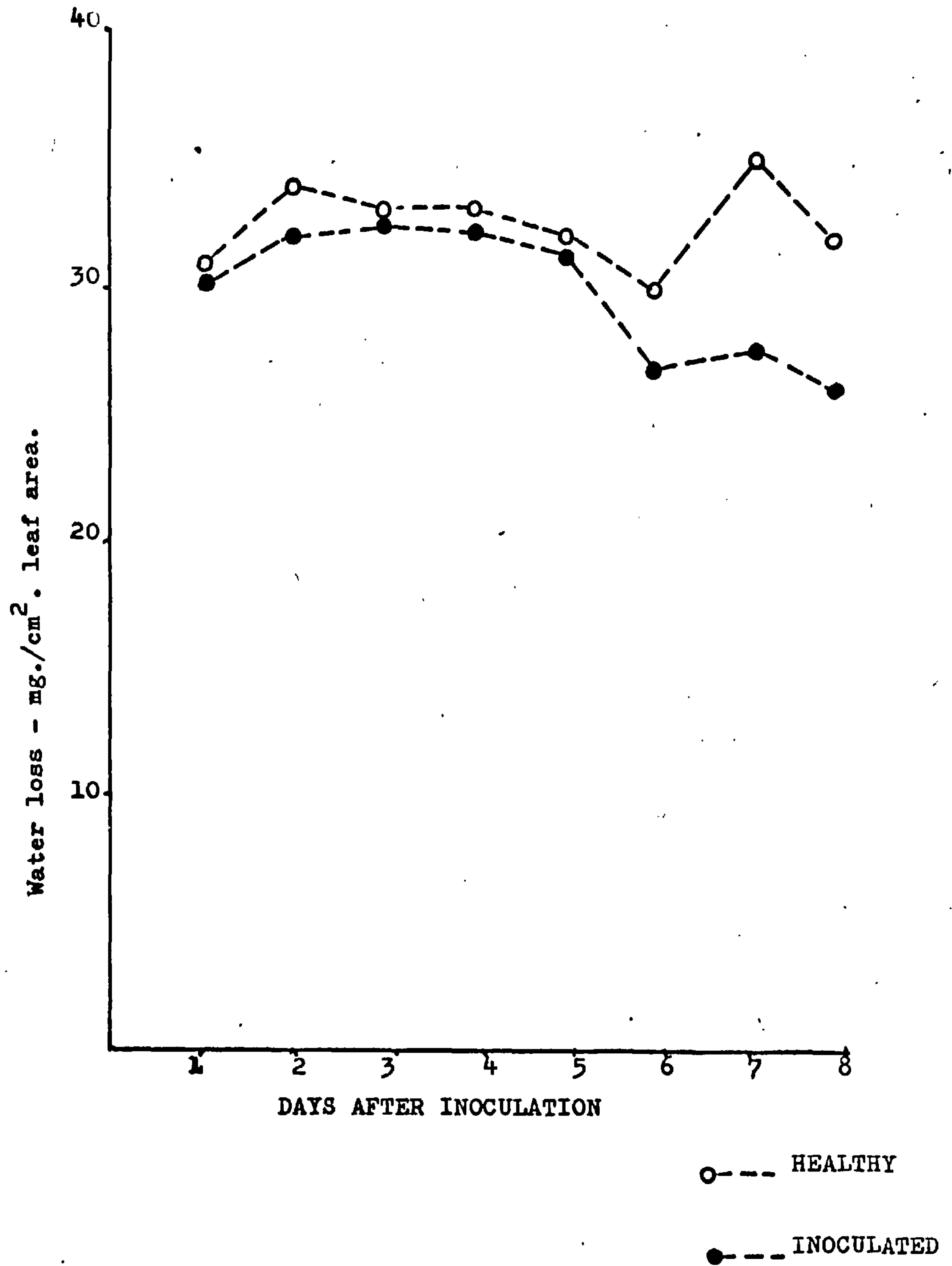
Table 19 Water loss from healthy and inoculated plants with primary leaves only

Days after inoculation	Water loss (mg/cm^2 leaf area)		Percentage reduction Inoculated
	healthy	inoculated	
1	34	30.5	1.6
2	34	32	5.8
3	33	33	0
4	33	32	3.03
5	32	31.5	4.6
6	30	27	10
7	35	27	22.8
8	32	26	18.7

Average of 5 replicates

Fig 10

WATER LOSS FROM HEALTHY AND INOCULATED PLANTS ; PRIMARY LEAVES ONLY.



The rate of transpiration of inoculated plants was lower than that of uninoculated plants throughout the experiment. There was the same somewhat erratic behaviour from day to day.

3. Transpiration of inoculated cuttings

The foregoing two experiments showed that the inoculated plants lost less water than the controls. But it was not clear whether or not this was due to reduced conduction, so the following experiment was done to investigate this.

Eighteen-day old cuttings with primary leaves only were used, with their buds removed when they were 12 days old. Twenty-four cuttings were inoculated with spore suspension (0.5 x 10^5 spores per ml.). The controls were placed in heat inactivated spore suspension. After 4-6 hours, when each cutting had taken up a ml. of the suspension, they were taken out and their bases were trimmed and washed under water. Each cutting was then placed in vials containing half strength nutrient solution and kept in the growth cabinet. From the second day, 3 inoculated and 3 control cuttings were taken out daily to measure their transpiration rates for the next 24 hours. They were placed in 30 ml. vials containing 20 ml. of water and closed with aluminium foil caps with central holes. The vials with the cuttings were

weighed and then placed in the growth cabinet. After 24 hours they were weighed again. The leaf areas were measured as before. The cuttings were taken out of the vials and the water left in each vial was measured. From the results the rate of transpiration and the rate of absorption for 24 hours for each cutting was determined. This was repeated for 8 days until pronounced symptoms appeared in the inoculated cuttings. The results are presented in Fig. 11 and Table 20.

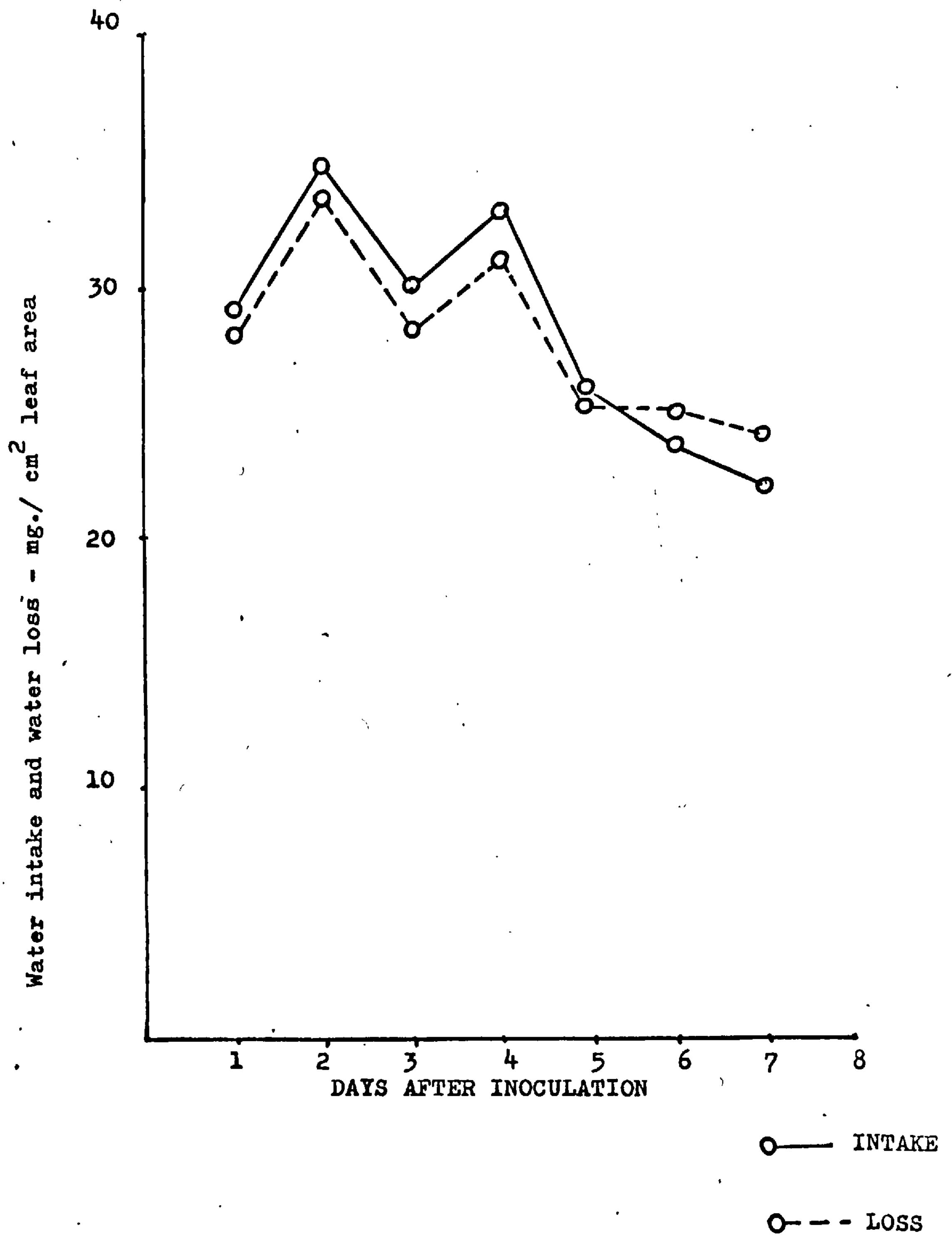
Table 20 Water intake and water loss of healthy and inoculated cuttings

Days after inoculation	Healthy			Inoculated		
	Intake	Loss	Loss as percentage of intake	Intake	Loss	Loss as percentage of intake
1	31	30	96.8	29	28	96.6
2	35	34	97.2	35	33.5	95.8
3	31	29	93.6	30	28	93.4
4	34	33	97.1	33	31	93.9
5	28	27	96.5	26	25	96.2
6	30	28.5	95	26.5	25.5	96.3
7	34	32	94.2	24	25	104.1
8	32	31	96.9	22	24	109.1

Average of 3 replicates

Fig. 11

WATER INTAKE AND WATER LOSS OF INOCULATED CUTTINGS



The results show that there was a gradual reduction in the rate of transpiration as well as in the rate of absorption in the inoculated cuttings. But, the reduction in the rate of transpiration was not paralleled by reduction in the rate of absorption. At no time during the course of the experiment did the transpiration rate of inoculated cuttings exceed that of controls, but the ratio of transpiration to absorption was higher for the inocaulated cuttings than it was for controls. In other words, the rate of water loss of inoculated cuttings slightly exceeded the rate of absorption just before symptom development, and this, presumably, is why the plants wilt.

4. Transpiration in detached leaves

a. Monofoliate leaves (Primary leaves)

The transpiration rate of detached monofoliate leaves from infected and healthy plants during the period between inoculation and disease development was studied in this experiment. Eighteen-day old plants with 2 monofoliate leaves and 2 trifoliate leaves were root inoculated by the usual method. After inoculation, they were planted in vermiculite and kept in the green house. There were 27 inoculated and 27 control plants. After every 24 hours, the monofoliate leaves of 3

inoculated and 3 control plants were detached from the plant for studying rates of transpiration and absorption for a 24 hour period. They were placed in 30 ml. vials containing 20 ml. of water and closed with aluminium foil caps with central holes. After weighing the vials with the leaves, they were kept in the growth cabinet. Twenty-four hours later they were weighed again. Leaves were then taken out and their areas were measured as in the previous experiments. The water left in the vials was measured and the amount taken up was determined. From the results obtained, the transpiration and conduction rate per cm^2 of leaf area were determined. This experiment was continued for 9 days until initial wilt symptoms appeared in the inoculated plants. The results are presneted in Fig.12 and Table 21.

Fig.12

WATER INTAKE AND WATER LOSS OF MONOFOLIATE LEAVES FROM INOCULATED PLANTS.

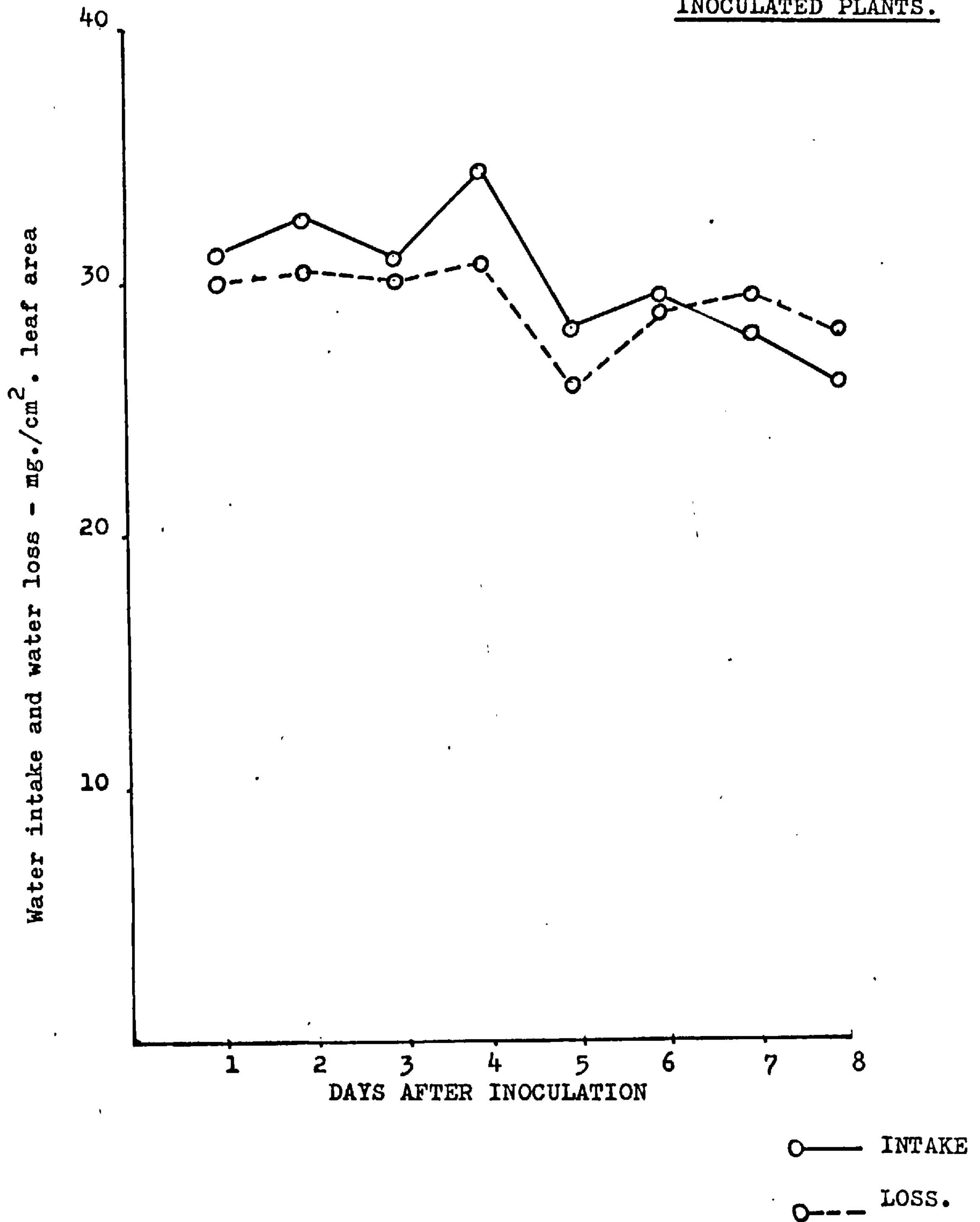


Table 21 Water intake and water loss of monofoliate leaves [illegible] healthy and inoculated plants

Days after inoculation	Healthy			Inoculated		
	Intake	Loss	Loss as percentage of intake	Intake	Loss	Loss as percentage of intake
1	3[illegible]	31	96.9	31	30	96.8
2	30	29.5	98.4	32.5	30.5	93.9
3	32	30.2	94.4	31	30	96
4	33	32.5	98.5	34	30.5	89.8
5	29	29	100	28	26	92.9
6	31	30.5	98.4	29.5	29	9[illegible]
7	32.5	31.5	97	28	29.5	105.3
8	34	33	96.9	26	28	107.6

Average of 3 replicates

1. Trifoliate leaves (ist and 2nd trifoliate leaves)

This experiment for studying the transpiration and conduction of trifoliate leaves was set up as in the previous experiment except that 21-day old plants were used, so that fully opened trifoliate leaves could be obtained. There were fifty inoculated and fifty control plants. The transpiration and conduction rates of 5 each of the 1st and 2nd trifoliate leaves were studied daily. The experiment was conducted for 10 days until initial wilt symptoms appeared in the 1st trifoliate leaves. The results obtained are presented in Fig. 13 and summarized in Table 22.

Fig.13

WATER INTAKE AND WATER LOSS OF TRIFOLIATE LEAVES FROM INOCULATED PLANTS

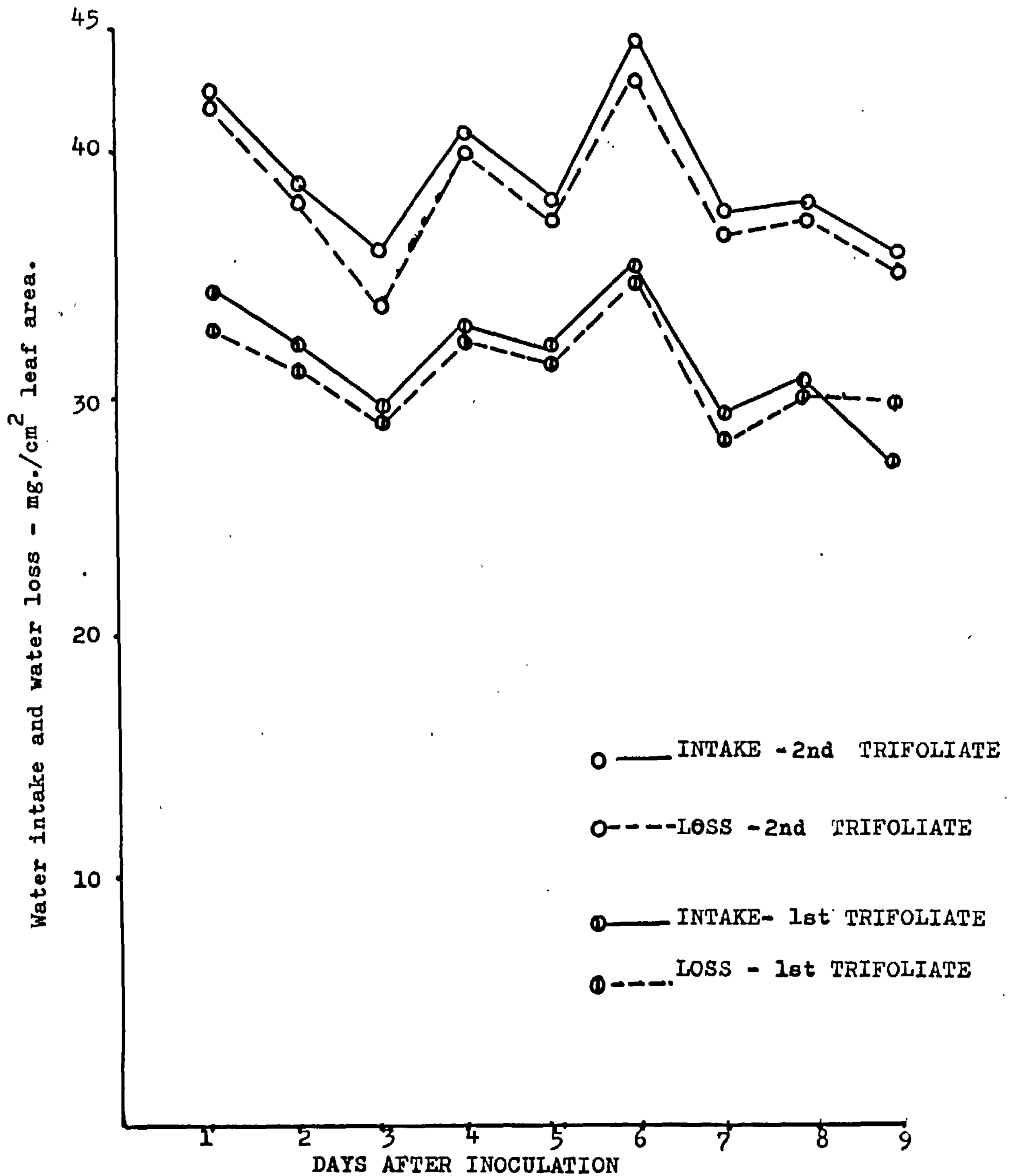


Table 22. Water intake and water loss of Trifoliate leaves from Healthy and inoculated plants.

Leaf No.	Days after inoculation	HEALTHY Intake	HEALTHY Loss	HEALTHY Loss as percentage of intake	INOCULATED Intake	INOCULATED Loss	INOCULATED Loss as percentage of intake
Tr.1	1	33	32	96.9	34	32.5	95.6
	2	31.5	30	95.3	32.5	31	95.4
	3	30	29.5	95	29.5	29	94.9
	4	34	33	97.1	35	34	97.2
	5	31.5	30.5	96.9	32	31	96.9
	6	37	36	97.3	33.5	32.5	97.1
	7	31	30	96.8	29	28.5	98.3
	8	33	32	97	30	30	100
	9	32	31.5	98.5	27.5	30	109
Tr.2	1	43	41.5	96.6	42.5	42	98.9
	2	38	37	97.4	39	37.5	96.2
	3	37	36.5	98.7	36	33.5	92.1
	4	42	40.5	96.5	41	40	97.6
	5	40	39	97.5	38	37	97.4
	6	44	43.5	99	45	43	95.6
	7	37	37.5	96.2	37	36	97.3
	8	39	38	97.5	38	37	97.4
	9	40	38.5	96.3	36	35	97.3

Tr.- Trifoliate leaf

Average of 5 replicates each

It is evident that the transpiration and absorption rates of leaves from inoculated plants gradually decreased until 7 days after inoculation, the amount of water taken up and lost corresponded very closely, though in general, slightly more water was taken up than was lost. But on the 8th day with the appearance of wilt symptoms in the primary leaves, the rate of water loss slightly exceeded the rate of absorption, though it did not exceed that of healthy leaves. A similar increase in the transpiration over absorption was noticed in the 1st trifoliate leaves also on the 9th day, prior to wilting (Fig. 13).

It is difficult to say how significant is this increase in the rate of water loss at this stage. As this happens almost simultaneously with the appearance of wilt symptoms, it cannot be regarded as the factor responsible for wilting. It is not known whether the cell permeability of the infected leaves has been affected at this stage or not.

.5. Effect of infection on conduction

In the foregoing experiments it was found that the infected plants absorbed less water than the healthy plants. This next experiment was done to see whether the decrease in absorption was due to nonfunctioning of

the vessels and if so, to what extent.

Eighteen-day old plants with 2 primary and 2 trifoliate leaves were used. They were root inoculated by the usual method with mycelial suspension. After inoculation they were planted in vermiculite and kept in the green house. There were 30 inoculated and 30 control plants. From the second day 3 each of inoculated and control plants were taken at random for studies on conduction. They were cut at ground level, and the lower end of each was again cut under water before placing in vials containing a solution of 0.1 per cent eosin in water. Each cutting was allowed to take up the dye for 90 seconds under conditions favouring normal transpiration in the green house and then the lower inch of the stem was cut and kept in water for free hand sectioning.

Thin, free-hand sections of the stem were taken and mounted in glycerine for counting of stained and unstained vessels. All stained vessels were considered as conducting.

This was done daily for 10 days after inoculation. When plants developed initial wilt symptoms after 8 to 9 days, their symptomatic leaves were removed before placing in the dye. The corresponding leaves of controls were also removed. The results are summarized in Table

Table 23 Percentage of functioning vessels in inoculated and control plants

Treatment	Days after inoculation									
	1	2	3	4	5	6	7	8	9	10
Control	92	91	93	95	93	90	95	82*	76**	78 **
Inoculated	90	93	94	90	91	89	86	79*	67**	65**

* 1 leaf removed

** 2 leaves removed

Average of 3 replicates (Sections taken ½ inch from the base).

These results show that the proportion of non-conducting vessels is low even after 10 days and it seems improbable that this factor would contribute significantly to the wilting of inoculated plants. But the experiments on transpiration did show that the absorption rate of water by infected plants is affected at the time of wilting. So, it appears that factors other than vascular occlusion is responsible for the reduction in the rate of absorption in infected plants

..6. Effect of infection on stomatal mechanism

The gradual reduction in the rate of conduction and transpiration suggested a study of the condition of stomata between inoculation and symptom development.

This was done by taking impressions of stomata with silicone rubber by a technique adapted from that of Zelitch and Walker,(1962). Five ml. of silicone rubber (silicoset, I.C.I., England) was mixed and stirred well with 0.2 ml. of curing agent D., (I.C.I., England) to the mixture of a thick consistancy. It was then spread evenly over 1 sq.cm. of the underside of the leaf. After 5 to 10 minutes when the silicoset was dry, it was peeled carefully from the leaf surface. Then the inner surface was painted with a thin film of cellulose acetate solution (commercial colorless nail varnish diluted with $\frac{1}{3}$ vol. acetone). After another film was dry, it was stripped off with forcep from the silicone rubber. The film was then mounted on a glass slide in a drop of water, and examined under a microscope atx 1000. The percentage of open stomata per mm.^{2} of leaf surface was determined. The stomata of 4 regions taken at random of the leav surface of one monofoliate and one trifoliate leaf of each infected and healthy plant were examined for studying the stomatal

pattern. Three inoculated and three healthy plants were examined daily (at 2 p.m.) from the 5th day after inoculation until the 9th day when pronounced symptoms appeared on the primary leaves. The results of these observations are summarised in Table 24.

Table 24. Percentage of functioning stomata per mm.^{2} of leaf surface in inoculated and control plants

Days After Inoculation	Treatment	No. of stomata/mm.^{2}	No. of opened stomata/mm.^{2}	Percentage functioning stomata
5	Control	112	107	95.5
	Inoculated	127	120	94.4
6	Control	121	110	95.8
	Inoculated	118	109	92.3
7	Control	124	118	95.1
	Inoculated	131	126	96
8	Control	138	131	95
	Inoculated	120	113	94.1
9	Control	130	120	93
	Inoculated	129	122	94.5

Average of 3 replicates

This shows that the reduction in the rate of water intake in infected plants and cuttings is not due to closing of the stomata and conflicts with the findings of Dimond and Waggoner (1953) who found closed stomata in drought-hardened and diseased leaves of tomato.

C. Studies with culture filtrate

1. This experiment was designed to see whether the filtrate from cultures of the fungus on liquid SCA has any effect on disease development. It was found in earlier experiments that cuttings developed typical wilt symptoms when inoculated with a spore suspension and responded to infection in the same way as intact plants. So, in this and most experiments with culture filtrates, cuttings were used in the place of intact plants.

Ten cuttings from 18 day old plants each having 2 primary and 2 trifoliate leaves were used in this experiment. They were cut at ground level with a wet razor blade and after trimming 5 mm. from the base under water, five cuttings were placed in cell free filtrates which were obtained from 10 day old liquid cultures. The filtrates had been sterilized by micropore filtration. The other five cuttings were placed in water as controls. All cuttings were kept in the lighted constant temperature cabinet and were examined daily for their reaction to the treatment.

The primary leaves of all cuttings in culture filtrate became flaccid in 24 hours and developed

typical wilt symptoms and pronounced necrosis in the intervenal regions of the leaves by the second day of treatment. The trifoliate leaves also developed wilt symptoms by the third day, and all the cuttings treated with culture filtrate wilted severely within 6 days. The results of this experiment are given in Table The disease symptoms were assessed on the basis of wilting and degree of necrosis.

Table 25 <u>Necrosis and wilting indices per leaf of cuttings treated with culture filtrate</u>

Disease Symptoms	Days After Treatment						
	1	2	3	4	5	6	7
Necrosis	O	M	M	M	E	E	E
Wilting	0.5	1.4	2	2.8	3.5	3.8	4

M - Moderate

E - Extensive

Average of 5 replicates

Yellowing and vein clearing were not very prominent in any of the cuttings in this experiment; probably because the cuttings wilted within a very short period.

2. Effect of treating cuttings with different dilutions of culture filtrate

It was found in the foregoing experiment that filtrates from 10 day old cultures were highly toxic to cuttings and caused severe wilting within 6 days. This experiment was designed to determine the extent to which the filtrate could be diluted and still be toxic to cuttings.

Twenty-one 18 day old cuttings each with 2 primary and 2 trifoliate leaves each were used in this experiment. The filtrate was obtained from 10 day old cultures of liquid SCA medium. After sterilizing by micropore filtration, five dilutions of the filtrate were prepared by adding 4, 9, 24, 49 and 99 times its volume of nutrient solution (½ strength) to it. The solutions were placed in vials of 40 ml and three cuttings were placed in each of the solutions. There were three controls and these were placed in ½ strength nutrient solution. All cuttings were kept in the lighted constant temperature cabinet and were examined daily for disease development. The disease symptoms were assessed on the basis of wilting. The results are given in Table 26.

Table 26 Wilting indices per leaf of cuttings treated with different dilutions of filtrate

Strength of Filtrate	Days After Treatment							
	1	3	5	7	9	11	13	15
1:4	0.4	1.8	3.2	3.8	4	4	4	4
1:9	0.25	1.5	2.6	3	3.4	3.9	4	4
1:24	0	0	0.1	0.6	1.2	1.7	2.4	2.8
1:49	0	0	0	0	0.3	0.8	1.4	1.6
1:99	0	0	0	0	0	0	0	0.2

Average of 3 replicates

3. Effect of culture filtrate on blockage

In the previous experiment, it was found that filtrates were toxic to cuttings which developed disease symptoms very similar to those in root inoculated plants, even when they were diluted 25 times. The following experiment was done to see if filtrates blocked the translocating elements.

The filtrate was prepared and sterilized as in the last experiment. It was diluted 25 times with ½ strength nutrient solution. The diluted filtrate was then placed in 30 ml. quantities in 40 ml. vials.

Twenty cuttings from 18 day old plants were cut at ground level with a wet razor blade and after trimming the base under water, twelve cuttings were placed in the diluted filtrate. The other eight were kept as controls in ½ strength nutrient solution. All cuttings were then kept in the lighted constant temperature cabinet until the end of the experiment. Five days after treatment and then at 2-day intervals, the stem bases of treated and untreated cuttings were examined microscopically after sectioning and staining as in expt. 6 (Section A). The vessels with gels and gums, tyloses and vascular discoloration were counted and percentages of vascular occlusion or discoloration were determined. These observations are summarized in Table 27.

Table 27 Percentages of vessels occluded or with discolored walls in cuttings treated with culture filtrates

	Days After Treatment				
	5	7	9	11	13
Gels and Gums	0	23	30	45	48
Tyloses	0	0	0	4	7
Vascula Discoloration	14	25	52	66	70

Average of 3 replicates

The results showed that a large percentage of vessels were affected when treated with culture filtrate.

4. Effect of dialysed culture filtrate on disease development

This experiment was designed to determine whether the disease symptoms produced in cuttings by culture filtrate were caused by high or low molecular weight substances.

Cell free filtrates from 10 day old cultures were used: 5 ml. were placed in dialyses tubing in 600 ml. glass distilled water (6 tubings/beaker). The beakers were kept at 4°C. The water in the beaker was changed 4 times each 24 hours. The filtrates were tested for toxicity after 24, 36 and 48 hours dialysis, and after sterilization by micropore filtration.

Toxicity was assessed on the basis of the wilting that developed in 18 day old cuttings treated with the filtrate. Controls were placed in distilled water or in undialysed filtrate. Three cuttings were used for each treatment. They were kept in the growth cabinet and examined daily for disease development. The results are given in Table

Table 28 Wilting indices per leaf of cuttings treated with dialysed and undialysed filtrates

Days after Treatment	Undialysed Filtrate	Dialysed Filtrate Duration Of Dialysis - Hours 24	36	48
1	0.5	0.1	0.1	0
3	2	0.2	0.1	0
5	3.4	0.4	0.2	.1
7	3.9	0.5	0.2	.1
9	4	1.0	0.2	.1
11	4	1	0.3	.1

Average of 3 replicates

From these results it is evident that the substance or substances responsible for wilting are dialysable and that after 48 hours dialysis, the filtrate loses its toxicity almost completely.

The effect of these filtrates on vascular occlusion or discoloration was also determined. Free hand sections from the bases of these cuttings were taken at the end of the experiment and stained as in expt. 6 (Section A). The results are given in Table

Table 29 Percentages of vessels occluded or with discolored walls after treatment with dialysed or undialysed filtrate

	Undialysed Filtrate	Dialysed Filtrate		
		Duration of Dialysis In Hours		
		24	36	48
Gels and Gums	42	35	38	31
Vascular Discoloration	58	14	9	7

Average of 3 replicates

These results show that the substance or substances that cause vascular occlusion and discoloration are not dialysable. Even after 48 hours dialysis, the filtrate caused vascular occlusion and discoloration in the base of cuttings. But the presence of such substances in culture filtrates may not be significant because vascular occlusion by gels or gums is not an important feature of this disease and vascular discoloration was found only in vessels occupied by the pathogen and these are few during the early stages.

5. Effect of water containing dialysed substances on disease development in cuttings

This experiment was designed to see whether the water against which the filtrate was dialysed was toxic to cuttings.

A cell free filtrate collected from 10 day old cultures was placed in 5 ml. quantities each in dialysis tubing ($5\frac{1}{2}$" x $5\frac{1}{4}$"). Each tubing with the filtrate was then placed in a glass tube (6" x 7/10") containing 25 ml. of glass distilled water; 150 ml. of culture filtrate was treated in this way at 4°C. The water in the tubes was changed once during 24 hours dialysis and it was stored at 4°C. After 24 hours dialysis, the water containing the dialysed substances and the dialysed filtrate were sterilized separately by micropore filtration and tested for their toxicity. The solutions were taken in 30 ml. quantities in 40 ml. vials and 18 day old cuttings were placed in the solutions; one cutting per vial. Five cuttings were used for each treatment and five were placed in glass distilled water as controls. All cuttings were then kept in the growth cabinet and examined daily for symptom development.

The cuttings in water containing dialysed substances developed wilt symptoms within 24 hours and by the fifth

day wilting and yellowing were very prominent in the primary leaves. Vein clearing and occasional necrosis were also observed. The cuttings in the dialysed filtrate developed slight wilt symptoms by the third day but all recovered eventually. After 5 days, all cuttings were placed in nutrient solution in 160 ml. bottles. Within the next 7 days, all cuttings except those treated with water containing the dialysed substances developed plenty of adventitions roots and grew normally. (Plate 5). Root formation was almost inhibited in all plants of the other set.

The results of this experiment are summarised in Table 30 . The toxicity of the test solutions was assessed on the basis of wilt symptoms in treated cuttin

Table 30 Wilting indices per leaf of cuttings treated with dialysed filtrate and water containing dialysed substances

Test Solutions	Days After treatment				
	1	3	5	7	9
Dialysed Filtrate	0	0.2	0.3	0.2	0.1
Water with Dialysed Substances	0.1	0.7	1.2	1.5	1.9

Average of 5 replicates

Plate 5

C - Control (Cutting in Water)

DCF - Cutting in Dialysed Culture filtrate

WDS - Cutting in water containing Dialysates.

The results of these experiments confirm that the substances responsible for wilting, yellowing, vein clearing and necrosis in beans are of low molecular weights and dialysable.

6. Effect of culture filtrate on transpiration of cuttings

Having found that the culture filtrates were toxic to cuttings and that they contain dialysable substances that induce disease symptoms, their effect on transpiration was now assessed.

Ten 18-day old cuttings with primary leaves only were used. Buds of these cuttings had been removed earlier. Filtrates were obtained from 10 day old cultures. After sterilizing by micropore filtration, they were diluted 25 times with ½ strength nutrient solution and were placed in 40 ml. vials. The vials were closed with rubber bungs having central holes in each. Cuttings were placed in the filtrate through the hole of the bung; one cutting per vial. Controls were placed in ½ strength nutrient solution. Five cuttings were used in each treatment. The vials with the cuttings were weighed on a balance ('Mettler, K 7') before placing inside the growth cabinet and then daily at 4 p.m. The loss in weight caused by transpiration of each cutting was recorded daily. After 5 days, when

wilt symptoms began to appear in one of the replicates, all cuttings were taken out and their leaf areas were determined. As the primary leaves had attained full size before setting up this experiment, areas were measured only at the end. From the daily loss in weight of each unit, the water loss by unit area of leaf surface was determined. The results of these are summarised in Table 31.

Table 31 Water loss from healthy and culture filtrate created cuttings

Days after treatment	Water loss (mg/cm^2 leaf area		Percentage increase or reduction - treated
	Healthy	Treated	
1	32	34	+ 6.2
2	37	41.5	+ 12.1
3	34.5	39	+ 13.04
4	36	31.5	- 12.5
5	35	30	- 14.2
6	34	23	- 32.3

Average of 5 replicates

It is evident that there was a considerable increase in transpiration of cuttings in culture filtrates during the first two days of treatment. Transpiration then fell suddenly. After 3 days, the rate of transpiration was considerably reduced, though no external disease symptom had become apparent at this time.

This experiment was repeated with some modifications with the object of studying the ratio of transpiration to absorption in culture filtrate treated cuttings. Here again, cuttings with primary leaves only were used. The experiment was set up as before with 18 cuttings in culture filtrate and 18 in water as controls. After 24 hours 3 control and 3 culture filtrate treated cuttings were taken out and placed in 30 ml. vials containing 20 ml. of water for determining the transpiration-absorption rates for the next 24 hours. Each unit was weighed at the beginning and then after 24 hours and from the difference in weights, the water loss for 24 hours per cm^2 leaf area was determined. From the quantity of water left in the vial, the water taken up by each cutting was also determined. This was repeated after every 24 hours for six days. The results are summarised in Table 32 and presented in Fig.14.

It is evident from these results that the rate of transpiration of culture filtrate treated cuttings increased the rate of absorption and the transpiration-absorption balance was somewhat upset during the first two days, and then on the sixth day just in time with symptom development.

Although the absorption rate was also high for the first two days of increased transpiration, it was not proportional to the loss. Whether the increase in the rate of water loss was due to change in the permeability of cells or not is unknown.

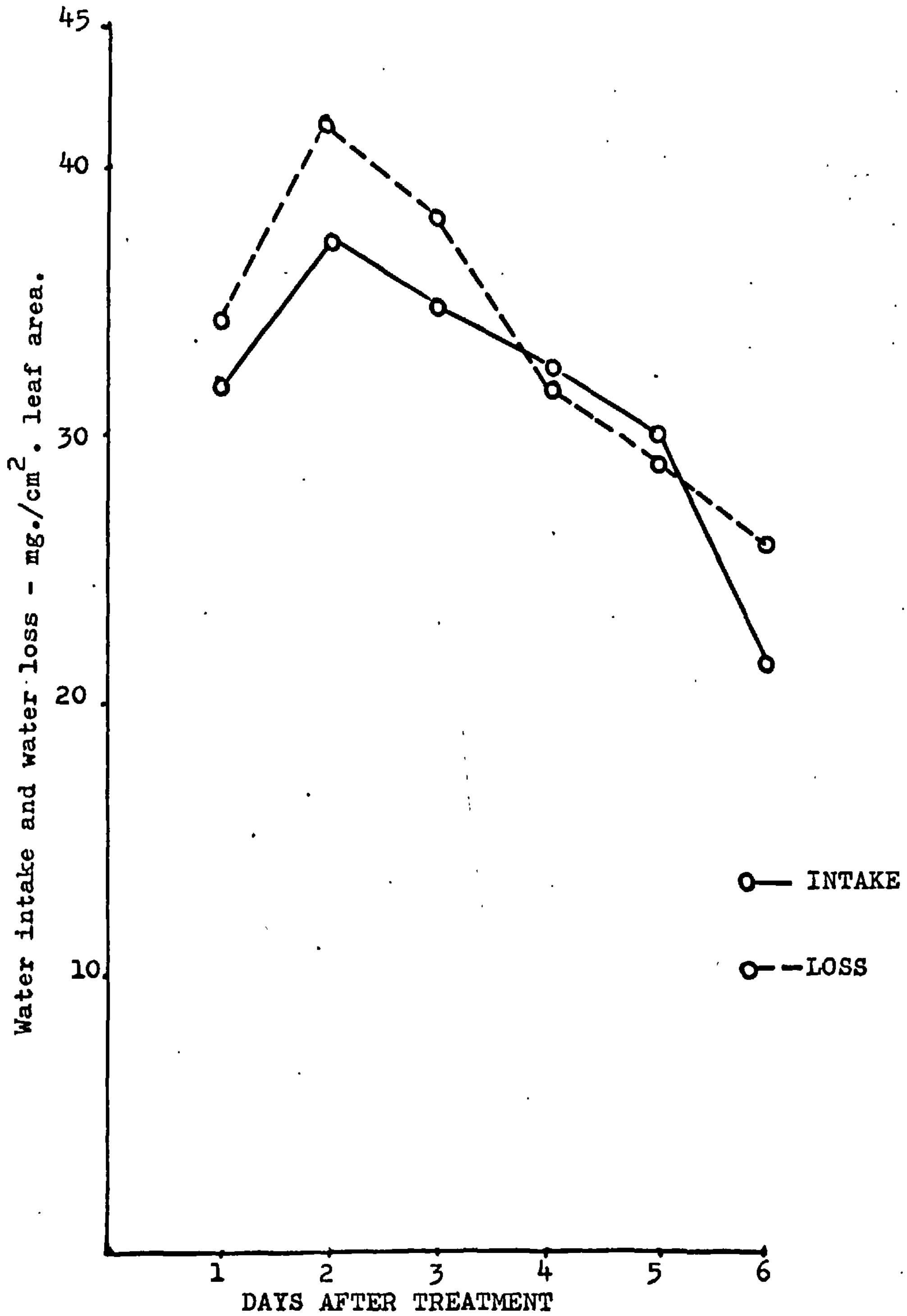
Table 32 Water intake and water loss of healthy and culture filtrate treated cuttings

Days after treatment	Healthy			Treated		
	Intake	Loss	Loss as percentage of intake	Intake	Loss	Loss as percentage of intake
1	34	33.4	98.3	32	34	106.2
2	37	36	97.3	37	42.5	114.8
3	36	35.5	98.7	35	37.5	107.1
4	35	34	97	32.5	32	98.5
5	33	32	97	30	29	96.7
6	36	34.5	96	21.5	25.5	118.6

Average of 3 replicates

Fig.14

WATER INTAKE AND WATER LOSS OF CULTURE FILTRATE TREATED CUTTINGS



Effect of culture filtrate on respiration of cuttings

Filtrates from 10 day old cultures were used. They were sterilized by micropore filtration before diluting it with ½ strength nutrient solution. In experiment 1, (Section C), it was found that culture filtrate diluted 25 times with nutrient solution induced disease symptoms in cuttings similar to those caused by spore suspensions. So, in this experiment, the filtrate was taken in 1:24 dilution.

Eighteen day old cuttings with 4 leaves were placed in the diluted filtrate and controls were placed in ½ strength nutrient solution. All were then kept in the growth cabinet.

As the cuttings absorbed the culture filtrate solution, their respiration rate was determined daily in a Warburg apparatus as given under 'Materials and Methods'. Fifteen leaf disks from each cutting were used for measuring the rate of oxygen uptake. Three healthy and three filtrate-treated cuttings were used on each occasion. The respiration was measured for 2½ hours daily and at the end of this period, the leaf disks were dried in an oven and weighed. From the dry weights obtained, the rate of oxygen uptake per mg. dry rot of leaf tissue was determined.

Table 33 Respiration of healthy and culture filtrate treated cuttings

O_2 uptake ul/mg. dry wt./hour

Days after treatment	Healthy	Treated	Percentage increase or reduction - treated
1	3.65	3.6	- 1.3
2	3.8	4.1	+ 7.8
3	3.6	4.4	+ 22.2
4	3.7	4.9	+ 32.4
5	3.7	5.6	+ 51.3
6	3.6	3.3	- 8.3

Average of 3 replicates

Fig.15

RESPIRATION OF HEALTHY AND CULTURE FILTRATE TREATED CUTTINGS

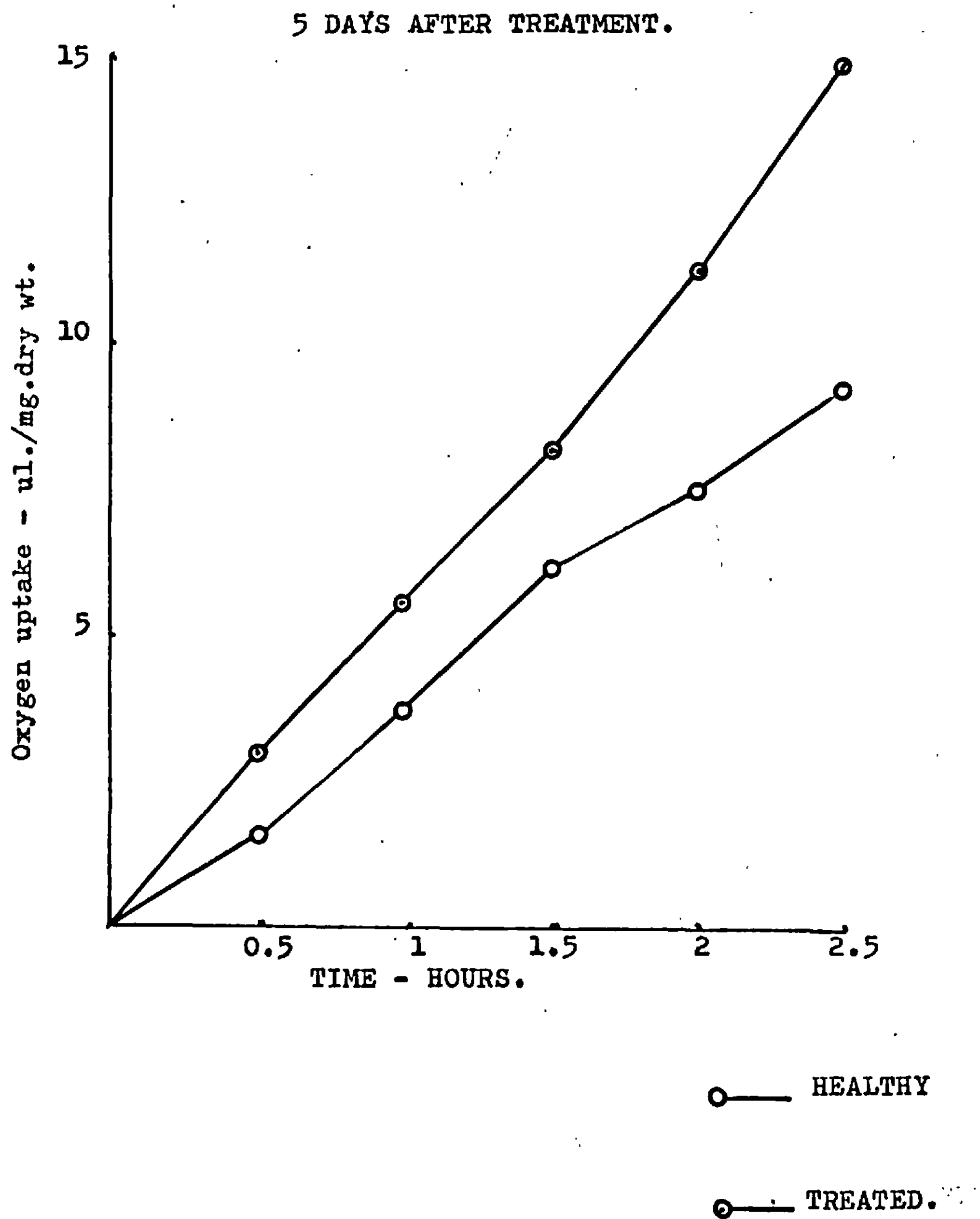
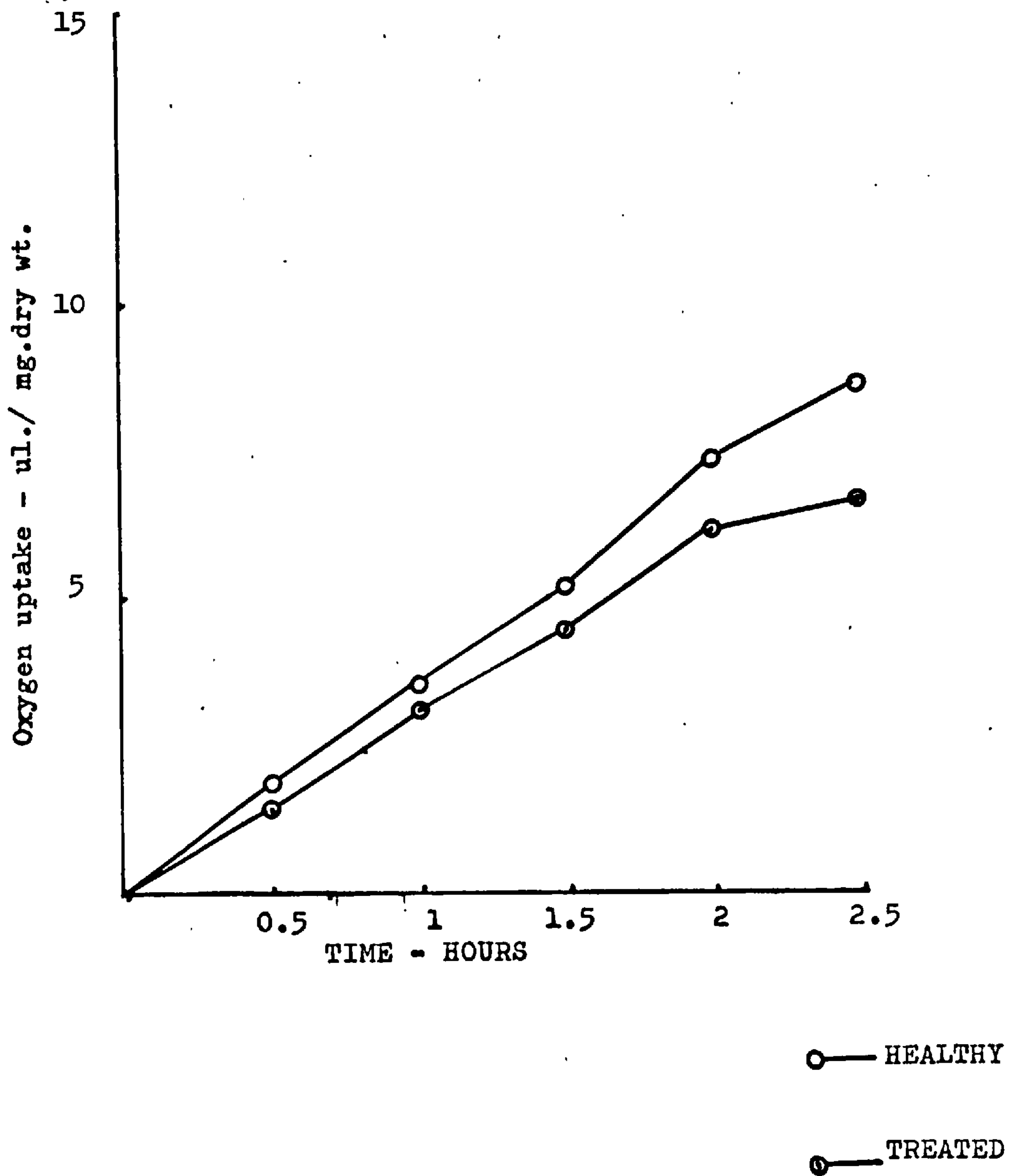


Fig.16

RESPIRATION OF HEALTHY AND CULTURE FILTRATE TREATED CUTTINGD

6 DAYS AFTER TREATMENT.



This experiment was continued for six days until wilt symptoms appeared. Dry weight of leaf tissue was determined. The results are presented in Figs. 15 and 16 and Table 33.

The results of this experiment show that there was an increase in the rate of respiration as from the second day after placing in the culture filtrate solution. This increase in the rate of respiration went up steadily until symptom development, and then it fell suddenly as in the naturally infected beans, suggesting that substances that are responsible for enhanced respiration are produced *in vivo* and *in vitro*.

8. Growth of fungus in plant exudate - tracheal sap

It was found by anatomical studies (Section A) that the fungus was mostly confined to the vessels of the host until at a late stage of infection. This means that it depends on the tracheal sap of the host for its nutrition. This experiment was designed to see whether the fungus would grow in the tracheal sap *in vitro*.

The tracheal sap was collected from 24-26 days old healthy plants as described under 'Materials and Methods'. Fresh sap was used in this experiment and it was sterilized by micropore filtration before taking in Erlenmeyer flasks. The sap was supplemented with 0.1

per cent glucose and 20 ml. of the sap glucose medium was placed in each flask. Three other media were used as controls; tracheal sap without glucose, water with glucose, and liquid SCA. All media were inoculated with 0.1 ml. spore suspension (1×10^6 spores/ml.) prepared by the usual method. All cultures were then incubated at 25°C. After 10 days incubation, the mycelia from each culture were collected on muslin and washed several times under running water. The filtrate collected was stored at -20°C, to be used later. The mycelia were placed in aluminium foil and dried in moving air at 70°C for 24 hours. After drying mycelia were cooled over $cacl_2$ and weighed to 0.1 mg. The dry weights of mycelia obtained from cultures on liquid SCA, tracheal sap-glucose, tracheal sap, and water-glucose were 26, 19, 5 and 0.2 mg. per 20 ml. of media, (average of 3 replicates). This shows that the fungus grows satisfactorily well on tracheal sap when supplemented with glucose. Accordingly, the filtrate obtained from this culture was tested for its toxicity. This was done as in experiment 1, (Section C). The filtrate was used undiluted after sterilization by micropore filtration and placed in 40 ml. vials. Eighteen-day old cuttings with 4 leaves were used. Controls were set up in sap containing 0.1 per cent glucose; in

filtrate from SCA cultures and also in water. There were five controls for each. The cuttings were kept in the growth cabinet. They were examined daily for disease development. The disease symptoms were assessed on the basis of wilting.

It was found that all cuttings in the sap-filtrate developed disease symptoms similar with those of cuttings in filtrate from SCA, but symptom development was delayed and less severe. After 9 days when these cuttings were placed in nutrient solution, they gradually recovered. The results are shown in Table 34.

Table 34 Wilting indices per leaf of cuttings treated with sap-filtrate

Treatment	Days After Treatment						
	1	3	5	7	9	11	13
Water	0	0	0	0	0	0	0
Sap-Glucose	0.1	0.2	0.1	0	0	0	0
SCA Culture Filtrate	0.4	1.8	3.2	3.8	4	4	4
Sap-Culture Filtrate	0	0.5	1	1.4	1.7	1.6	1.5

Average of 5 replicates

In this experiment, controls in sap-glucose showed slight wilting during the first 5 days but they soon recovered.

9. Effect of feeding inoculated cuttings with glucose or galacturonic acid

The aim of this experiment was to see whether the intensity of disease development would increase when inoculated cuttings are fed with carbohydrate because it had found in the previous experiment that the fungus grew well in tracheal sap supplemented with glucose.

Eighteen day old cuttings with four leaves each were used. They were prepared for spore inoculation as in experiment 8, (Section A). Spore suspensions contained 0.5 x 10^5 spores per ml. The spore suspensions were placed in 4 ml. quantities in calibrated 10 ml. tubes and cuttings were placed in these suspensions. For controls, heat inactivated spore suspensions were used. Eighteen cuttings were used of which 9 were controls. All cuttings were kept in the growth cabinet. Four to six hours later, when each cutting had taken up 1 ml. of the suspensions, they were transferred into 160 ml. bottles containing ½ strength nutrient solution. After trimming 1 cm. off their bases and washing under water. Twenty-four hours later, three

inoculated and three control cuttings were taken out and placed in 0.1 per cent glucose solution and another set of 3 inoculated and 3 controls were placed in galacturonic acid. (pH.6.5). After 24 hours all cuttings were put back in the nutrient solution and examined daily for disease development. The disease symptoms were assessed on the basis of wilting. The results of this experiment are summarised in Table 35.

Table 35 Wilting indices per leaf of inoculated cuttings fed with glucose or galacturonic acid

Treatment		Days After Inoculation							
		1	3	5	7	9	11	13	15
Inoculated with	1) Spore Suspensions	0	0	0	0.4	0.6	1.2	2.4	2.8
	2) Spore Suspensions & Glucose	0	0	0	0.2	0.4	1	2.2	2.4
	3) Spore Suspensions & Galacturonic Acid	0	0	0	0.1	0.3	1	1.8	2.1
Controls In:	1) Dead Spore Suspensions	0	0	0	0	0	0	0	0
	2) Dead Spore Suspensions & Glucose	0	0	0	0	0	0	0	0
	3) Dead Spore Suspensions & Galacturonic Acid	0	0	0	0	0	0	0	0

Average of 3 replicates

The results show that glucose or galacturonic acid did not increase the rate or severity of disease development of inoculated cuttings. If anything, both substances lessened the severity of symptom expression, contrary to what was expected. It is possible, though not certain, that the carbohydrates might have been consumed and assimilated by the cuttings rather than by the fungus.

10. <u>Effect of feeding inoculated cuttings with casamino acids</u>, (casein hydrolysates)

This experiment was designed to see the effect of feeding inoculated cuttings with nitrogenous substances. The casamino acids for this purpose were obtained from Oxoid Ltd., England.

The experiment was set up exactyly as in the previous one. Eighteen-day old cuttings with four leaves each were used. Six cu tings were inoculated and six were kept as controls. All cuttings were placed in 160 ml. bottles after trimming and washing their bases under running water. After 24 hours, 3 inoculated and 3 control cuttings were fed with 0.1 per cent casein hydrolysate solution in water and then placed back in the nutrient solution. They were examined daily for disease development and the symptoms were assessed on

the basis of wilting. The results are given in Table 36.

Table 36 Wilting indices per leaf of inoculated cuttings fed with casein hydrolysates

Treatment		Days after Inoculation							
		1	3	5	7	9	11	13	15
Inoculated with	1) Spore Suspensions	0	0	0	0.6	.8	1.5	2.5	2.9
	2) Spore Suspensions & Casein Hydrolysates	0	0	0.5	1	1.6	2.2	2.8	3.5
Controls In	1) Dead Spore Suspensions	0	0	0	0	0	0	0	0
	2) Dead Spore Suspensions & Casein Hydrolysate	0.1	0.1	0.1	0.2	0.1	0.1	0	0

Average of 3 replicates

As is evident from the results of this expt. (Table 36), the inoculated cuttings treated with casein hydrolysates developed wilt symptoms earlier than the spore inoculated untreated cuttings. This suggests that nitrogenous substances increased the incidence of wilt, possibly by favouring the production of substances responsible for wilting.

Studies with fusaric acid and indole-3-acetic acid

Because the infected plants consistantly showed symptoms such as vein clearing, necrosis, stunting and premature yellowing of the leaves suggesting the action of a toxin, it was thought appropriate to test the effect of fusaric acid which has been reported in fusarium infected cotton (Lakshminarayanan and Subramanian, 1955; Kalyanasundaram and Venkata Ram, 1956) and banana (Page, 1959).

Fusaric acid is a 5 n butylpyridene-2-carboxylic acid with a molecular weight of 179 and melting point 107°C, (Gaumann, 1957). It interferes with the vital processes of the cell including the permeability of the plasma membranes and is reported to withdraw calcium ions from the calcium pectate of the middle lamellae, transforming the water insoluble calcium pectate into water soluble pectin compounds (Gaumann, 1958). Tomato cuttings treated with fusaric acid (150 mg./Kg. fresh wt.) develop typical wilt symptoms and necrosis (Keon *et al*., 1957).

Effect of fusaric acid on disease development in bean cuttings

A solution of pure synthetic fusaric acid (1mg./10ml.) was prepared in glass distilled water. It was then placed in quantities of 20 ml. in 30 ml. calibrated vials. Twelve 18-day old cuttings each weighing 8 to 10 . . were then placed in the solutions. The same number of cuttings used as controls were placed in glass distilled water in vials. All cuttings were kept in the growth cabinet. When they had absorbed the fusaric acid solution from 2 to 20 ml. they were taken out and after removing 1 cm. of the stem bases under water, they were placed in 160 ml. bottles containing ½ strength nutrient solution and replaced in the growth cabinet. From the volumes of solution absorbed and the original fresh weights of cuttings, the quantity of fusaric acid taken up by each was determined. The cuttings were left in the growth cabinet and were examined daily for their reactions to the treatment.

Cuttings treated with fusaric acid developed wilting, necrosis, twisting of the petioles, ribbing of the stem and yellowing from 12 hours to 5 days after their removal from the solution. The intensity of symptom development depended on the amount of solution

absorbed by each cutting. Those that absorbed 150 to 200 mg./Kg. fresh wt. (15 to 20 ml. solution), wilted severely with extensive necrosis even before removing from the solution. Their petioles were twisted and their stems were ribbed. The cuttings collapsed and wilted completely in 3 to 4 days. Cuttings that had taken up 100 to 150 mg./Kg. fresh wt. (10 to 15 ml. solution) developed severe wilt symptoms and extensive necrosis within 2 to 3 days. Twisting of the petioles and shrinking of the stem were not very severe in this case. The cuttings that had absorbed 50-100 mg./Kg. fresh wt. (5 to 10 ml. solution), also developed wilting and necrotic symptoms. The foliar symptoms that developed in these cuttings resembled those of spore inoculated cuttings. Cuttings that had absorbed (20-50 mg./Kg. fresh wt.) showed some flaccidity of leaves at first but they recovered eventually. The results are summarized in Table 37.

Table 37. Disease symptoms in cuttings treated with various dilutions of fusaric acid solution

(4 days after treatment)

Fusaric acid solution taken up.(mg/Kg. fresh weight)	Symptoms	Intensity of Symptoms	Wilting/ Yellowing index per leaf
150 to 200 mg.	Wilting	Severe	3.8/leaf
	Necrosis	Extensive	
	Twisting of Petioles	Severe	
	Ribbing of stem	Severe	
100 to 150 mg.	Wilting	Severe	2.5/leaf
	Necrosis	Moderate	
	Twisting of petiole	Mild	
	Ribbing of stem	Mild	
50 to 100 mg.	Wilting	Moderate	1.5/leaf
	Yellowing	Slight	0.5/leaf
	Necrosis	Slight	
20 to 50 mg.	Wilting	Slight	0.2/leaf

Average of 3 replicates

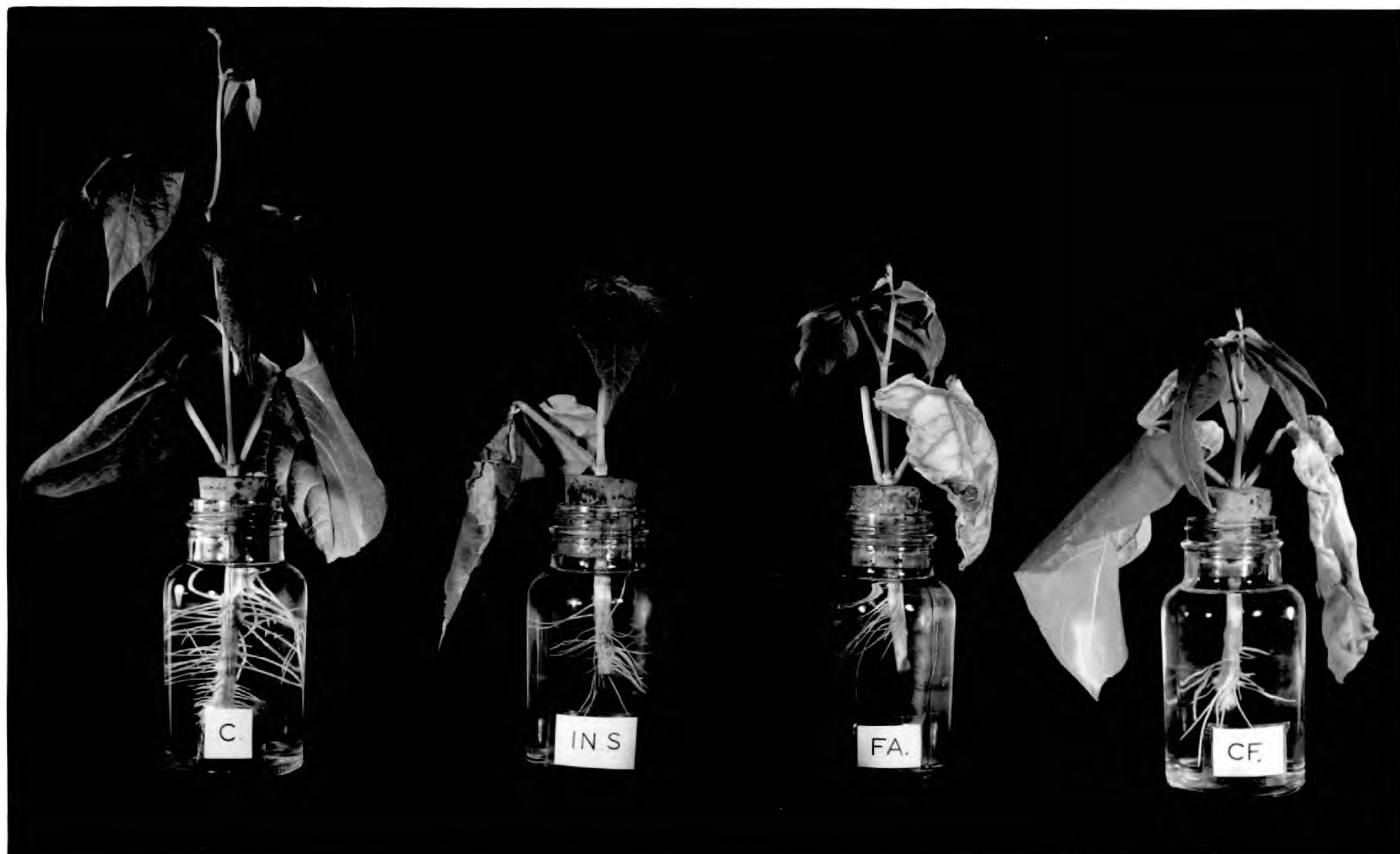


Plate 6.

C - Control

IN.S - Inoculated with spore suspension

FA - Treated with Fusaric Acid

CF - Treated with Culture filtrate

Cuttings that had absorbed approximately 100 mg./Kg fresh wt. showed yellowing. But no vein clearing. Adventitions root formation was inhibited in all cuttings as in spore inoculated cuttings. (Plate 6).

2. <u>Effect of fusaric acid on condution and blockage</u>

In the experiment described above cuttings developed severe wilt symptoms at 100 mg. or more fusaric acid per Kg. fresh wt. This next experiment was designed to study the effect of the fusaric acid on blockage of xylem. In order to obtain a cumulative and more natural course of disease development, more diluted fusaric acid solution (1 mg./20 ml. water) was used with 18-day old cuttings weighing 8 to 10 gm. and having four leaves. There were 15 cuttings in fusaric acid solution and fifteen cuttings in glass distilled water as controls. All cuttings were kept in the growth cabinet. As the cuttings absorbed the fusaric acid solution they were taken out and the lower 1" of the stem was removed for sectioning.

Free hand sections of the segment were taken and stained to show gels, gums and tyloses. The results are shown in Table 38.

Table 38 Percentage no. of vessels occluded in fusaric acid treated cuttings

Occluded with:	Fusaric acid taken up (mg/Kg. fresh wt.) 5	10	15	20
Gels	0	3	12	15
Tyloses	0	0	0	2

Average of 3 replicates

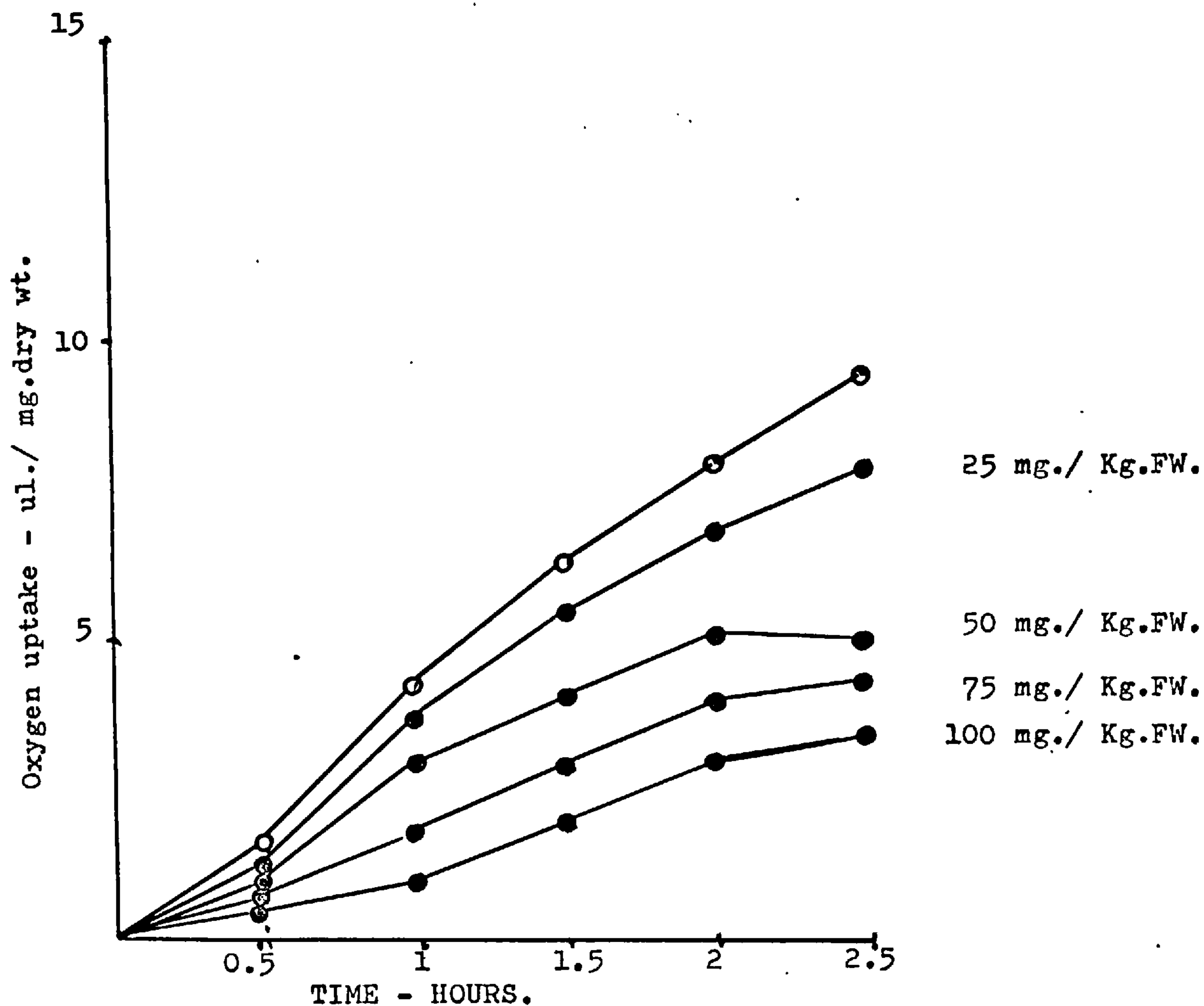
The results show that there are only very few vessels which have been occluded by gels or gums.

3. Effect of fusaric acid on respiration

The respiration rate of fusaric acid treated cuttings was studied in a Warburg apparatus as for infected plants and culture filtrate treated cuttings. Eighteen-day old cuttings with four leaves and weighing 8 to 10 gm. each were used, with fusaric acid (10 mg./ 200 ml. glass distilled water). As fusaric acid is reported to cause maximum injury to leaves at pH 6 or more (Gaumann, 1957), the pH of the solution was adjusted to 6.5 with N/10 NAOH. The solution was then placed in quantities of 30 ml. in 40 ml. calibrated vials. The

FIG. 17

RESPIRATION OF CUTTINGS TREATED WITH DIFFERENT QUANTITIES OF FUSARIC ACID.



FW - Fresh weight

o HEALTHY

● TREATED WITH FUSARIC ACID.

cuttings after taking their individual weights were placed in the solution. Twenty cuttings were placed in the fusaric solution and twenty in glass distilled water. as controls. All cuttings were kept in the growth cabinet. Then, three each of control and fusaric acid treated cuttings were removed after approximately 5 ml. of the solution (25 mg./Kg. fresh wt.) had been taken up by each cutting. Leaf disks were taken from the primary leaves and their respiration rates were measured as in the earlier expts. on respiration. This was repeated after approximately 10, 15 and 20 ml. of the solution had been taken up by the cuttings. The respiration rate in each case was measured for 2½ hours. The results of these observations are presented in Fig. 17 As is evident from these results, fusaric acid in concentrations of 25 mg. and above per Kg. fresh. wt. of plant redu e respiration.

D.4. Effect of Fusaric acid on transpiration

As in Expt. 6 (SECTION C) on transpiration of cuttings treated with culture filtrate, cuttings with only primary leaves were used. Their buds had been removed 5 to 6 days earlier.

A 1 mg./40 ml. solution of fusaric acid at pH 6.5 (NAOH) was used 30 ml. in calibrated vials, closed with

aluminium foil caps with central holes. Eighteen-day old cuttings with primary leaves only and weighing approximately 5 gms. were placed in the solution; one cutting per vial. Controls were placed in glass distilled water. There were five cuttings in the fusaric acid solution and five controls. All were then kept in the growth cabinet. Each unit was weighed at the time of setting up the experiment and then daily until wilt symptoms appeared. At the end of the experiment each cutting was taken out and the leaf area was measured. From the leaf area and the daily loss in weight, the daily rate of transpiration per cm^2 of leaf area was determined. From the original fresh weight of the cuttings and the quantity of solution taken up each day, an approximate estimation of the amount of fusaric acid that had been absorbed by each cutting was made. The r-sults are graphically presented in Fig. 18 and summarised in Table 39.

Table 39 Water loss from healthy and Fusaric acid treated cuttings

(mg/cm^2 leaf area)

Days after treatment	Healthy	Treated	Percentage increase or reduction - treated
1	33	34	+ 3.03
2	36	36	0
3	34	39	+ 14.7
4	37	44	+ 18.9
5	38	39	+ 2.6
6	37.5	31	- 17.3

Average of 3 replicates

This experiment was repeated with some modifications, primarily with the object of studying the ratio of transpiration to absorption in fusaric acid treated cuttings. The experiment was set up as in the previous experiment, except that 24 cuttings were placed in the fusaric acid solution and 24 in glass distilled water as controls. All cuttings were kept in the growth cabinet. After 24 hours, 3 control and 3 fusaric acid treated cuttings were taken out and placed in 30 ml. vials containing 20 ml. of water for determining their transpiration/absorption rates for the next 24 hours. Each unit was weighed at the beginning and then after 24 hours and from the difference in weights, the water loss for 24 hours was determined. Their leaf areas were measured as in the previous experiments and the rate of transpiration per $cm.^2$ of leaf area was determined. From the quantity of water left in the vial, the water taken up by each cutting was determined. This experiment was repeated after every 24 hours for 6 days. From the quantity of fusaric acid solution left in each vial and the weight of the cutting, the amount of fusaric acid that had been absorbed by each cutting was also determined. The results of these observations are presented in Fig.18 and Table 40.

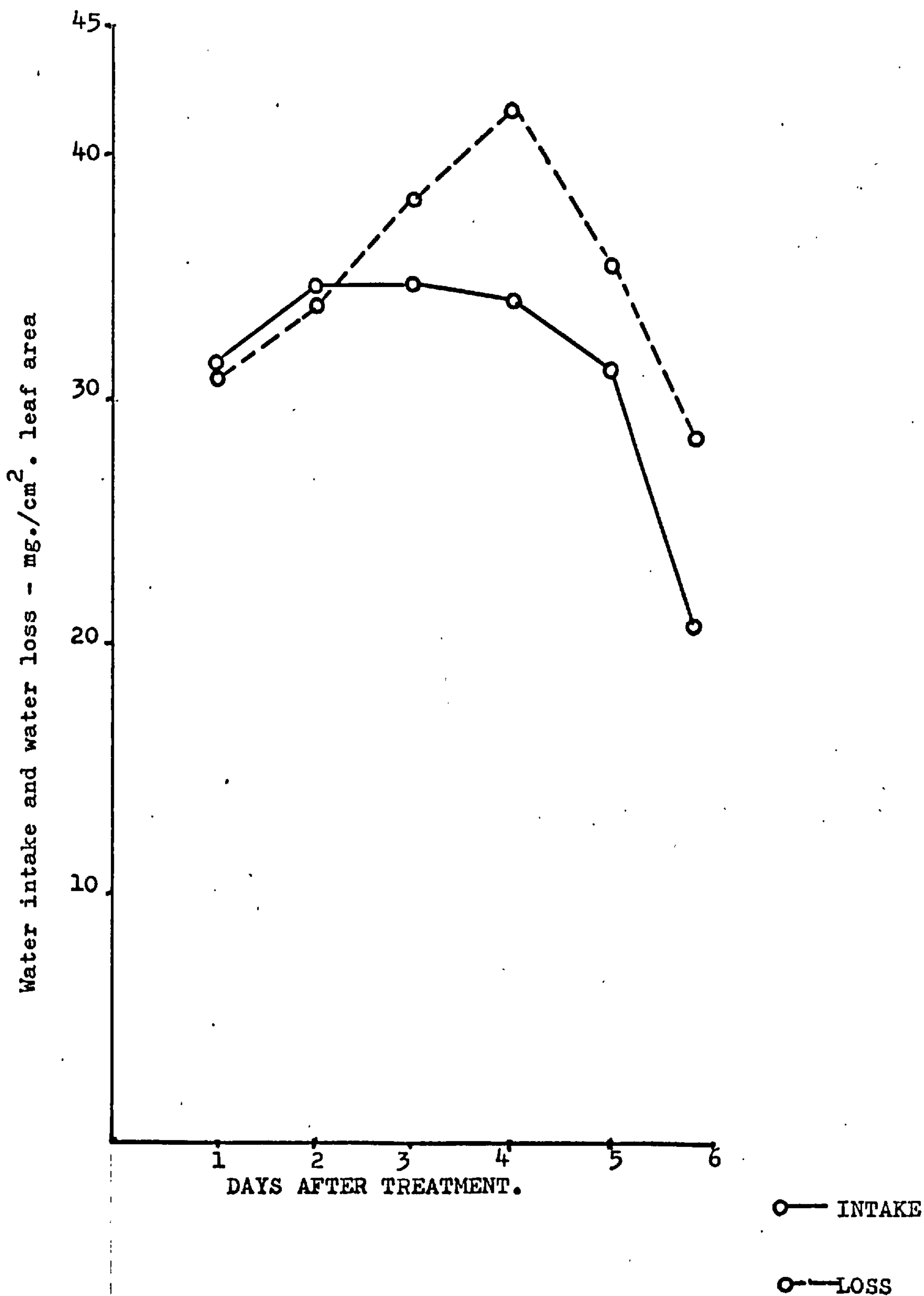
Table 40 Water intake and water loss of healthy and Fusaric acid treated cuttings. (mg/cm^2 leaf area)

Days after treatment	Healthy			Treated		
	Intake	Loss	Loss as percentage of intake	Intake	Loss	Loss as percentage of intake
1	32	31	97	32	31.5	98.5
2	41	39	95.2	35	35	100
3	36	35	97.3	35	37.5	107.1
4	38	37.5	98.7	34.5	42	121.7
5	41	39.5	96.4	31.5	35.5	112.6
6	37	35.5	96	21	28.5	135.7

Average of 3 replicates

Fig.18

WATER INTAKE AND WATER LOSS OF FUSARIC ACID TREATED CUTTINGS



As is evident from the results, the transpiration of fusaric acid treated cuttings exceeded absorption when the cuttings had taken up (50 mg. or more per Kg. fresh weight) of the solution. From then onwards, there was a steady rise in the rate of transpiration and a steady fall in the rate of absorption.

5. Identification of fusaric acid in culture filtrates

Filtrates obtained from 10-day old culture of *Fusarium oxysporum* on liquid casamino acid medium was used. It was centrifuged at 10,000 r.p.m. for 10 minutes to remove cells and mycelial fragments. Two hundred ml. culture filtrate was adjusted to pH 4 with N/10 HCI, and extracted 5 times with aliquots of 40 ml. ethyl acetate. The ethyl acetate extracts were then dried in a rotary evaporator at 40°C. The residue was taken up in 10 ml. 95 per cent ethanol.

The ethanol extract was spotted on chromatogram paper, (Whatman No.1) separately and also with 0.0001 M. solution (17.9 mg./litre) of pure fusaric acid solution. Spots were applied from an "Agla" syringe, 1 ul. to 4 ul. per spot, and dried with a hair dryer. Spots were then complexed with copper by adding 1 ul. of 0.001 M. copper sulphate. The papers were dried for a further 30 minutes and developed in n-butanol-acetic acid water

(120/30/50) at 22°C by the ascending technique. After 4 hours the papers were dried and sprayed with 0.1 per cent rubeanic acid in acetone when the fusaric acid/copper complex developed as dark bluish spots. The Rf. of the spots from culture filtrates were 0.88 the same as that of pure fusaric acid.

The result was confirmed by the identification of fusaric acid by U-V absorption. The absorption peaks of a 0.0001 M. solution of pure fusaric acid in ethanol and the ethanol extract obtained from culture filtrate were determined with a Beckman DU spectrophotometer. An absorption peak at 272 mu was obtained for both the substance in the culture filtrates and the synthetic product.

Estimation of quantity of fusaric acid

By comparing the size and colour intensity of the various spots developed on the chromatogram the quantity of fusaric acid present in the culture filtrate was estimated and found to be approximately 20 mg./litre of filtrate.

6. Identification of fusaric acid in diseased plants

The extracts for these studies were taken from root inoculated plants; 10 days after inoculation when they had developed pronounced disease symptoms. Hundred gm. of entire inoculated plants were used. They were extracted with a roller mill as described under 'Materials and Methods'. The juice obtained (about 60 ml.) was filtered through 3 layers of muslin and then centrifuged 10 times at 3000 r.p.m. The pH of the supernatant (6.5) was adjusted to 4 with N/10 HCl. The acidified juice was extracted six times with 20 ml. aliquots of chloroform, in a separating funnel. The chloroform extract was dried in a rotary evaporator at 35°C and the residue taken up in 10 ml. 95 per cent ethanol. Extracts of healthy plant materials were prepared in the same way.

The extracts were then spotted at the origin of chromatogram paper (Whatman No.1) with an "Agla" syringe in quantities varying from 1 ul to 10 ul. Pure fusaric acid solution in ethanol (0.0001 M.) was spotted separately and also with the extracts in quantities of 1 ul. per spot. All spots were then complexed with 1 ul. of copper sulphate solution (0.001 M.). The papers were then treated as before. with 0.1 per cent rubeanic acid in acetone until dark bluish spots appeared. After drying

the Rf. values were determined as before and it was found that extracts from diseased plants contained a substance of the same Rf. (0.88) as pure fusaric acid.

The quantity of fusaric acid was now estimated; 60 ml. juice (100 gm. fresh wt. of plant) contained approximately 0.9 mg. of fusaric acid (9 mg. per Kg. fresh wt.).

This shows that fusaric acid is present in the diseased plant. But it is difficult to say how significant is this much amount of fusaric acid in an infected plant because it was found in the earlier experiments that at least 100 mg./Kg. fresh weight of fusaric acid is needed to cause permanent disease symptoms. Although, lower quantities caused increased transpiration and an upset in the transpiration/absorption balance, cuttings treated with 50 mg. or less per Kg. fresh weight of plant, recovered when removed from the solution and placed in water. The significance of fusaric acid in inoculated plants must, therefore, remain problematical.

Identification of indole acetic acid

Infected plants beside increased respiration, also show abnormal swelling of the stem bases, hyperplasia, in xylem, tyloses and in certain cases epinasty, symptoms which can be induced by excess auxins. This suggested tests for the presence of auxin IAA in diseased beans.

The technique for the identification of IAA was adapted from that of Smith (1960). The extract was prepared from fresh entire plant collected 10 days after inoculation. About 10 gm. was used. After washing and removing surplus water, the juice was extracted as in the previous experiment; 60 ml. was filtered through 3 layers of muslin and extracted in a separating funnel 4 times with 20 ml. petroleum ether each time. The ethanolic phase was made alkaline with 0.1 ml. of ammonia and then dried in the rotary evaporator at 40°C. The evaporation flask was then rinsed with 10 ml. of ethanol. After addition of another 0.1 ml. of ammonia the ethanol was blown off by air from an air cylinder. The residue was dissolved in 0.4 ml. water. The solution was then spotted at the origin of chromatogram paper, (Whatman No.1). Extracts from healthy plants prepared exactly as above, and IAA solutions (1 mg./10 ml.) in 50 per cent aqueous

acetone were spotted next to the extract from infected plants as markers. The papers after drying were developed for 12 hours at 22°C by an ascending technique using isopropanol-ammonia-water (200/10/20) as solvent. The developed papers were dried and then dipped in a mixture of 1 vol. 10 per cent dimethyl-amino benzaldehyde (W/v) in concentrated HCI to 4 vol. acetone in a dip tray. The papers were again dried until coloured spots appeared on the paper.

The Rf. values of the spots appeared were determined and it was found that the spots from the extracts of plant material did not have the same Rf. as that of pure IAA, suggesting that IAA was not present in the infected plant at this stage (10 days after inoculation) of infection.

DISCUSSION

In a study of the relation of symptom development to the presence of the pathogen, it was found that foliar symptoms always appeared in advance of the pathogen. Although the pathogen entered the plant as early as 3 days after inoculation, its further spread up the stem, especially beyond the region of the hypocotyl was delayed until the 6th or the 7th day after inoculation and by this time the first symptoms of the disease; wilting or flaccidity of the leaves, had already appeared in the primary leaves. On the 9th day, when the symptoms were quite prominent at least 80 per cent of the vessels of the hypocotyl and roots and 85 to 100 per cent of the vessels of other regions were free of fungus. No trace of fungus in any of the vessels of the petiole, midrib or veins, was found at this stage. Even in vessels containing hyphae, it seemed unlikely that the obstruction caused by them would be sufficient to cause significant impediments to water flow. This suggests that factors other than physical blockage of the vessels by mycelium are responsible for the induction of disease symptoms. These findings are in agreement with those of Ludwig (1952), Dimond and Waggoner (1954), and Threlfall (1957).

Plugging by gels or gums or granular material was also found to be very infrequent. The maximum percentage of vessels occluded by these substances was 7 at the 9th day after inoculation when pronounced symptoms were apparent. Occlusion of vessels by gels and gums of pectinacecus origin is reported to play a major role in obstructing water flow in Fusarium wilt of tomato plants (Ludwig, 1952; Pierson et al., 1955; Scheffer and Walker, 1953; Gothoskar et al., 1955; Waggoner and Dimond, 1955), in Fusarium infected banana roots (Beckman et al., 1962), and in Fusarium infected cabbage (Melhus et al., 1924). But Chambers, Henrietta and Corden (1963), questioned the importance of such plugs in reducing the efficiency of water transport to any degree. Even if it does affect the efficiency, low percentage of vessels occluded by these substances in bean wilt indicates that occlusion does not play a major role in the induction of symptoms.

When cuttings were allowed to conduct a dye solution there was practically no difference in the proportion of stained vessels between healthy and infected cuttings. The fact that most of the vessels of the infected cuttings had conducted the dye further excludes the possibility of vessels occlusion as a major factor in disruption of water flow in the xylem.

Vascular discoloration and deposits of a brown substance were found to be a common feature of this disease but its role in affecting the efficiency of conduction or normal functioning of the vessels is probably dubious because these substances appeared only after the symptoms of water shortage had developed. Vascualr discoloration was always associated with the fungus and it occurred only at a late stage. In contrast, symptoms reflecting water shortage appeared in advance of the pathogen. Henreitta, Chambers and Corden (1963), suggested that the deposits of brown substances formed in the intercellular spaces between the vessels and xylem parenchyma make the vessles non-functional by sealing off the pits thus preventing lateral movement of water. Such substances do occur in bean wilt, but appear only when severe wilt symptoms had developed.

Other abnormal anatomical changes such as development of hyperplasia, tyloses or vessel collapse were also not very evident in infected beans and would not seem to contribute materially to obstruction through the vessels. Such of these abnormalities that were observed became apparent only after the symptoms had developed, so that they cannot be regarded as causal factors.

The sum of this evidence would seem to exclude the

possibility that reduced conduction is due to vascular obstruction in the xylem.

Despite the sparse occurence of pathogen, tyloses, hyperplasia, gels and gums and other occluding materials in the vessels, there was a gradual reduction in the rate of conduction and transpiration 4 to 5 days after inoculation. The reduction in the rate of conduction was found to be not associated with closure of stomata as in physiological wilting because at least 90 to 95 per cent stomata remained opened during the day in the leaves of infected plants at a time when there was reduced flow of fluid in the xylem.

In healthy leaves also the number of opened stomata was within this range. A reduction in the rate of water intake was quite apparent in inoculated cuttings, and in detached leaves of infected plants. The reduction in the rate of water intake in the detached leaves is significant because the petiolar vessels were free of pathogen.

Although in general the rate of water loss of an infected plant was less than that in a healthy plant, rate of loss exceeded slightly intake in infected cuttings and detached leaves 5 to 8 days after inoculation. Whether this was due to changes in the permeability of the cells of the leaf blade as suggested by Gaumann (1957)

is not clear. But that it occurs suggests the action of some metabolites. Experiments with cell free culture filtrate support this view.

When a cutting was allowed to conduct cell free filtrate it developed typical wilt symptoms. Increased transpiration was observed in cuttings treated with cell free filtrate diluted as much as 50 times. The rate of water intake was also considerably reduced. About 40 to 50 per cent vessels became blocked with gels and the vessel walls were discolored. When the filtrate was dialysed against water, the filtrate lost its toxicity but it still caused vessel blockage and vascular discoloration. This means, of course, that the wilt inducing substance and the substances responsible for vessel blockage and vascular discoloration are different. This was confirmed by the fact that cuttings wilted when they were allowed to conduct the water against which the filtrate was dialysed.

When the fungus was incubated in the tracheal sap collected from healthy plant, supplemented with 0.1 per cent glucose, it grew satisfactorily. The filtrate from this culture was as toxic to cuttings as the filtrate from the culture of fungus on a full nutrient medium supporting good growth. This shows that in a simulated

vascular environment the fungus produces metabolites which are toxic to cuttings and this can be regarded as a significant result.

A low molecular weight substance was detected in culture filtrate and also in diseased plants and was identified as fusaric acid by paper chromatography and by UV-spectrophotometry. The quantity of fusaric acid present in the culture filtrate was sufficient (20 mg. per litre) to cause disease symptoms in cuttings. But a difficulty arises when we consider the quantity of fusaric acid present in the infected plant. The yield from 100 g. fresh wt. of severely infected plants was 3.5 mg. which was too low to cause wilt symptoms to develop because it was found that at least 100 mg. per kg. fresh wt. fusaric acid is required to induce typical wilt symptoms in beans.

The fact that fusaric acid was detected only in very small amount in diseased plants does not rule out the possibility that it plays a role in the development of disease. It may be produced in sufficient quantities in the infected plant to cause wilt but its ability to chelate with iron or other ions might have made it difficult to extract or detect.

Even assuming that fusaric acid <u>is</u> produced in

sufficient quantities in the infected plant and that some factors made it difficult to extract or detect, then a satisfactory explanation for the increased respiration of infected plants is still required. From 4 to 5 days after inoculation, the infected plant was found to respire at a higher rate than the healthy plant until symptom development. With the appearance of symptom development, the respiration rate fell suddenly. A similar increase in the rate of respiration was also observed in inoculated cuttings. The decrease in the rate of respiration with symptom appearance may not have much significance but the increase in the rate of respiration before expression of symptoms indicates a change in the normal metabolism of the plant. Whether this change has a direct role in inducing symptom development or not is not known. But, the fact that these changes take place in regions away from the infection site and also before any symptom development, suggests the possibility that some diffusible metabolite other than fusaric acid is involved, and that fusaric acid, even if present in quantities sufficient to cause wilt symptoms, is not the only factor responsible for disease symptoms in beans. IAA was detected in severely infected bean plants, but its role in causing wilt symptoms is, however, doubtful.

No IAA could be detected in infected plants even 10 days after inoculation. Moreover, the symptoms of epinasty, pith hyperplasia, tylose formation, abnormal adventitions rooting characteristically induced by indole acetic acid, were not prominent in bean wilt. The abnormal swelling of the stem base in spore inoculated cuttings and the general dwarfing of the infected plant may be caused by IAA. But more proof is needed for this.

There is, however, enough evidencws to show that the substances involved in the production of many of the symptoms are of low molecular weight. The occurence of foliar symptoms in advance of pathogen, the absence of mycelium in the petiole or lamina, reduction in the rate of conduction inspite of occlusion-free vessels and open stomata, the increase in the rate of water loss over intake, the enhanced respiration, vein clearing and necrosis; all are evidences to support this view.

SUMMARY

Twenty-two varieties of bean plants were tested for their reaction to infection by *Fusarium oxysporum* and it was found that all were susceptible to infection. In most cases, the disease developed 5 to 10 days after inoculation and the plants died completely in another 2 to 3 weeks.

The most common symptoms of the disease were found to be wilting and yellowing of the leaves. Vein clearing and intervenal necrosis were also quite frequent. Inoculated plants were stunted with fewer leaves than healthy plants. Leaf area was also considerably reduced.

Injury to the roots made at the time of inoculation caused symptoms to develop considerably earlier but injury was not found to be essential for infection and the later development of severe symptoms.

Plants of different age groups responded to infection in the same way and there was no difference in the time or severity of symptom expression.

When inoculated plants were grown under high humidity, symptom expression was delayed considerably.

On plating infected plant material from different parts of the plant at different stages of infection on

nutrient agar, it was found that the fungus entered the plant as early as 3 days after inoculation, but that further spread up the stem was slow until 7 to 10 days after inoculation.

Studies made with free hand and microtome sections of plant material taken from different regions of the plant at different stages of infection revealed the following:

a) Symptoms developed well in advance of the pathogen.

b) Systemic invasion of the host took place only at a late stage of infection.

c) The maximum percentage of vessels invaded by fungus when symptoms were fully developed was less than 20 and even in colonized vessels the lumen was not blocked completely so as to obstruct the flow of water.

d) In most cases, the petioles were free of the fungus and the maximum percentage of occlusion of petiolar vessels by hyphae was 7 and this too was found only in severely wilted leaves.

e) Vascular discoloration was found only in vessels containing hyphae and was always associated with the fungus.

f) Hyperplasia was found in the hypocotyl and first and second internodal regions of infected plants when symptoms were well developed.

g) Tylose formation was not a prominent feature of this disease. In sections of 9-day old infected plants, they were rarely present. However, they were found in fairly large numbers in infected plants after 12 days.

h) Gels and gums were not formed in sufficient quantities to block the vessels. The maximum percentage of vessels showing gels or gums was less than 10 in plants after 9 days and when they were showing typical wilt symptoms.

i) The fungus was confined to vessels until the host was killed or severely diseased.

Cuttings from root inoculated plants taken 6, 7 and 8 days after inoculation developed typical wilt symptoms 2 to 3 days later when grown in nutrient solution. Anatomical studies of bases of these cuttings revealed that hyphae were sparsely distributed and probably insufficient to cause obstruction to water flow. This suggested the presence of some substance moving into the leaves in advance of the pathogen to cause wilting.

When cuttings from inoculated plants were placed in a dye solution and kept under conditions favouring normal transpiration, most of the vessels were found to have taken up the dye.

Cuttings inoculated with spore suspensions developed typical wilt symptoms; the severity of symptom development depended on the size of the inoculum.

The growth of lateral roots was considerably reduced in inoculated plants and cuttings.

Spore inoculated cuttings did not show any marked increase in the severity of disease when they were fed with glucose, galacturonic acid or casamino acids.

The respiration rates of inoculated plants were found to increase steadily in inoculated plants from the 4th day after inoculation until symptom development. With the appearance of symptoms, the respiration was reduced.

The transpiration of intact plants, cuttings and detached leaves was found to decrease gradually after inoculation. But, in cuttings and detached leaves the rate of transpiration exceeded the rate of absorption, though it did not exceed that of healthy cuttings and leaves.

There was a similar reduction in the rate of

absorption in the inoculated cuttings and detached leaves of infected plants.

Culture filtrates obtained from 10-day old cultures was toxic to cuttings, even when diluted 50 times. Cuttings treated with cell free filtrates developed typical wilt symptoms.

When a culture filtrate was dialysed against water its toxicity was lost after 24 to 48 hours, so that toxicity was due to dialysable substances.

Cuttings placed in the water against which the filtrate was dialysed developed typical wilt symptoms.

Anatomical studies of the stem bases of culture filtrate treated cuttings showed the presence of gels and gums in 20 to 50 percent of vessels. Discoloration of vessel walls was also very prominent.

Cuttings treated with a culture filtrate respired at a higher rate before symptom development.

A similar increase in the rate of transpiration also was noticed, 2 days after treatment, but the rate of transpiration fell considerably before symptom development. A simultaneous fall in the rate of absorption was also noticed.

When leaf disks were floated in xylem exudate from healthy and infected plants, in Warburg flasks,

those in sap from infected plants respired at a higher rate suggesting that the sap was toxic. The fungus grew satisfactorily in the xylem exudate from healthy plants when supplemented with 0.1 per cent glucose and incubated in the dark.

The filtrate obtained from the sap-glucose culture after 10 days incubation was found to be toxic, inducing typical wilt symptoms.

Fusaric acid was detected in culture filtrates. A 10-day old culture was found to contain about 20 mg. per litre.

When cuttings were treated with fusaric acid solution at 100 mg. or more per kg. fresh wt., they wilted, the severity of wilting depended on the concentration. Higher concentration caused shrinking of the stem and twisting of petioles which are not typical features of bean wilt. Typical symptoms were developed when cuttings were treated with 100 to 150 mg. per kg. fresh wt.

When cuttings were treated with fusaric acid at the above rate, they transpired more than did healthy cuttings.

Fusaric acid at concentrations sufficient to cause disease symptoms, reduced respiration.

Fusaric acid was detected in diseased plants by paper chromatography. The maximum amount detected in severely infected plants was 3.5 mg. per 100 gm. fresh wt. (35 mg. per kg. fresh wt.). But experiments with fusaric acid had shown that at least 100 mg. per kg. fresh wt. is needed to cause typical disease symptoms in beans.

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