SHORT TITLE

VERTICILLIUM WILT OF TOBACCO

by

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HOST-PARASITE RELATIONSHIPS IN VERTICILLIUM WILT OF TOBACCO

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A

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ABSTRACT

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HOST-PARASITE RELATIONSHIPS

IN VERTICILLIUM WILT OF

TOBACCO

Establishment of Verticillium dahliae in tobacco leaves was followed by a new technique, examining discs excised with a microsampler. Invasion of the leaf occurred long before symptoms appeared. Distribution of the fungus within the vessels was irregular. "Action in advance" of the pathogen did not occur. Chlorosis did not appear until after invasion of the secondary veins. The pathogen seldom colonized the finest veinlets nor did it invade the intercellular spaces of the lamina until necrosis developed. V. dahliae was present in desiccated leaves as torulose resting hyphae.

Xylem sap from infected or healthy, resistant or susceptible tobacco plants was not toxic to hyphae or conidia but altered the growth form of the fungus. Substances with cytokinin-like activity as well as senescence-accelerators were present in xylem sap.

All tobacco cells appear resistant to V. dahliae provided their vitality is maintained.

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INTRODUCTION

The successful cultivation of flue-cured tobacco (Nicotiana tabacum L.) requires particular soil types and climatic conditions. There is only one area in New Zealand, around the Motueka valley in Nelson province, where these conditions are found. This area, approximately 6,000 acres, produces about one half of the country's tobacco requirements. This crop represents an important saving in overseas exchange.

Because tobacco land is at a premium and the farms are small and highly capitalized with plant and machinery, monoculture has been practised by tobacco farmers in this area for many years. Therefore diseases which attack the crop jeopardize the whole industry.

Tobacco mosaic virus was a problem at first and this was eventually controlled by better hygiene (Thomson 1944). Later black root-rot caused by Thielaviopsis basicola Berk. and Br. was troublesome but the introduction of resistant cultivars from U.S.A. allowed tobacco culture to continue (Wright and Biss 1967).

More recently there has been a steady increase in the infestation of the tobacco fields by Verticillium dahliae

Kleb. (Thomson and McLeod 1959). This pathogen was first isolated from a few wilted plants with chlorotic leaves from two separate locations in the 1939-40 season (Curtis 1940). By 1959, 70 acres were affected by Verticillium to varying degrees, 30 acres so badly that production was no longer an economic proposition (Thomson and McLeod 1959). In 1964, when the last detailed survey was made, 442 acres of the total tobacco area of 5,878 acres were affected, 77 heavily enough to be put out of production (N.Z. Tobacco Research Station Annual Report, 1965). It has been estimated more recently (P. Hamilton, personal communication) that the area where Verticillium is a problem may be increasing at the rate of 1% per annum.

New Zealand is the only country in the world where Verticillium is a problem in tobacco production. Apart from monoculture, another contributing factor to the problem has been the standard practice of the New Zealand grower of chopping up the crop remains and discing these under at the end of the season. Stems and unpicked leaves from diseased plants are returned to the soil by this procedure and this has undoubtedly helped build up inoculum in the tobacco fields.

Initial infections apparently arose from domestic and market gardens where potatoes and tomatoes had been grown before tobacco. The rapid increase in incidence of Verticillium wilt between 1959 and 1964 may have resulted from the demand for more tobacco, encouraging its cultivation on land previously used for growing these crops. Spread of the pathogen to uninfested land probably occurred by the transfer of soil during cultivation, by the movement of men and machinery from one area to another, by occasional flooding of the Motueka river and by the transplanting of seedlings started in infested soil.

Verticillium in other high-value crops has been controlled successfully by soil fumigation with chloropicrin or mixtures of chloropicrin and methyl bromide. In the past small areas of tobacco land were treated with chloropicrin and to a lesser extent with formalin. The area fumigated with these chemicals increased to 117 acres in 1964-5 but then it declined to 30 and then to 7 acres in the two following years (N.Z. Tobacco Research Station Annual Reports, 1966 to 1968). There were several reasons for the failure of the fumigation of tobacco land. It was expensive, and to be economical it had to provide some control for more than one season. Treatment in many cases gave no

marked reduction in disease in the following crop, partly because of farm practices and partly because soil and temperature conditions were rarely optimum when the fumigant had to be applied. The increase in available nitrogen after treatment made the next crop too rank to harvest and sometimes this effect was carried over into the second season. The chlorine released from the chloropicrin added to that which had built up in the soil from fertilizers and from rainfall in an area close to the sea, made the chlorine content of the leaf so high that it would not burn. Soil fumigation is now recommended only for use in preventing spread from small areas of infestation and not for controlling the disease once it has become established over a large area.

The development of resistant varieties offered the best hope of control under the circumstances. Improvements in inoculation and evaluation techniques permitted rapid progress towards this goal (Wright 1968a). A range of resistance was found in the available cultivars of N. tabacum.

It was found that the inheritance of resistance to Verticillium in tobacco was controlled by additive factors

(Wright 1968a). These appeared to operate mainly through the root system (Gibbins and Wright 1968). There was also a maturity effect, young shoots were much more resistant than older shoots (Wright 1968b).

Despite all the literature on this pathogen in other crops, it was uncertain what the fungus was doing inside the host and what constituted resistance. More information was required on the role of the root system in resistance. More detail was required on the establishment of the fungus in the host especially in the leaves. It was necessary to investigate the hypothesis that all vigorous tobacco cells were resistant to Verticillium and that the fungus proliferated and symptoms in the leaves appeared only after the host tissues began to senesce (Wright 1969). It was also necessary to attempt to identify the bodies which had been observed to pass through the cell walls of tobacco and tomato leaf parenchyma near the pathogen (Wright and Abrahamson 1970).

These problems were the basis of the work reported in this thesis.

REVIEW OF THE LITERATURE

Many review articles and some books have dealt with vascular wilt diseases. Most authors discussed the subject in a generalized way (Sadasivan 1961; Beckman 1964; Dimond 1970) while others paid greater attention to specific topics such as toxins (Braun and Pringle 1959; Ludwig 1960), water deficiency (Subramanian and Saraswathi-Devi 1959), enzymes (Wood 1960) or the biochemistry of host-parasite relations (Goodman, Kiraly and Zaitlin 1967). Likewise many books on plant pathology within recent years have summarized the available knowledge on wilt diseases (Agrios 1969; Garrett 1970; Gäumann 1950; Rubin 1963; Walker 1969; Wood 1967 and others).

Most reviewers and authors attempt to explain the nature and cause of the various symptoms associated with wilt diseases, as well as the nature of resistance and the related phenomenon of host preference.

Rudolf (1931) in a comprehensive review discussed all aspects of Verticillium wilt of crops including history, taxonomy, etiology, host range, distribution and host-parasite relations. Panton (1964) also dealt with various aspects of Verticillium wilt, including the controversy over whether

V. dahliae Kleb. should be considered a distinct species and the role of fungal enzymes in symptom expression. Sackston (1972) reviewed the recent literature on host reaction to Verticillium infection, including the effect of environment, and the inheritance and mode of action of disease resistance, emphasizing that action in advance does not appear to occur in wilts caused by Verticillium, as it does in some other wilt diseases.

Symptoms

Rudolf (1931) described what might be considered to be typical symptoms, although he pointed out that there was considerable diversity in symptom expression in the various hosts.

The first symptoms usually occur on the lower leaves as slight chlorosis. Chlorosis becomes more general until the leaves are necrotic. Discoloration of the leaves usually progresses up the affected stem in acropetal succession. In some varieties but not all, wilting of all or part of the leaf may occur, while in others the infected leaves may turn dull green without becoming chlorotic. Severe infection of

some hosts such as hop or cotton ends in defoliation. In both these crops mild and severe forms of the disease occur (Isaac and Keyworth 1948; Schnathorst and Mathre 1966). Newly formed leaves may be smaller than normal and new shoots may grow from the base of badly affected plants. All leaves on a host may have symptoms or they may appear on one side of the plant, one shoot or limb or even one side of the midrib. Chlorosis does not usually occur until the pathogen is present in the leaf veins (Rudolf 1931; Talboys 1958b; Hall and Busch 1971; Sackston 1972). Rudolf referred to workers who found that symptoms did not appear until the fungus had penetrated the vessel walls and entered the parenchyma tissue.

Plants with Verticillium wilt are generally stunted and the internodes shortened. The stems and roots of most plants with the disease show no external symptoms but some annuals may have black cankers on roots which contain the fungus.

Discoloration of the vessels of affected plants may be brown, brownish black, brownish red, grey, yellow or green and may be traced from the roots to the leaf petioles at the top of the plant in a continuous line. The discoloration often intensifies as the disease runs its course.

Hosts may or may not die as a result of Verticillium infection. Apart from maples, mature trees are seldom killed although infected first year shoots may die (Rudolf 1931).

The Plugging of the Host Vessels

Much of the basic work on histology and etiology of vascular wilt diseases had been done before 1928 (Rudolf 1931); for example, the concept of reduced water supply to the leaf tissue being directly responsible for symptoms was put forward by Smith in 1896 (Walker 1969). He reached this conclusion from histological evidence which showed mechanical blocking of the vessels and later workers confirmed his findings (Klebahn 1913; Melhus et al. 1924; Waggoner and Dimond 1954; Dimond 1966).

Rudolf (1931) reviewing the work of others and more recently Pegg and Dixon (1969), have concluded that the severity of symptoms does not necessarily depend upon the amount of mycelium in the vessels. Severe symptoms may result from sparse colonization of the vascular tract or lighter symptoms may follow heavy invasion. Rudolf cites the work of Klebahn who considered that growth of mycelium in the vessels eventually led to crowding, restriction of water flow, and the onset of symptoms. Waggoner and Dimond (1954)

concluded that mycelium in the vessels may contribute to, but may not necessarily be the sole cause of, resistance to sap flow.

According to Rudolf (1931) tylose formation in infected plants has been observed by many workers starting with Reinke and Berthold in 1879. Wardlaw (1931) working with Fusarium wilt of banana concluded that tyloses and hypertrophy in general, were the result of physiological disturbances. The formation of tyloses and hypertrophy are considered to be the result of hyperauxiny (Grieve 1939; Pegg and Selman 1959; Goodman et al. 1967). The result of tylose formation is uncertain; some consider that it plays a part in resisting invasion of the host by confining the pathogen, whereas others believe it is not important in resistance and may contribute to symptoms by blocking the vessels (Talboys 1958b, 1964; Beckman 1964, 1966; Pegg and Dixon 1969; Dimond 1970).

Butler (1911) concluded that gel formation in fruit trees resulted from enzymic dissolution of the middle lamella and primary walls. Ludwig (1952) found a gel-like material formed in infected tomato vessels which he believed contained pectin and reduced the sap flow in these ducts. Later Scheffer et al. (1956), Wood (1961) and many others have concluded that pectinases and cellulases from the pathogen

liberate material from the vessel walls which increases sap viscosity and plugs the vessels. These enzymes may be either inducible or constitutive and their role is in invasion rather than nutrition. The ability to form enzymes has been related to aggressivity in Verticillium isolates (Dimond 1970).

Like tylose formation, the deposition of gums in the vessels of infected plants has long been associated with Verticillium wilt (Rudolf 1931). It is thought that gum is formed by the combination of oxidized and polymerized phenolic compounds with gels (Dimond 1970). Recently Cyaninski et al. (1971) with the electron microscope, studied the formation of a substance which these workers believed to be gum, by the companion cells of invaded vessels.

Toxins

Hutchinson (cited by Dowson 1922) found that Pseudomonas solanacearum (Smith) Dowson, produced metabolites which when injected into tobacco produced wilting, whereas vessels blocked with wax did not. Bewley (1922) with Verticillium wilt of tomato and Brandes (1919) with banana wilt, also concluded that toxins were important. For many years the belief that toxins were transported in the transpiration stream and were responsible for symptoms, as well as host preference,

dominated research on vascular wilt diseases. The main proponents of this hypothesis were Gäumann and his co-workers (1950) with the toxins lycomarasmin and fusaric acid. In 1953 Dimond and Waggoner pointed out that lycomarasmin did not occur in diseased plants and was a product of autolysis of old cultures. Microbes produce a variety of phytotoxic metabolites but to evaluate their significance in vascular wilt diseases is difficult. Toxic substances have been detected in infected tissue but they are now considered to be of secondary importance (Dimond 1970).

Invasion and Colonization

Rudolf (1931) described the work of Reinke and Berthold, who found that conidia would not germinate on potato tubers but actively growing mycelium would invade the roots. He also discussed the work of Van der Meer who reported that the penetration of potato and cucumber seedling roots by Verticillium occurred under sterile conditions. She showed that following penetration the inner walls of the epidermal cells had cone-shaped protuberances directly opposite the points of entry.

Since this early work others have observed the invasion of intact host roots by Verticillium spp. (Bewley 1922; Isaac 1946; Talboys 1958a and many others).

Talboys (1958a) with hop and Griffiths and Isaac (1966) with tomato were able to follow early colonization of the root by Verticillium reporting both inter- and intra-cellular invasion, the formation of lignitubers within the cells, and the deposition of lignin and suberin on the host cell wall. Griffiths and Isaac observed hyphae entering the xylem vessels through the pit membranes 4 days after inoculation.

The progress of mycelium through the vessels and the formation of fungal spores in them were also observed by early workers (Rudolf 1931). Fusarium bud spores were noticed forming in the vessels of cabbage by Gilman in 1916. Dimond (1970) credits the work of Yoshi (1933) as being the first to show the importance of spore movement in the dispersal of vascular wilt fungi within the host. This was later demonstrated by Banfield (1941) and Pomerleau (1968) in trees; Scheffer and Walker (1953) in Fusarium wilt of tomato; Sewell and Wilson (1964) in Verticillium wilt of hops and Garber and Houston (1966) in cotton. However, Mahanty (1970) was of the opinion that dispersal by conidia may not be important in tobacco.

With the exception of some bacterial wilt diseases, and in some cases with fungal wilts, confinement to the vascular tract until the surrounding tissue is moribund, is a characteristic of vascular wilts. Ernst Hallier in 1878 was the first

to draw attention to this feature (Rudolf 1931; Beckman 1964).

Klebahn (cited by Rudolf 1931) considered that Verticillium inhabits the vessels of the host as a saprophyte, eventually crowding them so that nutrient translocation is inhibited and the host cells die of starvation. Wood (1961) found that Verticillium would grow only slowly in tracheal fluid from tomatoes without added carbohydrate. Schnathorst et al. (1964 and 1966) found that germination of conidia and growth of the pathogen was inhibited by tracheal sap from an inoculated resistant cotton cultivar but not by uninoculated plants or infected susceptible plants. Sinha and Wood (1968) with tomato and Taylor (1968) with tobacco could find little or no difference in growth of the fungus in tracheal fluid from infected or uninfected, resistant and susceptible plants.

Resistance of the Host to Colonization

Rudolf (1931) considered that the conical protuberances seen in potato and cucumber root cells by Van der Meer may be defense reactions of the host cells. Talboys (1958a) concluded that the initial infection of hops by Verticillium was checked by lignification of the epidermal and cortical cell walls and that exclusion of the fungus from the pericycle was apparently associated with deposition of suberin on the endodermal walls.

This initial check was self perpetuating, as invasion by the limited amount of fungus which reached the vascular tract, stimulated the formation of tyloses in the vessels ahead of the fungus, thus blocking its progress (Talboys 1958b). This was Talboys' explanation of the determinative role of the root system postulated by Keyworth (1953). The work of Griffiths and his coworkers (Griffiths and Lim 1964; Griffiths and Isaac 1966) with several species of host and Verticillium confirmed the findings of Talboys. There appeared to be a relationship between the virulence of the pathogens and their ability to invade the roots.

Talboys had observed that V. albo-atrum may form resting hyphae before and during invasion and Griffiths observed that V. dahliae may form microsclerotia before and after entering the root.

Garber and Houston (1966) showed that epidermal invasion of the roots of cotton seedlings by Verticillium occurred within 24 hours of contact, but most hyphae did not reach the vascular region, and those that did were retarded in growth or were destroyed. The few that penetrated the xylem did so within 3 days of initial infection of the root, regardless of the level of tolerance of the host. The time and amount of infection were closely related to the mass of

invading fungi. After the xylem had been entered, rapid colonization was attributed to the movement of conidia in the xylem sap. These workers found that susceptible seedlings had more spores near the stem apex than resistant seedlings and they postulated that resistance was related to the inhibition of spore germination in the vessels of the leaves (Garber and Houston 1967).

Presley and Taylor (1969) found that intact end walls of xylem vessels in cotton seedlings restricted the colonization of the shoot by conidia and this contributed to resistance in both susceptible and resistant cotton varieties. In older plants the walls had disappeared and conidia were able to move to the terminal parts of the plants much more readily.

Snyder et al. (1946) concluded that in resistant tomato stems the lateral movement of Fusarium from one vessel to the next was restricted. Scheffer and Walker (1954) inoculated tomato cuttings with Fusarium bud spores by allowing the spores to flow into the vessels. In the resistant plants the pathogen was confined to the primary vessels and was restricted in lateral movement. The fungus disappeared from the upper stem of resistant plants whereas in susceptible ones the fungus gradually invaded the vessels of the host. Keyworth (1953) reached similar conclusions with Verticillium

wilt of hops. The pathogen was restricted in its colonization of the vessels more in resistant than in susceptible cultivars and in both, confinement was by active resistance of the host tissue. This resistance could be overcome by using very high inoculum levels.

The active formation of barriers such as gels, gums, tyloses, and the lignification and suberization of root cell walls, are believed to be defense reactions by the host to invasion. These plus the presence of xylem end walls are known to be effective in slowing down the colonization of the vascular system. Related to this, inoculum potential is considered to be important in the establishment of vascular wilt pathogens in the host and subsequent rate of disease development. High levels of inoculum potential seem to be related to overcoming active resistance of the root cells (Keyworth 1953; Tolmsoff and Young 1957; Garber and Houston 1966; Müller 1969).

Most workers agree that the vascular discoloration which is characteristic of vascular wilt diseases (Rudolf 1931; Beckman 1964) results from the oxidation and polymerization of phenolic compounds through the action of phenolases, and many workers have postulated that the pathogens are confined to the vessels by the fungitoxic properties of these pigments

(Davis, Waggoner and Dimond 1953; Dimond 1970). Resistance to invasion has been associated with the ability of the host to form polyphenolic substances which are responsible for blocking the enzyme activity of the host (Deese and Stahmann 1963; Goodman et al. 1967).

Bell (1969) reviewed some of the literature related to disease resistance and concluded that mechanical barriers and the blocking of pathogen enzymes could not entirely explain differences in resistance found in cotton cultivars. He believed that the rate of formation of gossypol-related terpenoids and condensed tannins was the key to Verticillium resistance in this crop. Beckman (1966 and 1971) postulated that wound type responses come from phenol-containing cells scattered throughout host tissue. These cells respond to stimuli initiated by the invading pathogen.

Grafting experiments have been utilized by many workers to determine the site of resistance to wilt pathogens and their conclusions may be summarized by the statement that both root and stem tissue have resistance but the root in some way has a determinative effect on disease development (Heinze and Andrus 1945; Keyworth 1953; Sewell and Wilson 1964; Fronek 1965). Berry and Thomas (1961) found that resistance to Verticillium wilt of mint was greater in the stem than in the root. In this

host, leaf symptoms occur in basipetal succession. Gibbins and Wright (1968) showed that in tobacco the influence of the root system was quantitative and predictable. Three-quarters of the resistance in the cultivars they studied was conditioned by the root system and this ratio was constant in all cultivars at all stages of growth.

To explain the results obtained from studies on Verticillium wilt of tobacco, Wright (1969) postulated that the shoot and root systems both influence the integrity of the leaf cells and that in tobacco it is the loss of vitality of these cells which determines the difference between resistance and susceptibility.

This hypothesis may be supported by the work of Maine (1960) who concluded that resistance to P. solanacearum in tobacco resulted from some substance produced in the leaves in the light. Gothaskar et al. (1955) postulated that resistance to Fusarium wilt in tomato was closely linked with labile substance(s) formed continuously by the host cells.

Inheritance of Resistance

Variety and species differences in resistance to vascular wilts have been found in almost every crop in which widespread screening has been made. Early work in selecting

crops for resistance to wilt diseases was carried out by E.F. Smith before 1900 and W.A. Orton had selected varieties of cotton, melons and cowpeas resistant to Fusarium during the decade 1899-1909 (Walker 1969). Walker also described the classical work of Jones and Gilman in developing cabbages resistant to Fusarium wilt. Two forms of resistance were found. The first was controlled by a single dominant gene and prevented the fungus from invading the roots. The second was quantitatively inherited and controlled mechanisms which slowed down the rate of spread of the pathogen within the vessels. This form of resistance was markedly influenced by temperature and the evidence suggested that it was the pathogen not the host which responded to temperature changes (Walker 1969).

Putt (1964) reviewed the literature on the breeding behaviour of crops with resistance to Verticillium and found that there was no consistent pattern among the various host species. Recessive, dominant and quantitative factors were found in plants with field resistance and in some individual plants at least two systems were present (Bowen 1965).

Resistance to one vascular wilt pathogen may give the host resistance to others (Lucas 1965) and also to unrelated diseases (Sappenfield1963). The presence of a second pathogen

has been known to lower resistance (McKeen and Mountain 1960).

Recent understanding of how genes operate and how they are controlled (Jacob and Monod 1961) suggests that resistance is stimulated by the activities of the pathogen and that the pathogen is influenced by the host response (Beckman 1966). Most resistance requires the activation of gene operons which control the formation, or increased synthesis, of compounds such as polyphenols (Hadwiger and Schwochau 1969). According to Bell (1969) the evidence suggests that gene activation occurs in vascular wilts after minor injury. Large inoculations cause permanent cell damage, inducing a different response. Talboys (1958b) reported that tylose formation was inversely related to the quantity of fungus within the vessels.

Nutrition has been associated with disease development and it has been postulated that there is an interplay between nutrition and the equipose of genes (Garber 1956; Roberts and Smith 1968). Albersheim et al. (1969) have suggested that dissolution products from the xylem wall may or may not stimulate the pathogen and that this is the basis of host specificity. Linskens (1968) suggested that induced substances passed from one host cell to the next to depress genes and also from the host to the pathogen. He postulated that the "mutual gene regulation theory" explained action in advance and specificity.

Nomenclature and History of *Verticillium dahliae*

Verticillium species are members of the Fungi Imperfecti, order Moniliales, family Moniliaceae. Although six species of *Verticillium* are listed as being pathogenic to plants, the two main causal organisms of *Verticillium* wilt are *V. dahliae* and *V. albo-atrum* (Isaac 1967; Hawksworth and Talboys 1970). According to Rudolf (1931) the genus was created by Nees von Esenbeck in 1816 and named from the whorled conidiophore branches on which ovoid, hyaline, usually single celled, uninucleate, conidia arise. Some confusion arose after Corda set up a new genus *Acrostalagmus* in 1830, based on the formation of spore clusters on the conidiophores. In 1854 Hoffman showed that there was no real difference between the two.

Rudolf (1931) described how Ernst Hallier (1878) isolated a fungus pathogenic to potato which he thought was *Pleospora polytricha* Tuls. Hallier gave a good description of the morphology and etiology of this organism which, from his drawings, appears to have been *V. dahliae*. Reinke and Berthold (1879) accepted Hoffman's conclusions when they named *V. albo-atrum* the following year.

Klebahn (1913) isolated a fungus from dahlia which he considered sufficiently different to be given specific rank.

Rudolf (1931) and many other workers since then, have considered V. albo-atrum and V. dahliae to be the same species (Smith 1965; Isaac 1967). Hastie (1971 and personal communication) has found that some isolates of the two will hybridize but there may be some incompatibility between the two genomes. Heterokaryosis, high frequency of mitotic recombination, and anastomosis may explain the variability in pathogenicity and morphology found in Verticillium species (Hastie 1967; Orellana 1969).

Host Range of Verticillium Species

From the first decade of the 20th century there has been a steady increase in the number of reports of crops being attacked by Verticillium and in the geographic distribution of problems caused by this pathogen. Rudolf (1931) recorded the isolation of Verticillium from over 120 species and Engelhard (1957) reported that isolation had been made from over 250 species, although not all of these showed symptoms. Smith (1965) listed 38 plant species from which Verticillium had been isolated in New Zealand.

Verticillium Wilt of Tobacco

Averna-Sacca in a short report published in 1922, stated that V. albo-atrum had attacked tobacco plants in Brazil

(Rudolf 1931). From the description given by this worker Rudolf doubted whether the fungus was in fact Verticillium. Wolf (1935) reported that V. albo-atrum had caused wilting in tobacco plants in Tennessee and Hopkins (1956) isolated a species of Verticillium from a few tobacco plants with root-rot-like symptoms; this was the first report of this disease from Africa. Recently G. Evans (personal communication) isolated V. dahliae from wild Nicotiana suaveolens in Australia but there were no reports of commercial tobacco being involved. McLeod and Thomson (1959) had found this species was susceptible to the pathogen. Lucas (1965) reviewed the literature on Verticillium with special reference to the problem in New Zealand. He emphasized the similarity between the different wilt diseases of tobacco caused by Fusarium oxysporum (Schlect) Wr. var. nicotianae Johnson, P. solanacearum and V. dahliae.

In a series of papers, Thomson and McLeod described V. dahliae as a pathogen of tobacco including symptoms, spread of the disease in New Zealand tobacco fields, and initial attempts to control the problem by soil fumigation and by breeding for resistance.

Symptoms in Tobacco

Thomson and McLeod (1959) pointed out that the most outstanding characteristic of this disease in tobacco is the orange color of the infected leaves. Before chlorosis occurs one or more leaves may wilt, particularly in hot weather. This is followed by interveinal areas of the leaves turning bright orange. As the disease progresses, the tissue becomes brown with an orange border between the living and dead areas. Usually symptoms appear first after flowering, on one or two leaves low down on the plant, although they may occur at any height. Eventually there is a complete breakdown of all leaves.

Wright described other symptoms which may occur in inoculated tobacco plants (Wright 1966; Wright and Biss 1968). These were delayed flowering, decrease in dry matter content, retardation of growth, an increase in the number of leaves formed, shortening of the internodes, uneven growth of the lamina and shortening of the midrib.

Breeding for Resistance

McLeod described screening work for resistance in N. tabacum cultivars and other Nicotiana species and also the initial breeding work (McLeod and Thomson 1959). The wild

Colombian variety, given the introduction number TI 448 A by the U.S. Department of Agriculture, did not show symptoms until the fifth year of trial. This cultivar had become the basis for control of Granville wilt (P. solanacearum) in the U.S.A. and it was also found to have resistance to Fusarium wilt (Clayton and Smith 1942; Clayton 1958). The high field resistance of TI 448 A, Amarello and Kentucky 34 to Verticillium was believed by McLeod to be controlled by multiple genes, because when crossed with more susceptible varieties the resistance was not transferred to the progeny. Wright (1968a) with genetic experiments confirmed that inheritance of resistance was controlled by quantitative factors.

Establishment of Verticillium Within Tobacco Plants

Wright (1968b) by using heavy inoculation found V. dahliae could enter uninjured roots of both resistant and susceptible tobacco seedlings. Mahanty (1970) used much smaller plants under sterile conditions and was unable to observe penetration of the roots by the pathogen. From this he concluded that V. dahliae was unable to enter the roots of tobacco seedlings until the root tissue had become moribund.

Wright (1968b) allowed conidia to be taken into the vessels of seedlings and by isolation techniques was able to show that most of the conidia had been destroyed within 24

hours and that this effect was more marked towards the top of the stem than the base. After 2 months the pathogen had been kept in check at the base of the stem in the resistant variety but had increased up the stem in the susceptible variety.

Mahanty (1970) repeated this work using histological methods for observing the pathogen. He found that within 8 to 12 hours conidia had been trapped by gums in the vessels and after 4 days most of the spores were dead in the resistant plants but mycelium could be seen trapped in gums within the vessels of the susceptible variety. After 5 weeks he could find no fungus at the top of resistant plants and at the base its growth had been limited. In the susceptible cultivar the fungus was well up the stem and tended to have greater lateral spread.

Mahanty concluded that, lateral growth of the fungus was restricted in the vascular tissue, abundant sporulation did not occur in the vessels of tobacco and that first gums and later the formation of tyloses were the mechanisms of resistance in this crop.

MATERIALS AND METHODS

Host Cultivars

Plants used in this work were some of the tobacco cultivars (Nicotiana tabacum L.) studied by the author in field experiments in New Zealand. These included:

Virginia gold (VG), susceptible to Verticillium wilt.

Giant (G), a mutant form which arose spontaneously in a commercial crop of VG. The G plants flower under short daylength and if not debudded in the field, will continue to grow producing 40 or more leaves, compared with about 18 on VG. When G plants are infected with Verticillium, chlorosis appears on the lower leaves and progresses in acropetal succession but the upper 15 or so leaves do not show symptoms even though they may be extensively invaded by the pathogen.

A breeding line from North Carolina, N.C.2512 (NC), which has some genes derived from N. plumbaginifolia Viv. and has field tolerance to Verticillium. In the United States this line has resistance to several root rots as well as to vascular wilt organisms (Lucas 1965).

A selection derived from McNairs 121 (MN) which has a very high level of resistance to Verticillium dahliae (Wright 1968a).

In a few trials the sunflower cultivar (Helianthus annuus L.) Sunrise was used as well as the eggplant (Solanum melongena L.) variety Black Beauty and the cotton cultivar (Gossypium hirsutum L.) Stardel.

Growing Conditions

Tobacco plants were grown in controlled environment cabinets at 25°C day temperature and 13°C night, with a day-length of 14 hours and 17,500 to 20,000 lux light intensity (1600-1800 ft-c). The light was from cool white V.H.O. fluorescent tubes supplemented with incandescent bulbs. These conditions were close to those occurring in New Zealand in January when Verticillium symptoms first appeared in the field. Occasionally plants were grown in the greenhouse with much poorer control of the environment. Symptoms and relative resistance of the cultivars infected with Verticillium in the growth cabinets and the greenhouse were similar to those observed in the field in New Zealand.

The Pathogen

The isolate of Verticillium dahliae Kleb. from tobacco used in most of this work was that designated V3 by Wright and Biss (1968). Other isolates used included V58 from sunflower (Devaux and Sackston 1966) and four V. dahliae isolates from cotton.

Inoculation

Cultures for root dip inoculation were grown on potato dextrose agar (P.D.A.) in petri dishes for 14 days at laboratory temperature (about 22°C). Inoculum was prepared by comminuting one petri plate culture per 100 ml of distilled water for 3 minutes in a Waring blendor.

Plants 12 weeks old were removed from flats or pots, their roots carefully washed in tap water and dipped briefly into the suspension of spores, mycelial fragments and microsclerotia. The plants were repotted in a mixture of soil, sand and peat and placed in growth cabinets or on a greenhouse bench.

Symptoms

The term "symptoms" in this thesis refers only to chlorosis and necrosis of the leaves unless stated otherwise (Figure 1).

FIGURE 1

FIGURE 1

Leaves of inoculated tobacco cv. Hicks growing in the field. The leaves have chlorosis (C) and necrosis (N) of the interveinal areas, typical of "tiger-stripe" symptoms.

FIGURE 1

FIGURE 1.

Histology

Observations were made on material fixed in F.A.A. (40% formaldehyde 10 cc; glacial acetic acid 5 cc; ethyl alcohol 50 cc and water 35 cc) infiltrated and embedded in paraffin wax. Sections were usually cut 15 μ thick and stained in a mixture of acid fuchsin (0.1 g) and malachite green, (0.25 g) in 100 ml of 25% alcohol (Sass 1968; Mahanty 1970).

Most observations were made on fresh material mounted in water or in a lactophenol-picric acid staining solution (Smith 1969) modified by the author as follows:

Phenol crystals	10.0 g
Lactic acid	10.0 g
Glycerol	20.0 g
Distilled water	10.0 g
Orange G	0.5 g

To this excess picric acid was added until saturation and after filtering, a few drops of sudan III (0.5 g in 100 cc of 80% alcohol) were added. This stain gave good differentiation between pathogen and host cells.

Leaf parenchyma cells were studied by cutting discs (8.2 mm) from the lamina with a No. 4 cork borer. These

discs were pressed gently between two strips of adhesive cellulose tape. When the pieces of tape were pulled apart quickly, the epidermis of one side of the disc adhered to one strip of tape and the parenchyma and vessels to the other. The tissue was removed from the tape by gently working a wet, sharp razor blade between the sticky surface and the cuticle. The material was then mounted on a microscope slide and stains added for specific studies.

The location, quantity and form of the pathogen in the vessels of the leaf were determined by a technique developed for the purpose. Tissues were surface sterilized for 10 seconds in a 30% solution of commercial bleaching agent (containing approximately 2% free chlorine) and then were rinsed in sterile distilled water. Sections of the lamina or vein tissues were cut aseptically with a No. 16 disposable hypodermic needle with the point removed and the edges sharpened. The tissue being sampled was placed on a pad of sterile filter paper and the sterilized "micro cork-borer" was pushed through cutting out a disc 0.86 mm in diameter. A fine wire was fitted inside the needle to eject the discs. This method was used successfully with tobacco, sunflower, cotton, and eggplant leaves.

Five discs were placed in a row approximately 4 mm apart on a microscope slide and covered with a 22 mm circular cover slip broken in half. The row of discs was approximately 2 mm from the straight edge of the cover slip. When in position, the underside of the cover slip was flooded with either distilled water from an eye dropper or in some experiments with other solutions delivered from pasteur pipettes. Two lots of 5 discs were arranged on each slide and the slides were placed on moist filter paper in petri dishes and covered. The samples were usually incubated on a laboratory bench for 24 hours.

V. dahliae grew from the cut ends of the vessels, usually within 3-6 hours. In heavily infected material growth was profuse after 15 hours. The fungus was sometimes retarded if bacterial contamination was high, therefore when it was not possible to surface sterilize tissue before removing the discs, 5 p.p.m. streptomycin were added to the water. The streptomycin did not affect the growth of V. dahliae but did check bacteria.

The amount of fungal growth was measured on a Reichert, Visopan projection microscope. The growth from each disc was scored on a scale of 0 to 5, with 5 for profuse growth from the cut veins and edges of the disc. The total

number of hyphae growing from the vessels was counted where possible and the longest hyphal strand was measured from the edge of the disc to the tip of the hypha. Where more than 50 hyphae grew from a single vessel it was not possible to count the strands and the rating of 0 to 5 gave the most consistent assessment of growth.

Discs which did not have fungus growing from them after 24 hr incubation were plated on P.D.A. Very rarely was V. dahliae isolated from samples from which it did not grow out during the normal incubation in water. Likewise where there was any doubt about the identity of the fungus growing from a disc sample, the sample was plated for positive identification of the fungus.

To follow the pathogen along the midrib and the main lateral veins (Fig. 2) and to estimate the quantity of V. dahliae present in these, a technique was used which had been successful in previous work (Wright 1969). The midrib was cut from the leaf blade, the vascular bundle was removed from the surrounding parenchyma with forceps and scalpel and then the bundle was sectioned transversely into 3 mm lengths. The sections were placed in order along microscope slides and covered with medium from an eye dropper. The composition of this medium which will be termed "cellulose agar" was described

FIGURE 2

Diagram of the veins in a tobacco leaf. On the left hand side of the midrib the lateral and secondary veins have been drawn in. On the right hand side the tertiary veins also are shown. The fine veins of the vascular network are not illustrated in this diagram. The broken lines show how the leaf was divided up into four areas, namely the tip margin TM, tip centre TC, basal margin BM, and basal centre BC. The circles on the lateral veins of the tip margin indicate the sites from which lateral vein samples were taken. The areas indicated by the arrows from I are the points where intercellular inoculations were made.

The figure was adapted from Avery (1933).

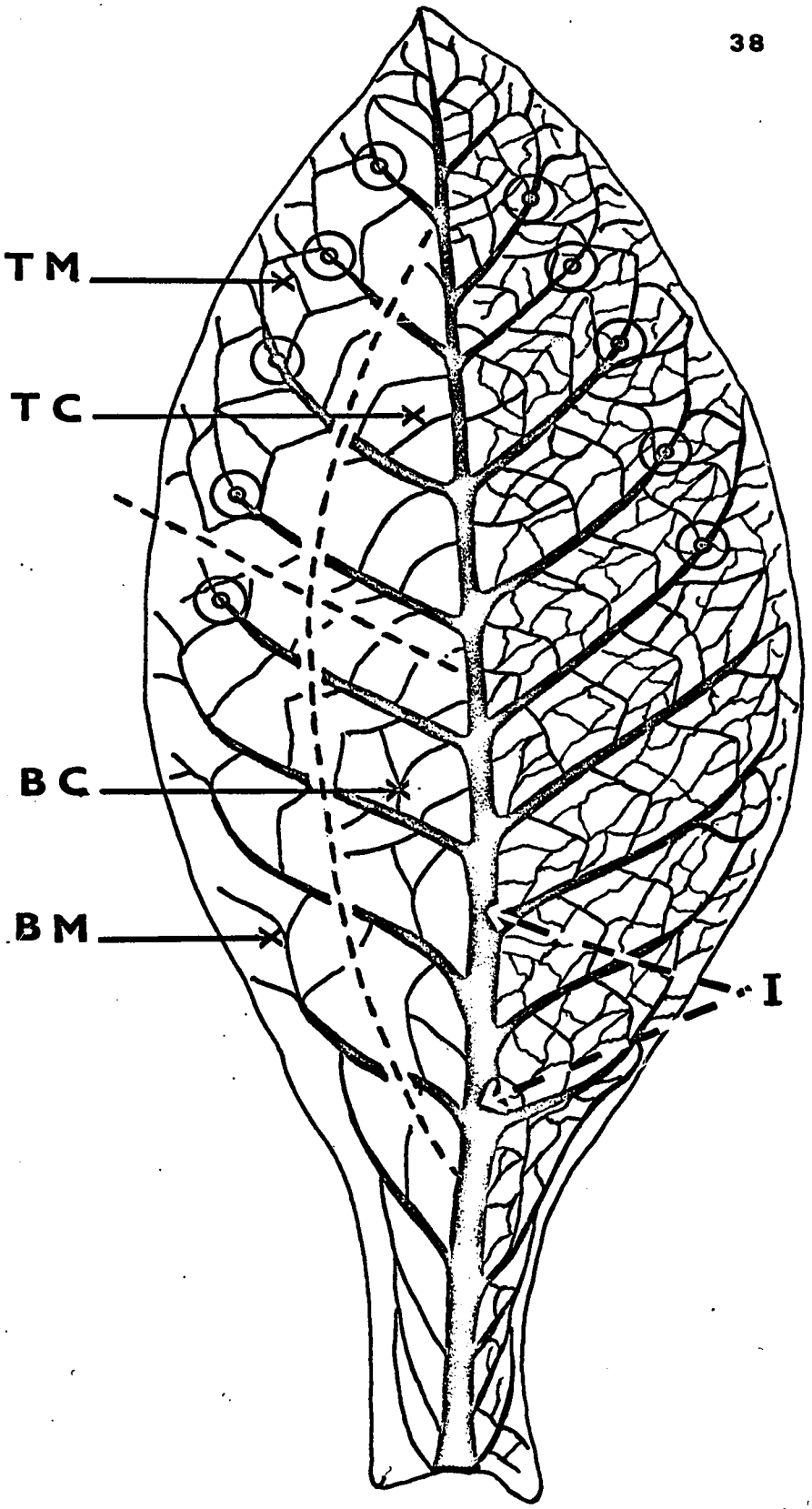


FIGURE 2

in the paper by Wright (1969) containing the following:

Distilled water	500	ml
P.C.N.B.	50	p.p.m.
Neomycin	30	p.p.m.
Streptomycin	30	p.p.m.
Novobiocin	30	p.p.m.
Biotin	10	p.p.m.
Agar	5	g
Carboxymethyl cellulose sodium salt[1/]	1	g
Citrus pectin N.F.[2/]	1	g
KH_2PO_4	1	g
KCl	1	g
$NaNO_3$	0.5	g
$MgSO_4$	0.25	g

The prepared sections were incubated on moist filter paper in petri dishes for one week before assessment under a stereoscopic microscope. The lateral veins were treated in the same way with the exception that the vascular bundles were not removed from the surrounding parenchyma.

1/ Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.

2/ Sunkist Growers, Inc., Ontario, California, U.S.A.

The work of Wright and Abrahamson (1970) on V. dahliae and its relationship to disease symptoms in tobacco leaves, left several questions unanswered. Of particular interest were the unidentified bodies these workers found passing through host parenchyma cell walls in the vicinity of vessels containing the pathogen. This work also mentioned the uncertainty about which of the different cells in microsclerotia of Verticillium dahliae germinated to give rise to new hyphal growth. To study the particulate exudates from the hyphae and the germination of individual microsclerotial cells, the fungus was grown in a clear medium containing 50% glycerine in distilled water (V/V). Microscope slides were placed on moist filter paper in petri dishes, the glycerine-water mixture was added to the slide from an eye dropper before being autoclaved for 20 min at 15 lb p.s.i. The medium was seeded with conidia by running a loopful of spore suspension along the centre of the slide. Germination took place within two days and observations could usually be made within a week.

Single cells from microsclerotia were scraped with a razor blade from dried out agar cultures which had been held at room temperatures for 5 years. These cells germinated slowly after about 14 days.

The glycerine-water mixture was also used with conidia labelled with a fluorescent brightener and the growth studied under ultra-violet light. An America Optical Co. Fluorolume Illuminator was used as the light source (Li 1966).

The spores were labelled by growing V. dahliae for 14 days in liquid Czapek's medium (Tuite 1969) containing 400 p.p.m. "Calcofluor white M 2 R New" (disodium salt of 4, 4' - bis (4 - anilino - 6 - diethylamino - 5 - triazin - 2 - ylamino) - 2, 2' stilbene - disulphonic acid), (Darken1962; Tsao 1969). It was hoped that this stable, non-toxic labelling of the pathogen could be used to follow the fungus within the tissues and vessels.

Variations of the techniques described above and special procedures not mentioned are outlined at the beginning of the sections dealing with the work in which they were used.

The methods for testing the significance of skewness and kurtosis of the frequency distributions of fungus growing from sample discs, are those described by Snedecor and Cochran (1969). Likewise the analysis of variance of the factorial experiments follows the method outlined by these authors.

LOCATION AND FORM OF VERTICILLIUM DAHLIAE IN INFECTED TOBACCO LEAVES

Introduction

Vascular wilt diseases may affect their hosts in various ways. The most obvious symptoms of Verticillium wilt of tobacco occur in the leaves and may include wilting, uneven growth of the lamina, and shortening of the midrib. As in other hosts the most common symptoms are chlorosis and necrosis of the interveinal areas (Fig. 1).

There is much evidence to suggest that wilting and chlorosis result from blocking of the vessels or breakdown of cell membrane permeability, induced by metabolic products of the pathogen carried to the cells of the lamina (Dimond 1970).

Earlier work with tobacco has shown a significant correlation between the amount of V. dahliae in various parts of the plants and the severity of leaf symptoms. There was also evidence that there were changes in the quantity of the fungus present in the various stages of leaf degeneration leading to necrosis (Wright 1969).

Hall and Busch (1971) found V. dahliae in chrysanthemum leaves up to 12 days before symptoms appeared. They showed

that there was a relationship between symptom expression, the quantity of fungus present and the permeability of leaf cells. Their methods like those of Wright (1969) with tobacco did not indicate the distribution of the fungus in vessels or other tissues of intact leaves, nor the nature of the propagules which gave rise to the colonies counted in estimating the amount of fungus present.

The purpose of the work described here was to follow the establishment of the pathogen, to determine its form, location, and concentration in the leaves in relation to chlorosis and necrosis.

Materials and Methods

Five 8-week-old seedlings of each of the 4 tobacco cultivars were inoculated by the root-dip method, planted in 15-cm clay pots and transferred to a controlled environment cabinet. The leaves were first sampled 4 days after inoculation, then at five 2-week intervals until the plants were approximately 75 cm in height and the VG plants were flowering.

As symptoms usually appeared first near the leaf margins at the centre or at the distal end, 5 main lateral veins nearest the tip on each side of the midrib were sampled approximately 2 cm from the margin (Fig. 2). There were

usually 8 to 10 lateral veins on each side of the midrib. Since the samples were taken from the same location in all leaves, differences in the number of vessels in the veins were minimized. All viable leaves were sampled and when a leaf was sampled more than once, each succeeding disc was taken nearer the midrib than the previous one. The wounds healed quickly and had no apparent adverse effect upon the leaf.

Selected leaves at different stages of chlorosis and necrosis were taken from other plants grown in the cabinets and these were studied in greater detail. The midribs and lateral veins were evaluated after taking sections in order along the bundles and covering these with cellulose agar. Fine veins, secondary and tertiary veins were sampled with the "micro-sampler" and incubated in water. By taking the discs in sequence along the veins it was possible to follow the progress of the fungus in individual bundles.

To determine whether the fungus was confined solely to the vessels or was in the intercellular spaces, samples were taken to include fine veins and secondary veins from adjacent areas of green, chlorotic and necrotic tissue of VG leaves. These were incubated in water, and in xylem sap from 5 uninfected plants of VG and five of MN. Ten sample discs of each of the different tissues were incubated in each of

the 3 liquids for 3, 6, 12 and 24 hours respectively. Control samples were placed immediately without incubation in glacial acetic acid and 95% alcohol, 2:3 (GAA). After the discs had been incubated for the desired period the liquid under the cover slip was withdrawn with filter paper and replaced with GAA clearing solution. All the slides were kept in petri dishes on moist filter paper. After 3 days the GAA was replaced with chloral hydrate (2:1 W/V in water) for a further 3 days. Chloral hydrate was changed from time to time as it became diluted and was replaced finally by lactophenol containing picric acid, orange G and sudan III, before the cover slips were sealed. Similar tissues were fixed in FAA, embedded in paraffin wax, sectioned, mounted and stained.

Results and Discussion

Establishment of the Pathogen Within the Leaf.

All VG and G plants developed severe leaf symptoms and plants of G variety did not flower but grew to a height of about 44.1 cm (Table 1) before being dwarfed by the effects of the pathogen. The root system of these plants, although white and apparently healthy, was markedly reduced in size compared to those of the other cultivars. Confinement of the root systems in the pots may have resulted in the dwarfing

Table 1. Height, number of infected leaves and total number of leaves in 4 tobacco cultivars inoculated with Verticillium dahliae.

Cultivar	Height (cm)	Number of leaves		
		With chlorosis and necrosis	From which V. dahliae was isolated	Total per plant
G	44.1[1/]	18.2	23.8	30.4
VG	77.9	15.2	18.2	23.6
NC	79.3	1.0	2.4	24.0
MN	80.1	1.0	2.0	23.8
SE of means	3.7	1.4	2.1	1.7

1/ All values are means of 5 plants.

and extreme symptoms compared to those observed under field conditions (Wright 1969).

The NC and MN plants developed few symptoms and these were mostly on the lower leaves. The fungus was isolated from leaves (below the fifth) in 4 of the 5 NC plants. One of these plants remained symptomless and the pathogen was not recovered from it until the last sampling, when it grew from samples taken from the upper leaves. V. dahliae was isolated from only 2 of the MN plants, in one from a lower leaf, and in the second from upper leaves which later developed symptoms. Confinement of Verticillium to the lower leaves which fall from the plant had been noted in MN under field conditions (Gibbins and Wright 1968).

The fungus was recorded from leaves showing no symptoms 61 times during the trial. Usually within 14 days these leaves had light to medium chlorosis and after 28 days severe symptoms. Occasionally in the upper leaves symptoms did not appear until the second sampling after the pathogen was first detected (28 days) whereas on the same plants in the lower leaves severe symptoms appeared within the 14 days between samplings. Verticillium was usually isolated from leaves 3 or 4 above those with symptoms but on one plant it was found in the lateral veins of the seventh leaf above the highest showing chlorosis.

Some leaves had marked chlorosis on one side of the midrib but remained green on the other side. In these almost every sample (23 out of 25) taken from the veins of the chlorotic side contained the pathogen, whereas not a single culture was obtained from the 25 samples taken from the green side at the time they were first sampled.

In the G plants the upper leaves often had shortened midribs, uneven growth of the lamina, or the leaves were small in relation to their position on the stem. The pathogen was not usually isolated from the lateral veins of such leaves when they were first sampled. More detailed study of similar leaves showed that the fungus was present in the midribs and in the lateral veins near the midribs but not at the ends nearest the margin. Leaves showing uneven growth of the lamina without any chlorosis contained the fungus in the midrib vascular bundle on the side with reduced growth. V. dahliae had apparently influenced the development of the lamina without proceeding far out into the lateral veins. This suggests interference with the nutrient supply to the developing lamina cells or perhaps some other influence on the part of the pathogen at a distance from the proliferating cells.

The Pathogen in the Midrib of the Leaf.

The midribs of over 20 leaves with symptoms were examined by plating small sections in sequence. The vessel walls were discoloured brown or black and some of the smaller vessels were often blocked with brown, black or red coloured material which was sometimes granular in appearance. Most of the fungus was concentrated in the vessels on the edge of the midrib vascular bundle and in lateral vein traces but it was also present in the central vessels. Brown coloured sap was found in almost all the vessels of the midrib of an invaded leaf, although the large vessels in the centre of the bundle were relatively clear of discoloured sap and fungus. Parenchyma cells nearest the midrib were occasionally filled with pigmented material and the intercellular spaces between the smaller parenchyma cells surrounding the midrib were frequently filled with black material.

Similar observations were made from the lateral veins, namely, the vessel walls were discoloured, the sap was brown and the vessels were full of fungus. In samples from some leaves that had heavy pigmentation in the vessels, the fungus was slow growing or did not grow at all from the sections where this material had infiltrated the agar.

Plating also revealed fluctuations in the concentrations of the fungus in both the midrib and lateral veins. However, the technique of covering the tissue with agar did not show up the variability in fungal concentration as well as the disc technique, as the tissue samples were larger and the incubation time much longer.

Distribution of the Fungus in the Vessels of the Leaf in Relation to Symptoms.

Growth of the fungus from the cut ends of the lateral veins of leaves with symptoms was extremely variable and the number of hyphae growing from the cut end of a single bundle ranged from 0 to well over 50. For example, of 360 discs from VG leaves with marked chlorosis, 44 contained no fungus while from 63 there was so much growth that it was not possible to count the individual hyphal strands.

To illustrate this variability more clearly and at the same time show tendencies towards high or low scores in some of the tissue, the data are presented as frequency distributions in the tables below and in the Appendix (indicated by 'A'). Where the distributions were obviously not normal, χ^2 tests for normality were not calculated. Where appropriate, the tests for skewness and/or kurtosis were made (Snedecor and Cochran 1969).

Ten lateral vein disc samples were taken from 150 leaves, showing a range of symptoms, including abnormal growth of the lamina, wilting without chlorosis, and tiger striping. For the first instance the data were examined from all the leaves regardless of the type of symptom expression and then the results from leaves with the same type of symptoms were analysed separately.

The mean score and S.D. for the 1500 samples were 2.48 ± 1.10 and distribution of the scores was significantly different from a normal one ($P = .05$). The test for kurtosis was highly significant ($P = .01$), with too many low and too many high scores. From the same material a highly significant correlation ($r = + 0.689$ **) was obtained between the growth score on a 0 to 5 basis and the mean number of hyphae growing from a lateral vein. Likewise there was a good correlation ($r = + 0.564$ **) between the length of the longest hypha and the mean number of hyphae growing from a vein. As it was not possible to count accurately the number of individual hyphae when over 50 were growing from one vein end, these were recorded as 50. Consequently the means for the number of hyphæ growing from lateral veins are estimates only and the actual mean might be expected to be slightly higher.

When the growth of fungus from samples of the lateral veins of leaves showing typical chlorotic and necrotic symptoms were considered separately, the data presented in Table 2 were obtained. The distribution of the scores was not significantly different from a normal one. There was a tendency toward small numbers of hyphae and reasonable uniformity of growth from both sides of a single lateral vein. However, there was some variability as may be seen from the distributions and the variances.

The results of growth from samples taken from symptomless leaves for comparison, are presented in Table 3. The fungus was absent from many sampling sites and present in very small amounts in most of the rest, although high concentrations were observed in a few instances. The data were similar for samples from symptomless leaves, and green leaves which were wilting without chlorosis.

Concentration of the Pathogen in Relation to Symptom Expression.

Growth scores were slightly higher from the lateral veins of green areas of leaves with chlorosis and necrosis than from green symptomless leaves.

Table 2. Frequency distributions of fungal growth scores, number of hyphae (mean of two ends of a vein) and difference between the number of hyphae growing from each side of lateral veins from 64 tobacco leaves with chlorosis and necrosis.

Score 0 - 5	Frequency %	Number of hyphae per vein	Frequency %	Differences in hyphae between ends	Frequency %
0	16.5	0.5 - 10.0	27.4	0	1.9
1	13.7	10.5 - 20.0	25.3	1 - 5	45.5
2	14.5	20.5 - 30.0	21.7	6 - 10	26.9
3	27.0	30.5 - 40.0	12.2	11 - 15	15.2
4	15.6	40.0 - 50.0	6.6	16 - 20	5.6
5	12.7	> 50.0	6.8	> 21	4.9
Number of samples	640		468		468
Mean	2.49		19.03		7.55
Variance	2.59		154.72		36.24
S.E. of mean	0.06		0.58		0.28
Normality χ^2 = 7.79, 3 df (N.S.)			-		-
Skewness	-		1.21 (P = .01)		0.63 (P = .01)

Table 3. Frequency distributions of fungal growth scores and number of hyphae growing from lateral veins of 37 symptomless leaves and 10 green leaves which wilted.

Growth score (0 - 5)	Frequency %		Mean number of hyphae per vein	Frequency %	
	Symptomless leaves	Wilted leaves		Symptomless leaves	Wilted leaves
0	44.6	48.5	0.5 - 10.0	61.5	68.1
1	27.7	30.7	10.5 - 20.0	24.5	18.2
2	11.7	9.9	20.5 - 30.0	7.0	6.8
3	12.5	6.9	30.5 - 40.0	3.0	2.3
4	1.4	3.0	40.5 - 50.0	3.0	2.3
5	2.1	1.0	> 50.5	1.0	2.3
Number of discs examined	370	100		201	86
Mean	1.05	0.88		10.33	7.15
Variance	0.98	1.28		119.52	104.6
S.E. of mean	0.05	0.11		0.77	1.10
Skewness	1.16 (P=.01)	0.48 (P=.05)		2.03 (P=.01)	2.84 (P=.01)

The concentrations of fungus in leaves with severe symptoms varied from green to chlorotic to necrotic tissue. As reported in earlier work (Wright 1969) concentrations were highest in the brown areas (Table 4). There was a marked increase in the number of hyphae per vein with increasing severity of symptoms and similar trends in concentration were observed in samples taken from secondary and fine veins (Table 5).

There was great variability in the distribution of the fungus in lateral, secondary and fine veins in tissues showing uniform discoloration and "hot spots" appeared to occur at random. Even within the diameter of the disc it was possible to have a "hot spot" on one side and a much lower count on the other (Fig. 3). The impression gained was that the pathogen was suppressed more or able to proliferate to a greater degree in some sections of the vascular tract than in others. This would agree with the findings of Beckman (1966) working with Fusarium wilt of bananas. Talboys (1958b) also commented on the variability in fungal concentrations of V. albo-atrum in hop leaves, although he attributed this to the blockage of the vessels by tylose formation.

When 0.5% aqueous eosin was taken up by detached tobacco leaves with symptoms, the dye usually flowed into the

Table 4. Comparison of frequency distributions of fungal growth scores and number of hyphae growing from lateral veins of 30 infected leaves with chlorosis and necrosis.

Growth score	Frequency %		
	Green areas	Chlorotic areas	Necrotic areas
0	41.5	19.1	11.8
1	20.0	10.1	10.8
2	11.8	11.0	14.1
3	11.1	18.8	17.7
4	8.2	18.4	11.8
5	7.4	22.6	34.0
Mean score	1.88	2.75	3.09
Variance	4.20	3.28	3.13
S.E. of mean	0.18	0.10	0.12
Skewness	0.98 (P=.01)	-	-
Kurtosis	-	1.71 (P=.01)	1.83 (P=.01)

(Continued)

Table 4. Continued.

Mean number of hyphae per vein	Frequency %		
	Green areas	Chlorotic areas	Necrotic areas
0.5 - 10.0	47.4	19.5	23.5
10.5 - 20.0	21.8	23.5	20.2
20.5 - 30.0	11.5	17.2	18.9
30.5 - 40.0	7.7	15.3	9.8
40.5 - 50.0	10.3	14.0	9.8
> 50.5	1.3	10.5	17.8
Mean number	14.91	26.10	28.09
Variance	76.00	270.93	310.48
S.E. of mean	0.99	1.00	1.27
Skewness	1.03 (P=.01)	-	-
Kurtosis	-	1.89 (P=.01)	1.23 (P=.01)

Table 5. Frequency distributions of fungal growth scores from 150 random sample discs taken from veins of 15 leaves with chlorosis and necrosis.

	Frequency %					
	Green areas		Chlorotic areas		Necrotic areas	
Growth score	Secondary veins	Fine veins	Secondary veins	Fine veins	Secondary veins	Fine veins
0	44.7	66.7	18.0	58.7	47.3	38.0
1	14.7	11.3	12.7	9.3	5.3	22.7
2	13.3	6.0	11.3	8.7	6.0	8.7
3	8.0	8.6	14.0	10.0	6.0	9.3
4	11.3	6.0	18.7	6.7	10.7	9.3
5	8.0	1.3	25.3	6.7	24.7	12.7
Mean score	1.51	0.71	2.79	1.17	2.01	1.69
Variance	2.56	1.80	3.39	3.03	4.67	2.86
S.E. of mean	0.13	0.11	0.15	0.14	0.18	0.15
Skewness	0.70 (P=.01)	1.53 (P=.01)	-	1.12 (P=.01)	0.35 (P=.05)	0.72 (P=.01)
Kurtosis	-	-	1.45 (P=.01)	2.84 (P=.01)	1.35 (P=.01)	2.05 (P=.01)

(Continued)

Table 5. Continued.

Frequency distributions of number of hyphae from 150 random sample discs taken from secondary veins of 15 leaves with chlorosis and necrosis.

Mean number of hyphae	Green areas	Chlorotic areas	Necrotic areas
0.5 - 10.0	43.2	28.4	22.7
10.5 - 20.0	33.5	23.1	24.0
20.5 - 30.0	14.8	19.2	12.0
30.5 - 40.0	4.9	10.0	5.3
40.5 - 50.0	1.2	6.2	1.3
> 50.5	2.4	13.1	34.7
Mean number	14.08	22.30	28.01
Variance	80.00	247.12	324.00
S.E. of mean	0.99	1.38	2.08
Skewness	2.01 (P=.01)	0.66 (P=.01)	-
Kurtosis	-	-	1.37 (P=.01)

FIGURE 3

A. Verticillium dahliae hyphae which have grown from a fine vein into the leaf parenchyma just before necrosis. From cleared necrotic tissue without incubation. B. Growth of the fungus from a "hot spot" after incubation of a disc from necrotic tissue placed in McNairs xylem sap for 6 hours. (X390).

FIGURE 3

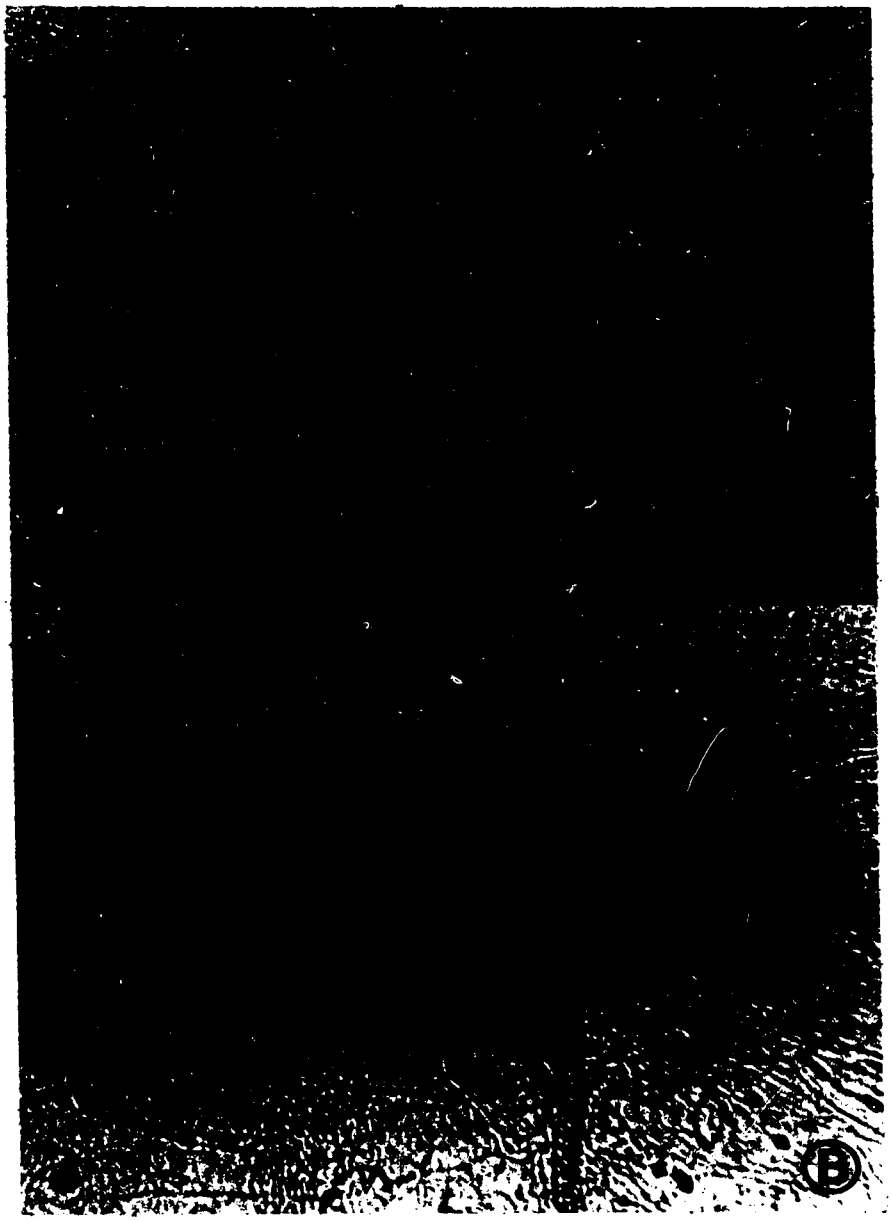


FIGURE 3

veins of some green areas but not into those about to show symptoms or into the veins of tissue that was already chlorotic. Growth of the fungus was not affected by the eosin and sampling along secondary veins of similar tissue with and without the eosin showed that the pathogen was absent or in very low amounts in veins which contained the dye but in nearby veins without dye the fungus was present in large amounts. This would suggest the vessels were blocked, yet if the lateral, secondary or tertiary veins were severed so that large or small lamina areas were apparently isolated from the main vascular network, there was still no chlorosis in either old or young leaves of uninfected plants. This indicated that vessel blockage alone was not responsible for chlorosis in tobacco leaves. This is in agreement with the findings of Talboys (1958b) with hop leaves.

By taking the samples in sequence it was possible to determine the quantity of fungus in individual veins passing through green, chlorotic and necrotic lamina. V. dahliae was usually present in the secondary veins 3 to 4 days before chlorosis and had built up to large quantities, 2 days before chlorosis appeared. As the secondary veins are connected to two lateral veins (Fig. 2) the fungus may invade the secondary veins as a few hyphae from one but usually from both lateral veins.

There were often short gaps (1 to 2 mm) in these secondary veins from which it was not possible to isolate the pathogen. This may be the result of short distance translocation of conidia but it may also be the result of defense mechanisms of the plant killing sections of the advancing pathogen.

Like the secondary veins, the tertiary veins may be invaded from one or both ends from the secondary veins, to which they are joined and again gaps may occur with no fungus, during the early stages of colonization. Usually the tertiary veins were well invaded when chlorosis of the lamina occurred.

The finest veins were colonized from both the secondary and tertiary vessels but mainly from the latter to which most are connected directly. Invasion of the finest veins took place at random and was usually not heavy (Table 5). Most fine veins were not invaded at all, especially those furthest from the tertiary veins. Talboys (1958b) had also noticed this in hop leaves. However, occasionally even veinlets containing a single vessel were invaded (Fig. 4).

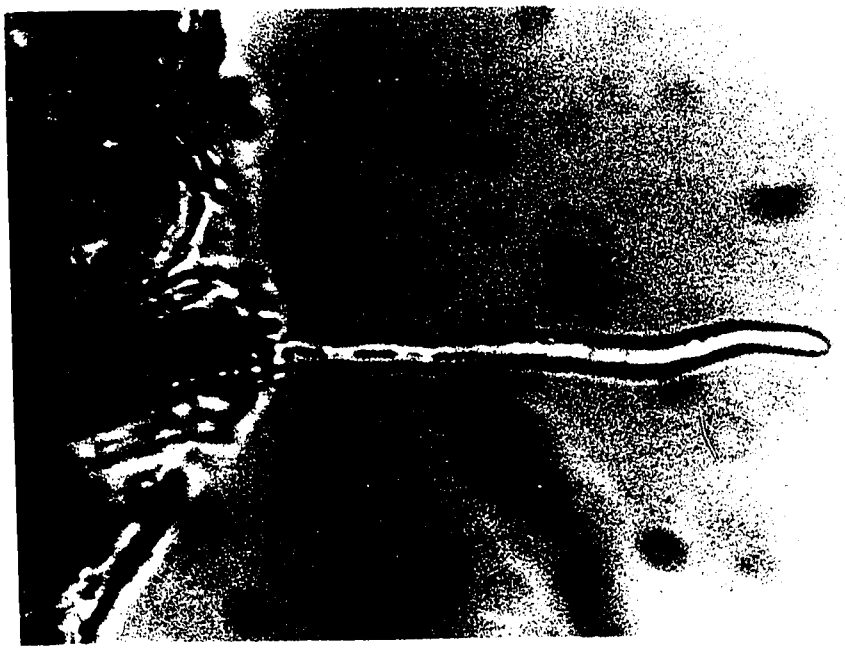
It is difficult to explain the high proportion of samples without fungus in the secondary veins and also the lateral veins of necrotic tissue compared with those from

FIGURE 4

Growth of Verticillium dahliae from a fine veinlet consisting of a single vessel. (X925).

FIGURE 4

FIGURE 4



chlorotic areas of the leaf. Possibly some hyphae in these veins die as the lamina becomes necrotic, leaving pockets of resting cells in some areas. One third of the samples from the secondary veins in the necrotic tissues contained "hot spots" which could have been such pockets (Fig. 3).

Growth of the Pathogen Out of the Xylem Vessels.

When discs of green, chlorotic and necrotic tissue from infected leaves were incubated for different periods in water and xylem sap and then cleared, the following observations were made. In tissue straight from the plant the pathogen was not observed outside the vessels of green or chlorotic leaf but was clearly visible inside the vessels especially those of the secondary veins. The hyphae inside the vessels were growing as winding, relatively unbranched strands, with rectangular shaped cells 3 μ wide by 10 μ long (Fig. 5). In contrast to the green and chlorotic tissue, in many of the samples from necrotic lamina the fungus was outside the vessels in the intercellular spaces of the parenchymatous tissue, as dark torulose hyphae (Fig. 6). The microsclerotia which might have been expected were not observed. Fixed and stained sections confirmed this (Fig. 7). Garber and Houston (1967) detected Verticillium in the vessels of cotton leaves with symptoms but rarely in the vessels of leaves without symptoms.

FIGURE 5

Verticillium dahliae growing in vessels of tobacco leaf. A. Fungus has undulating appearance. B. Hypha within a vessel with secondary thickening. (X910).

FIGURE 5

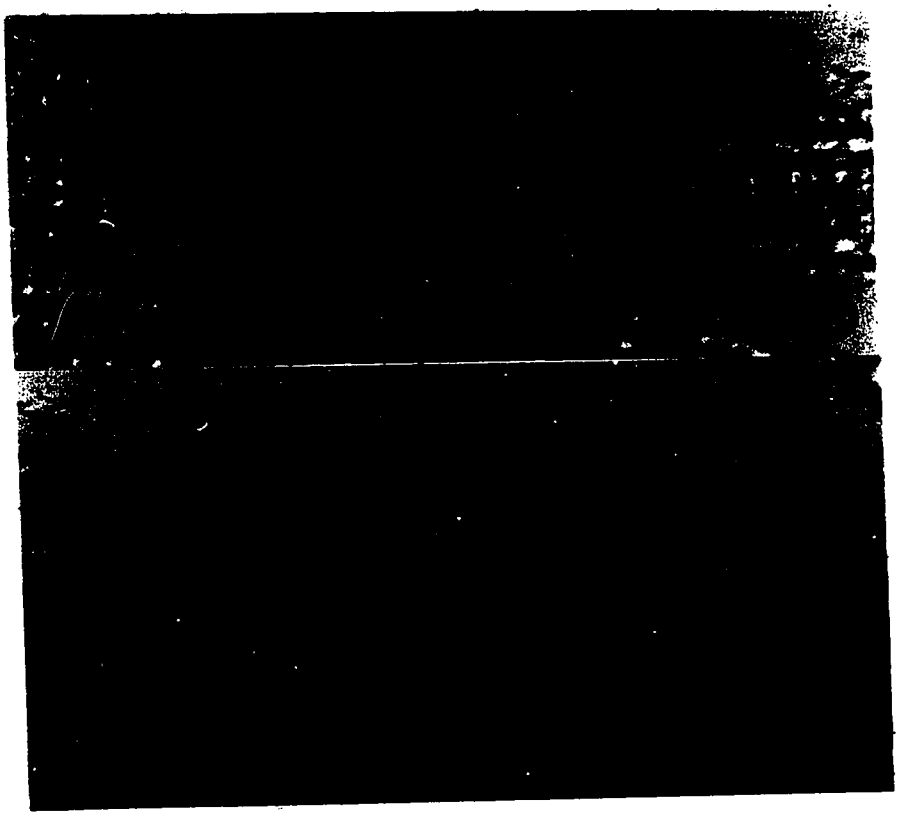


FIGURE 5

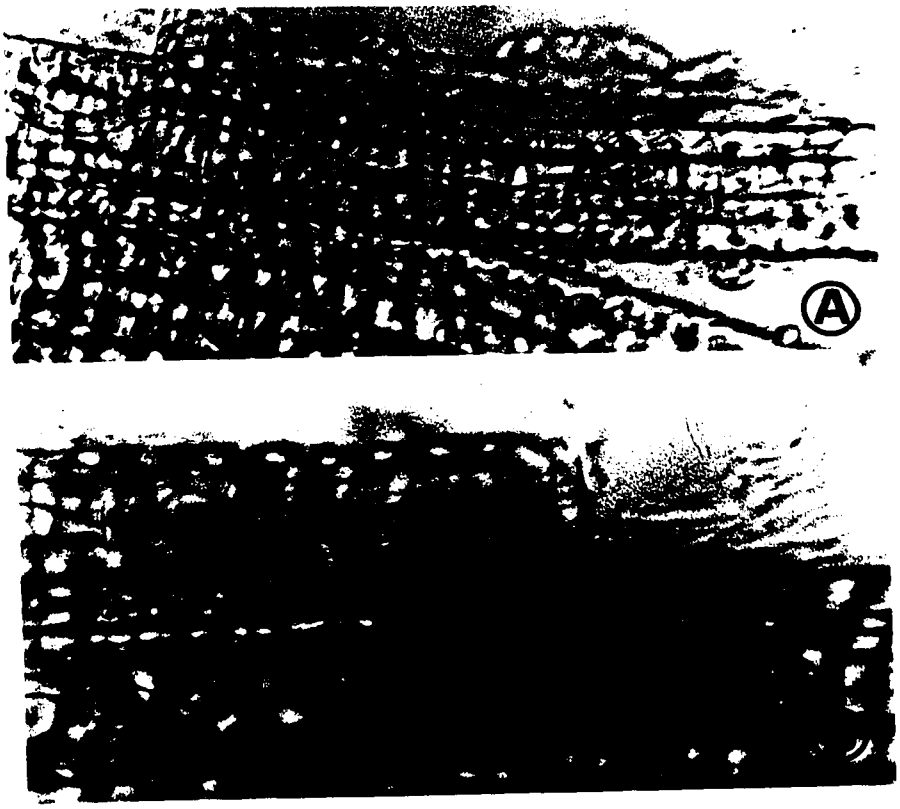


FIGURE 6

Cleared necrotic tissue containing rectangular cells of torulose hyphae. Arrows indicate swelling of a hypha where it has passed through a tracheid pit. (X970).

FIGURE 6

FIGURE 6

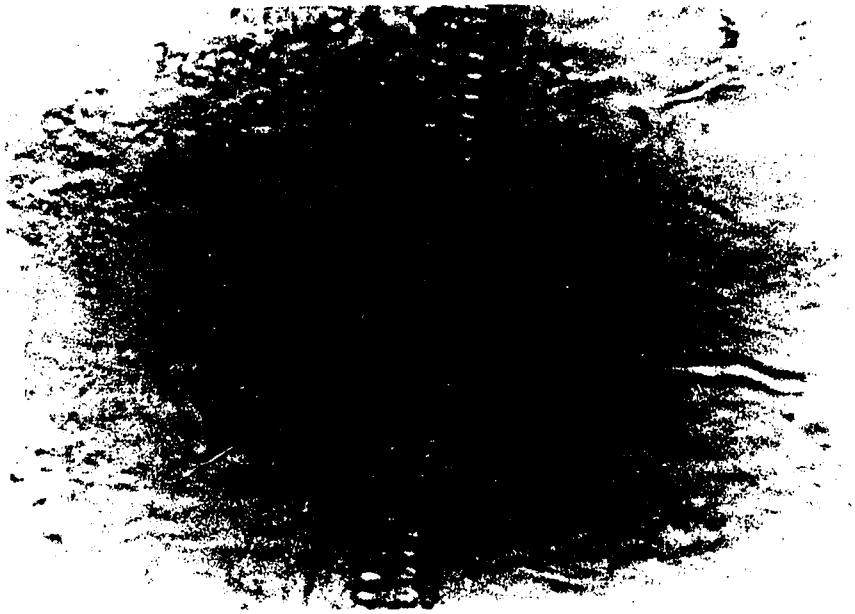


FIGURE 7

Necrotic tissue showing extensive growth of Verticillium dahliae before necrosis occurred. Section of embedded and stained necrotic tissue. Arrow indicates thick hyphal strand. (X775).

FIGURE 7

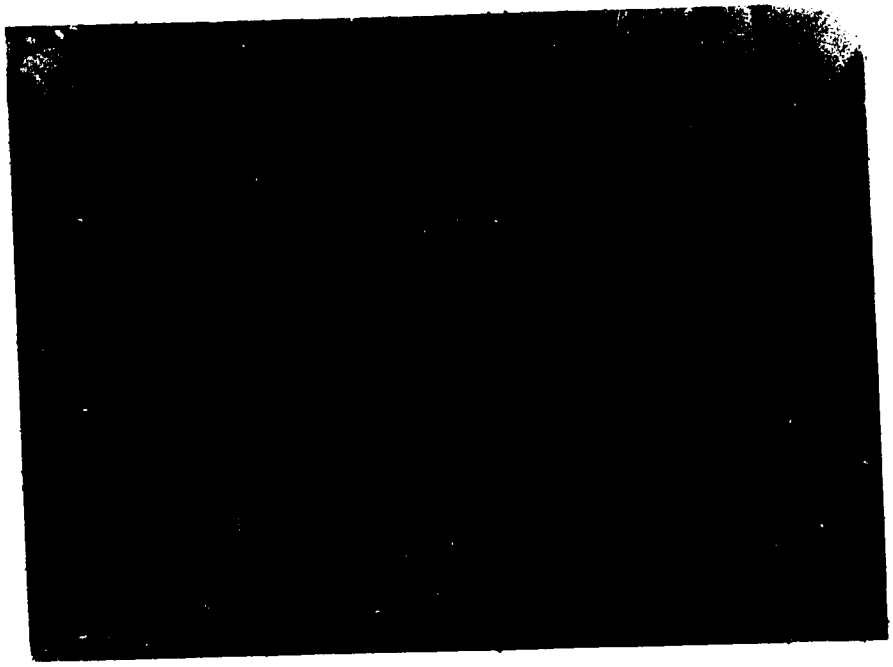


FIGURE 7

The fungus was seldom found by these workers within the parenchyma of the leaves even when discoloration was pronounced.

The cleared tissue samples supported the observations made with the sampling experiments, that there was an uneven distribution of the pathogen in the vessels with heavy concentrations in localized areas (Fig. 3 B). There was no obvious difference in the rate of growth between tissues in the respective xylem saps and between these and water. Clearing the samples after they had been incubating 3 hours in these solutions, showed that growth had started from the ends of the vessels but not through the vessel walls, in the green and chlorotic tissue. After 6 hours the fungus was starting to grow through the pits of the vessels in the green tissue and there was profuse growth through the vessel walls of the chlorotic material. After the 6 hour incubation period, there was good growth of fungus in necrotic samples, from the vessels as well as from the pathogen already in the intercellular spaces. With longer incubation periods there was progressively more growth from tissues in all treatments.

Brinkerhoff *et al.* (1968) found that in cotton leaves mycelium, conidium-like and chlamydospore-like bodies and sometimes microsclerotia occurred prior to abscission. In attached leaves which died from *Verticillium* wilt, microsclerotia

may develop during wet weather. They observed that the fungus was able to survive in abscissed leaves which had been powdered, for 28 months but it was dead after 36 months.

To obtain some information on the possible longevity of the mycelia in necrotic tobacco leaf, samples were taken from leaves which had been killed by the pathogen six months before and which had remained in a dry condition on plants in a greenhouse. After 24 hours in water, the number of discs which had V. dahliae growing from them was not as great as from material which had been necrotic less than a fortnight. However, the pathogen was still easily recoverable from lateral veins and even interveinal areas. Clearing revealed no microsclerotia. Hawksworth and Talboys (1970) stated that resting mycelia were found only in association with microsclerotia in this species of Verticillium. The results of the work reported here suggest that hyphae in the infected xylem and in the intercellular spaces of necrotic leaf were resting mycelia without microsclerotia.

Discussion of the Disc Sampling Technique.

The disc technique was useful for estimating the relative amounts of fungus in the veins of the leaf lamina. It was based on the reasonable assumption that the hyphal

cells within the vessels responded to the stimulus of water to the same degree. Figure 8 illustrates how the individual hyphal cells gave rise to new hyphae each of which was apparently capable of growing out through a tracheid pit. Because of this type of regrowth it was not possible to determine the actual number of hyphae present in any vascular bundle before the sample had been taken from the host. Figure 9 shows the growth of the fungus from a vessel through the intercellular spaces of tobacco lamina after 24 hours incubation in water. The profuse growth often seen from the edges of some sample discs is illustrated in Figure 10.

The variability of fungal concentration found in the samples taken from tissue with uniform symptoms may be due to the fact that not all veins were invaded, and during establishment there were gaps where no fungus was found. Also there was variability in fungal concentration along the vessels and in the final stages of necrosis the pathogen may disappear from some of the finer veins.

Preliminary samplings of infected cotton, sunflower and eggplant leaves revealed no major differences in distribution patterns or form of the fungus from those found in infected tobacco leaves.

FIGURE 8

Growth of Verticillium dahliae from consecutive cells of hyphae from the vessels of a tobacco leaf. A. Tissue in water 3 hours. (X990). B. Tissue in water 15 hours. (X925).

FIGURE 8

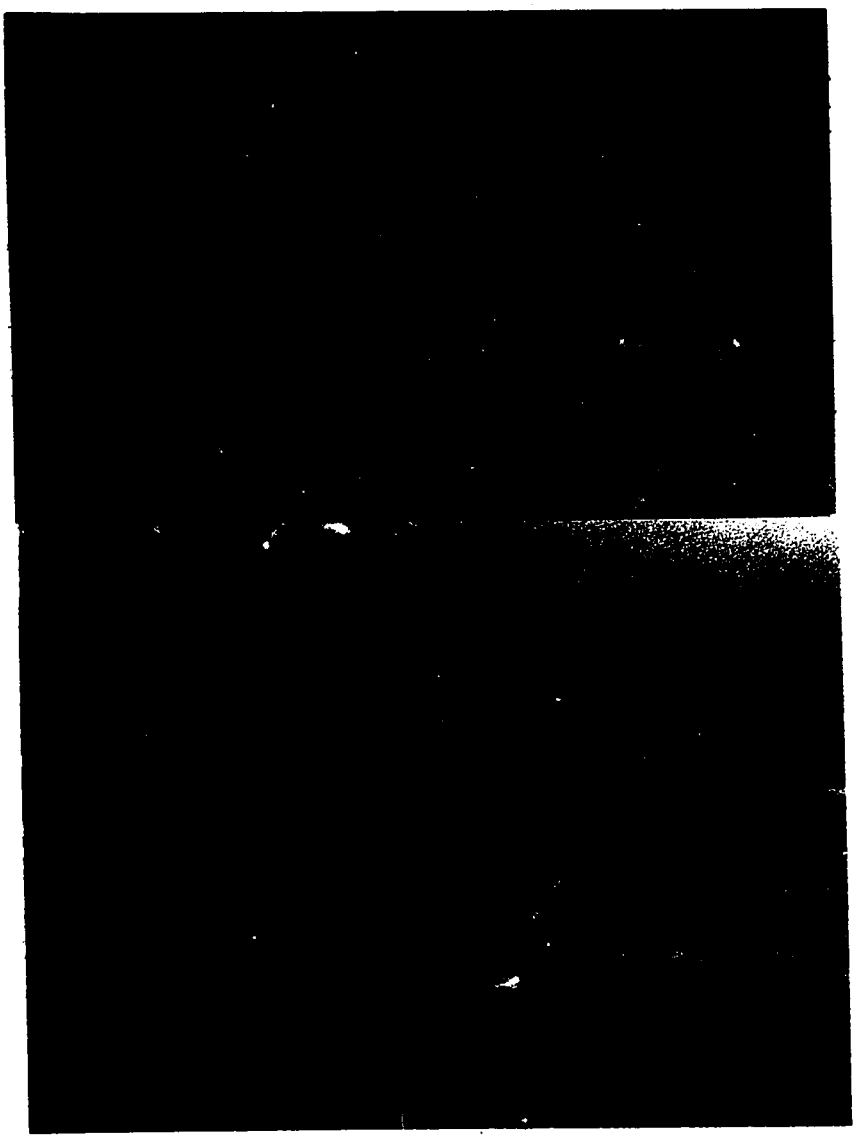


FIGURE 8

FIGURE 9

Profuse growth of Verticillium into intercellular spaces, from a tertiary vein in green lamina tissue about to become chlorotic. The sample had been incubated in water for 24 hours before the epidermal cell layer was removed. Arrows (1) indicate hyphae growing between cells (2) swollen chloroplasts in the centre of the host cells. (about X300).

FIGURE 9

FIGURE 9

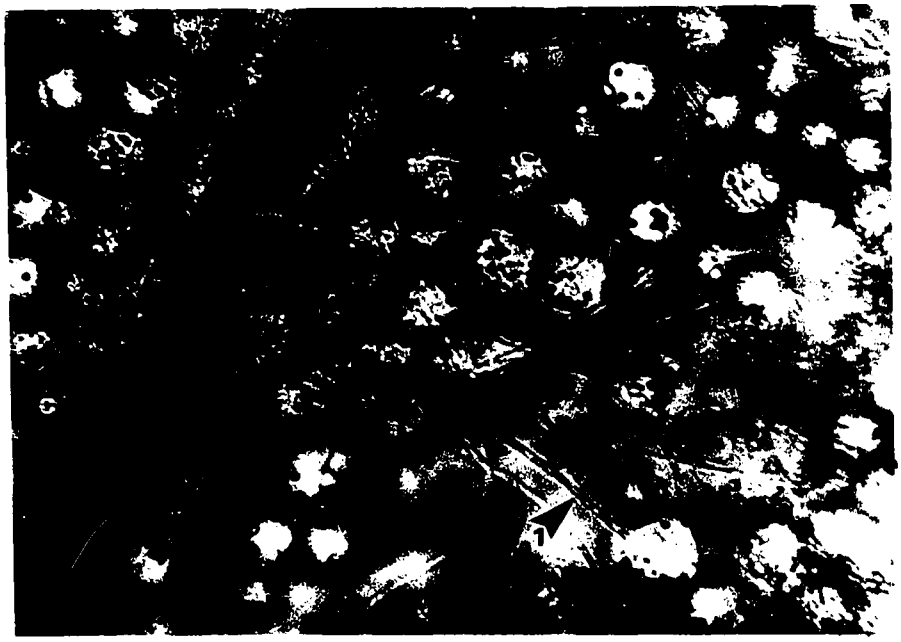


FIGURE 10

Profuse growth of hyphae from the edge of a sample disc from necrotic lamina after 12 hours in water. (X775).

FIGURE 10

FIGURE 10

~~COMPARISON OF~~ THE EFFECTS OF FLOWERING AND SEED FORMATION UPON SYMPTOM EXPRESSION IN TOBACCO PLANTS INFECTED WITH *V. DAHLIAE*

Tolmsoff (1960) working with potatoes, established that under a range of conditions *Verticillium* wilt symptoms did not appear in the leaves until tubers had set. Also after tuberization the roots became progressively more susceptible. Harrison and Isaac (1968) found that by delaying the maturity of potato plants, resistance to *V. dahliae* was increased, supporting the evidence of Busch and Edgington (1967), who associated *Verticillium* wilt symptoms with tuberization in this crop. Busch and Schooley (1970) found that in chrysanthemum plants grown under short-day conditions, symptoms did not appear in the leaves until the buds had formed. Hall and Busch (1971) found a rapid build up of the fungus in the leaves after bud formation in this host.

Under field conditions, naturally infected tobacco does not usually show *Verticillium* symptoms until after flowering. Late-maturing cultivars are more resistant than early ones and in some seasons late varieties appear to be unaffected by the disease (McLeod and Thomson 1959). After flowering there is a very rapid increase in leaf symptoms in susceptible

varieties (Fig. 11). This may be related to flower and seed formation although in tobacco culture the flowerheads are removed early in their development and symptoms appear progressively more rapidly after their removal. Wright (1969) found that short-day tobacco cultivars, which do not flower and continue to form leaves throughout the growing season, may have 13-15 heavily infected, though symptomless, leaves at the top, regardless of the total number of leaves on the plants. The evidence suggests that physiological changes associated with reproduction may influence symptom expression.

A trial was carried out by the writer of this thesis on the Verticillium isolation area at the Tobacco Research Station, Riwaka, New Zealand, during the 1967-8 season. The following four tobacco lines were used. 1. Normal Virginia gold (normal, day-neutral). 2. Mammoth or Giant Virginia gold which requires short days to induce flowering. 3. Normal Kutsaga 51 (day-neutral). 4. Mammoth Kutsaga 51 (short-day). All of these cultivars were known to be very susceptible to Verticillium (Wright 1968a).

Each of the four lines was subjected to two treatments:- a. The plants had their apical meristem removed (topped) after the usual 15 or so leaves had been formed on the main stem, prior to flowering, and all lateral growth was removed by hand

FIGURE 11

Percentage of leaves and plants with symptoms at different periods after the inoculation of two tobacco cultivars with different levels of resistance.

1 = percentage of MN leaves showing symptoms.

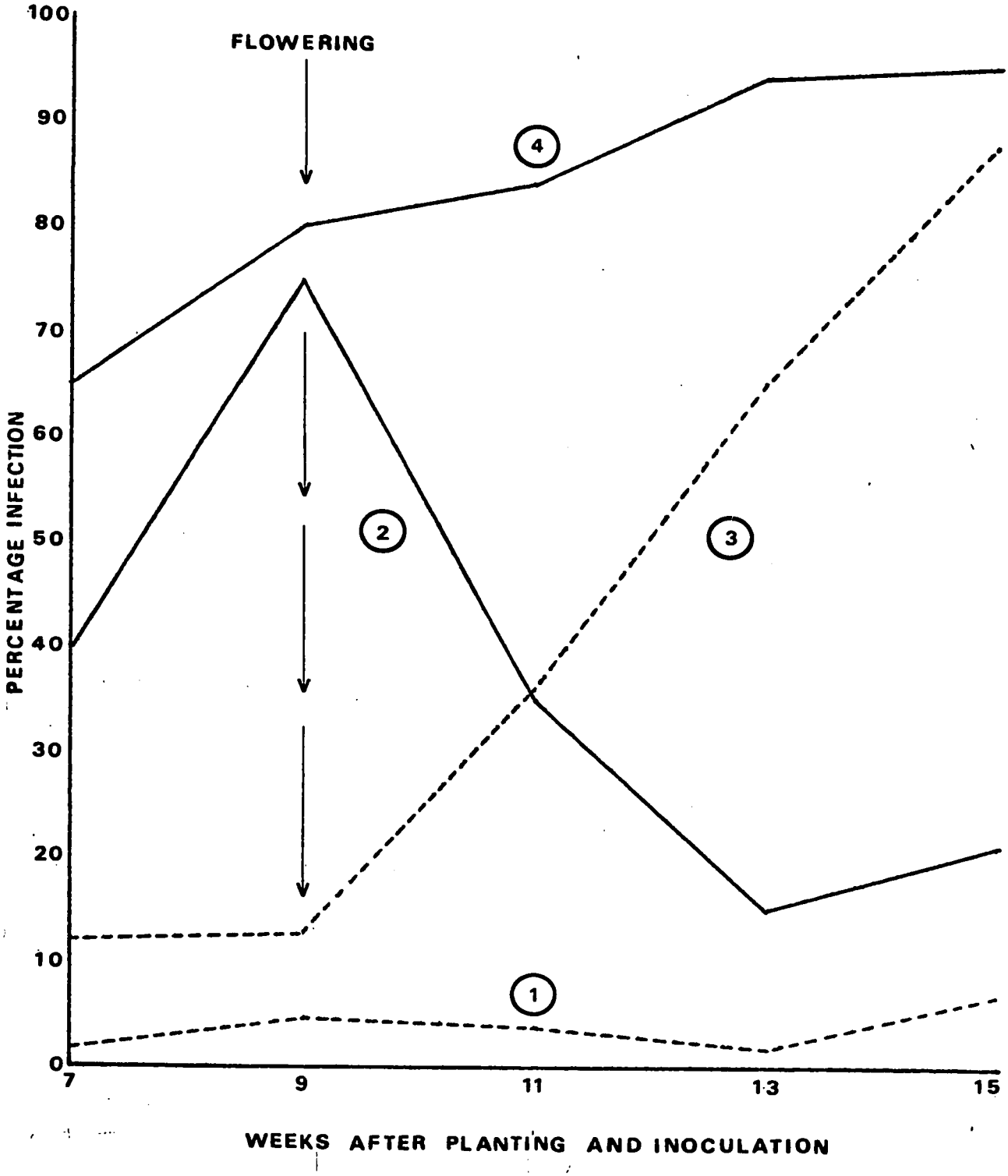
2 = percentage of MN plants showing symptoms. The percentage of plants with symptoms declines sharply after flowering because the lower leaves which show early symptoms drop from the plants after becoming necrotic.

3 = percentage of VG leaves with symptoms. This rises sharply after flowering until finally every leaf becomes chlorotic and necrotic.

4 = percentage of VG plants with symptoms. The number showing early infection is high and this increases to 100% towards the end of the growing season.

Data from Gibbins and Wright (1968).

FIGURE 11


100
90
80
70
60
50
40
30
20
10
0
PERCENTAGE INFECTION
FLOWERING
1
2
3
4
7
9
11
13
15
WEEKS AFTER PLANTING AND INOCULATION

as it appeared. b. The apical meristem was not removed so that the day-neutral plants formed flowers and seeds; the short-day plants did not flower but continued to form leaves until there were 40 or more on each plant by the end of the growing season.

The 8 treatments were arranged in 4 randomized blocks with 20 plants per plot. All plants were inoculated and scored for symptom expression as described previously (Wright 1968a). The number of leaves with symptoms and the total number of leaves on each plant were counted 4 times over a period of 9 weeks starting 21 days before flowering and ending 44 days after flowering, when almost all leaves had symptoms except on the short-day untopped plants.

Statistical analysis was carried out on the raw data by Dr. M.P. Griffin, Applied Mathematics Division, D.S.I.R., Wellington, New Zealand. He analysed the data by normal analysis of variance, and by a Wilcoxin non-parametric rank test. He determined levels of significance between any two treatments by the Wilcoxin test done on the 40 plants of the two in each replication, 20 plants of each treatment being compared. The result was adjusted to the normal with unit variance and these were added over the four replications. This method gave results similar to the normal analysis of variance.

The short-day and day-neutral plants of each variety were similar in appearance until flowering. There were marked differences between the two varieties, VG being a broad-leaved and K 51 narrow-leaved tobacco. The inheritance of the short-day effect was governed by a simple Mendelian recessive gene (unpublished data). It may be assumed that the only differences between the parent cultivar and the mammoth mutant were related to the physiology of flowering, and therefore differences in symptom expression were also related to this.

Kutsaga 51 plants were significantly ($P = 0.001$) more infected than the corresponding VG plants, both topped and untopped, except at the first date, when there was no significant difference between the levels of infection in the short-day plants of the two cultivars (Table 6).

The day-neutral plants were significantly ($P = 0.001$) less infected than the corresponding normal plants with the following exceptions. 1. At the first scoring the situation was reversed in the VG plants where the mammoth plants had significantly ($P = 0.001$) more symptoms than normal plants. 2. At flowering there was no significant difference between normal and mammoth VG plants. 3. At the last observation the difference between the K 51 day-neutral topped and

Table 6. Leaf infection in inoculated day-neutral and short-day tobacco plants at different periods during the growing season.

Cultivars	Treatments	Date of assessment			
		Jan. 15 (Flowering)	Feb. 5	Feb. 23	Mar. 21
Virginia gold (Day neutral)	Topped	39[1/]	26	43	84
	Flowered	45	28	46	83
Virginia gold (Short day)	Topped	58	26	32	66
	Not topped and no flowers	61	28	25	47
Kutsaga 51 (Day neutral)	Topped	76	61	80	98
	Flowered	75	63	81	99
Kutsaga 51 (Short day)	Topped	51	40	52	93
	Not topped and no flowers	58	45	44	66

1/ All figures are percentages of infected leaves per plant. Treatments were of 20 plants per plot arranged in 4 randomized blocks.

short-day topped was significant only at the 5% level ($P = 0.05$).

There were no significant differences between the topped and untopped plants of any of the lines of tobacco except that the short-day topped plants had significantly more symptoms than the corresponding untopped ones at the third ($P = 0.01$) and final ($P = 0.001$) observations.

From these results it may be concluded that the formation of flowers and seeds as such did not influence symptom expression. Where the apical and lateral buds were removed as they formed, the symptoms appeared at the same time and were just as severe as in the plants that were allowed to form full seed heads and lateral growth. However, the physiological changes which occurred after the stimulus to form flowers may have had a marked effect upon symptom expression. There were differences in symptom expression between the plants conditioned to form flowers and the short-day plants and these were apparent even before bud formation. This may be seen from the significantly lower level of infection (delay in symptoms) in the mammoth plants which were not conditioned to form flower heads and which had been kept at the same height as the normal plants by topping. Approximately 14 days after the final notes were recorded there was little difference between the day-neutral plants

and the topped mammoth plants, indicating that failure to become physiologically conditioned to form flowers had merely delayed symptoms. In this trial the influence of this delay was apparent for over six weeks.

The untopped ~~day-neutral~~ short-day plants continued to grow with symptomless leaves at the top. At the time the last notes were taken there were 19.2 symptomless leaves on the VG mammoth plants and 13.8 on the Kutsaga 51 ($P = 0.01$) and 18 days later the respective numbers were VG 15.6 and Kutsaga 13.6 (N.S.) (Wright 1969).

RESISTANCE OF TOBACCO LEAF CELLS TO V. DAHLIAE

Invasion of Detached Leaves by V. dahliae.

When mature infected tobacco plants were cut from the root system just above ground level and placed in water, the leaves continued to develop symptoms in acropetal succession in the same manner as when they were still attached to the root system. It was not known whether the time of symptom expression was related to the time of invasion or the age of the leaf being invaded.

Three consecutive symptomless leaves above others showing symptoms, were removed from each of 5 resistant and 5 susceptible tobacco plants just coming into flower. The leaves were between the 6th and 9th along the stem of plants which had formed approximately 18 leaves. Uninoculated control plants grown under the same conditions had similar sets of 3 leaves detached and paired with the corresponding infected leaves.

As the leaves were detached, 2 cm of petiole was cut from the base of each leaf and the vascular bundle removed and cut into 2 mm lengths on microscope slides. The sections were covered with cellulose agar and incubated in petri

dishes for one week. Disc samples were taken from 10 lateral veins of each leaf at the time of detachment and again 14 days later (Fig. 12).

The leaves were placed with their petioles in beakers of distilled water which was renewed each day to minimize bacterial contamination. At the start of the trial and at the end, 14 days later, the leaves were compared with Ridgeway's standard colour charts (Rayner 1970).

The lowest leaves were greenish yellow (No. 16 on the colour chart) when picked and the upper ones were mainly herbage green (No. 17). After 3 days the lowest leaves from all 5 infected VG plants were slightly wilted and chlorotic patches were developing in the interveinal areas. Slight chlorosis was seen on only 2 of the infected MN leaves.

One week after detachment there was marked chlorosis on all the lowest, infected VG leaves and on 3 of the next leaves up but none on the uppermost leaves. On the MN leaves chlorosis had appeared on only one of the lowest, two of the middle, and none of the upper leaves. All controls were green and healthy.

After 14 days, 14 of the 15 leaves from infected VG plants had some necrosis (Table 7) and most of these had

FIGURE 12

Detached infected VG leaves (Plant 4) showing lateral vein sampling sites.

FIGURE 12

FIGURE 12

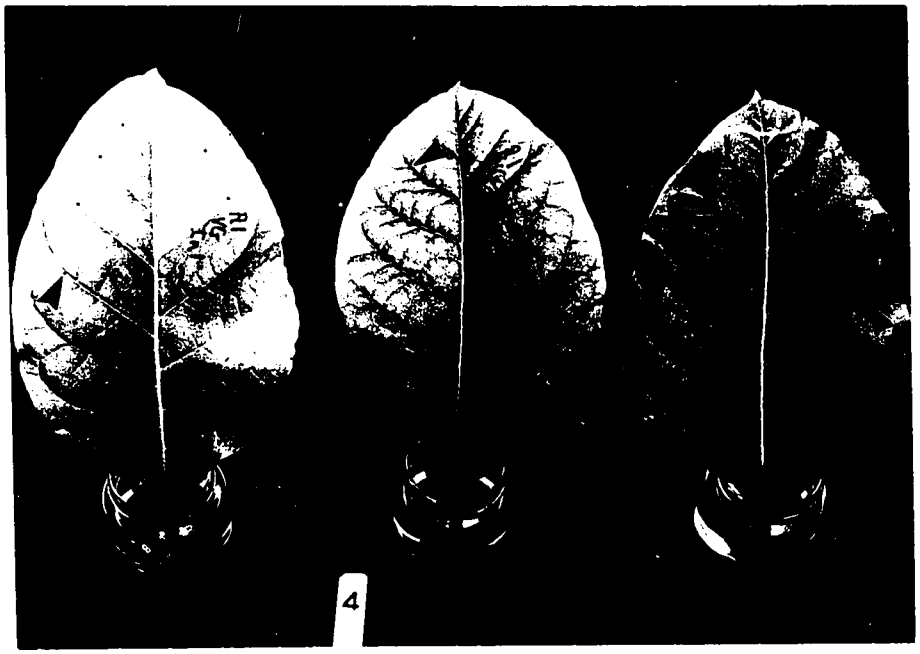


Table 7. Development of Verticillium and changes of colour in detached leaves from inoculated plants.

Sample		Growth score and S.D. of fungus from petiole at detachment (0-5 basis)	No. of lateral veins with fungus (out of 10)		Colour of leaf	
Plant	Leaf		At detachment	14 days after detachment	At detachment	14 days after detachment
VG-1	L[1]	4.0 ± 1.4	2	7	16[2]	N
	M	1.8 ± 1.1	0	9	16	68 + 46 + N
	U	2.7 ± 1.2	0	6	17	68 + 46 + N
-2	L	1.4 ± 0.8	2	6	16	N
	M	4.9 ± 0.1	3	10	16	N
	U	0	0	0	17	16
-3	L	2.8 ± 0.8	0	8	16	N
	M	2.3 ± 1.0	4	10	17	N
	U	2.4 ± 1.1	2	8	17	68 + 46 + N

(Continued)

Table 7. Continued.

Sample		Growth score and S.D. of fungus from petiole at detachment (0-5 basis)	No. of lateral veins with fungus (out of 10)		Colour of leaf	
Plant	Leaf		At detachment	14 days after detachment	At detachment	14 days after detachment
VG-4	L[1]	3.4 ± 1.3	6	10	16[2]	N
	M	1.3 ± 1.1	0	9	17	16 + 14 + N
	U	1.8 ± 0.9	2	10	69	16 + 14 + N
-5	L	2.1 ± 1.3	2	6	16	N
	M	2.6 ± 1.3	4	8	16	N
	U	3.5 ± 0.9	1	10	16	68 + 14 + N
MN-1	L	0	0	0	16	15
	M	2.0 ± 1.4	0	6	16	N
	U	0.1 ± 0.9	0	0	16	16

(Continued)

Table 7. Continued.

Sample		Growth score and S.D. of fungus from petiole at detachment (0-5 basis)	No. of lateral veins with fungus (out of 10)		Colour of leaf	
Plant	Leaf		At detachment	14 days after detachment	At detachment	14 days after detachment
MN-2	L[1]	0	0	0	17[2]	16
	M	0.3 ± 1.0	0	0	17	16
	U	0	0	0	69	16
-3	L	1.7 ± 1.0	2	8	16	N
	M	3.9 ± 1.0	1	10	17	N
	U	2.0 ± 1.6	0	10	17	68 + N
-4	L	0.3 ± 0.7	0	10	17	16 + 14 + N
	M	1.5 ± 1.1	2	8	69	16 + 14 + N
	U	0.4 ± 0.9	0	7	69	16 + 14 + N

(Continued)

Table 7. Continued.

Sample		Growth score and S.D. of fungus from petiole at detachment (0-5 basis)	No. of lateral veins with fungus (out of 10)		Colour of leaf	
Plant	Leaf		At detachment	14 days after detachment	At detachment	14 days after detachment
MN-5	L[1/]	0	0	0	16[2/]	16 + 15
	M	0	0	0	16	16
	U	0	0	0	16	16

1/ Leaf position. L = Lower, M = Middle, U = Upper of three leaves.

2/ Figures refer to colours on Ridgeway's charts. 69 = Dark green; 68 = Greyish yellow green; 17 = Herbage green; 16 = Greenish yellow; 46 = Straw; 15 = Sulphur yellow; 14 = Pure yellow; N = Necrotic.

"tiger stripe" symptoms typical of Verticillium (Fig. 1). At this stage the controls had turned paler (No. 16) but were still healthy.

The pathogen was not present in samples from any control leaves. Isolations made from the petioles of infected leaves at the time they were picked showed that distribution of the fungus was variable along the midrib vessels that were examined. There were sometimes gaps of several millemeters where Verticillium was not present. In some instances it was isolated from one side and not the other, or in different concentrations on one side compared to the opposite side. As was suggested above for establishment of the fungus in the secondary veins, this variation may have been the result of short distance establishment by conidia or else elimination of the pathogen in some areas by the defenses of the host. The petioles of the lowest leaves did not always have the highest concentration of fungus at the time they were detached.

Over the 14 day period there was a marked increase in the number of lateral veins from which the pathogen could be isolated in infected VG leaves and also in the sets of leaves from 2 infected MN plants which had a high level of fungus in the petioles at the start of the trial.

Usually the lowest leaves showed symptoms first and after 7-14 days these were the most severely affected. The lower leaves in the control series lost their colour first.

It was not possible to judge from this type of experiment whether the time of symptom development was related to greater establishment of the pathogen in the lower leaves at the time they were picked or to the age or physiological condition of the leaves.

Inoculation of Detached Leaves with Conidia.

To overcome possible effects of differences in the initial level of inoculum in detached leaves, leaves from healthy uninoculated plants were allowed to take up a predetermined number of conidia.

A suspension of conidia was prepared from 14-day-old cultures grown on P.D.A. The suspension was centrifuged twice at 3000 r.p.m. (1470 g) for 20 min and the pellet resuspended in sterile distilled water. The final concentration was adjusted to 500,000 spores per ml.

The experiment was conducted with two isolates of V. dahliae, V3 from tobacco and V58 from sunflower. The sunflower isolate had been shown in other experiments to induce only

mild symptoms in tobacco after very heavy root-dip inoculation.

The petioles of detached leaves were placed in distilled water for 1 hour to dilute xylem sap in the vessels. They were then placed in the conidial suspension in sunlight at 30°C until each leaf had imbibed 0.5 ml to provide an inoculum level of 250,000 conidia per leaf. Control leaves were placed in distilled water. All leaves were kept in a cabinet for the duration of the experiment under the conditions outlined in Materials and Methods. Throughout the trial period the leaf petioles were kept in distilled water which was renewed daily.

Leaves were selected from VG and MN plants approaching the flowering stage. One set of leaves (old) was picked from near the base of the plants while a second set (young) was taken from near the top. Leaves within the two sets were matched for size.

Two leaves from each treatment combination were sampled immediately after spore uptake and 3 days after. Old leaves were sampled again at 10 days and young leaves at 24 days after inoculation. The 10-day interval was chosen because the old inoculated leaves were deteriorating at this stage

and the 24-day period because the younger inoculated leaves and the controls were senescing.

To sample the leaves the vascular bundle of the midrib and lateral veins were removed aseptically, sectioned into 2 mm lengths in sequence, and covered with cellulose agar.

To determine which areas of the leaves were colonized first each side of the midrib was divided into four sections as illustrated in Fig. 2. Secondary and fine veins were sampled by taking 10 discs of each from each of the four areas. Five discs of each type were taken from the corresponding areas on opposite sides of the midrib. These discs also were covered with agar and all sections and discs were incubated for one week before examination.

In all leaves (old and young, MN and VG) the growth of the fungus at the first sampling showed that the conidia had lodged in greatest numbers at the base of the petiole and progressively less along the vessels of the midrib for one half to two thirds of its length. The pathogen was also found in the first 3 or 4 lateral veins near the midrib. A few isolated pockets were present right at the tip of the midrib and in some of the lateral veins at the leaf margins

and in a few secondary veins. It was of interest to note that it was possible for conidia to move to the extremities and that when this occurred they tended to lodge in pockets. There was no difference in the lodgement pattern between V58 and V3.

After 3 days there was no obvious external difference between inoculated leaves and the controls; both groups appeared to be healthy. The fungus had spread further along the midrib and into some of the lateral veins nearer the centre of the inoculated leaves. Increased colonization of the lateral veins at this stage was particularly marked in the young leaves of both cultivars inoculated with V3.

Ten days after inoculation the old control leaves were becoming senescent and the inoculated old leaves had interveinal chlorosis and necrosis typical of Verticillium wilt (Fig. 1). The midribs had been colonized by V3 along their entire length as had all the lateral veins and secondary veins near the centre of the lamina. The V58 isolate was still confined to the lateral and secondary veins near the base of the leaf and had not grown along the midrib to any degree. Samples were taken from the old but not from the young leaves as the young leaves were similar in appearance to the controls with only slight pale coloration and no sign of chlorosis.

Twenty four days after inoculation, the young leaves, both the inoculated and control, were senescing with no obvious difference in outward appearance between the two groups. The only exceptions were the VG leaves inoculated with V3 which showed some slight necrosis in the interveinal areas.

In the young VG leaves inoculated with V3 the fungus had spread right to the tip of the midrib, to the end of every lateral vein, and was isolated from every secondary vein sample (80 samples) and almost every fine vein (73 samples out of 80). By contrast the young VG leaves inoculated with V58 as noted before had no obvious Verticillium wilt symptoms and although the fungus had grown along the midrib it was absent or in low concentrations in most of the lateral veins. It was also found in very few secondary vein samples and in only 4 out of 80 fine vein samples.

All young leaves of MN were similar to the controls in appearance. In those inoculated with V3, the pathogen had colonized the vessels to the tip of the midrib and there was heavy infection the full length of all lateral veins. The secondary veins were also well invaded but there was only sporadic colonization of the fine veins (14 out of 80 samples). In the 2 MN leaves inoculated with V58, the

pathogen had not colonized the end of the midrib but in one it was present in isolated pockets near the tip. Most of the lateral veins also remained uninvaded. Few cultures were isolated from the secondary veins except in the basal area between the lateral veins which had been colonized. Very few cultures were isolated from fine veins of young MN leaves inoculated with V58.

In the young inoculated leaves the lower two fifths of the petiole and midrib had become black after 24 hours in water. The controls did not show this blackening and were green and apparently normal. This discoloration extended well out into the parenchyma from the vascular bundle of the midrib. There was severe necrosis of the parenchyma cells nearest the vessels and a heavy deposition of dark material in these cells. In MN leaves inoculated with V58, only the midrib and a few cells nearby had become blackened and necrotic. When young VG and MN leaves which had been inoculated with V3 but not included in the sampling were placed in 0.5% aqueous eosin, the dye was able to move slowly into the secondary and fine veins but how the eosin bypassed the blocked vessels was not determined.

When the pigmented tissue was dissected out fungal hyphae could be seen within the vessels. Vascular bundles

from these midribs when plated produced little or no growth of V. dahliae and the agar became brown. Growth was progressively better along the midrib as the pigmentation among and around the vessels became less. When some of the pigmented sections which had produced no fungus after the normal incubation period, were teased out and covered with fresh cellulose agar, profuse growth resulted within 7 days. On the other hand the growth was still poor on similar sections which had been left in the original agar for the 14 days. The pathogen was still growing through the pigment in the tissue and agar, but very slowly. Apparently the growth of the fungus was checked although it was not killed. Similar observations were made from sections of both VG and MN inoculated with V58 and V3.

Several conclusions may be drawn from these results. Conidia were taken up readily by the detached leaves and lodged along the vessels in a fairly definite pattern. Distribution was not uniform throughout the vessels. Although the tobacco and sunflower isolates originally were distributed in similar patterns in the midrib and lateral veins, subsequent colonization of lateral and secondary veins by the sunflower isolate was much more restricted than that of the tobacco isolate.

Young and old leaves received the same massive dose of inoculum yet the old leaves showed symptoms in 10 days and the young leaves were still relatively symptomless after 24 days, in spite of extensive colonization by the pathogen. In the old leaves symptoms appeared a few days before the controls became senescent. In spite of heavy invasion, symptom expression had obviously been delayed. In the young leaves symptoms were very light and these appeared about the same time that the controls were senescing. There appeared to be two forms of resistance. There was the relative absence of gross symptoms until senescence, which was particularly noticeable in young leaves and occurred in spite of heavy colonization. Then there was resistance to the colonization of the V58 isolate. The extensive colonization of the MN leaves by V3 was unexpected.

In the vessels of the petiole and lower midrib of young detached leaves, the fungus was apparently checked but not killed by the deposition of dark pigmented material. This material completely blocked the vessels and filled the parenchyma cells around the vascular tract. Eosin was able to bypass vessels blocked in this way perhaps by plasmodesmata which were shown by Livingston (1935) to connect cells throughout the tobacco plant.

Without the influence of xylem sap from the root system detached young leaves were able to organize a defence against Verticillium within the limits of the reserves available, and the parenchyma cells did not develop premature chlorosis even when the vessels nearby had been invaded by the pathogen.

Flooding the Intercellular Spaces of Tobacco Leaf Lamina with Conidial Suspensions.

From the sampling of vessels of infected tobacco leaves and from the work with cleared material it appeared that V. dahliae was confined to the vessels by the activity of the surrounding companion cells. The purpose of the work described in this section was to determine, if possible, what would take place if Verticillium were placed outside the vascular tract of the leaf. Several workers have reported that Verticillium conidia may germinate and enter intact leaves, producing symptoms (Sackston 1959; Provvidenti and Schroeder 1959; Thanassoulopoulos and Hooker 1970).

If the parenchyma cells surrounding the vessels were responsible for keeping the pathogen confined, then flooding the intercellular spaces of leaves with conidia should give the pathogen an advantage and symptoms might be expected to result from this. If the pathogen did grow and cause symptoms

when placed outside the vessels, it would also indicate that the vessel walls were an important barrier to the fungus.

Conidia introduced into the leaf could be reisolated by plating but the problem remained of assessing host-pathogen interaction within the system over a short period. Clearing the tissue after flooding was not successful, nor did the procedure of introducing spores labelled with fluorescent brightener or the removal of the cuticle reveal anything of note, except that some conidia disappeared and others germinated.

The technique of sampling with small discs, used repeatedly throughout this work, was again found to be useful. Discs (0.86 mm diameter) were removed from the lamina after intercellular flooding and incubated as described previously. The number of hyphae that grew from the edge of each disc was counted and used to estimate fungal survival.

After preliminary trials had shown that the technique gave consistent and reproducible results, three experiments were conducted.

The first experiment was planned to compare the effects of attached leaves and detached leaves upon the conidia in the intercellular spaces and also to determine, if possible, changes in the viability of the conidia with time. VG and V3

spores only were used in this experiment, a 3 x 3 factorial design with 5 replications.

Test plants were raised in the greenhouse until just before flowering and after inoculation were transferred to controlled environment cabinets for the duration of the trial. Two leaves, one of which was used as a control, were chosen from the 10th to 12th positions on the stems of VG plants about to flower. After inoculation the leaves of 5 plants were left attached and the leaves from 10 others were removed. Half of these were kept with their petioles in distilled water while the remainder were placed in inflated plastic bags.

Suspensions of washed conidia were prepared and the concentration adjusted to 2 x 10^5 per ml.

Intercellular spaces of tobacco leaves may be flooded by inserting a hypodermic needle (1 ml, disposable plastic syringe fitted with a No. 26 needle) into the parenchyma of the midrib just above the point where this tissue joins the leaf blade (Fig. 2). Flooding the lamina in this way does not appear to damage the leaf. Klement (1963) used this technique successfully to study the resistance reactions of tobacco to several parasitic and saprophytic Pseudomonads.

The area chosen for flooding was between two lateral veins towards the base of the stem (Fig. 13). Two control areas on the opposite side of the leaf were flooded with (a) water and (b) some of the suspension which had been autoclaved at 15 lb p.s.i. for 30 min. The water-soaked areas were readily seen by the deeper colour and were outlined with a felt pen (Fig. 12). Control leaves were inoculated with water and autoclaved spore suspension only to compare the effect of inoculation on the whole leaf as well as on inoculated zones within leaves.

At the time of inoculation, holes were made in some VG leaves with the micro-sampler up to 2 cm from the site of injection. Droplets which formed in these openings were collected and haemocytometer counts showed that the spore concentrations were similar to those of the suspensions injected. Hanging drops were prepared from the conidial suspensions and after 24 hours incubation the original V3 suspension had 66.0 $\pm$ 4.4% germination whereas those that had passed through the leaf had 68.5 $\pm$ 2.7%. When this was repeated with V58 in VG leaves the corresponding figures were 73.1 $\pm$ 5.2% and 75.1 $\pm$ 3.9%. Apparently quick passage through the leaf had not affected germination.

FIGURE 13

Detached tobacco leaves (controls) after intercellular inoculation with autoclaved conidial suspension and water. An "old" leaf on the left and a "young" leaf on the right. The flooded areas are outlined by the black lines and arrows indicate sites from which samples have been removed.

FIGURE 13

FIGURE 13

The results of the first experiment are presented in Table 8 and the statistical analysis in Table A 2. Differences in the number of hyphae growing from the disc samples at different periods after inoculation were highly significant ($P = .01$) and much greater than differences due to leaf treatments, although these were also highly significant. Although the results show that the conidia were adversely affected within a few hours of inoculation, the pathogen was not completely destroyed in any of the inoculated leaves and was recovered in samples from all the leaf treatments after 14 days.

With the leaves left on the plants it was possible to isolate the fungus from inoculated areas weeks afterwards, although only slight chlorosis developed in these areas. These samples showed that V. dahliae was present in small amounts and usually it grew from the cut veins suggesting that the pathogen was surviving or growing better within the vessels than in the intercellular spaces.

After being in plastic bags for 14 days the leaves had deteriorated and there was a large amount of V. dahliae in the intercellular spaces as well as in the veins. Mycelium with verticels covered the surface of the necrotic areas.

Table 8. Growth of Verticillium (V3) from sample discs of VG tobacco leaves at different periods after flooding intercellular spaces with a conidial suspension.

Period after inoculation (hours)	Number of hyphae growing from each disc (Mean of 50 discs)			
	Leaves attached to plant	Detached leaves in plastic bags	Detached leaves in water	Mean
4	52.6[1/]	24.4	35.8	37.6
24	10.0	11.4	9.7	10.4
120	1.0	3.7	0.9	1.9
Mean	21.2	13.2	15.5	16.6

L.S.D.(.05) = 5.70

L.S.D.(.01) = 8.94

1/ All figures are means of 50 discs, 5 replications of 10 discs in a 3 x 3 factorial arrangement. Analysis of variance given in Table A 2.

Visible changes in the inoculated areas of the leaves are summarized in Table 9. Leaves inoculated with conidia became chlorotic much faster than the controls. The presence of the pathogen precipitated chlorosis even though the quantity of fungus had been quickly reduced (Table 8). In the detached leaves in bags the tissues which had received the conidial suspension remained turgid, though chlorotic, for several days after the surrounding areas had become necrotic, as if the presence of the Verticillium in some way had been instrumental in keeping them alive.

At the end of the trial, 5 days after inoculation, the pathogen could not be found in samples from the control areas nor from outside the inoculated areas.

Two further experiments were conducted to compare the responses of both "young" and "old" leaves to V3 and V58 over the three time intervals, 4 hours, 24 hours and 120 hours. The design for both experiments was 2 x 2 x 3 factorial with 4 replications. In one trial VG plants were used as the hosts and leaves were chosen in the 5th leaf position (old) and in the 14th to 15th leaf position (young). The other trial was the same except that MN plants were used. In these experiments the inoculated leaves were left attached to the plant.

Table 9. Symptoms on VG tobacco leaves inoculated by flooding intercellular spaces with suspensions of Verticillium (V3) conidia.

Period after inoculation (hours) and treatment area	Attached leaves		Detached leaves			
			Petioles in water		In plastic bags	
	Inoculated[1]	Control[2]	Inoculated	Control	Inoculated	Control
48 Leaf[3]	None	None	Wilting and	None	Flaccid	Flaccid
Control areas[4]	None	None	chlorosis	None	Flaccid	Flaccid
Inoculated	Slight	-	Chlorosis	-	Slight	-
area[5]	chlorosis				chlorosis	
72 Leaf	None	None	Chlorosis	None	Chlorosis	Flaccid
Control areas	None	None	Chlorosis	None	Chlorosis	Flaccid
Inoculated area	Slight	-	Marked	-	Marked	-
	chlorosis		chlorosis		chlorosis	

(Continued)

Table 9. Continued.

Period after inoculation (hours) and treatment area	Attached leaves		Detached leaves			
			Petioles in water		In plastic bags	
	Inoculated[1]	Control[2]	Inoculated	Control	Inoculated	Control
96 Leaf[3]	None	None	Pale yellow	None	All leaves yellow and cells collapsing	Slight Chlorosis
Control areas[4]	None	None	Pale yellow	None		
Inoculated area[5]	Slight chlorosis	-	Orange	-	Deep yellow and cells turgid	-
120 Leaf	None	None	Yellow	Slight chlorosis	Collapsing	Chlorosis
Control areas	None	None	Yellow	Slight chlorosis	Collapsing	Chlorosis
Inoculated area	Slight chlorosis	-	Orange	-	Deep yellow and turgid	-

(Continued)

Table 9. Continued.

Period after inoculation (hours) and treatment area	Attached leaves		Detached leaves			
			Petioles in water		In plastic bags	
	Inoculated[1/]	Control[2/]	Inoculated	Control	Inoculated	Control
336 Leaf	None	None	Chlorotic	Slight chlorosis	Necrotic	Necrotic
Control areas	None	None	Chlorotic			
Inoculated area	Slight chlorosis and some necrosis where samples taken	-	Necrotic	-	Necrosis darker brown than rest of leaf. Verticels on surface of leaf	-

(Continued)

Table 9. Continued.

1/ Inoculated leaves = Leaves which had areas inoculated by flooding the intercellular spaces with a conidial suspension.

2/ Control leaves = Leaves which had areas of the lamina flooded with water and autoclaved conidial suspension only.

3/ Leaf = Symptom expression on a leaf outside the inoculated areas.

4/ Control areas = Areas of the lamina adjacent to inoculated areas but which were flooded with water or autoclaved conidia only.

5/ Inoculated areas = Areas of the lamina inoculated by intercellular flooding with conidia.

The results of the two experiments are presented in Tables 10, A 3 and A 4. Although the two cultivars were treated separately the results are very similar. Differences between isolates, upper and lower leaves as well as the times after inoculation are all highly significant ($P = .01$). The V58 inoculation gave less recovery of fungus than V3 and the check in growth of both isolates was more marked in the upper than in the lower leaves.

In the old leaves particularly, there was sometimes a much larger number of hyphae than normal recovered from a single disc. From one disc from a lower VG leaf inoculated with V3, 136 hyphae grew out. This could have been the result of conidial aggregation at this site because it is not known whether spore flow or settlement was uniform over the whole flooded area. However, it could have resulted from differential reaction from the host tissue.

It was not possible to determine whether the growth after the longer periods came from conidia which had remained ungerminated in the intercellular spaces or from hyphae, however, growth from vessels in the inoculated zone appeared to be from hyphae.

Table 10. Growth of Verticillium from sample discs of VG and MN leaves at different periods after the intercellular spaces had been flooded with conidial suspensions of V3 and V58.

Period after inoculation (hours)	VG Lower leaves			VG Upper leaves		
	V3	V58	Mean	V3	V58	Mean
4	57.5 1/	34.0	45.8	9.7	3.4	6.6
24	10.1	8.0	9.1	0.5	0.6	0.6
120	3.9	2.6	3.3	0.4	0.2	0.3
Mean	23.8	14.9	19.4	3.5	1.4	2.5
L.S.D. (.05) =	4.69					
L.S.D. (.01) =	7.77					

	MN Lower leaves			MN Upper leaves		
	V3	V58	Mean	V3	V58	Mean
4	46.2	22.2	34.2	3.1	2.0	2.6
24	14.3	5.4	9.9	1.1	0.9	1.0
120	3.5	0.9	2.2	0.6	0.2	0.4
Mean	21.3	7.2	15.4	1.6	1.0	1.3
L.S.D. (.05) =	5.88					
L.S.D. (.01) =	9.76					

1/ All figures are means of 40 discs, 4 replications of 10 discs arranged as a 2 x 2 x 3 factorial design. Analysis of variance given in Tables A 3 and A 4.

After 14 days in both VG and MN lower leaves, areas inoculated with both V3 and V58 showed some chlorosis and necrosis, and in the upper leaves, slight chlorosis.

It may be concluded that the germination of conidia injected into the intercellular spaces was checked after the conidia had been in the leaves for only a few hours. The fungus was not eliminated and survived in the intercellular spaces. It also became established in the vessels, probably at the time of injection and proliferated from there to produce generalized symptoms.

Relationship Between the Amount of Host Tissue and Its Proximity to the Pathogen.

The results of the work with detached leaves suggested that the suppression of the fungus within the vessels of the leaf may be an active and continuing process. There was also evidence that some areas of tissue may not be as effective in checking the pathogen as others and that in detached leaves nutrient reserves may be important for prolonged control of the fungus. If these hypotheses are correct then a given quantity of host tissue should be required to suppress a given amount of pathogen.

Healthy leaves from the top and base of the stem were selected from uninoculated VG plants and surface sterilized. Circles of lamina were cut with a No. 4 cork borer (8.2 mm diameter) from the leaves under aseptic conditions and a second hole, 2.0 mm was cut from the centre of the circles to form a doughnut-shaped ring of lamina.

Test pieces cut from secondary veins of VG leaves showing severe symptoms were placed in the centre of a ring of host tissue on a microscope slide. The material was then covered with a 15 mm circular cover slip and gently flooded with sterile distilled water, xylem sap or other chemicals such as the respiratory inhibitors 2, 4-dinitrophenol (10^{-5} M) or potassium fluoride (2×10^{-4} M). The resulting growth of fungus was scored after 24 hours incubation in the same way as in earlier experiments.

The results of the first experiments were clear cut. When surrounded by a circle of either young or old leaf lamina and flooded with water the fungus would not grow or grew poorly (Fig. 14). With eight replications of eight discs (64 discs per treatment) the control test discs without the circle of lamina around them averaged a score of 4.97 (scored on a 0 to 5 basis). The test discs surrounded by

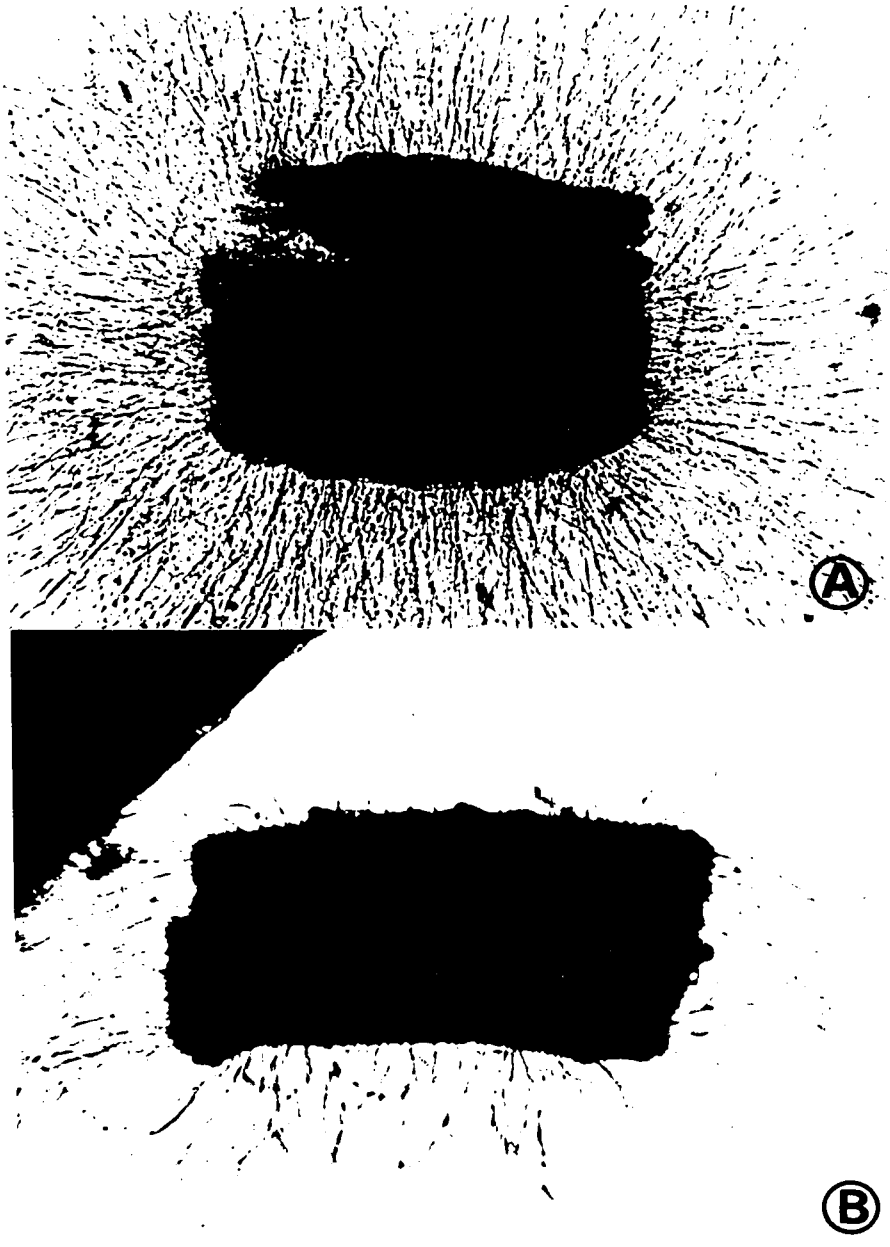
FIGURE 14

A. Profuse growth of Verticillium dahliae from a leaf piece of infected tobacco xylem. Incubated in water for 24 hours under a cover slip but not surrounded by tobacco lamina.

B. Poor growth of fungus from a test piece of infected tobacco xylem. Incubated in the centre of a ring of tobacco leaf lamina. Usually there was no growth from similar test pieces.

FIGURE 14

FIGURE 14



old leaf lamina averaged 0.22 and those with young leaf circles 0.05 (Table A 5).

If the test disc surrounded by the lamina ring was incubated in water without a cover slip, the fungus was able to grow (Tables 11 and A 6).

These results suggest that the pathogen was checked by something formed by the host tissue but this was either not formed or not active when the cover slip was not applied. Several hypotheses could be tested to explain these results. The inhibitor may be active only in the absence of oxygen or perhaps carbon dioxide produced by the host tissue may have been trapped under the cover slip and affected the fungus either directly or by altering the pH. (pH of the liquids was tested and found to be the same with or without cover slips, 5.5 to 6.0). The concentration of the inhibitory substance may have been increased within the confines of the cover slip or perhaps with ample oxygen the fungus was able to overcome the toxicity of the system.

When the respiratory inhibitors 2,#4-dinitrophenol (10^{-5} M), potassium fluoride (2×10^{-4} M) or thiourea (10^{-3} M) were used instead of water over the incubation period similar results were obtained. The inhibitors at these concentrations

Table 11. Growth of fungus from test discs in water with and without lamina circles and with and without cover slips.

Treatment	Score (0 - 5)	Length of longest hypha (mm)
Control, no lamina circles, no cover slips	4.79[1/]	1.26
Green lamina circles, no cover slips	3.25	1.15
Green lamina circles with cover slips	0.21	0.05
Autoclaved lamina circles, no cover slips	4.25	0.91
Autoclaved lamina circles with cover slips	4.50	1.14
L.S.D. (.05)	0.67	0.43
L.S.D. (.01)	0.93	0.59

1/ All figures means of 24 discs. 4 replications of 6 discs per replicate. Analysis of variance in Table A 6.

did not prevent the fungus from growing from the test discs nor did they overcome the inhibitory effects of the lamina circles when these were covered with slips.

In other experiments it was found that if the test disc was closely surrounded by a ring of 6 uninfected lamina discs of the same size as the test disc (0.86 mm diameter) (Fig. 15, position 3) there was no growth or very little growth of the pathogen. If the 6 host discs were moved out one diameter from the central position (position 4, Fig. 15) there was some growth but it was not as good as the control (Table 12). With a single healthy disc in close proximity to the test disc there was good germination and growth. With 12 discs under the same cover slip but in a circle one diameter away from the test disc, growth was again inhibited. In this experiment each arrangement was covered with a slip.

The degree of inhibition was related to the proximity as well as the number of host lamina discs.

The system composed of a test disc surrounded by a close ring of host discs under a cover slip was again tested with respiratory inhibitors in the system. Again at the concentrations used the test discs produced the same growth of fungus as the controls in water and growth was markedly

FIGURE 15

Diagram of the arrangements of test discs containing Verticillium dahliae and challenge discs of green tobacco lamina under cover slips.

● = Test disc containing fungus.

o = Challenge discs of tobacco lamina.

FIGURE 15

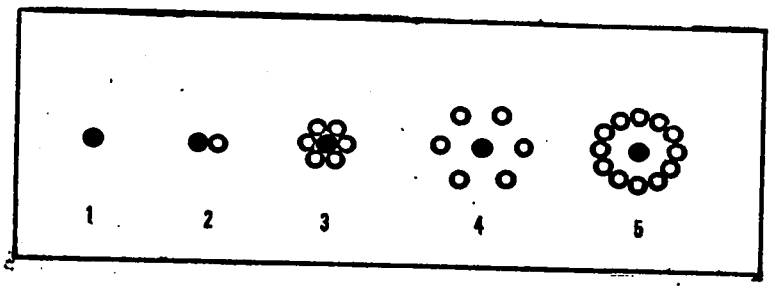


Table 12. Growth of fungus from test discs in water surrounded with host lamina discs incubated in water.

Treatment	Score (0 - 5)	Length of longest hypha (mm)
Test disc alone	5.00[1/]	1.10
Test disc surrounded by 6 host discs touching it	0.25	0.07
Test disc surrounded by 6 host discs at one diameter	2.58	0.60
Test disc surrounded by 12 host discs at one diameter	0.08	0.02
L.S.D. (.05)	0.72	0.21
L.S.D. (.01)	1.04	0.30

1/ Mean of 12 discs. 3 replicates of 4 discs.

checked when the test disc was closely surrounded by host tissue. The inhibitors had not affected the fungus nor had they altered the inhibitory effect of the host tissue.

The trial was repeated using ethanol (0.1%) and the reducing agents ascorbic acid (2.7×10^{-3} M) and sodium sulphite (2.7×10^{-4} M). At 2.7×10^{-3} M sodium sulphite completely inhibited the growth of the fungus but was only slightly inhibitory at 2.7×10^{-4} M. When old leaf tissue was used as challenge discs with 0.1% alcohol the fungus was able to grow but not as well as in the controls. The reducing agents at the concentrations used did not overcome the inhibitory effect of the host tissue but did affect the pathogen to some degree.

The results indicate that a strong inhibitor is produced by tobacco leaf cells, which either does not move far from the cells, or else has to be present at a certain threshold concentration before it becomes effective.

If the inhibitor was an oxidized polyphenol, the reducing agents might be expected to prevent its formation, however, this did not occur and it may be concluded that the inhibitory effect of the system may not be due to oxidized metabolites.

Just why the respiratory inhibitors did not stop the formation of the fungal inhibitor by the host cells cannot be explained by the author. Perhaps higher concentrations of these chemicals may have been effective. Further work should be done using this technique with other respiratory inhibitors and at other concentrations.

CHARACTERISTICS OF TOBACCO XYLEM SAP

Cytokinin-like Activity of Xylem Exudate from the Roots of Tobacco Plants Infected with V. dahliae.

It was postulated that leaf cell vitality influenced symptom expression and that this in turn had been affected by transportable factor(s) from the root system (Wright 1969). To test this hypothesis the cytokinin-like activity, of tobacco xylem sap from the roots, was studied.

The work was done in collaboration with Dr. D. Eastwood, a post-doctoral fellow at Macdonald College.

Kulaeva (1962) had found that cytokinin formed in the roots of tobacco was transported to the leaves where it influenced leaf metabolism. Cytokinin activity has been reported from the sap of several plant species (Weiss and Vaadia 1965; Yoshida et al. 1971) and cytokinins have been found in the root systems of some but not all plants (Shibaoka and Thimann 1970).

Plants subjected to physical stress such as increased temperature or water deficit may have a reduced concentration of these hormones in the sap stream (Itai and Vaadia 1971).

Cytokinins are known to stimulate transpiration by inducing opening of the stomata (Luke and Freeman 1968). As smaller amounts of cytokinins leave the root system during water stress, and there is also increased breakdown of cytokinins in the leaves, transpiration is reduced and water is conserved. As the root system is known to play an important part in the Verticillium wilt-tobacco syndrome and other wilt diseases, the possible implication of these compounds may be important.

Misaghi et al. (1969) working with cotton found that in infected plants, the injection of kinetin or benzyladenine did not delay yellowing of the leaves but had the opposite effect and accelerated the process in a synergistic fashion. These workers concluded that infection with Verticillium had reduced the compounds with cytokinin-like activity from two in healthy plants to one in infected plants.

Materials and Methods.

Three-month-old tobacco seedlings of MN and G were inoculated by the root dip method and control plants had their roots dipped in water. After inoculation the plants were grown in sterilized soil in the greenhouse for a further 7 weeks until just before flowering. Symptoms were then present on the lower leaves of both cultivars. The plants were

sectioned 4 cm above the soil and the sap collected into U-shaped capillary tubes. Collection took place over a 30 hour period. There was great variability in the time and rates of sap flow among the plants. Exudates were stored at 4°C in glass vials for a short period before extraction. Root systems were washed free of soil blotted dry, and stored in polythene bags at -15°C. Five plants of each cultivar were used for each treatment and the experiment was performed twice.

Extraction procedures.

The root exudate was adjusted to pH 2.0 with 0.02 M HCl and extracted successively with diethyl ether (3 x 1 vol) and ethyl acetate (3 x 1 vol). The pH was then adjusted to 8.0 with 0.02 M NH_4OH and ethyl acetate extraction was repeated. The fractions including the aqueous phase were dried at 50°C under reduced pressure. Residues were taken into a few drops of 90% (V/V) ethanol for paper chromatography or into a known volume of 0.005 M citrate phosphate buffer (pH 5.6) for use in bioassays. All solvents were distilled immediately before use and other chemicals used were A.C.S. standard.

Part of the root system was used to estimate dry weights. The remainder was homogenized at 4°C in 90% methanol (V/V) in a Waring blender, (10 ml solvent per gm fresh weight of root)

and incubated for 16 hours at 4°C. Methanolic extraction was repeated at room temperature for 1 hour and the debris was extracted with warm water. Bulked extracts were filtered and brought to the water phase at 50°C under reduced pressure. The water phase was then fractionated in the same way as the xylem exudates.

Bioassay procedures.

Solutions obtained from the organic solvent extraction were made up to 10 ml in citrate phosphate buffer and senescence retarding activity was evaluated by a modification of the technique used by Osborne and McCalla (1961). Discs were cut with a No. 4 cork borer from young barley leaves (Hordeum vulgare L.). In each test 10 discs were placed in 2 ml of solution and control discs were incubated in buffer solution. Incubation was carried out with the discs placed on filter paper soaking in the test solution because when the discs were put directly into a test liquid some sank and retained more chlorophyll than those which floated (Kende 1964). Chlorophyll was extracted with 90% (V/V) acetone saturated with $KHCO_3$. Extracts were clarified by centrifugation and the supernatant solutions were brought to a constant volume. Light absorbance of the chlorophyll extracted was read at 660 nm in a Bausch and Lomb "Spectronic 20" spectrophotometer, against an acetone

blank. Loss of chlorophyll in the different solutions was expressed in relation to the loss in buffer alone and results were compared with a curve determined from known amounts of kinetin (6 - furfuryl - amino - purine).

Cytokinin activity.

Solutions which retarded senescence were then tested for cytokinin activity. The method used was based on the finding that cytokinins will induce betacyanin formation in the hypocotyls of Amaranthus seedlings grown in the dark (Bamburger and Mayer 1960; Bigot 1968; Piatelli et al. 1971). Seeds of A. retroflexus L. were soaked in concentrated H_2SO_4 for up to 45 sec then thoroughly washed in distilled water. This surface-sterilized the seed and increased germination from < 10% to > 90%. After germination in the dark, 50 uniform seedlings were transferred to filter paper soaked with 2 ml of buffered residue (5 m M citrate-phosphate buffer) plus 0.5 ml of 0.4 mg/ml l-tyrosine and grown for another 2 days at 25°C under dark conditions. Betacyanins were extracted by homogenizing the seedlings in ice water with a glass homogenizer. The homogenates were incubated at 4°C for 0.5 hours and centrifuged at 13,500 r.p.m. for 20 min. The pellet was extracted again and the combined supernatent solutions adjusted to 10 ml. The light absorption of the solutions was read at 540 n m against a distilled water blank.

Measurements of the amount of absorbance from betacyanin formed by the Amaranthus seedlings germinated in known quantities of kinetin, were used to draw a reference curve. By comparing this curve with the absorbance of betacyanin formed when the seedlings were grown in the root extracts and exudates it was possible to convert the cytokinin activities of these to kinetin equivalents. Activity could then be expressed as ng of kinetin equivalents per ml of exudate or per g dry weight of tissue.

Paper chromatography.

After tests for cytokinin activity had been completed the aqueous phases from each set of plants and treatments were pooled and the solutions concentrated 40 fold. Ethanolic solutions of the residues were line loaded onto 4 cm x 20 cm strips of Whatman 3 MM paper which had been pre-washed with the developing solvent, butan-1-ol; glacial acetic acid; water (4: 1: 1 by volume). The dried chromatograms were cut transversely into 10 equal strips from 0.5 cm below the origin up to the solvent front. The strips were then used to determine cytokinin activity. Other strips were eluted with water and tested for possible toxicity to V. dahliae using the infected test disc technique described above.

Results and Discussion.

Taylor (1968) had found the sap from inside the xylem vessels of mature tobacco stems to be distinctly brown in colour whereas the sap collected from the roots in this work was colourless.

The roots of infected plants were significantly smaller than those from non-infected plants (P = 5%). The quantity of exudate collected varied widely from plant to plant and there appeared to be no relationship between size of root system, variety of tobacco or infection and the yield of exudate (Table 13).

In extractions from both xylem exudates and root tissue only the water phase contained senescence retarding activity, the other fractions increased the loss of chlorophyll from the barley leaf discs (Figs. 16 and 17). This finding was supported by the results of the Amaranthus bioassay where the water phase showed cytokinin-like activity whereas the other fractions had no such activity and adversely affected the growth of the seedlings.

Other workers have not been successful in removing cytokinins from the water phase with alkaline ethyl acetate especially from xylem exudate (Taylor and Luckwill 1967). The failure to recover cytokinins in the alkaline ethyl acetate

Table 13. Fresh weight of roots and volume of exudate collected from uninfected and *V. dahliae* infected tobacco plants over a 24 hour period.

	VG		MN		
	Uninfected	Infected	Uninfected	Infected	SE
Fresh weight of roots (g)[1/]	11.98	7.14	10.10	8.12	1.78
Exudate per g fresh weight (ml)[1/]	0.30	0.31	0.28	0.18	0.24

1/ Mean of five plants.

FIGURE 16

Senescence retarding and accelerating activity of different fractions from the xylem exudate of infected and uninfected VG and MN plants.

A = direction of increased chlorophyll loss by barley leaf discs incubated in the fraction, compared with the loss in buffer solution.

R = direction of reduced chlorophyll loss by barley leaf discs incubated in the fraction, compared with the loss in buffer solution.

1 = ether fraction 2 = acid ethyl acetate fraction

3 = alkaline ethyl acetate fraction 4 = water phase

5 = buffer solution.

FIGURE 16

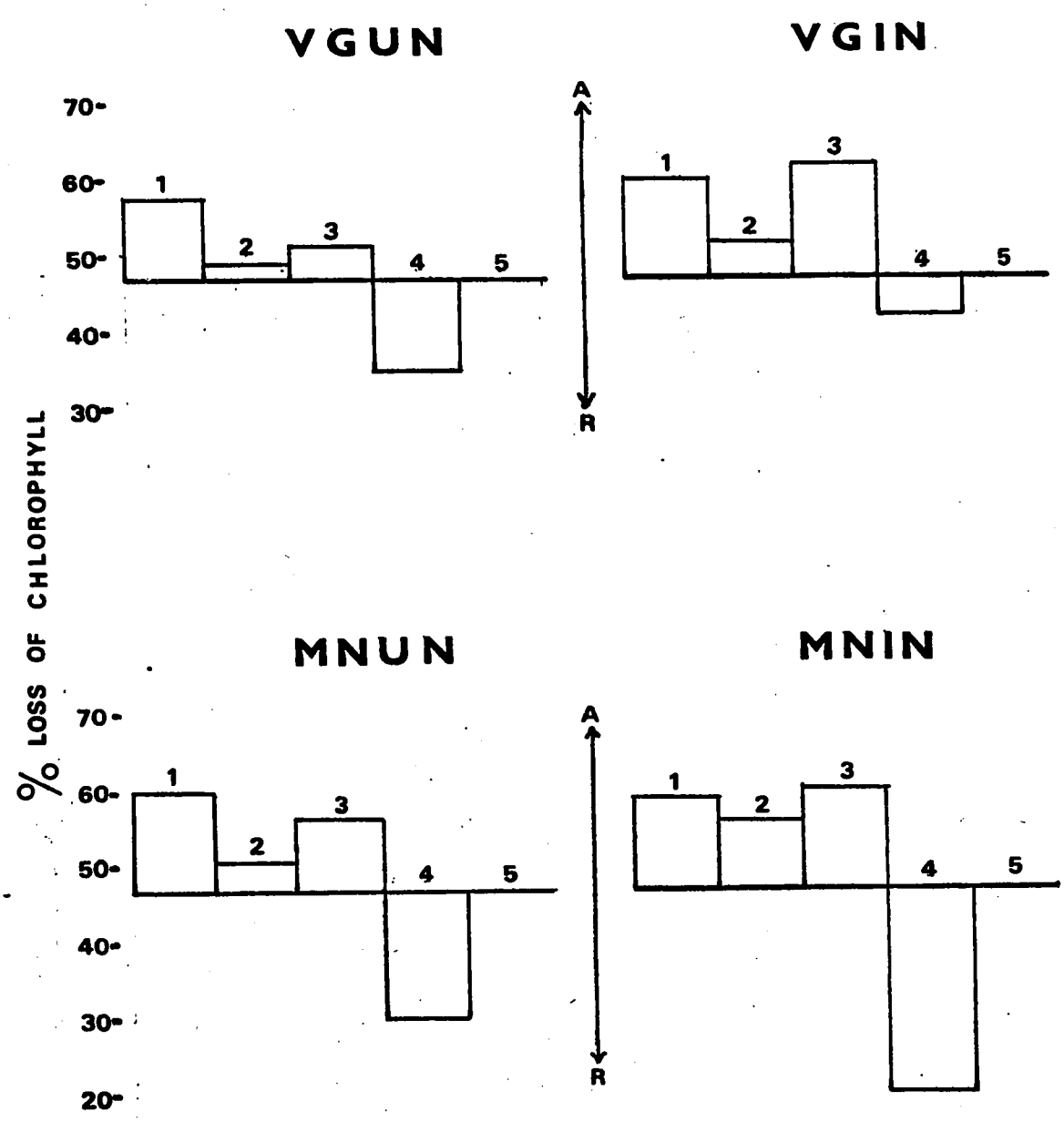

VGUN
VGIN
MNUN
MNIN
% LOSS OF CHLOROPHYLL
70-
60-
50-
40-
30-
20-
A
R
1
2
3
4
5

FIGURE 17

Senescence retarding and accelerating activity of different fractions of extracts from tobacco roots.

A = direction of increased chlorophyll loss by barley leaf discs incubated in the fraction, compared with the loss in buffer solution.

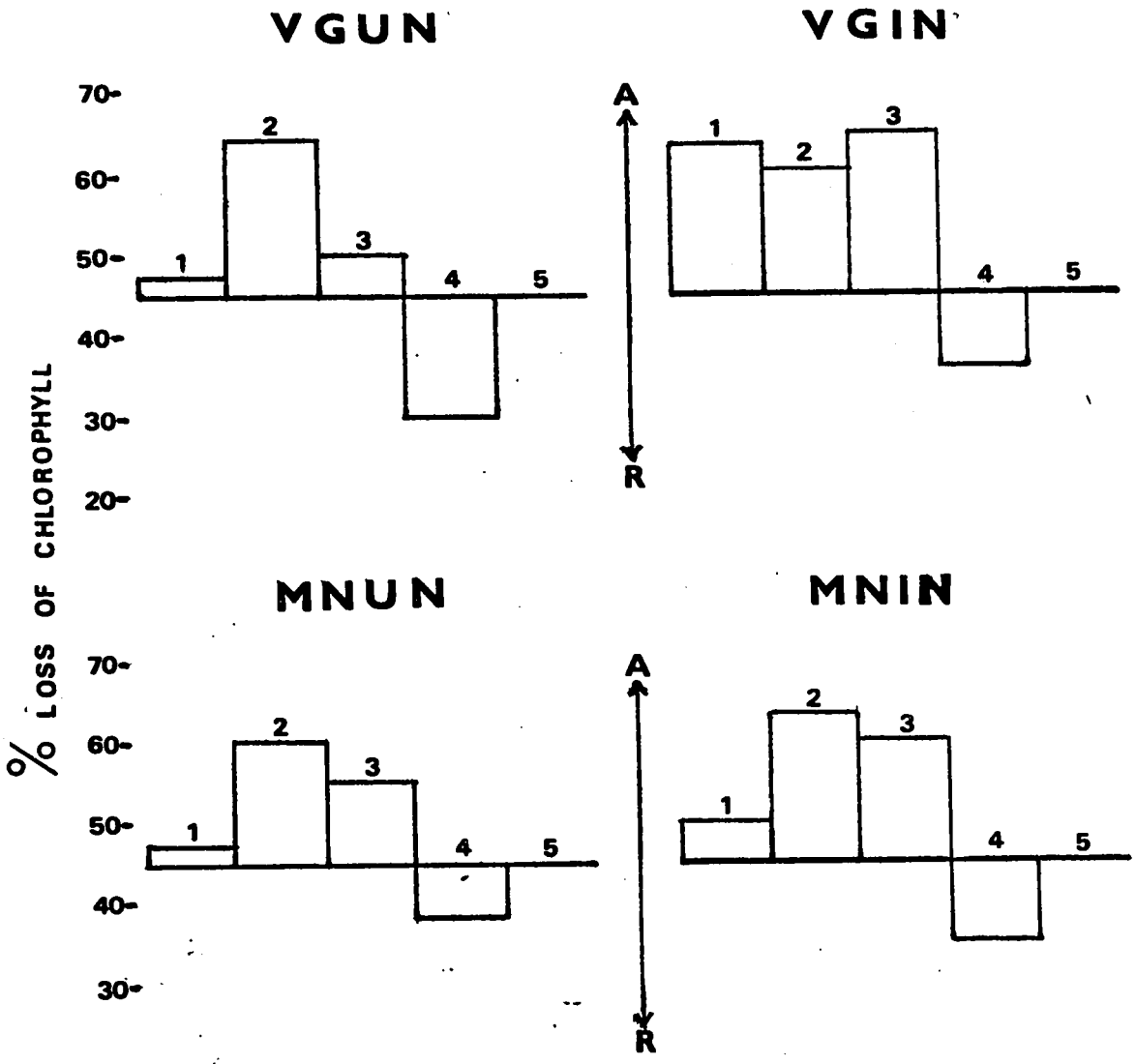
R = direction of reduced chlorophyll loss by barley leaf discs incubated in the fraction, compared with the loss in buffer solution.

1 = ether fraction 2 = acid ethyl acetate fraction

3 = alkaline ethyl acetate fraction 4 = water phase

5 = buffer solution.

FIGURE 17


VGUN
VGIN
MNUN
MNIN
% LOSS OF CHLOROPHYLL
70-
60-
50-
40-
30-
20-
1
2
3
4
5
A
R

may be related to the stage of growth of the plants. After finding no cytokinins in the alkaline ethyl acetate fraction this was not investigated further and after acid ethyl acetate extraction the pH of the water phase was adjusted to 7.0 before concentration under reduced pressure.

There were high levels of cytokinin activity in the xylem exudate from infected resistant plants even though the root system was relatively small. Conversely the infected susceptible plants had larger root systems but very low cytokinin activity in the xylem sap (Table 14). This was not related to flowering in this instance as they were short-day plants and were not conditioned to form flowers. This result would suggest that the resistant plants may maintain normal transpiration and viability of the leaf cells even though the plants are infected. Although symptoms were present on the lower leaves of the resistant plants they were not as marked as those on the susceptible ones: It was possible that different cytokinin levels may be found at different stages of growth of the host and at different levels of symptom expression.

Paper chromatography.

The chromatograms of the xylem exudate samples all showed strong fluorescence at Rf 0 to 0.10 and Rf 0.20 to 0.30 and faint fluorescence at Rf 0.55 to 0.60. In addition the MN

Table 14. Cytokinin-like activity from exudates and extracts of root tissue.

	VG		MN		
	Uninfected	Infected	Uninfected	Infected	SE
Dry weight of root system (g)[1/]	2.11	2.57	2.05	1.46	0.97
Kinetin equivalents/ml sap (ng)	23.09	12.14	18.93	20.11	0.76
Kinetin equivalents/g dry weight of root tissue and SD	13.17 ± 1.95[2/]	8.77 ± 2.21	9.83 ± 1.60	13.70 ± 3.85	

1/ Mean of five plants. 2/ Standard deviations were calculated independently because of loss of some samples. 3/ Analysis of variance and tests of significance are given in Tables A 7 and A 8.

uninfected samples had faint fluorescence at Rf 0.75 to 0.82 and this band showed up strongly in infected MN exudate but was not found in either of the VG samples.

The root extracts were treated in the same way as the xylem exudates. Under U.V. light several fluorescent bands were clearly visible. The extracts from the root stocks of both uninfected and infected VG showed similar fluorescent patterns, a brown-yellow colour at Rf 0 to 0.10 and faint fluorescences at Rf's 0.15 to 0.18 and 0.22 to 0.25. Strong fluorescent bands were present at Rf 0.35 to 0.55 and 0.72 to 0.88.

In contrast the chromatogram of the MN uninfected and infected root extracts differed both from each other and from the chromatograms of the VG extracts. A brown-yellow colour was again present at Rf 0 to 0.10 but faint fluorescence was seen at 0.18 to 0.20 and a strong fluorescence at Rf 0.38 to 0.55 in both MN samples. The uninfected MN sample gave strong fluorescence at Rf 0.78 to 0.88 and the infected MN sample faint fluorescence at Rf 0.62 to 0.71 and strong fluorescence between Rf 0.84 to 0.88.

Results of Amaranthus bioassays of chromatogram strips.

The cytokinin-like activity of the chromatographed water phase fractions was estimated by growing Amaranthus

seedlings on the chromatograph paper in the dark and measuring betacyanin formation as before. The absorbance values for the different fractions and Rf values of these are presented in Figure 18. The total cytokinin-like activity of the different treatments (areas under the histograms) reflect the results shown in Table 14. There was no apparent pattern in the activity at the different Rf's except that there was a tendency for high activity at the low Rf values, 0.1 to 0.2 for the xylem exudates and 0 to 0.1 for the root extracts. In the infected MN xylem exudate this activity was highest at Rf 0.2 to 0.3 and extended up to Rf 0.5.

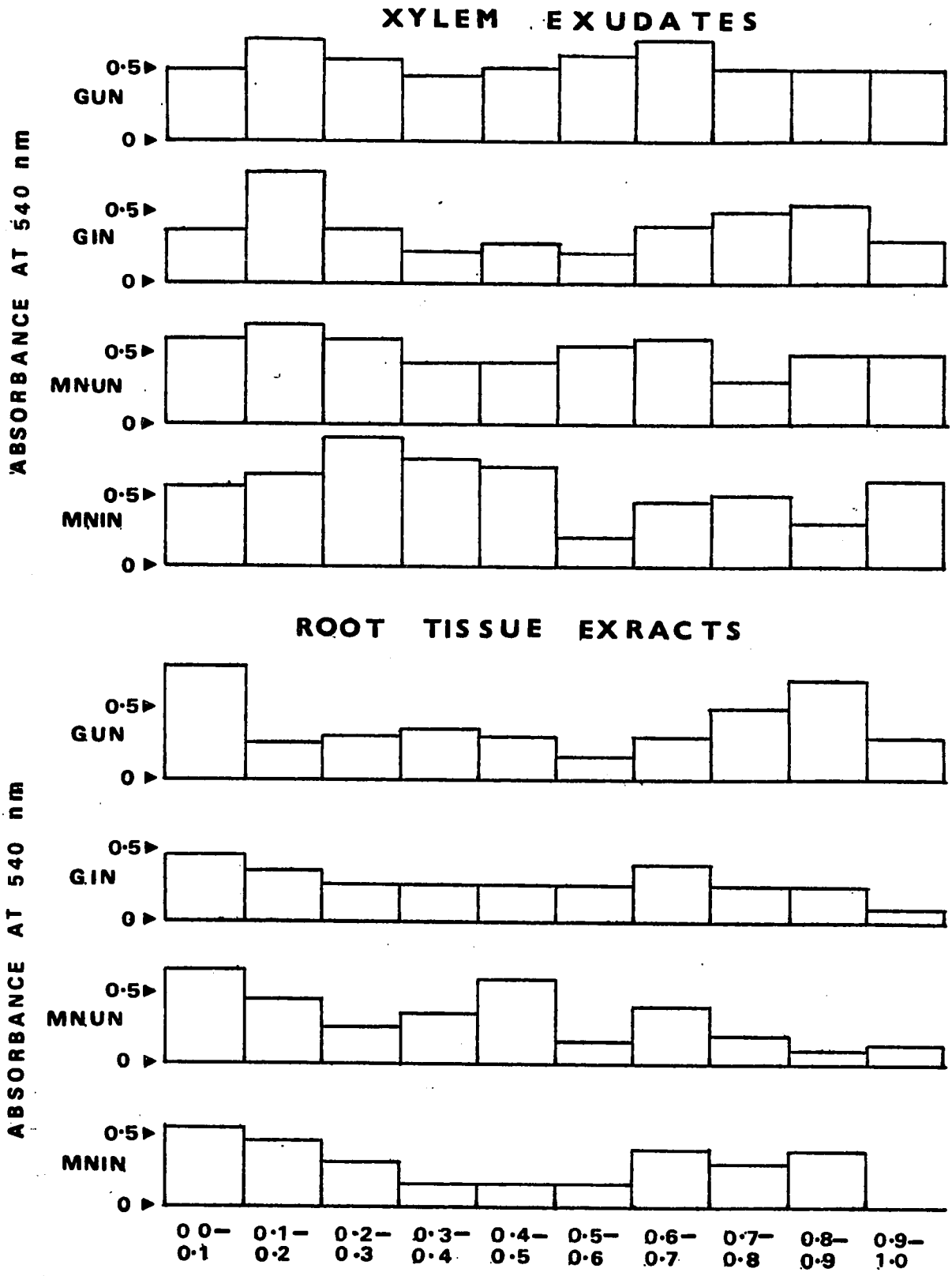
The effect of the fractions upon growth of the fungus.

The chromatograms from the water phase of the root tissue extracts were divided into three, Rf's 0.180 to 0.233; 0.333 to 0.500 and 0.733 and 0.866. These were eluted with water and tested for their effect upon the growth of the fungus with the disc technique. There were four replications of 10 discs per treatment. The results showed no significant retardation or stimulation of growth from any of the eluted solutions. Earlier the different fractions from xylem exudate had been tested for their effect upon the growth of V. dahliae. The germination of spores and the growth of fungus from infected lamina test discs, were both erratic but it was later found

FIGURE 18

Cytokinin-like activity of chromatographed water phase fractions from xylem exudates and root tissue extracts. Seedlings were germinated on chromatogram strips of the water phase fractions of xylem exudates and root tissue extracts of infected and uninfected VG and MN plants. Absorbance of light at 540 nm from betacyanin formation in Amaranthus seedlings was used to estimate relative cytokinin-like activity at the different Rf values.

FIGURE 18


XYLEM EXUDATES
ABSORBANCE AT 540 nm
0·5
GUN
0
0·5
GIN
0
0·5
MNUN
0
0·5
MNIN
0
ROOT TISSUE EXRACTS
ABSORBANCE AT 540 nm
0·5
GUN
0
0·5
GIN
0
0·5
MNUN
0
0·5
MNIN
0
0 0— 0·1— 0·2— 0·3— 0·4— 0·5— 0·6— 0·7— 0·8— 0·9—
0·1 0·2 0·3 0·4 0·5 0·6 0·7 0·8 0·9 1·0
Rf's OF CHROMATOGRAPH STRIPS ON WHICH AMARANTHUS GROWN

that a buffer solution used may have had an adverse effect. However, none of the fractions completely inhibited growth and all produced the twisted growth characteristic of the pathogen growing in unfractionated xylem sap. The acid ether fraction inhibited growth more than the water phase or acid ethyl acetate. This suggested that if the xylem sap exerted a subtle effect upon the growth of the hyphae, the substance that was responsible for this may be found in the acid ether fraction of the xylem sap.

The Effect of Tobacco Xylem Exudates and Nutrient Solutions Upon the Growth of *Verticillium dahliae*

The mechanism of resistance to *Verticillium* wilt in tobacco is not understood. In some way the root system determines the time of symptom expression in the leaves. The quantitative nature of this phenomenon and its predictibility at different stages of growth suggests that a continuous process is in operation (Gibbins and Wright 1968).

Inoculum potential is known to be important in this crop and the degree of invasion related to the level of symptom expression (Wright and Biss 1968; Wright 1969). In heavily infested soils and at high inoculum levels in pots, the pathogen

appears to be able to enter tobacco roots without difficulty. Differences in susceptibility to Verticillium are a reflection of differences in growth of the fungus within the cultivars. It is possible that after invasion, resistance in some cultivars may depend upon the availability of a better supply of nutrients or other factors which enable parenchyma cells surrounding the vessels to keep the fungus in check more effectively than in susceptible cultivars. It is also possible that the xylem sap itself has a direct effect on the growth rate of the fungus.

Taylor (1968) studied the effect of sap from tobacco stems on conidial germination and subsequent growth of V. dahliae. The work reported here was designed to study the effect of xylem exudates from the root and various nutrient solutions on the growth of the fungus from within the host vessels.

Materials and Methods.

Fifteen seedlings each of VG and MN were inoculated by the root-dip method and a further 15 were dipped in water as controls. The plants were grown in 10 cm clay pots in the greenhouse in three randomized blocks of five plants of each treatment. Just before flowering the xylem exudate was

collected for 24 hours as described previously. After collection the sap was passed through 0.2 μ millipore filters into sterilized test tubes and was used immediately after collection.

To test the effect of liquids upon fungal growth, the fungus is usually grown in the solutions in agitated flasks or germination and growth of conidia are evaluated by the hanging drop technique. The observations made on cleared infected tobacco leaf discs suggested that V. dahliae may be present in a different form to that found in culture. The disc technique therefore, offered a way to study the responses of the fungus, as it exists in the plant, to various test solutions.

One of the initial problems was to obtain invaded tissue which would give uniform growth, as the pathogen is not distributed evenly throughout the vessels. Test discs were taken from the secondary veins of chlorotic laminae within 1 cm of the lateral veins and the quantity and uniformity of the fungus at the prospective sampling sites was established by preliminary sampling. In other trials it was found that the vascular bundles from the petioles of infected sunflower leaves provided excellent test discs for evaluating the effect of solutions upon the sunflower isolate.

The advantage of this technique was that only very small quantities of the test solution were needed to flood the test discs.

Conidial germination and fungus growth were studied in xylem exudates, Czapek's broth, potato dextrose broth and V8 juice (Tuite 1969). Water was used as the control.

Results.

The effect of the xylem saps was tested in three experiments with similar results. A typical set of results is presented in Table 15. The various xylem saps had no apparent adverse effects upon the germination of the hyphae or conidia. If phytoalexin-like effects were operative as found in cotton (Schnathorst et al. 1968), the techniques used here were not sensitive enough to detect them. Death of conidia detected in the vessels of young tobacco seedlings (Wright 1969; Mahanty 1970) was apparently not attributable to a direct effect of the xylem sap.

There was, however, a marked difference in the form of growth of the fungus in the sap compared with that in water (Figs. 19, 20). In water at least over the first 24 hours, growth tended to be relatively straight with little branching.

Table 15. Growth of Verticillium hyphae after 24 hours, and germination of conidia after 18 hours, in xylem exudates of tobacco.

Source of xylem exudate	Growth score of hyphae (0 - 5)	Length of longest hyphae (mm)	Germination of conidia (%)
VG Inoculated	4.8[1/]	1.21[1/]	89.2[2/]
VG Uninoculated	4.8	1.15	92.6
MN Inoculated	4.9	1.19	92.0
MN Uninoculated	4.9	1.00	92.4
Water control	4.9	1.20	-
L.S.D. (.05)	0.9	0.15	4.0

1/ Means of six replicates with five discs per replicate.

2/ Mean of five fields of approximately 40 conidia per field. Analysis of variance given in Tables A 9 and A 10.

FIGURE 19

Effect of liquid media on growth form of *Verticillium dahliae* from infected tobacco leaf discs. A. Relatively straight hyphae in water. B. Much-branched, twisted hyphae in xylem exudate from infected McNairs plants. (X460).

FIGURE 19

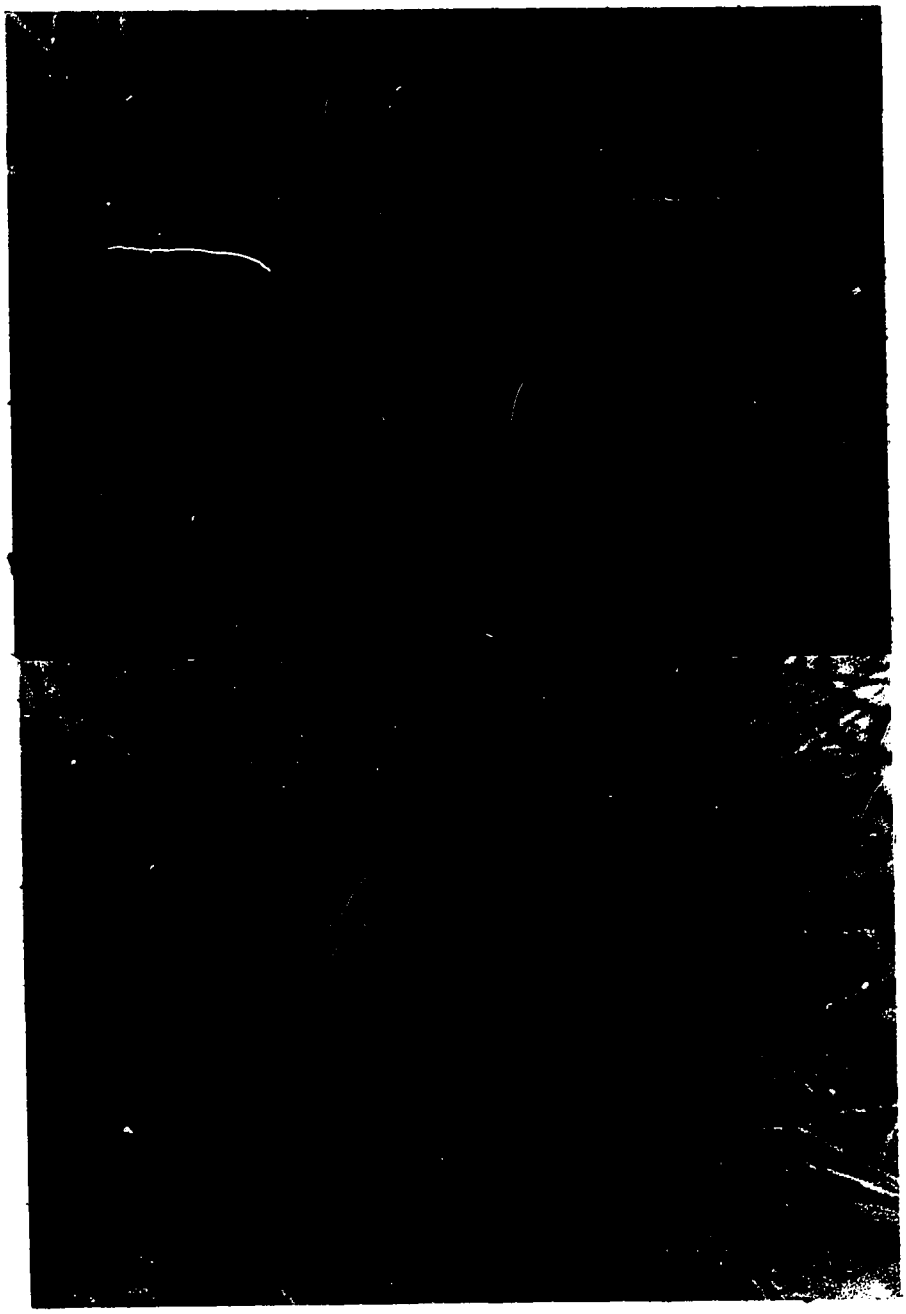


FIGURE 19

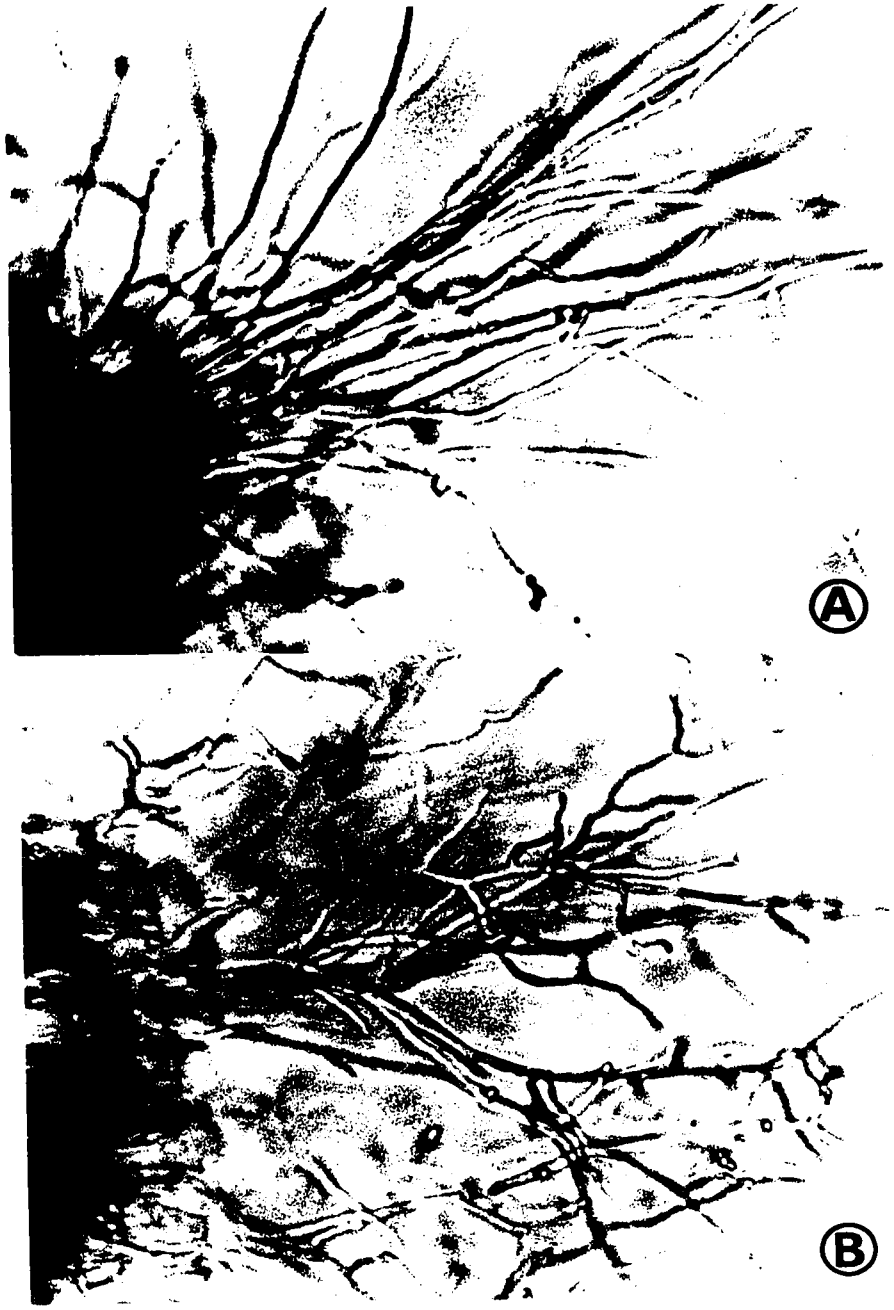


FIGURE 20

An enlargement of Fig. 19. A. Growth of Verticillium dahliae in water with relatively straight hyphae. B. Growth into McNairs infected xylem sap with branching at a much sharper angle than in water (arrows). In sap the hyphae grow in an undulating fashion. (X925).

FIGURE 20

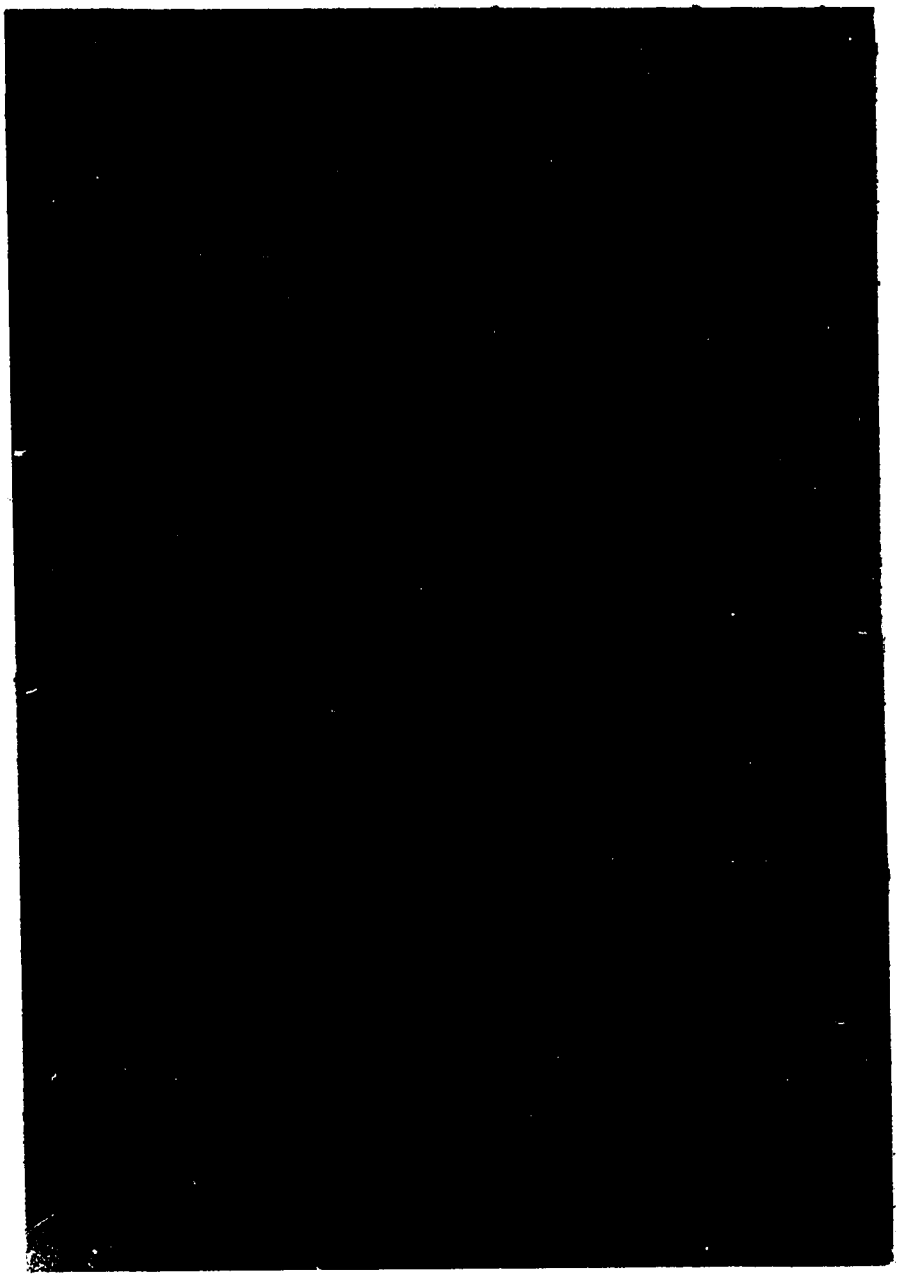


FIGURE 2

Growth in xylem sap was much-branched and had a characteristic twisted form.

There was no significant difference in the percentage germination of conidia in the xylem saps after 18 hours incubation. However, the germ tubes were thicker, longer, more wavy and more branched than when the spores were germinated in water (Fig. 21). This may have been a matter of nutrition although tobacco xylem sap was shown to be low in nutrients (Taylor 1968).

The effects of various nutrient solutions on the growth from test discs and the germination of conidia are shown in Table 16. There was significantly less growth in potato dextrose broth than in the control or other solutions. The fungus was apparently sensitive to something in this medium.

The growth form of V. dahliae in the nutrient solutions was rather similar to that when the test discs were incubated in xylem exudate. Growth in the different nutrients was no better than in water and only Czapek broth increased the length of the hyphae. However, all of the nutrient solutions gave significantly wider germ tubes than water (Table 16). The rapid growth of the pathogen from the xylem vessels when placed in water in this way suggests that the fungus is drawing on

FIGURE 21

Effect of various solutions on the form and extent of growth of Verticillium dahliae germ tubes.

Top row: Much branched wavy growth in xylem sap from infected resistant plants.

Middle row: Normal germination in water.

Bottom row: First two drawings, germination in V8 juice and last sample, tobacco isolate in sunflower xylem sap.

Drawings were made free-hand using X400 magnification of the microscope. Measurements are in microns.

FIGURE 21

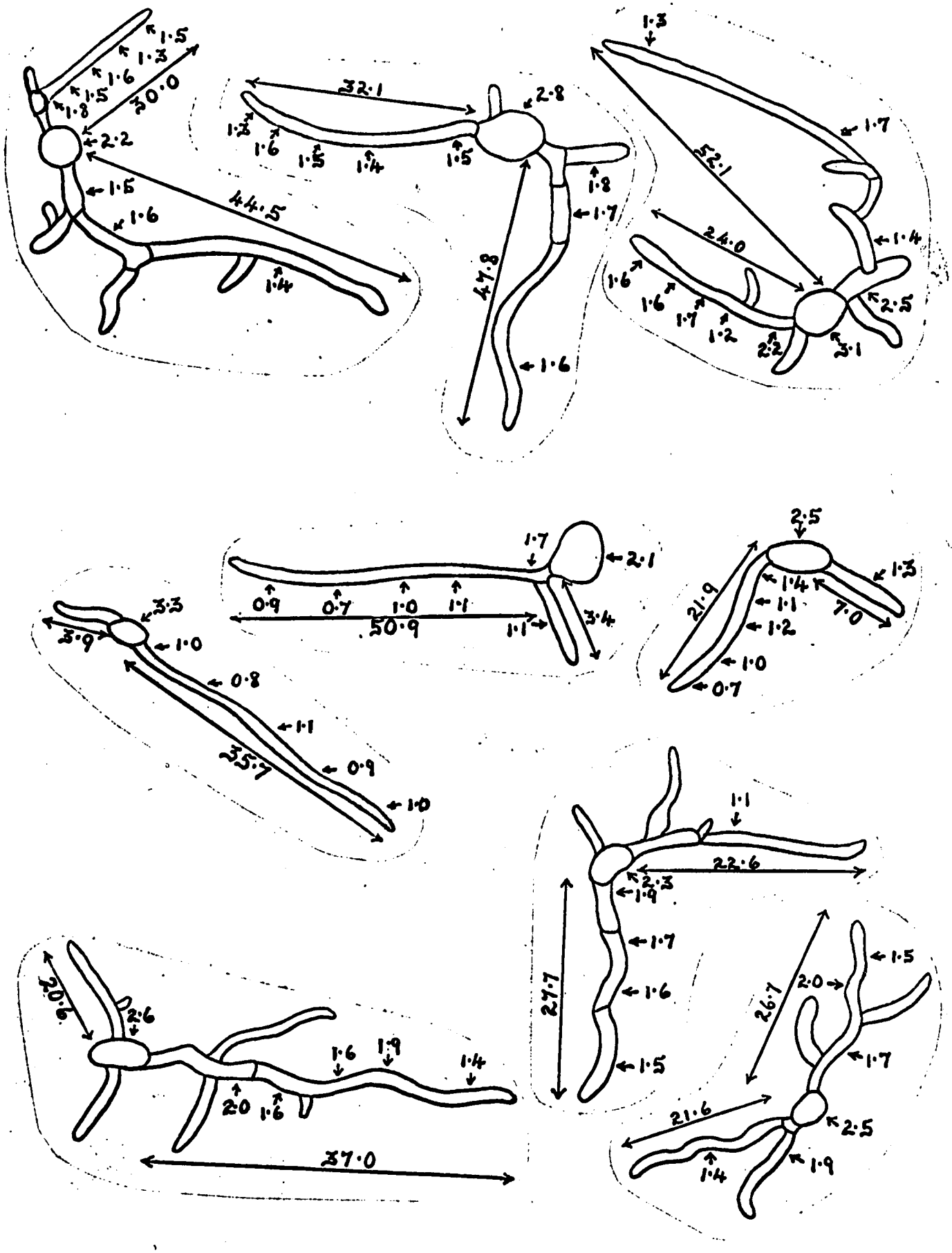

1·5
1·3
1·6
1·5
1·8
30·0
2·2
1·5
1·6
44·5
1·4
32·1
1·3
1·6
1·5
1·4
1·5
2·8
1·8
1·7
47·8
1·6
1·3
1·7
52·1
24·0
1·4
1·6
1·6
1·7
1·2
2·2
2·5
3·1
1·7
2·1
0·9
0·7
1·0
1·1
50·9
1·1
3·4
2·5
1·3
1·4
21·9
1·1
1·2
7·0
1·0
0·7
3·3
3·9
1·0
0·8
1·1
35·7
0·9
1·0
1·1
22·6
2·3
1·9
1·7
1·6
27·7
1·5
2·0
2·6
1·6
1·9
1·4
2·0
1·6
37·0
1·5
2·0
26·7
1·7
21·6
2·5
1·4
1·9

Table 16. Growth of Verticillium dahliae hyphae after 24 hours and germination of conidia after 18 hours in nutrient solutions.

Nutrient	pH	Growth of hyphae		Germination of conidia	
		Score (0 - 5)	Length of longest hyphae (mm)	Germination (%)	Width (μ)
PD broth	5.9	2.9[1]	0.37[1]	78.2[2]	1.48[3]
Czapek broth	7.3	4.8	0.86	85.8	1.57
V8 juice	6.1	4.2	0.70	88.4	1.60
Sunflower sap (infected plants)	6.5	4.8	0.82	-	-
Water	-	4.8	0.79	80.4	0.93
L.S.D. (.05)		0.4	0.07	3.4	0.20
L.S.D. (.01)		0.5	0.10	4.7	0.27

1/ Means of six replicates with five discs per replicate.

2/ Mean of five fields of approximately 40 conidia per field.

3/ Mean of five measurements on each germ tube. 10 germ tubes measured. Analysis of variance given in Tables A 11, A 12 and A 13.

nutrient reserves for this growth. It also suggests that the water either dilutes some inhibitory factor or else it acts as a stimulant to proliferation. Isaac and Mac Garvie (1966) concluded that water alone was sufficient to stimulate the germination of microsclerotia.

Xylem sap from sunflowers inoculated with V58 and showing symptoms did not check the growth of the tobacco isolate, any more than did sap from infected tobacco plants.

The Effect of Some Chemicals Upon the Growth of *V. dahliae* from Xylem Vessels

Much of the evidence presented in this work indicated that the growth of *Verticillium* is retarded within the vessels and the fungus is confined there by the activities of the xylem parenchyma cells. One of the problems associated with the study of vascular wilts is that dynamic interactions which may take place between the host and pathogen, cannot be examined as they occur.

To determine the importance of certain reactions taking place between various kinds of hosts and pathogens, workers have used chemicals which were believed to affect the activities of either the host or the pathogen. In this way

it was hoped to obtain information on resistance mechanisms and/or pathogenicity.

As a preliminary to doing similar work with Verticillium wilt of tobacco some compounds mentioned in the literature were tested using the leaf disc technique.

Materials and Methods.

Senescence retarding compounds.

Samborski et al. (1958) ~~increased~~ retained resistance to rust by floating detached wheat leaves in chemicals which retarded senescence. Channon et al. (1968) applied nickel sulphate to detached cabbage cotyledons and reduced the spread of Peronospora, apparently by inducing physiological changes in the host tissue.

The senescence retarding compounds used here were, kinetin in 0.01 M citrate phosphate buffer (pH 6.1), 20 p.p.m.; kinetin in water 2 p.p.m. and 20 p.p.m.; benzimidazol, 120 p.p.m.; nickel sulphate 49 p.p.m. and 490 p.p.m.

Systemic fungicide.

Recently Hall and Busch (1971) used benomyl to delay Verticillium wilt symptoms in chrysanthemum by inhibiting the growth of the fungus within the leaves.

In the present work benomyl was tested at three concentrations 0.2 p.p.m., 2 p.p.m. and 20 p.p.m.

Narcotic.

Müller and Behr (1949) anaesthetized potato leaves to break down resistance to Phytophthora.

With the disc technique ethanol was used at 0.1%, 0.5% and 1.0%.

Inhibitors of pectic enzyme formation.

Patil and Dimond (1968) found pectinase production by Verticillium was reduced in the presence of glucose.

In the experiments here glucose at 1800 p.p.m. and galacturonic acid 1060 p.p.m. (pH 5.7) were used. Sodium methyl cellulose was also included to observe the effect it had upon the growth of Verticillium.

Respiratory inhibitors.

Gothaskar et al. (1955) found that respiratory inhibitors broke down the resistance of tomato plants to Fusarium and Maine and Kelman (1961) obtained similar results with respiratory inhibitors when working with bacterial wilt (P. solanacearum) of tobacco.

The respiratory inhibitors used here were 2,4-dinitrophenol, 1.8 p.p.m.; potassium fluoride, 18.8 p.p.m. and thiourea, 76.0 p.p.m.

The fungus was also grown in water and xylem exudates from uninoculated VG and MN plants under different oxygen regimes using the technique of Brouzes and Knowles (Personal communication). Five test discs were mounted on a small glass slide and covered with a narrow cover slip. The liquids were applied from pasteur pipettes and each slide was placed in a test-tube sealed with a rubber cap. The test-tube was evacuated through a hyperdermic needle inserted into the cap and then filled with the appropriate proportions of argon and oxygen to give a range of oxygen tensions with the test materials. Each treatment was replicated 3 times.

Results and Discussion.

In preliminary experiments no growth of fungus was recorded when the test discs were placed in $NiSO_4$, 490 p.p.m.; alcohol, 1.0% and 0.5% or benomyl, 20 p.p.m.

The results of two different experiments are presented in Tables 17 and 18. Growth was retarded with kinetin in 0.01 M citrate-phosphate buffer, however, when the buffer alone was tested, poor growth again resulted (Table 17).

Table 17. Growth of Verticillium dahliae from test discs into various solutions.

Compound	Growth Score[1/] (0 - 5)	Length of longest hyphae[1/] (mm)
Alcohol (0.5%)	0	0
Benomyl (2 p.p.m.)	0.20	0.01
Phosphate-citrate buffer	0.56	0.11
Kinetin in buffer (20 p.p.m.)	1.52	0.30
Benzimidazole (120 p.p.m.)	1.84	0.33
Nickel sulphate (49 p.p.m.)	3.48	0.51
Water	4.20	0.84
L.S.D. (.05)	0.41	0.09
L.S.D. (.01)	0.55	0.12

1/ Mean of 25 test discs arranged in five replicates of five discs. Replicate totals and analysis of variance in Table A 14.

Table 18. Growth of Verticillium dahliae from test discs into various solutions.

Compound	Growth Score[1/] (0 - 5)	Length of longest hyphae[1/] (mm)
Benomyl (2.0 p.p.m.)	0.36	0.01
Benomyl (0.2 p.p.m.)	2.40	0.48
Water	4.20	0.90
Alcohol (0.1%)	4.64	1.23
Kinetin in water (2 p.p.m.)	4.84	0.94
Kinetin in water (20 p.p.m.)	4.88	1.03
L.S.D. (.05)	0.45	0.15
L.S.D. (.01)	0.61	0.21

1/ Mean of 25 test discs arranged in five replicates of five discs. Replicate totals and analysis of variance in Table A 15.

Kinetin was later dissolved in warm water and was found to stimulate growth of the pathogen (Table 18). At the concentrations used both benzimidazole and $NiSO_4$ (49 p.p.m.) retarded growth compared with the control.

The fungus was very sensitive to ethanol. It was completely inhibited by the two higher concentrations used in the experiments, yet stimulated by the solution containing 0.1%. The hyphae growing in this were long and had relatively few branches (Table 18 and Fig. 22).

At the lowest concentration used, 0.2 p.p.m., benomyl reduced growth (Table 18) and affected the growth form of the fungus (Fig. 23). After standing in 20 p.p.m. benomyl for 7 days, 54 leaf discs were rinsed in distilled water and plated on P.D.A. The pathogen grew from only 16 of these which suggested that some but not all the Verticillium had been killed.

Galacturonic acid at the concentration used in the experiments reduced growth to approximately one half that in water. There was no significant difference between glucose, the sodium salt of methyl cellulose, or water (Table 19).

The good growth of fungus in the respiratory inhibitors (Table 20) was unexpected and suggested that the growth of the

FIGURE 22

Long, relatively unbranched growth of Verticillium dahliae after 24 hours incubation in 0.1% ethanol. (X60).

FIGURE 22

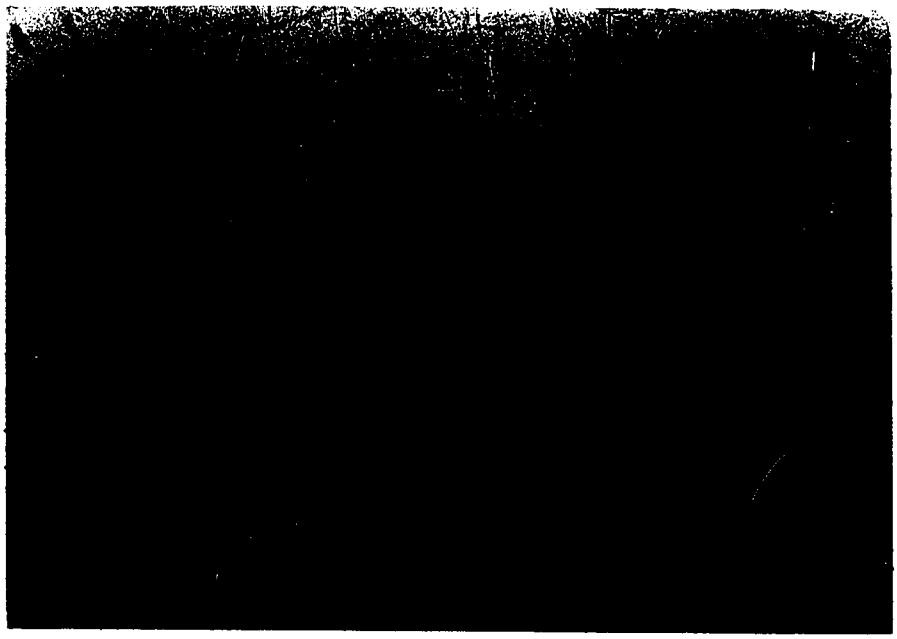


FIGURE 22

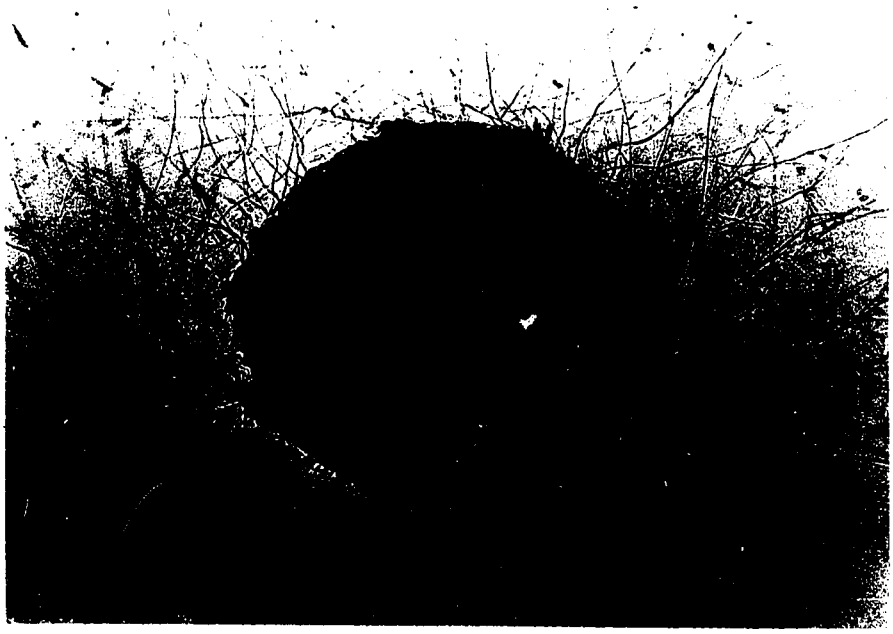


FIGURE 23

Inhibited growth of Verticillium dahliae after 24 hours incubation in 0.2 p.p.m. benomyl. (X60).

FIGURE 23

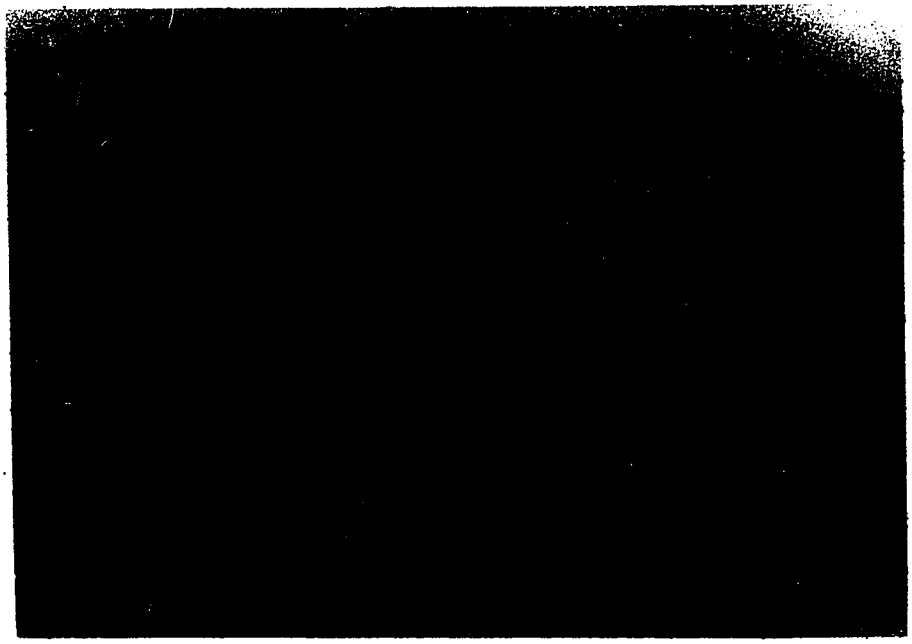


FIGURE 23

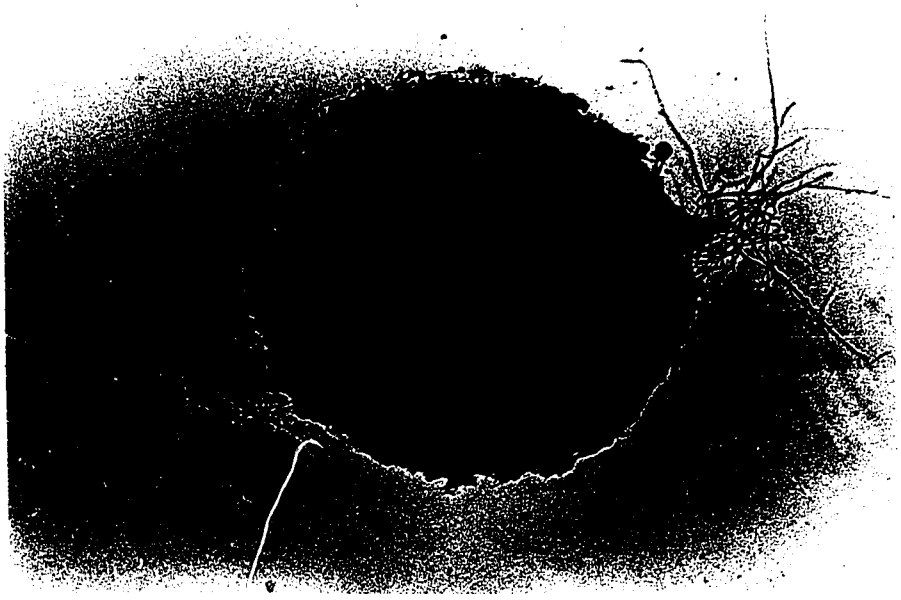


Table 19. Growth of Verticillium dahliae from test discs into various solutions.

Compound	Growth Score[1/] (0 - 5)	Length of longest hyphae[1/] (mm)
Galacturonic acid	2.40	0.56
Glucose	4.60	1.03
Water	4.72	1.05
Methyl cellulose	4.76	1.10
L.S.D. (.05)	0.62	0.19
L.S.D. (.01)	0.86	0.26

1/ Mean of 25 test discs arranged in five replicates of five discs. Replicate totals and analysis of variance in Table A 16.

Table 20. Growth of Verticillium dahliae from test discs into respiratory inhibitors.

Inhibitor	Growth Score[1/] (0 - 5)	Length of longest hyphae[1/] (mm)
2,4-D.N.P.	4.95	1.31
Thiourea	3.98	0.96
K.F.	4.95	1.16
L.S.D. (.05)	0.51	0.18
L.S.D. (.01)	0.61	0.25

1/ Mean of 40 test discs arranged in eight replicates of five discs. Replicate totals and analysis of variance in Table A 17.

pathogen may not depend on normal metabolic pathways. In the early rapid growth after being taken out of the host leaf and being placed in solution the fungus may be metabolizing reserve fats degraded by different metabolic pathways (West et al. 1967). This may also explain why glucose did not give a better response than water during the first 24 hours of growth.

The results obtained when the discs were placed in water under different oxygen tensions, are shown in Table 21. There was practically no growth when no oxygen was present but at 5% oxygen growth was good. There was no significant difference between the growth of fungus in water and the growth in xylem exudates in the different oxygen levels.

In the trials recorded above no attempt was made to determine optimum concentrations for growth or minimum concentrations for complete inhibition. Nor were variations in osmotic pressure, pH or the effect of other gases such as ethylene or CO_2 investigated.

The main conclusion to be drawn from these exploratory experiments is that the technique is suitable for studying V. dahliae in the form in which it is found within the host. Responses in both growth rate and growth form were detected in various liquids and different concentrations.

Table 21. Growth of Verticillium dahliae from test discs into water and xylem exudates at different levels of oxygen.

Oxygen (%)	Water		VG exudate		MN exudate	
	Growth Score (0 - 5)	Length of longest hyphae (mm)	Growth Score (0 - 5)	Length of longest hyphae (mm)	Growth Score (0 - 5)	Length of longest hyphae (mm)
0	0.07 1/	0.02	0.07	0.002	0	0
5	2.80	0.63	3.13	0.60	3.80	0.65
10	2.67	0.55	2.70	0.57	3.07	0.55
15	4.27	0.88	4.47	0.90	2.70	0.66
20	3.67	0.68	4.27	0.73	4.87	0.95
Control in air	4.07	1.03	4.33	0.93	3.87	0.85
L.S.D. (.05)	1.34	0.32				
L.S.D. (.01)	1.80	0.43				

1/ All figures are means of 15 discs arranged as a 3 x 6 factorial design, with three replications of five discs. Analysis of variance in Tables A 18 and A 19.

MISCELLANEOUS HISTOLOGICAL OBSERVATIONS

The Use of Conidia "Labelled" with Fluorescent Brightener

Darken (1962) demonstrated that the fluorescent brightener known as "fluor" was absorbed and transported by many bacteria, fungi and algae without affecting their viability. By labelling conidia and ascospores of Scleroderris with this substance, Skilling and Krogh (1969) were able to observe the invasion of the needle epidermis and growth of mycelium within bud and stem tissue of Pinus resinosa Ait. Patton and Johnson (1966) used the same technique to study the penetration of pine needles by Cronartium basidiospores and Tsao (1969) labelled the mycelium of Phytophthora parasitica Dast. for ecological studies in soil.

This non-toxic, stable biological marker, which has bright blue fluorescence under U.V. light, seemed to offer a method of following the progress of V. dahliae within the vessels of host plants. However, it was not possible to follow the pathogen within the vessels of tobacco because after a short period the brightener became associated with the vessel walls and these fluoresced, masking the fungus inside. In the vessels, and sometimes in culture, deposits

formed around hyphae and this markedly reduced fluorescence.

The seeding of glycerine-water medium with labelled conidia was useful for observing mycelial development. As centres of active growth gave the greatest fluorescence, it was suitable for observing such phenomena as anastomosis and intrahyphal hyphae. It was hoped that endoconidia would be more easily distinguished by using this technique. Even though bodies were observed that appeared to be the correct size and shape, it could not be proved that they were endoconidia and not vacuoles (Wright and Abrahamson 1970).

Discs from sunflower leaf infected with V58 were placed in water near drops of spore suspension containing V3 conidia labelled with 'fluor'. Within 24 hours anastomosis between the two isolates was clearly seen. The same procedure was repeated using V. dahliae from discs of infected tobacco leaf and labelled conidia from V. albo-atrum. Again anastomosis was observed. Hastie (1971) using conidia from autotrophic mutant isolates of V. dahliae and V. albo-atrum found that the two species will hybridize but there may be some incompatibility between the genomes.

Invasion of Tobacco Roots

Mahanty (1970) was unable to detect invasion of intact roots of very small tobacco seedlings by V. dahliae. As no

invasion had occurred one week after inoculation, he concluded that invasion did not take place unless the root was moribund or injured. This did not agree with the findings of Wright (1968b), therefore it was decided to investigate possible root invasion, by using a different method.

Clay pots (10 cm diameter) containing 10-week-old VG seedlings were supported on glass rods in a petrie dish, leaving 5 mm between the bottom of the pot and the dish. When the roots of the tobacco plants grew through the drainage hole into the space beneath the pot, the petrie dish was filled with a suspension of V. dahliae conidia (5×10^5 conidia per ml) for 24 hours. The suspension was then drained off. Water was added from time to time to the petri# dish for the next 6 days to keep the base of the pot moist. Roots were then removed, sectioned and mounted in water for microscopic examination without staining.

Mycelium and microsclerotia of V. dahliae were observed on the surface of the roots. Structures resembling lignitubers were observed projecting into the epidermal cells in contact with the hyphae and microsclerotia (Fig. 24). These penetration pegs showed strong autofluorescence under U.V. light (Fig. 24, A 4).

FIGURE 24

Invasion of tobacco root epidermal cells by Verticillium dahliae. A. In ultra violet light. B. In incandescent light. (X990).

1. Surface of invaded root 2. Microsclerotia which had formed on the root surface before penetration
3. Position of hypha near lignituber formation
4. Lignituber inside epidermal cell.

FIGURE 24

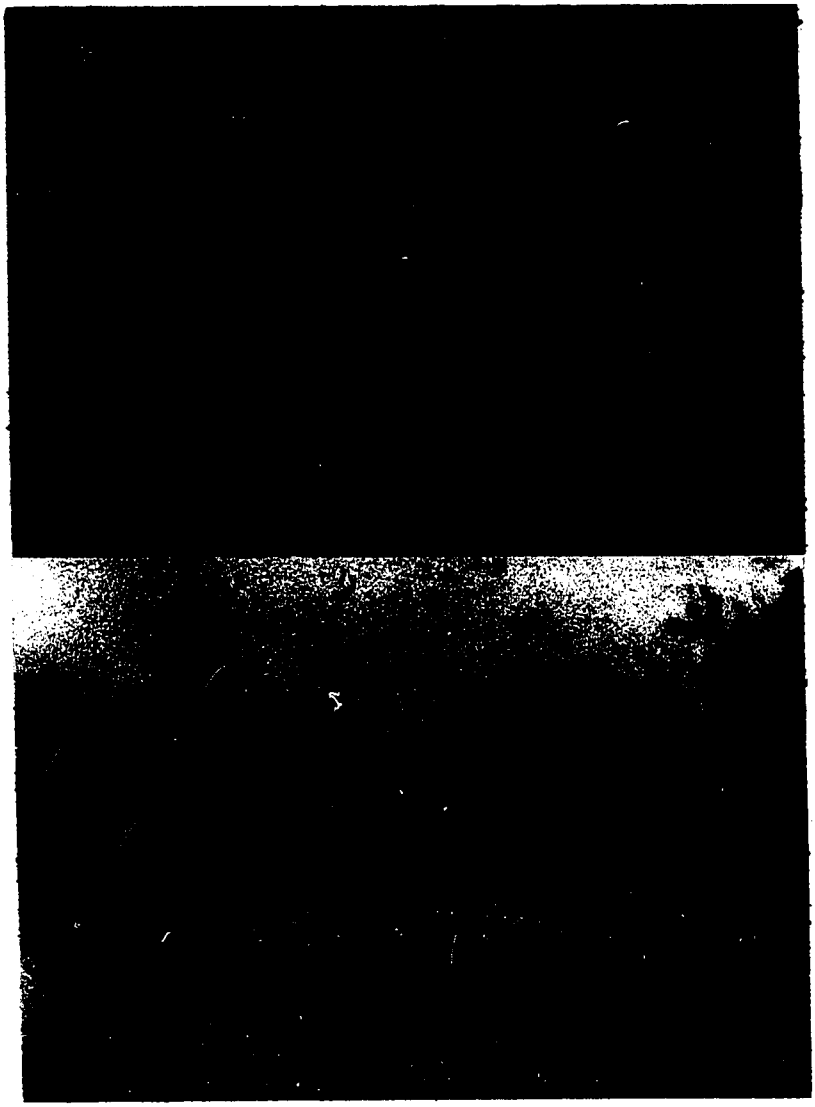
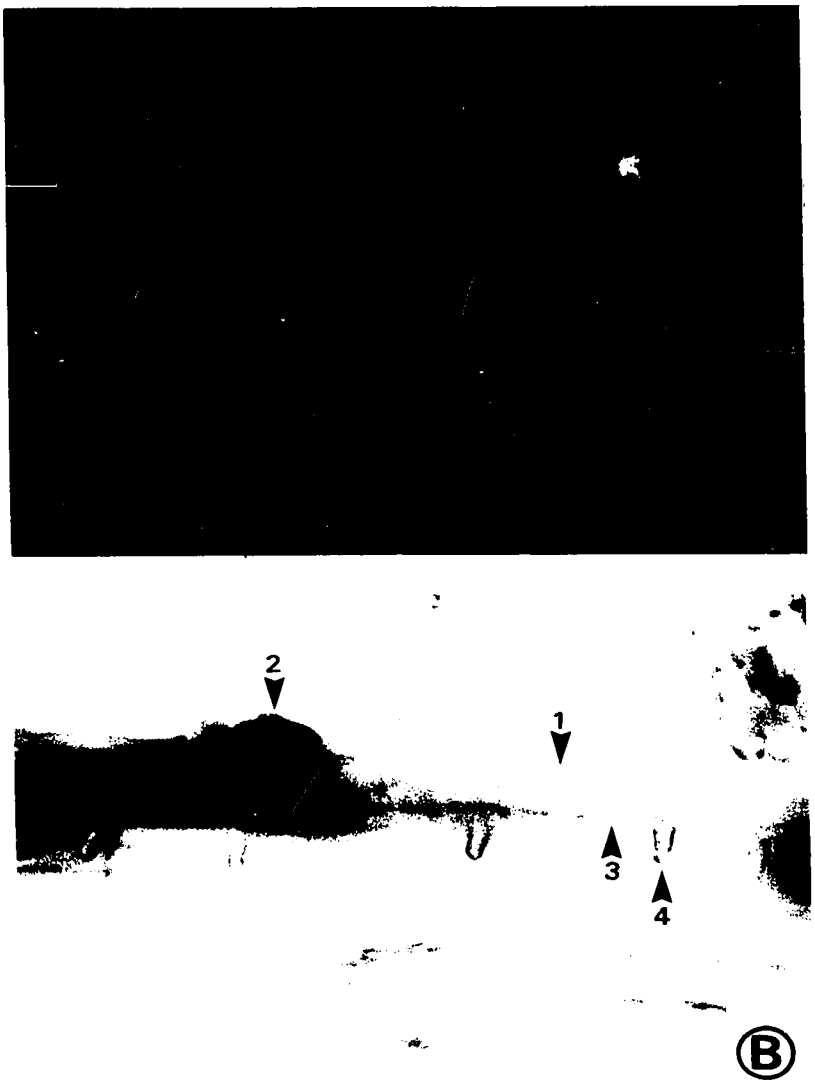


FIGURE 24



Most of the fungus on the roots was present as masses of torulose hyphae around and just behind the root cap (Fig. 25). The hyphae were approximately 3 μ in diameter with well-defined cell walls. The fungus invaded the epidermis beneath the mass of hyphal cells and grew intercellularly. Lignitubers were observed in the epidermal cells in contact with the hyphae. No penetration of the cortex or vascular tissue was observed. When the roots were cut into pieces 2 mm long and placed in water for 24 hours, however, V. dahliae grew out from vascular bundles near the invaded area. Identity of the fungus was confirmed by transferring the sections to P.D.A. This was evidence that invasion of intact tobacco roots may occur after growth of the pathogen on the root surface. Talboys (1958b) observed the formation of torulose hyphae of V. albo-atrum on the surface of hop root before invasion and Griffiths and Isaac (1966) noted the formation of microsclerotia before and during the invasion of tomato roots by V. dahliae. Many workers with other crops have concluded that Verticillium may enter uninjured roots, Isaac (1946) with sainfoin and Garber and Houston (1966) with cotton and many others.

FIGURE 25

Torulose hyphae of Verticillium dahliae formed just behind the root cap of growing tobacco root. (X990).

FIGURE 25

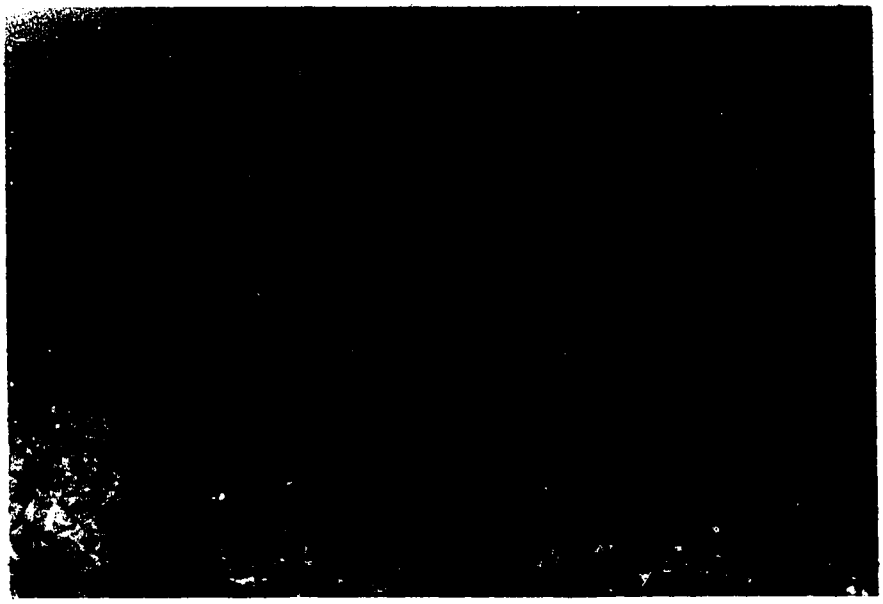


FIGURE 25

"Pockets" of Infection and "Active Propagules" from Mature Infected Tobacco Stems

The stems of mature tobacco plants which have shown severe symptoms may remain green and apparently healthy for many months after the leaves have died. Cultures from transverse sections of these stems show that in susceptible cultivars the fungus is confined to a narrow band of primary and secondary xylem vessels nearest the pith. In the more resistant varieties there may be sections along the stem from which the pathogen cannot be isolated (Wright 1966 and 1969). After drying the stems at a temperature which would check the growth of V. dahliae without killing it and then grinding the stem to a fine powder, Wright (1969) found that plated aliquots of the powdered stem gave rise to colonies of V. dahliae. A surprisingly large number of colonies developed even from the stem tissue of MN plants. To explain this apparent contradiction Wright postulated that the pathogen was present in these "old" stems as "isolated pockets".

To test this hypothesis the vessels of infected G plants were studied. The plants were 5 months old and the leaves had been necrotic for some time, although the stems were still green and sound. Individual vessels which showed brown discoloration were gently teased from the vascular

cylinder nearest the pith. These were mounted in water and examined without stains.

It was difficult to find mycelium in the vessels, even from vascular tissue which had dark discoloration. The pathogen was observed in an occasional pitted vessel and when it was present the vessels were usually packed full of hyphae. If the infected tracheids were incubated in water profuse growth resulted. It was very probable that these isolated vessels were the "pockets of infection" postulated by Wright and the large number of "active propagules" which gave rise to the colonies from pulverized stem may have been pieces of these hyphae broken up and dispersed by the grinding and sieving processes.

The Origin of Particulate Bodies Within the Intercellular Spaces of Tobacco Leaves Infected with *V. dahliae*

Wright and Abrahamson (1970) in their electron microscope study of *V. dahliae* infected tobacco leaves, reported the presence of unidentified particulate bodies within the intercellular spaces of the lamina. These bodies were found passing from the intercellular spaces through the cell walls of green and chlorotic tissue near vessels containing the fungus. The physical appearance of these particles was similar.

They were more or less circular or globular with irregular margins and about 2 μ in diameter, although the size was very variable. They were composed of concentric layers, some more densely staining than others and in most there were one or more empty spaces in the centre. Frequently the bodies were clumped in groups of three or four.

Because of the proximity of these particles to the pathogen and their apparent movement through the walls of cells about to become chlorotic, there was the possibility that they might be toxic products believed to be formed by the fungus, at least in culture (Stoddart and Carr 1966). However, as the leaf lamina becomes chlorotic all the cell layers lose colour more or less evenly and it does not seem physically possible for discrete bodies of this size formed by the fungus in the vessels, to pass into the intercellular spaces and through the walls of so many widely separated cells.

It was therefore of interest to determine the nature of these bodies. As a first step a study was made of particulate material formed by V. dahliae in culture. With the glycerine-water medium the fungus could be examined without the problem of extraneous artifacts. Globules, slightly smaller than the diameter of the hyphae (1.6 μ) were often seen forming behind the tip of rapidly growing hyphae. These

were smaller than the objects seen within the leaf. Similar material was seen on occasions near conidiophores and this was thought to be conglomerates of cellular material from burst conidia. Likewise debris from dead hyphal cells was frequently found in the medium. None of the particulate material arising near the fungus in culture appeared to be the unidentified bodies in the infected leaf.

Attention was then turned to the infected leaf. Within 3 days of inoculation of detached leaves with conidia, the chloroplasts of the petiole parenchyma cells near the xylem, enlarged and clumped together. At the same time dark deposits formed in some of the intercellular spaces (Figs. 26 and 27). When the tissue was cleared, round bodies with concentric layers were seen and some of these were embedded within the cell walls (Fig. 28). Most of these were similar in size to the bodies found in the electron microscope study but many were much larger (50 to 75 μ).

If the epidermal layer of cells is removed from diseased chlorotic lamina and the tissue stained with sudan III, large numbers of degenerating chloroplasts and fatty globules may be seen (Fig. 29). These may be found both within the cells and in the intercellular spaces and their size is very variable. In adjacent green tissue not yet showing symptoms the chloroplasts

FIGURE 26

Parenchyma cells from near the xylem of inoculated tobacco leaf. 1 = Deposit of pigmented material in an intercellular space. 2 = Swollen chloroplasts clumped together. (X990).

FIGURE 26

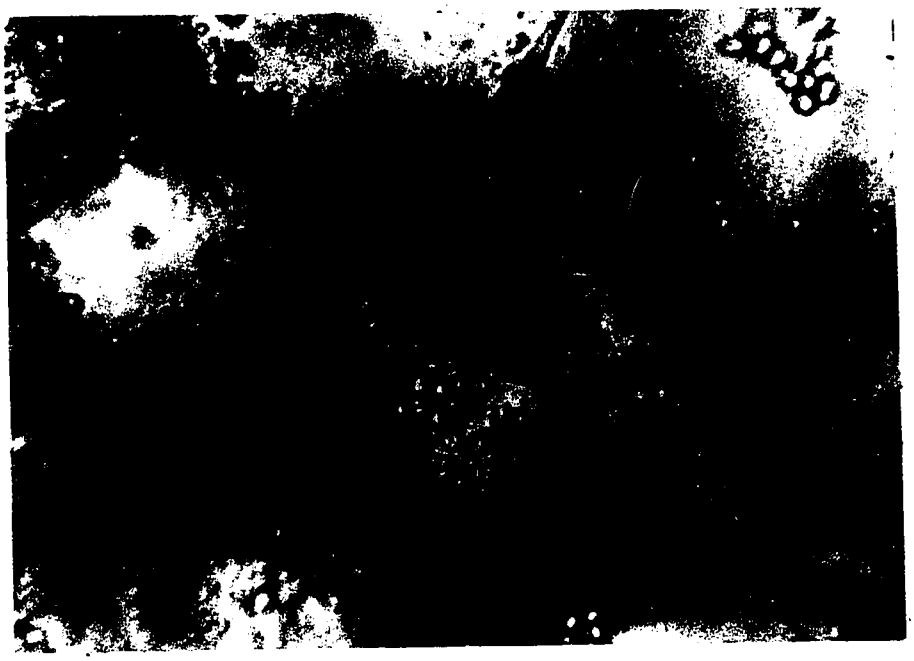


FIGURE 26

FIGURE 27

Deposition of pigmented material in intercellular spaces around fine veins of a leaf from a plant inoculated with Verticillium dahliae. (X495).

FIGURE 27

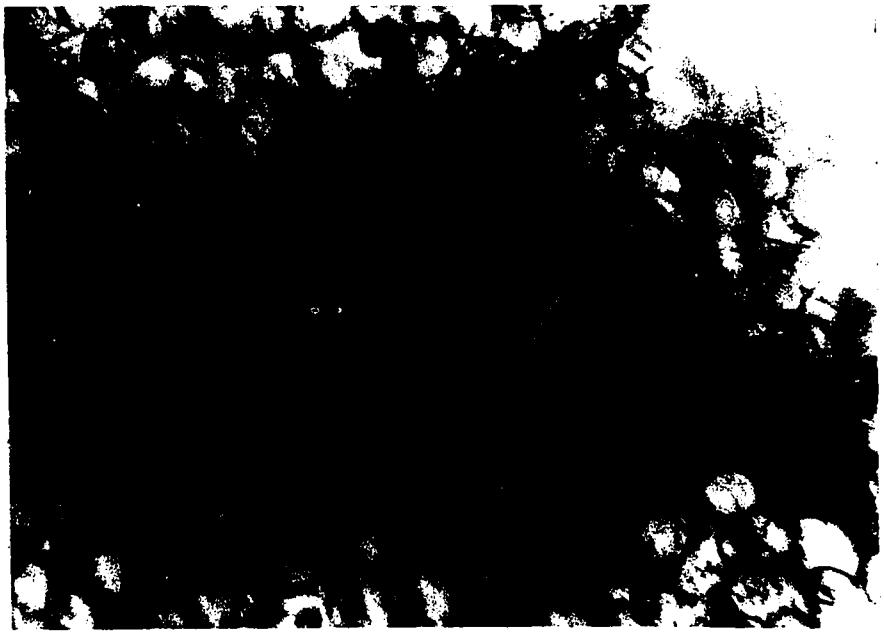


FIGURE 27

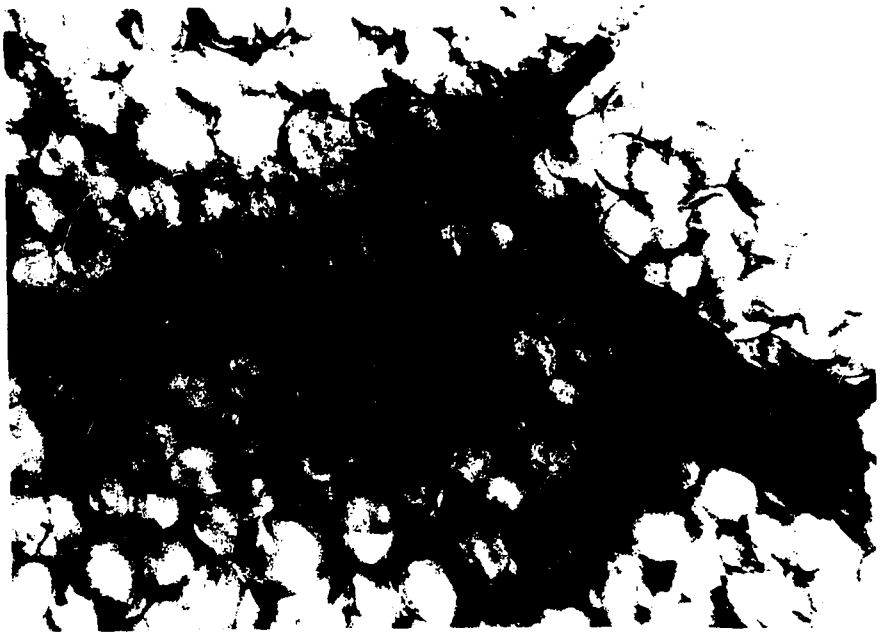


FIGURE 28

Body formed from lipoidal material which has passed through an epidermal cell wall before condensing. 1. Concentric layering in the body. 2. Host cell wall. (X990).

FIGURE 28

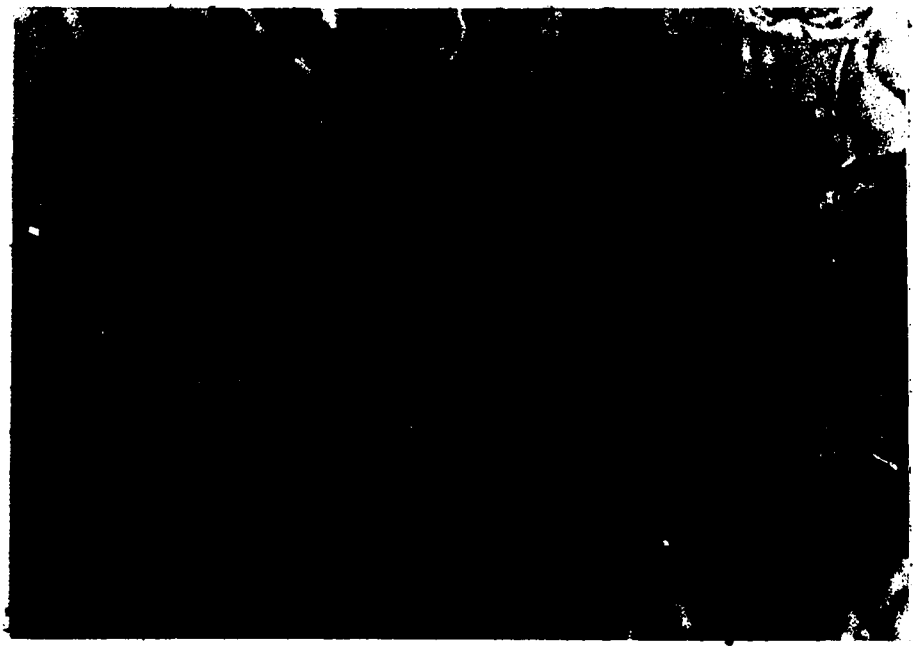


FIGURE 28

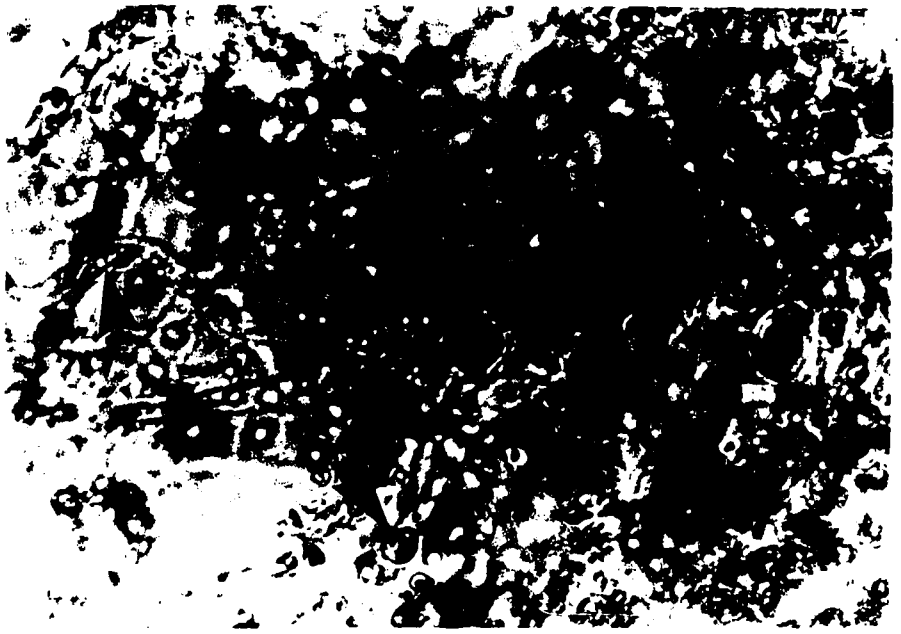
FIGURE 29

Fatty globules in infected tobacco leaf. The lamina was incubated in water for 24 hours before removal of the epidermis and stained with sudan III.

1 = Fatty globules. 2 = Hyphae of Verticillium dahliae.

FIGURE 29

FIGURE 29



appear normal. In senescing uninfected leaves degenerating chloroplasts also stain with sudan III but there are not as many present as in the diseased chlorotic tissue. Barnard (1960) working with cured tobacco leaf noted the presence of large numbers of fatty globules, averaging 5 μ in diameter, which stained with sudan III. He concluded that leaf pigments were located in these globules.

The characteristic orange colour of tobacco leaf infected with Verticillium (Thomson and McLeod 1959) may result from the formation of these lipoidal bodies in large numbers after the breakdown of the chloroplasts of relatively young green leaf and the formation of plastoglobuli.

The bodies observed by Wright and Abrahamson (1970) may also be degenerating chloroplasts which with large lipoidal content may be able to pass between the microfibrils of the cell wall as well as through the cell membrane. The dark concentric layers may be the remains of degenerated grana and the empty central areas the remains of degenerated starch grains. This hypothesis is supported by the fact that in the micrographs of Wright and Abrahamson these unidentified bodies were usually present near material, such as starch grains, which had come from degenerated chloroplasts.

The Germination of Microsclerotia

Several workers using light and electron microscopy have described microsclerotia as being composed of two types of cells, namely thin-walled hyaline and thick-walled pigmented cells (Gordee and Porter 1961; Nadakavukaren 1963; Schnathorst 1965). The two types were found in close association. More recently it has been found that the cytoplasm of the peripheral cells degenerates to leave the intact but non-functional hyaline cells around and embedded between the pigmented cells. The pigmented cells contain cytoplasm, complete with nucleus and organelles (Brown and Wyllie 1970; Griffiths 1970; Wright and Abrahamson 1970). All these workers concluded that the only difference in cell wall thickness between the two cell types was the deposit of pigmented material on the outside of some of them.

Many workers have observed the germination of the hyaline cells and also mycelium associated with the microsclerotia. Both of these are believed to be empty. The pigmented cells have not been seen germinating (Schnathorst 1965), yet the work with electron microscopy has shown that these cells have normal cytoplasm.

There may be several explanations for this apparent contradiction.

Wright and Abrahamson (1970) considered that there was no difference between the two types of cells and that some of the pigmented cells may also appear empty. Griffiths (1970) found that vacuoles in the cytoplasm increase in size until in some mature cells, the cytoplasm is limited to the peripheral region of the cell, although organelles are still visible. When the writer gently squashed microsclerotia under a cover slip in water containing sudan III, large quantities of lipoidal droplets were released and Bondartseva (1968) found that hyaline, as well as pigmented cells, contain lipids.

Dehydration in alcohol or acetone during preparation for microscopic study would remove lipoidal material from the cells and those with a high lipid content and attenuated cytoplasm may appear empty in the prepared sections. If this is the case, hyaline cells may in fact contain cytoplasm and be capable of germination.

Energy reserves in the form of fats in the cells would help insulate the cytoplasm and play a role in the resistant qualities and longevity of these cells.

Brown and Wyllie (1970), Griffiths (1970) and Wright and Abrahamson (1970) all reported intrahyphal hyphae in their studies on microsclerotia. Brown and Wyllie concluded that germination of microsclerotia took place through the pores

connecting the viable pigmented cells. Furthermore, intrahyphal growth through the dead hyaline cells would give the impression that germination had arisen from them. Nadakavukaren (1963) showed a micrograph (his Fig. 5) of an unidentified structure penetrating a pigmented cell from the outside. This growth does not appear to be coming from a pore but rather from an endoconidium-like body in an adjacent dead cell. Griffiths (1970) also showed the invasion of degenerated microsclerotial cells by mycelium from the outside and suggested that intrahyphal hyphae played a type of parasitic role in which nutrients were obtained by invasion from some cells to build up reserves in others.

Wright and Abrahamson (1970) found that the intrahyphal bodies had dense cytoplasm and cell walls. These were similar to the intrahyphal hyphae and endospores which Aubé and Pelletier (1968) described as probably arising from the fragmentation of hyphal protoplasm. If germination takes place from either hyaline microsclerotial cells or degenerated hyphal strands, it may be from these intrahyphal bodies protected within the walls of the dead cells. Figure 30 A shows the germination of what may be endoconidia inside hyphal cells from a five-year-old dried culture. This may explain the germination of hyphae associated with microsclerotia observed by Van den Ende (1959) and Schnathorst (1965).

FIGURE 30

Growth from microsclerotial cells. A. Germination of a hyphal cell from a five-year-old dried culture. Cells contain what may be endoconidia (arrowed). B. Germination of individual pigmented microsclerotial cells.

1. Germ tube growing from the cell. 2. Pigmented case has cracked prior to germination.

FIGURE 30

FIGURE 30

In the present work germination of individual pigmented cells was observed from scrapings of dried five-year-old cultures incubated on glycerine-water medium (Fig. 30 B). Pigmented cells appeared to swell prior to germination and the pigmented cases split apart and fell away as germination proceeded. Without the case the pigmented cells appeared similar to the hyaline cells. It is possible that earlier workers in their efforts to destroy adhering hyphae and conidia actually cracked or removed the pigmented casings from cells and these may have appeared hyaline when germinating.

It may be concluded, that hyaline and pigmented microsclerotial cells may in fact be the same and may germinate with or without the pigment case. Intrahyphal bodies from inside degenerated cells associated with microsclerotia may also germinate.

Penetration of Cell Walls by *V. dahliae*

The original drawings of *V. albo-atrum* by Reinke and Berthold (Rudolf 1931) showed the narrowing of the hyphae as they passed through potato cell walls and swelling when they had grown out the other side. Talboys (1958a) estimated that the penetrating hyphae narrowed to 0.5 μ as they went through root cell walls. Isaac (1946) found that the tips of *Verticillium*

hyphae may swell before entry. Talboys (1958c) had evidence of cellulase activity at the hyphal tip of Verticillium as did Griffiths (1971). With the invasion of cellulose membranes the hyphae may be constricted but there is no stress line as the hyphae push through, indicating that enzyme activity may be more important than mechanical pressure.

When infected tobacco leaves were left in running water for six days the trichomes had been colonized by hyphae of V. dahliae. The progress of the fungus out through the trichome walls could clearly be seen (Fig. 31). The hyphal tip approached the wall at an angle and after touching formed a small penetration peg. The hypha behind the peg began to swell and became more at right angles to the trichome wall. As the penetration hypha moved through the wall in some cases but not in others the wall began to bulge in front of the peg. In instances where the wall had bulged, when the peg broke through, a small rift could be seen in the wall in front of the penetration peg, indicating that at least some force had been applied to the wall by the peg. Once on the outside of the wall the hyphae bulged slightly before resuming normal growth. It may be concluded that penetration of these cell walls by V. dahliae was similar to that described for other pathogens (Walker 1969).

FIGURE 31

Penetration of tobacco trichome walls by Verticillium dahliae hyphae. 1. Swollen hyphae on inside of wall as tip emerges. 2. Bulging of trichome wall in front of penetrating hypha. 3. Swelling of hypha on both sides of trichome wall.

FIGURE 31

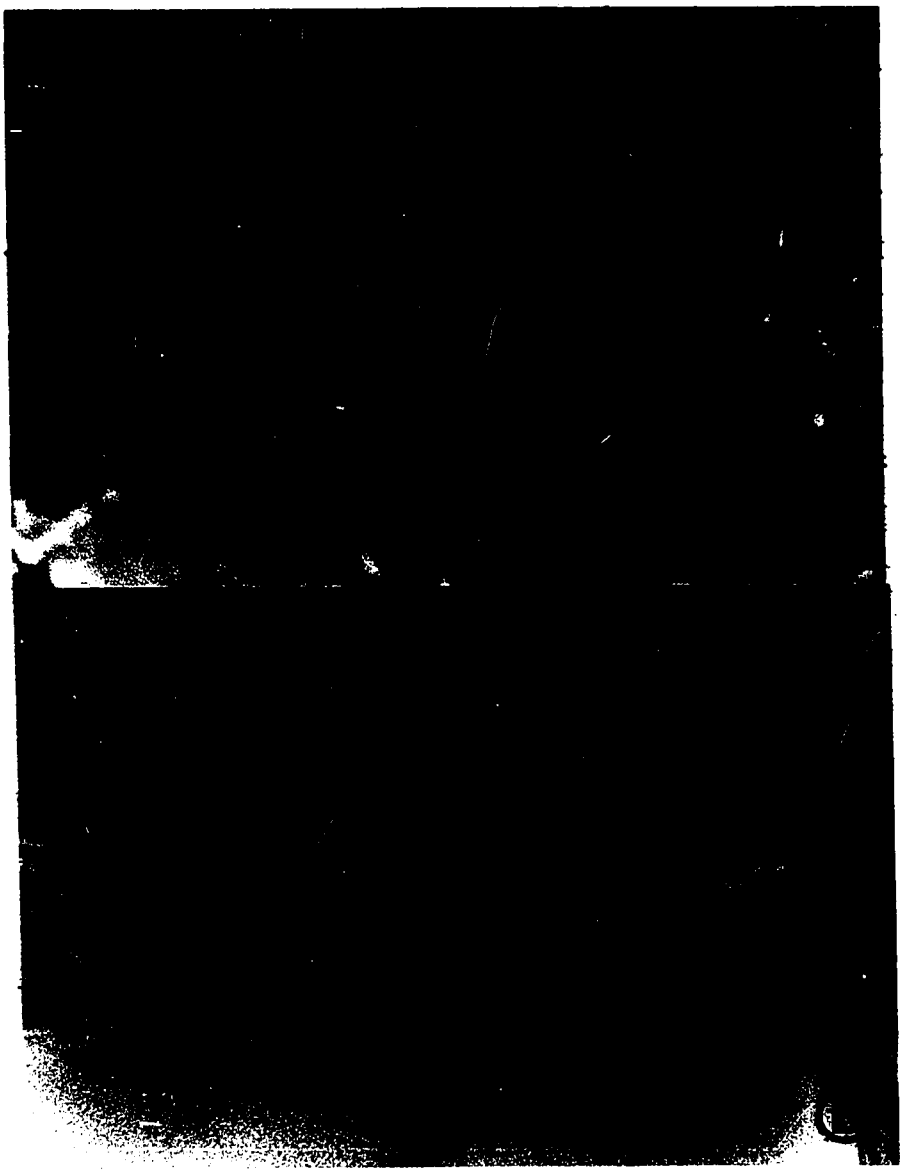
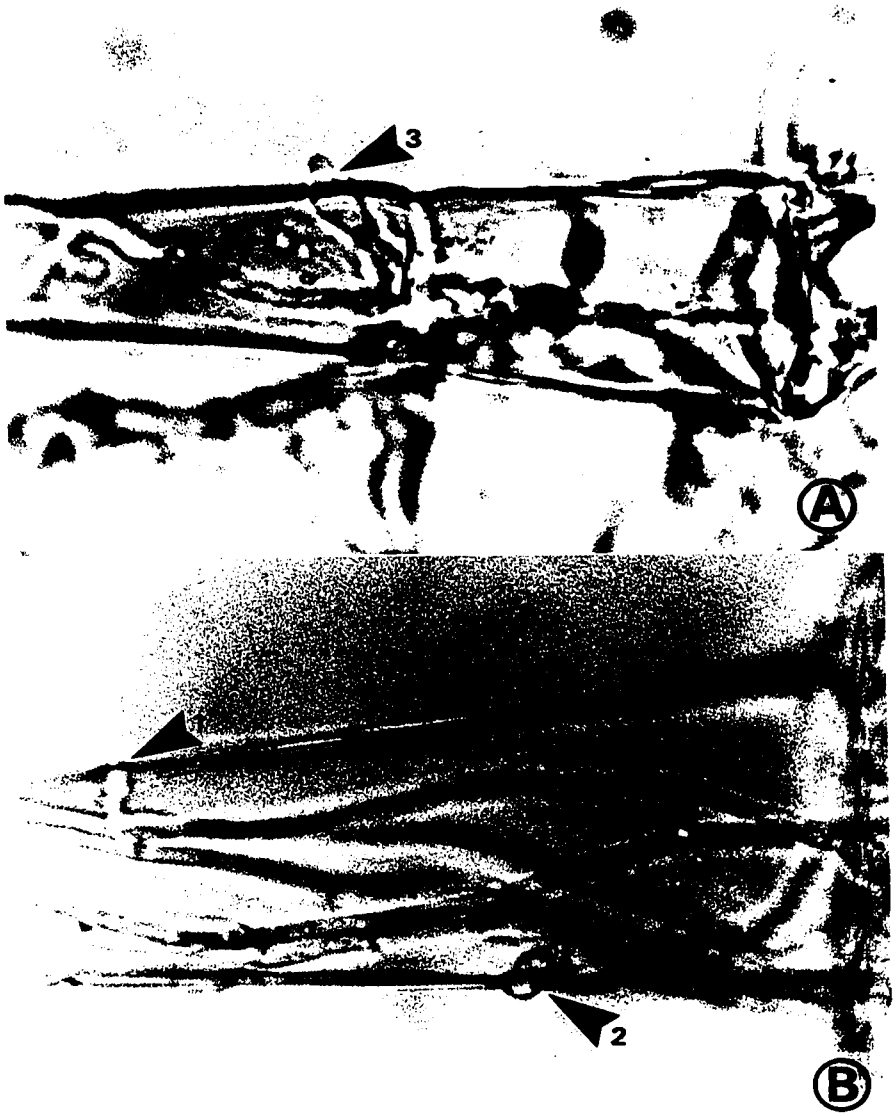


FIGURE 31



RESPONSE OF SUNFLOWER SEEDLINGS TO INOCULATION WITH CONIDIA OF V. DAHLIAE

Introduction

When tobacco seedlings with trimmed roots were placed in conidial suspensions, the spores were taken up and lodged in the taproot, stem and petioles. After 24 hours the quantity of fungus which could be cultured from the seedlings had been markedly reduced, particularly in the upper stem. As the plants developed the pathogen continued to be kept in check at the top of the stem but more so in MN than VG plants (Wright 1968b; Mahanty 1970).

Wright and Biss (1968) used this method to inoculate seedlings before planting them in the field. After 56 days 93% of the plants and 45% of the leaves had chlorosis. 86 days from planting only 27% of the leaves had symptoms, as the leaf number increased and the plants grew to maturity. After flowering symptoms developed rapidly and 70% of all leaves had symptoms, 106 days after inoculation.

Sackston (personal communication) suggested that sunflower seedlings treated in the same way would not recover from the inoculation as the tobacco had done prior to flowering.

The purpose of the experiment reported here was to repeat this form of inoculation with sunflowers and follow the build up of the pathogen in this host, from inoculation until severe symptoms developed.

Materials and Methods

Inoculum was prepared by growing the sunflower isolate of *V. dahliae*, V58 on P.D.A. for 16 days. This isolate formed few conidia in culture and 12 petri plates were used to make an initial suspension of 300 ml in distilled water. The conidia were washed twice by centrifuging the suspension at 3,000 r.p.m. (1470 g) for 15 minutes and resuspending the pellet in sterile distilled water. The suspension was finally adjusted to 8.5×10^5 conidia per ml. In the tobacco work a suspension of 4 to 5×10^6 conidia per ml was used.

Seedlings of the sunflower cultivar Sunrise, were raised in 10 cm pots in the greenhouse until they were approximately 10 cm high, with two pairs of true leaves, 14 days after sowing the seed.

At the time of inoculation the plants were carefully removed from the soil and their roots were gently washed. The roots of the seedlings were placed in the spore suspension and at the same time five plants were placed with their roots in

0.5% aqueous eosin. After 10 minutes in bright sunshine at 24°C, the dye could be seen in the veins of the leaves. The period taken for the eosin to reach the tips of the leaves was considered the shortest time it would take conidia to reach the upper parts of the plants. At this time three seedlings which had been in the inoculum for 10 minutes were removed and prepared for isolation of the fungus. Other sets of three plants were taken from the suspension and sampled after 4, 12 and 24 hours. After 24 hours the remainder of the seedlings were planted in soil, one plant in each 10 cm clay pot. The potted plants were transferred to a controlled environment cabinet with 16 hours daylight, light intensity 1200 ft-c from cool white VHO fluorescent tubes. Day temperature was 24°C and night temperature 20°C. Controls were treated in the same way except the roots were put in water instead of inoculum.

Groups of three plants were selected for plating at 3, 6, 9, 12, 15 and 18 days respectively from the start of inoculation.

To determine the amount of fungus in each seedling, they were surface sterilized in a commercial hypochlorite bleach to give 2% free chlorine, for 10 sec, then washed thoroughly in distilled water for 3 min. Lateral roots were removed from where they joined the taproot and then cut as a

bundle into 4 mm lengths. The taproot and stem were then sectioned into 2 mm lengths from the base upward and placed in sequence along microscope slides. Leaves were cut from the plants where the petiole joins the stem and the lamina was removed by cutting along the midrib so that the petiole and the midrib were left joined. This was then sectioned consecutively. Lateral veins were sampled by removing 10 discs at random with the microsampler. The fine veins were sampled by taking a single disc from each of five sites with the microsampler.

All sections and discs were placed on microscope slides and covered with warm cellulose agar delivered from an eye-dropper. The slides were placed in petri plates on moist filter paper and incubated at room temperature for 7 days.

The growth of V. dahliae from each section or disc was scored on a 0 to 5 basis according to colony size. Because of the variability in the sizes of the organs and the differences in sampling methods used the scores were added and an assessment value was given representing the infection in the organs as low, medium, or high at the particular sampling time.

Results

Both the controls and the inoculated plants showed the effects of transplanting, but by the third day they had all recovered and were forming new roots.

The results of the samplings are presented in Table 22. Conidia were not taken up during the first 10 min of immersion in suspension but after 4 hours the fungus was isolated from the vessels of the taproot and the stem. Sometimes there were gaps of up to 9 mm with no fungus between places where the spores had lodged. Often it was recovered from only one side of the stem. After 12 hours in the suspension the fungus was distributed more evenly in the vessels and was recovered from the midrib of one of the first pair of true leaves. After this sampling time, there was a steady increase in the amount of fungus in the taproots and stems and by the sixth day there were medium levels of fungus in the vessels of the stem.

Leaf symptoms which could be attributed to the pathogen were seen on the ninth day after inoculation. At this sampling there was a marked increase in the quantity of fungus throughout the whole plant. There was heavy colonization of the fine veins even in the third pair of leaves, which had just developed.

Table 22. Colonization of sunflower seedlings by Verticillium dahliae conidia.

	Hours in spore suspension			Days after inoculation started					
	4[1]	12	24	3	6	9[2]	12	15[3]	18
Seminal roots	0[4]	+	0	+	+	+	+	+	+
Taproots	+	+	+	++	+	+++	+	+	+
Stems	+	+	+	+	++	+++	++	+++	+++
First true leaves[5]									
Midribs	0	+	0	+	+	+++	++	++	++
Lateral veins	0	0	0	0	0	++	++	+	++
Fine veins	0	0	0	0	0	+	+	+	+

(Continued)

Table 22. Continued.

	Hours in spore suspension			Days after inoculation started					
	4[1]	12	24	3	6	9[2]	12	15[3]	18
Second leaves									
Midribs	0	0	0	+	+	+++	++	++	++
Lateral veins	0	0	0	0	+	++	+	++	++
Fine veins	0	0	0	0	0	+	+	+	+
Third leaves									
Midribs						++	+	++	++
Lateral veins						+++	+	++	+
Fine veins						++	0	+	+

(Continued

Table 22. Continued.

	Hours in spore suspension			Days after inoculation started					
	4[1/]	12	24	3	6	9[2/]	12	15[3/]	18
Fourth leaves									
Midribs								++	++
Lateral veins								+	++
Fine veins								0	+

1/ The roots were washed and stood in conidial suspension for 24 hours. Sample plants were removed at 10 min, 4 hours, 12 hours and 24 hours. No fungus was recovered from the sample which had been in the spore suspension for 10 min.

2/ After 9 days slight chlorosis of the leaves was seen. Also about this time the third pair of leaves developed.

3/ After 15 days flowerbuds were visible.

4/ Quantity of fungus which developed from samples of various tissues: 0 , no fungus; + , small amount of fungus; ++ , medium amount of growth; +++ , large amount of growth.

All plants sampled at each date were sectioned from the tip of roots to apex of stem and assessments were based on all sections of each organ of three plants.

After the ninth day the inoculated seedlings grew no taller but formed new leaves.

Flower buds were visible by the fifteenth day on both control and inoculated plants. By the eighteenth day the controls were 5 to 8 cm taller than infected plants. At the last sampling there was a tendency for higher scores towards the top of the plant than the base. The sections near the base had been colonized for the longest time and contained a very dark pigment which discoloured the medium. This appeared to affect the growth of hyphae from the stem sections near the base.

The pathogen was never isolated in large amounts from the seminal roots.

Thirty days after inoculation 30 seedlings which had been inoculated but not used for sampling, collapsed within a space of 48 hours. Most of these had a black canker above the cotyledonary node, while others had cankers nearer the base of the stem. In a third group that died, the stem remained intact but the lower leaves became black and the upper leaves developed chlorosis and wilted: In the latter group the root system had rotted away. Presumably each of these areas were weak points in the plants' defenses and collapsed

first. The fungus could be isolated in large amounts from the parenchyma tissue of stems and roots where the cankers appeared and in thin fresh sections the fungus could be seen growing both inter- and intra-cellularly.

Cankering and sudden, dramatic collapse of sunflower seedlings are not observed in routine inoculations with V. dahliae. In such routine inoculations, the roots are dipped in suspensions of conidia for very short periods, ranging from 30 secs to 5 mins (Sackston, personal communication).

There are several differences in these results from those obtained with tobacco seedlings. In tobacco the spores were taken up throughout the plant but were checked at the base and killed near the apex. In sunflower seedlings the conidia tended to be sporadic in distribution and lodged in the vessels near the base of the plants. This may have been related to the size of the conidia as V3 spores in another experiment, lodged further up sunflower stems than V58. There was no obvious destruction of conidia in the vessels of inoculated sunflowers and the pathogen progressed steadily throughout the vessels of the plants. In tobacco treated in this way symptoms remain slight and are confined to the lower leaves until after flowering. With sunflowers severe leaf symptoms appeared in all formed leaves within nine days of

inoculation, and this was 6 days before flowering. There was no obvious remission in sunflower, which is a characteristic of Verticillium wilt of tobacco. Death of the seedlings or cankers are not found in Verticillium wilt of tobacco and even when all the leaves have fallen from severely infected tobacco plants the stems may remain for months apparently healthy, with the fungus confined to the primary xylem and a few secondary xylem vessels, until the frosts kill the stem.

It may be concluded that sunflower is not as resistant to "sunflower" isolates of V. dahliae as tobacco is to "tobacco" isolates. Tobacco shows a characteristic pattern of disease development. If anything comparable to the remission phase in tobacco occurs in sunflower, it is certainly not as marked as it is in tobacco.

GENERAL DISCUSSION

Young intact roots of tobacco were invaded by V. dahliae. The formation of torulose hyphae and microsclerotia before penetration of the root and the intercellular invasion which followed, were similar to observations made on hop by Talboys (1958a) and by Griffiths and Isaac (1966) on tomato. It is not possible to explain why Mahanty (1970) was unable to observe invasion of intact tobacco roots, although this may be related to the small size of his seedlings. Very young seedlings may react differently from the somewhat larger plants used in this work.

Mahanty (1970) was unable to detect conidia in the vessels of tobacco and concluded that this may not be an important means of colonization in this host. The writer also has not observed conidia in tobacco vessels but has seen them form within three hours on hyphae growing from vessels in water. The discontinuous distribution of the pathogen observed in the early stages of invasion of the midrib and lateral and secondary veins suggests that colonization occurred by movement of conidia in the vascular system, in spite of failure to observe them in prepared sections of infected tobacco plants.

In the field tobacco reaches a height of about 180 cm from soil level, in 70 days or so after planting. Inoculated VG plants may have 90% of their leaves showing symptoms in 105 days (Gibbins and Wright 1968). Assuming that the plants become infected within a few days of planting and inoculation, the pathogen must move more than 17 mm per day to reach the upper leaves in this time. From the data in Table 15, the rate of extension of mycelium from vessels into xylem sap is about 1.2 mm per day. The spread of the pathogen cannot be by extension of the mycelium but must be by dispersal of conidia in the sap stream. Conidial movement has been observed in hops by Sewell and Wilson (1964) and in cotton by Garber and Houston (1966).

If the basis for rapid establishment of V. dahliae in tobacco is by conidial transport in the xylem sap, then resistance to invasion lies in the prevention of formation or germination of the spores. This was suggested by Garber and Houston (1966) for cotton. Mahanty (1970) found that conidia were trapped in gums and were destroyed within eight hours, but in the susceptible variety colonization, although retarded, was not completely inhibited.

The pathogen was always present in the secondary veins of leaves before chlorosis developed, and symptoms appeared

within 14 days of invasion of the lateral veins. Talboys (1968) concluded that Verticillium exerted toxic effects close to the parenchyma cells in hop leaf. "Action in advance" does not appear to occur in Verticillium wilts (Sackston 1972).

The evidence suggests that the onset of symptoms at the various leaf levels on the plant must be related to the time of establishment of the fungus in these leaves and this is a reflection of the "resistance" of the host. Susceptibility is related to the time when conidia are not suppressed but germinate and colonize the vessels where they lodge (Garber and Houston 1966).

There is evidence that in tobacco the time of colonization is closely related to the physiological condition of the tissue surrounding the vessels. This effect is most marked in young tobacco tissue. In the field, symptoms appear more or less evenly in acropetal succession in individual plants of a cultivar. As lateral growth of the fungus in the xylem is limited (Mahanty 1970), it is difficult to accept that the uniformity in symptoms results from uniform invasion and colonization from each side of every plant. Aghion-Prat (1965) demonstrated that a meristematic gradient occurred in tobacco stems from the apex towards the root. In tobacco leaves, symptoms appear first in areas which, according to

Avery (1933), are those which lose their meristematic activity first.

Young tobacco shoots seldom show Verticillium wilt symptoms even when the vessels are heavily colonized (Wright 1969). In seedlings which have taken up conidia in the vessels, the spores are destroyed within 24 hours at the apex but are not killed at the base of the stem (Wright 1968b). Scheffer and Walker (1953) obtained similar results with tomato cuttings inoculated with Fusarium bud spores. This may not be a generalized reaction, however, because sunflower seedlings which had taken conidial suspension into the xylem, showed severe symptoms at the apex on the ninth day after inoculation (six days before flowerbuds appeared) and were dead after 30 days. Death of the terminal (young) internodes and young hypocotyls is found with Verticillium infections in cotton (Bell 1969), and in mint symptoms occur in basipetal succession.

The physiological condition which influences establishment of Verticillium in tobacco is not necessarily confined to young growing tissue. McLeod and Thomson (1959) noted that late maturing cultivars tended to be resistant and in some seasons did not show symptoms at all. Many other workers with various crops have noted that Verticillium wilt symptoms increase rapidly at the time of reproduction and that this is

related to physiological changes in the host at this time (Busch and Edgington 1967; Harrison and Isaac 1968; Hall and Busch 1971). Changes which took place in tobacco at flowering advanced chlorosis in leaves and presumably the rate of establishment of the fungus by 6 weeks, compared with plants not conditioned to flower.

In tobacco it is the root system which controls the time of symptom expression (Gibbins and Wright 1968). The determinative role of the roots has been described in many vascular wilt syndromes (Heinze and Andrus 1945; Keyworth 1953; Fronek 1965). There was no apparent inhibition of growth of the fungus in tobacco xylem exudate although the growth form of the pathogen was affected. These findings were in agreement with Taylor (1968) working with tobacco and Sinha and Wood (1968) with tomato, who could find no toxicity in the saps from either infected or healthy, resistant or susceptible plants. Inhibition of conidial germination in sap from inoculated resistant cotton was reported by Schnathorst et al. (1968).

Senescence accelerating, as well as retarding, substances were found in the xylem exudate. There was more cytokinin-like activity in sap from resistant than susceptible cultivars and most from infected resistant plants. This result would support the hypothesis that the root system

maintains cell vitality in the shoot through factors transported in the tracheal fluid, as suggested by Wright (1969). The presence of senescence accelerating compounds in the sap suggests that a balance between the two types of controlling substances may exist in the plant. Recently Shibaoka et al. (1970) found that there was antagonism between amino acids such as L-serine, and kinetin in pea roots. The amino acids promoted proteolysis and these may have been among the senescence accelerating compounds found in this work.

Taylor (1968) found a fivefold increase in phenolase activity in sap from infected tobacco plants and suggested, as many others have done (Goodman et al. 1967) that these may oxidize polyphenols to form substances toxic to the pathogen. He found scopoletin in xylem sap of Verticillium-infected tobacco plants, supporting the work of Sequiera (1965) who studied tobacco infected with bacterial wilt.

It would be of interest to study the effects of scopoletin and amino acids such as L-serine upon the pathogen in the leaves, using the disc technique.

The build-up of Verticillium in the vessels of the midribs of newly formed tobacco leaves retards normal development, most probably by cutting off the supply of nutrients to the proliferating cells. A physiological effect, however,

should not be discounted, as a plant growth-inhibitor has been found in tobacco leaves after infection with P. solanacearum (Steadman and Sequeira 1969). Even though the young leaf may be malformed by the pathogen in the midrib, chlorosis does not occur until the fungus has invaded the finer veins of these leaves.

Chlorosis does not appear to result from a type of cell for cell reaction, that is, a given quantity of metabolite such as an enzyme or toxin producing a given amount of response in a given number of cells. The fungus may be very unevenly distributed in the vessels of laminae showing uniform chlorosis. It is difficult to visualize how the effects of such metabolites would be distributed to parenchyma cells so widely separated spatially. The fungus is confined to the veins far distant from the parenchyma cells. Transfer of such a metabolite would have to be through the plasmodesmata and the question of dilution of concentration would have to be considered.

There is a uniform response within the different host cell layers to invasion of the secondary vessels by V. dahliae and the band of chlorosis and necrosis which passes, sometimes at an even rate, across the lamina may do so from any direction. This suggests that chlorosis may be a controlled response on the part of the host.

The build-up of the fungus in "hot-spots" in the secondary and tertiary veins and the presence of single hyphae in the fine veinlets would cut off nutrients to the parenchyma cells. This was supported by the fact that eosin would not flow into areas of the lamina which were chlorotic or about to become chlorotic.

There may be a reduction in size and dry matter content of infected tobacco (Wright 1969) and reduction in leaf areas has been used to measure symptom expression in potato (Harrison and Isaac 1968) and tomato (Pegg and Dixon 1969). In plants showing symptoms, reduction in size may be the result of reduced photosynthetic area but retardation of growth before flowering may result from energy normally used for growth, being diverted to keep the pathogen in check. This was suggested by Maine (1960) working with bacterial wilt of tobacco.

In the leaves, because of vessel blockage, nutrients and raw material required to check the fungus may not be sufficient to form inhibitory substance and this may lead to a build-up of the fungus and to chlorosis. Lamina tissue nearest the main lateral veins and midrib may remain green and apparently unaffected long after the central area has become necrotic, even though the secondary veins supply both areas and the concentration of fungus is similar just before

chlorosis first appears. The areas which remain green would be connected to the reserves of the midrib and lateral veins by plasmodesmata.

From the evidence with tobacco there is the possibility that two types of resistance mechanisms may be involved. These may be differences in intensity of the same system or may be distinctly different systems. The first is expressed by the formation of toxic pigments and perhaps by death of cells in areas where the fungus builds up to a high threshold level, or when a heavy inoculum is introduced into the vessels.

The other may occur continuously and is related to the physiological condition of the particular tissue. In tobacco it is especially active in young tissue but in other crops such as cotton (Bell 1969), it may be more potent in older tissue. In tobacco it may take the form of a gel with phytoalexin-like activity (Mahanty 1970). This resistance mechanism is operative within a very short time, at least within a matter of hours. This type of reaction was encountered in the experiments with intercellular inoculation and those where the fungus was kept in check when surrounded by tobacco leaf lamina. The inhibitor is apparently active over a short distance and may be responsible for the "hot-spots" found in the leaf vessels. These build-up areas may be weak points

for the production of the inhibitor and may be the first areas colonized. Conversely conidia, lodging in areas where the formation of the substance(s) is very active, may be destroyed. The evidence supports the gel-and-tylosis hypothesis put forward by Beckman (1966).

This appears to be the system responsible for the different levels of resistance found in the different tobacco cultivars. It is not known what the relationship is between the physiological condition of the tissue, the precisely quantitative influence of the root system and the effectiveness of inhibitory substance(s) in controlling the rate of establishment of the pathogen in the vessels of tobacco. These aspects should form the basis for further research.

CLAIM TO ORIGINALITY

The invasion of intact epidermal cells of tobacco root by V. dahliae is reported for the first time. Both microsclerotia and torulose hyphae were formed before penetration of the root. Invasion was intercellular and penetration pegs were observed in host cells adjacent to the hyphae.

The invasion of tobacco leaves by the pathogen was studied using a new technique I developed and described, which allowed a quantitative estimation to be made of the fungus within the veins of the leaf. This is the first time this has been done.

The propagules within infected leaves from which cultures arise were identified as hyphal cells and a description of their "germination" is given.

V. dahliae was found to grow out of the vessels into the intercellular spaces only at necrosis. It remained in the intercellular spaces as torulose resting hyphae for some months. This is the first time that hyphae have been shown to act as resting bodies in V. dahliae.

Single, pigmented, microsclerotial cells from culture were observed forming germ tubes for the first time.

The xylem sap from the roots of tobacco plants was found not to inhibit growth of V. dahliae hyphae or conidia, but it did alter the form of growth of hyphae and germ tubes from germinating conidia.

My new technique, which allows the fungus to be studied in the form in which it exists within the plant, was used to observe responses to gases and to chemicals in solution. The technique may be useful for studying other vascular wilt fungi under a wide range of environmental conditions.

I was able to confirm my earlier hypothesis that all tobacco cells appear to be resistant to the tobacco isolate of V. dahliae provided that vitality of the cells is maintained.

LITERATURE CITED

Aghion-Prat, D. 1965. Floral meristem-organizing gradient in tobacco stems. Nature, London 207: 1211.

Agrios, G.N. 1969. Plant Pathology. Academic Press. New York and London. 629 pp.

Albersheim, P., T.M. Jones and P.D. English. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7: 171-194.

Aubé, C. and G. Pelletier. 1968. Formation of endospores in Verticillium albo-atrum R & B. Can. J. Microbiol. 14: 606-607.

Avery, G.S. 1933. Structure and development of the tobacco leaf. Amer. J. Bot. 20: 565-592.

Bamburger, E. and A.M. Mayer. 1960. Effect of kinetin on formation of red pigment in seedlings of Amaranthus retroflexus. Science, 131: 1094.

Banfield, W.M. 1941. Distribution by the sap stream of spores of three fungi that induce vascular wilt diseases of elm. J. Agr. Res. 62: 637-681.

Barnard, C. 1960. Leaf structure in relation to quality in flue-cured tobacco. Austral. J. Agr. Res. 11: 169-185.

Beckman, C.H. 1964. Host responses to vascular infection. Annu. Rev. Phytopathol. 2: 231-252.

Beckman, C.H. 1966. Cell irritability and localization of vascular infections in plants. Phytopathology, 56: 821-824.

Beckman, C.H. 1971. Mechanisms of resistance to vascular infection. International Verticillium Symposium. Wye Coll. (Abstr.).

Bell, A.A. 1969. Phytoalexin production and Verticillium wilt resistance in cotton. Phytopathology, 59:1119-1127.

Berry, S.Z. and C.A. Thomas. 1961. Influence of soil temperature, isolates, and method of inoculation on resistance of mint to Verticillium wilt. Phytopathology, 51: 169-173.

Bewley, W.F. 1922. Sleepy disease of tomato. Ann. Appl. Biol. 9: 116-134.

Bigot, M.C. 1968. Action d'adénines substituées sur la synthèse des bétacyanines dans la plantule d'Amaranthus caudathus L. Possibilité d'un test biologique de dosage des cytokinines. Compt. Rend. Acad. Sci., Paris, t 266, Série D: 349-352.

Bondartseva, M.A. 1968. Investigation of the process of development of the microsclerotial form of the causal agent of Verticillium wilt of cotton. Rev. Appl. Mycol. 47: 625.

Bowen, H.H. 1965. Breeding studies of Verticillium wilt resistance in Fragaria ananassa Duch. Diss. Abstr. XXVI: 5664.

Brandes, E.W. 1919. Banana wilt. Phytopathology, 9: 339-390.

Braun, A.C. and R.B. Pringle. 1959. Pathogen factors in the physiology of disease. Toxins and other metabolites. In: Plant Pathology Problems and Progress 1908-1958. Univ. Wisconsin Press. Madison. pp. 88-99.

Brinkerhoff, L.A., D. Sarojak, N. Hormchong and N.C. Rogers. 1968. Morphology and longevity of Verticillium albo-atrum in cotton leaves. Phytopathology, 58: 1044 (Abstr.).

Brown, M.F. and T.D. Wyllie. 1970. Ultrastructure of microsclerotia of Verticillium albo-atrum. Phytopathology, 60: 538-542.

Busch, L.V. and L.V. Edgington. 1967. Correlation of photoperiod with tuberization and susceptibility of potato to Verticillium albo-atrum. Can. J. Bot. 45: 691-693.

Busch, L.V. and H.D. Schooley. 1970. Environmental influence on symptom expression in Verticillium wilt of chrysanthemum. Can. J. Bot. 48: 1939-1941.

Butler, O. 1911. A study on gummosis of prunus and citrus, with observations on squamosis and exanthema of the citrus. Ann. Bot. 25: 107-154.

Channon, A.G. and R.J. Hampson. 1968. Laboratory tests of fungicides against Peronospora parasitica (Pers. ex Fr.). Fr. Ann. Appl. Biol. 62: 23-33.

Clayton, E.E. 1958. The genetics and breeding progress in tobacco during the last 50 years. Agron. J. 50: 352-356.

Clayton, E.E. and J.E. Smith. 1942. Resistance of tobacco to bacterial wilt (Bacterium solanacearum). J. Agr. Res. 65: 547-554.

Curtis, K.M. 1940. New Zeal. Tobacco Res. Sta. Cawthron Inst. Ann. Rep. 1939-40.

Czaninski, Y., M. Peresse, A.M. Catesson and M. Moreau. 1971. Modifications ultrastructurales du xyléme de l'oeillet infecte par le Phialophora cinerescens. Compt. Rend. Acad. Sci. In press.

Darken, M.A. 1962. Absorption and transport of fluorescent brighteners by micro-organisms. Appl. Microbiol. 10: 387-393.

Davis, D., P.E. Waggoner and A.E. Dimond. 1953. Conjugated phenols in the Fusarium wilt syndrome. Nature, London 172: 959-961.

Deese, D.C. and M.A. Stahmann. 1962-3. Formation of pectic enzymes by Verticillium albo-atrum on susceptible and resistant tomato stem tissues and on wheat bran. Phytopathol. Z. 46: 53-70.

Devaux, A.L. and W.E. Sackston. 1966. Taxonomy of Verticillium species causing wilt of horticultural crops in Quebec. Can. J. Bot. 44: 803-811.

Dimond, A.E. 1966. Pressure and flow relations in vascular bundles of the tomato plant. Plant Physiol. 41: 119-131.

Dimond, A.E. and P.E. Waggoner. 1953. On the nature and role of vivotoxins in plant disease. Phytopathology, 43: 229-235.

Dimond, A.E. 1970. Biophysics and biochemistry of the vascular wilt syndrome. Annu. Rev. Phytopathol. 8: 301-322.

Dowson, W.J. 1922. On the symptoms of wilting of Michaelmas daisies produced by a toxin secreted by a Cephalosporium. Brit. Mycol. Soc. Trans. 7: 283-286.

Engelhard, A.W. 1957. Host index of Verticillium albo-atrum R & B. Plant Dis. Rep. Suppl. 244: 23-49.

Fronek, F.R. 1965. Verticillium wilt resistance in potato. Diss. Abstr. XXVI: 607.

Garber, E.D. 1956. A nutrition-inhibition hypothesis of pathogenicity. The Amer. Natur. 90: 183-194.

Garber, R.H. and B.R. Houston. 1966. Penetration and development of Verticillium albo-atrum in cotton plants. Phytopathology, 56: 1121-1126.

Garber, R.H. and B.R. Houston. 1967. Nature of Verticillium wilt resistance in cotton. Phytopathology, 57: 885-888.

Garrett, S.D. 1970. Pathogenic root-infecting fungi. Cambridge Univ. Press. 294 pp.

Gäumann, E. 1950. Principles of plant infection. Trans. W.B. Brierly. Crosby Lockwood and Sons Ltd., London.

Gibbins, L.N. and D.S.C. Wright. 1968. Verticillium wilt of tobacco. VI. The influence of roots and stems on Verticillium wilt symptoms in tobacco. New Zeal. J. Agr. Res. 11: 789-796.

Goodman, R.N., Z. Kiraly and M. Zaitlin. 1967. The biochemistry and physiology of infectious plant disease. D. Van Nostrand. London. pp. 354.

Gordee, R.S. and C.L. Porter. 1961. Structure, germination and physiology of microsclerotia of Verticillium albo-atrum. Mycologia, 53: 171-182.

Gothoskar, S., R.P. Scheffer, M.A. Stahmann and J.C. Walker. 1955. Further studies on the nature of Fusarium-resistance in tomato. Phytopathology, 45: 303-307.

Grieve, B.J. 1939. Epinastic response induced in plants by Bacterium solanacearum E.F.S. Ann. Bot. 3: 587-600.

Griffiths, D.A. 1971. The fine structure of Verticillium dahliae Kleb. colonizing cellophane. Can. J. Microbiol. 17: 79-81.

Griffiths, D.A. 1970. The fine structure of developing microsclerotia of Verticillium dahliae Kleb. Arch. Mikrobiol. 74: 207-212.

Griffiths, D.A. and I. Isaac. 1966. Host-parasite relationships between tomato and pathogenic isolates of Verticillium. Ann. Appl. Biol. 58: 259-272.

Griffiths, D.A. and W.C. Lim. 1964. Mechanical resistance in root hairs to penetration by species of vascular wilt fungi. Mycopath. Mycol. Appl. 24: 103-112.

Hadwiger, L.A. and M.E. Schwochau. 1969. Host resistance -- an induction hypothesis. Phytopathology, 59: 223-227.

Hall, R. and L.V. Busch. 1971. Verticillium wilt of chrysanthemum: colonization of leaves in relation to symptom development. Can. J. Bot. 49: 181-185.

Harrison, J.A.C. and I. Isaac. 1968. Leaf area development in King Edward potato plants inoculated with Verticillium albo-atrum and V. dahliae. Ann. Appl. Biol. 61: 217-230.

Hastie, A.C. 1967. Mitotic recombination in conidiophores of Verticillium albo-atrum. Nature, 214: 249-252.

Hastie, A.C. 1971. Genetics and Verticillium. International Verticillium Symposium. Wye Coll. (Abstr.).

Hawkworth, D.L. and P.W. Talboys. 1970. Descriptions of pathogenic fungi and bacteria. Commonwealth Mycol. Inst., Kew. Set 26: 255-260.

Heinze, P.H. and C.F. Andrus. 1945. Apparent localization of Fusarium wilt resistance in Pan America tomato. Amer. J. Bot. 32: 62-66.

Hopkins, V.C.F. 1956. Tobacco diseases. Commonwealth Mycol. Inst., Kew.

Isaac, I. 1946. Verticillium wilt of sainfoin. Ann. Appl. Biol. 33: 28-34.

Isaac, I. 1967. Speciation in Verticillium. Annu. Rev. Phytopathol. 5: 201-222.

Isaac, I. and W.G. Keyworth. 1948. Verticillium wilt of hop. III. A study of the pathogenicity of isolates from fluctuating and progressive outbreaks. Ann. Appl. Biol. 35: 243-249.

Isaac, I. and Q. MacGarvie. 1966. Dormancy and germination of resting structures of Verticillium spp. Trans. Brit. Mycol. Soc. 49: 669-678.

Itai, C. and Y. Vaadia. 1971. Cytokinin activity in water-stressed shoots. Plant Physiol. 47: 87-90.

Jacob, F. and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

Jones, L.R. and J.C. Gilman. 1915. The control of cabbage yellows through disease resistance. Wisconsin Agr. Exp. Sta. Res. Bull. 38.

Kende, H. 1964. Preservation of chlorophyll in leaf sections of substances obtained from root exudate. Science, 145: 1066.

Keyworth, W.G. 1953. Verticillium wilt of hop. VI. The relative roles of root and stem in the determination of wilt severity. Ann. Appl. Biol. 40: 344-361.

Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic Pseudomonads. Nature, London 199: 299-300.

Kulaeva, O. 1962. The effect of roots on leaf metabolism in relation to the action of kinetin on leaves. Fiziol. Rast. 9: 229-239.

Li, G.C. 1966. Fluorescence microscopy of healthy and infected potato tuber. M.Sc. thesis, Dep. of Plant Pathol., McGill Univ., Montreal, Canada.

Linskens, H.F. 1968. Host-pathogen interaction as a special case of interactions between organisms. Neth. J. Plant Pathol. 74. Suppl. 1: 1-8.

Livingston, L.G. 1935. The nature and distribution of plasmodesmata in the tobacco plant. Amer. J. Bot. 22: 75-87.

Lucas, G.B. 1965. Diseases of tobacco. 2nd Ed. Scarecrow Press, New York and London. 778 pp.

Ludwig, R.A. 1952. Studies on the physiology of hadromycotic wilting in tomato plant. Macdonald Coll. J. Ser. Tech. Bull. 20.

Ludwig, R.A. 1960. Toxins. In: J.G. Horsfall and A.E. Dimond (Eds.) Plant Pathology, An Advanced Treatice. Academic Press, New York. pp. 315-357.

Luke, H.H. and T.E. Freeman. 1968. Stimulation of transpiration by cytokinins. Nature, London 217: 873-874.

Mahanty, H.K. 1970. A histological study of the pathogenesis of Verticillium dahliae Kleb. in tobacco. New Zeal. J. Agr. Res. 13: 699-709.

Maine, E.C. 1960. Physiological responses in the tobacco plant to pathogenesis by Pseudomonas solanacearum, causal agent of bacterial wilt. Thesis. Dep. of Plant Pathol., North Carolina State Coll., Raleigh, N.C.

Maine, E.C. and A. Kelman. 1961. The influence of reducing substances on resistance to bacterial wilt in tobacco. Phytopathology, 51: 491-492.

Melhus, I.E., J.H. Muncie and W.T.H. Ho. 1924. Measuring water flow interference in certain gall and vascular diseases. Phytopathology, 14: 580-584.

McKeen, C.D. and W.B. Mountain. 1960. Synergism between Pratylenchus penetrans (Cobb) and Verticillium albo-atrum R & B in eggplant wilt. Can. J. Bot. 38: 789-794.

McLeod, A.G. and R. Thomson. 1959. Verticillium wilt of tobacco. II. Field and glasshouse trials to determine resistance of varieties and species to Verticillium dahliae Kleb. New Zeal. J. Agr. Res. 2: 792-796.

Misaghi, J., J.E. DeVay and A. Ravenscroft. 1969. Cytokinin activity in cotton plants infected by Verticillium albo-atrum. Phytopathology, 59: 1041. (Abstr.).

Müller, H.L. 1969. Additional studies in Verticillium disease of alfalfa. Verticillium albo-atrum R & B. Phytopathol. Z. 65: 69-97.

Müller, K.O. and L. Behr. 1949. Mechanism of Phytophthora resistance of potatoes. Nature, 163: 498-499.

Nadakavukaren, M.J. 1963. Fine structure of microsclerotia of Verticillium albo-atrum R & B. Can. J. Microbiol. 9: 411-413.

Orellana, R.G. 1969. Relative virulence and selective pathogenesis among isolates of Verticillium from sunflower and other hosts in relation to sunflower wilt. Phytopathol. Z. 65: 183-188.

Osborne, D.J. and D.R. McCalla. 1961. Rapid bioassay for kinetin and kinins using senescing leaf tissue. Plant Physiol. 36: 219-221.

Panton, C.A. 1964. A review of some aspects of the wilt pathogen Verticillium albo-atrum. Acta Agr. Scand. 14: 97-112.

Patil, S.S. and A.E. Dimond. 1968. Repression of polygalacturonase synthesis in Fusarium oxysporum f. sp. lycopersici by sugars and its effects on symptom reduction in infected tomato plants. Phytopathology, 58: 676-682.

Patton, R.F. and D.W. Johnson. 1966. Penetration of pine needles by Cronartium basidiospores labelled with a fluorescent brightener. Phytopathology, 56: 894. (Abstr.).

Pegg, G.F. and G.R. Dixon. 1969. The reactions of susceptible and resistant tomato cultivars to strains of Verticillium albo-atrum. Ann. Appl. Biol. 63: 389-400.

Pegg, G.F. and I.W. Selman. 1959. An analysis of growth response of young tomato plants to infection by Verticillium albo-atrum. II. The production of growth substances. Ann. Appl. Biol. 47: 222-231.

Piattelli, M., G. de Nicola and V. Castrogiovanni. 1971. The effect of kinetin on amaranthin synthesis in Amaranthus tricolor in darkness. Phytochemistry, 10: 289-293.

Pomerleau, R. 1968. Progression et localization de l'infestation par le Ceratocystis ulmi dans l'orme d'Amerique. Phytopathol. Z. 63: 301-327.

Presley, J.T. and E.E. Taylor. 1969. Ontogeny of vessels influence the development of Verticillium in young cotton. Phytopathology, 59: 253-254.

Provvidenti, R. and W.T. Schroeder. 1959. Foliage infection of tomato and eggplant by Verticillium. Plant Dis. Rep. 43: 821-826.

Putt, E.D. 1964. Breeding behaviour of resistance to leaf mottle or Verticillium in sunflowers. Crop Sci. 4: 177-179.

Rayner, R.W. 1970. A mycological colour chart. Commonwealth Mycol. Inst., Kew.

Roberts, L.D. and W.E. Smith. 1968. Gene equipoise and pathogenicity. Nature, London 218: 574-575.

Rubin, B.A. and Y.V. Artsikhovskaya. 1963. Biochemistry and physiology of plant immunity. Macmillan Co., New York.

Rudolf, B.A. 1931. Verticillium hadromycosis. Hilgardia 5: 197-353.

Sackston, W.E. 1959. Verticillium albo-atrum on red clover (Trifolium pratense). Que. Soc. Prot. Plants Rep. 41: 116-120.

Sackston, W.E. 1972. Host response to infection by Verticillium. In: First International Symposium on Pathological Wilting in plants. Univ. of Madras, India. In press.

Sadasivan, T.S. 1961. Physiology of wilt disease. Annu. Rev. Plant Physiol. 12: 449-468.

Samborski, D.J., F.R. Forsyth and Clayton Person. 1958. Metabolic changes in detached wheat leaves floated on benzimidazol and the effect of these changes on rust reaction. Can. J. Bot. 36: 591-601.

Sappenfield, W.P. 1963. Fusarium wilt-root knot nematode and Verticillium wilt resistance in cotton: possible relationships and influence on cotton breeding methods. Crop Sci. 3: 133-135.

Sass, J.E. 1968. Botanical microtechnique. Iowa State Univ. Press. 3rd Ed. 228 pp.

Scheffer, R.P., S.S. Gothoskar, C.F. Pierson and R.P. Collins. 1956. Physiological aspects of Verticillium wilt. Phytopathology, 46: 83-87.

Scheffer, R.P. and J.C. Walker. 1953. The physiology of Fusarium wilt of tomato. Phytopathology, 43: 116-125.

Scheffer, R.P. and J.C. Walker. 1954. Distribution and nature of Fusarium resistance in the tomato plant. Phytopathology, 44: 94-101.

Schnathorst, W.C. 1965. Origin of new growth in dormant microsclerotial masses of Verticillium albo-atrum. Mycologia, 57: 343-351.

Schnathorst, W.C., D. Coplin, J.T. Presley and H. Carns. 1966. Inhibition of germination of conidia of Verticillium albo-atrum in tracheal fluid from infected, tolerant cotton plants. Phytopathology, 56: 899.(Abstr.).

Schnathorst, W.C. and D.E. Matre. 1966. Host range and differentiation of a severe form of Verticillium albo-atrum in cotton. Phytopathology, 56: 1155-1161.

Schnathorst, W.C., J.T. Presley and H.R. Carns. 1964. Properties of tracheal fluids from susceptible and resistant cottons and their relation to development of Verticillium albo-atrum. Phytopathology, 54: 906. (Abstr.).

Schnathorst, W.C., J.T. Presley and H.R. Carns. 1968. Factors affecting the development of Verticillium wilt in cotton plants as revealed by studies on tracheal fluids. Belt-wide Cotton Prod. Res. Conf. (Hot Springs, Arkansas) Proc.: 227-228.

Sequeira, L. 1965. Origin of indoleacetic acid in tobacco plants infected with Pseudomonas solanacearum. Phyto-pathology, 55: 1232-1236.

Sewell, G.W.F. and J.F. Wilson. 1964. Occurrence and dispersal of Verticillium conidia in xylem sap of hop (Humulus lupulus L.) Nature, London 204: 901.

Shibaoka, S. and K.V. Thimann. 1970. Antagonisms between kinetin and amino acids. Plant Physiol. 46: 212-220.

Sinha, A.K. and R.K.S. Wood. 1968. Studies on the nature of resistance in tomato plants to Verticillium albo-atrum. Ann. Appl. Biol. 62: 319-327.

Skilling, D.D. and N.K. Krogh. 1969. Infection of Pinus resinosa tissue by fluorescent-labelled spores of Scleroderris lagerbergii. Phytopathology, 59: 1050. (Abstr.).

Smith, E.F. 1899. Wilt disease of cotton, watermelon, and cowpea (Neocosmospora nov. gen.). U.S. Dep. Agr. Div. Veg. Physiol. and Pathol. Bull. 17.

Smith, G. 1969. An introduction to industrial mycology. 6th Ed. E. Arnold Ltd., London. 390 pp.

Smith, H.C. 1965. The morphology of Verticillium albo-atrum, V. dahliae and V. tricorpus. New Zeal. J. Agr. Res. 8: 450-478.

Snedecor, G.W. and W.G. Cochran. 1969. Statistical methods. 6th Ed. Iowa State Univ. Press. Ames, Iowa, U.S.A. 593 pp.

Snyder, W.C., K.F. Baker and H.N. Hansen. 1946. Interpretation of resistance to Fusarium wilt in tomato. Science, 103: 707-708.

Steadman, J.R. and L. Sequeira. 1969. A growth inhibitor from tobacco and its possible involvement in pathogenesis. Phytopathology, 59: 499-503.

Stoddart, J.L. and A.J.H. Carr. 1966. Properties of wilt-toxins produced by Verticillium albo-atrum R & B. Ann. Appl. Biol. 58: 81-92.

Subramanian, D. and L. Sarawathi-Devi. 1959. Water is deficient. In: J.G. Horsfall and A.E. Dimond (Eds.) Plant Pathology, An Advanced Treatice. Academic Press, New York. pp. 313-348.

Talboys, P.W. 1958a. Some mechanisms contributing to Verticillium resistance in the hop root. Trans. Brit. Mycol. Soc. 41: 227-241.

Talboys, P.W. 1958b. Association of tylosis and hyperplasia of the xylem with vascular invasion of the hop by Verticillium albo-atrum. Trans. Brit. Mycol. Soc. 41: 249-260.

Talboys, P.W. 1958c. Degradation of cellulose by Verticillium albo-atrum. Trans. Brit. Mycol. Soc. 41: 242-248.

Talboys, P.W. 1964. A concept of the host-parasite relationship in Verticillium wilt diseases. Nature, London 202: 361-364.

Talboys, P.W. 1968. Water deficits in vascular disease. In: Water deficits and plant growth. Vol. II. T.T. Kozlowski (Ed.). Academic Press, New York. pp. 255-311.

Taylor, R.E. and L.C. Luckwill. 1967. In: Plant growth regulators symposium. Phytochem. Soc. and Brit. Ass. of Ind. Chem.

Taylor, M.E.U. 1968. Resistance to Verticillium: the biochemical approach. Span, 11: 96-99.

Thanassoulopoulos, C.C. and W.J. Hooker. 1970. Leaf and sprout infection of potato by Verticillium albo-atrum. Phytopathology, 60: 196-203.

Thomson, R. 1944. Flue-cured tobacco growing in New Zealand. New Zeal. Dep. Sci. Ind. Res. Information Bull. Ser. 1.

Thomson, R. and A.G. McLeod. 1959. Verticillium wilt of tobacco. I. A new disease of tobacco caused by Verticillium dahliae Kleb. New Zeal. J. Agr. Res. 2: 785-791.

Tolmsoff, W.J. 1960. Relation of age of potato plants to infection by Verticillium albo-atrum. Phytopathology, 50: 86. (Abstr.).

Tolmsoff, W.J. and R.A. Young. 1957. Relationship of inoculum potential of Verticillium albo-atrum to development and severity of wilt in potatoes. Phytopathology, 47: 536.

Tsao, P.H. 1969. Vital fluorescent labelling as applied to soil ecological studies of Phytophthora parasitica. Phytopathology, 59: 16. (Abstr.).

Tuite, J. 1969. Plant pathological methods. Fungi and bacteria. Burgess Publishing Co. 239 pp.

Van den Ende, G. 1959. Untensuchungen Uber den Pflanzenparasiten Verticillium albo-atrum. Rev. Appl. Mycol. 38: 312-313.

Waggoner, P.E. and A.E. Dimond. 1954. Reduction in water flow by mycelium in vessels. Amer. J. Bot. 41: 637-640.

Walker, J.C. 1969. Plant Pathology. 3rd Ed. McGraw Hill. New York and London. 819 pp.

Wardlaw, C.W. 1931. The biology of banana wilt (Panama disease). III. An examination of sucker infection through root-bases. Ann. Bot. 45: 381-399.

Weiss, C. and Y. Vaadia. 1965. Kinetin-like activity in root apices of sunflower plants. Life Sci. 4: 1323-1326.

West, E.S., W.R. Todd, H.S. Mason and J.T. Van Bruggen. 1967. Textbook of biochemistry. 4th Ed. The Macmillan Co., New York. Collier - Macmillan Ltd., London. 1595 pp.

Wolf, F.A. 1935. Tobacco diseases and decays. Duke Univ. Press, Durham. North Carolina.

Wood, R.K.S. 1960. Pectic and cellulolytic enzymes in plant disease. Annu. Rev. Plant Physiol. 11: 299-322.

Wood, R.K.S. 1961. Verticillium wilt of tomatoes -- the role of pectic and cellulolytic enzymes. Ann. Appl. Biol. 49: 129-139.

Wood, R.K.S. 1967. Physiological plant pathology. Blackwell, Oxford. 570 pp.

Wright, D.S.C. 1966. Verticillium wilt of tobacco (a note). New Zeal. J. Agr. Res. 9: 448-451.

Wright, D.S.C. 1968a. Verticillium wilt of tobacco. V. Varietal screening for resistance to Verticillium dahliae Kleb., and mode of inheritance of resistance. New Zeal. J. Agr. Res. 11: 655-664.

Wright, D.S.C. 1968b. Verticillium wilt of tobacco. VIII. Movement of Verticillium dahliae conidia in tobacco plants. New Zeal. J. Agr. Res. 11: 803-811.

Wright, D.S.C. 1969. Verticillium wilt of tobacco.IX. The relationship between leaf symptoms and levels of infection in the organs of mature tobacco plants. New Zeal. J. Agr. Res. 12: 228-239.

Wright, D.S.C. and J.P. Abrahamson. 1970. Electron microscopic study of Verticillium dahliae Kleb. as a pathogen of tobacco (Nicotiana tabacum L.). New Zeal. J. Bot. 8: 326-343.

Wright, D.S.C. and J.M. Biss. 1967. Screening tobacco varieties for resistance to black root rot in New Zealand. Tobacco Sci. 164: 30-32.

Wright, D.S.C. and J.M. Biss. 1968. Verticillium wilt of tobacco. VII. Pathogenicity of Verticillium dahliae isolates. New Zeal. J. Agr. Res. 11: 797-802.

Yoshida, R., T. Oritani and A. Nishi. 1971. Kinetin-like factors in the root exudates of rice plants. Plant and Cell Physiol. 12: 89-94.

APPENDIX

Table A1. Frequency distribution of growth scores of *Verticillium dahliae* from lateral vein samples taken from tobacco leaves showing symptoms[1/].

x	f	%f	Expected frequency	Contribution to χ^2
0 - 0.5	1	0.67	3.59	2.38
0.6 - 1.0	16	10.67	5.26	5.56
1.1 - 1.5	18	12.00	9.72	0.53
1.6 - 2.0	24	16.00	14.47	0.16
2.1 - 2.5	22	14.77	16.36	0.15
2.6 - 3.0	14	9.33	17.48	3.80
3.1 - 3.5	29	19.33	14.17	1.88
3.6 - 4.0	13	8.67	9.37	0.05
4.1 - 4.5	9	6.00	5.05	0.18
4.6 - 5.0	4	2.67	3.33	0.13

$\bar{x}$ = 2.483

S.D. = 1.100

Normality χ^2 = 14.82 with 7 d.f. (P = 5%)

Skewness = $\sqrt{b_1}$ = 0.169 ± 0.200 (N.S.)

Kurtosis g_2 = -1.022 ± 0.40 and

b_2 = 1.978 (P = 1%)

(Continued)

Table A1. (Continued) Frequency distribution of growth scores of Verticillium dahliae from lateral vein samples taken from leaves showing symptoms[2/].

x	f	%f	Expected frequency	Contribution to χ^2
0	291	19.40	12.88	3.30
1	217	14.47	15.90	0.13
2	206	13.73	20.66	2.32
3	316	21.07	21.63	0.01
4	214	14.27	15.75	0.14
5	256	17.06	12.28	1.86

$\bar{x}$ = 2.476

S.D. = 1.744

Normality χ^2 = 7.76 with 3 d.f. (N.S.)

1/ Sample discs scored on a 0 to 5 basis. Frequency distribution is from mean scores of 10 discs from 150 leaves.

2/ Frequency distribution of 1500 samples taken from 150 leaves.

Table A2. Number of hyphae growing from sample discs from tobacco leaves at various periods after intercellular flooding with a suspension of Verticillium dahliae conidia.

Replicate	Treatment: Attached, Period after inoculation (hours): 4	Attached: 24	Attached: 120	In bag: 4	In bag: 24	In bag: 120	In water: 4	In water: 24	In water: 120	Total
1	52.5[1/]	8.6	0.5	27.4	7.0	5.8	46.0	8.5	1.3	157.6
2	49.1	9.4	1.1	23.4	11.6	3.9	29.9	13.8	0.6	142.8
3	45.2	12.3	0.9	22.5	14.4	3.4	34.0	7.2	0.9	140.8
4	54.5	11.3	1.6	23.8	14.3	3.4	33.8	12.6	1.5	156.8
5	61.7	8.3	0.9	24.7	9.6	1.9	35.4	6.3	0.4	149.2
Total	263.0	49.9	5.0	121.8	56.9	18.4	179.1	48.4	4.7	747.2

Analysis of Variance

Source	df	SS	MS	F	
Replicates	4	26.62	6.66	0.54	
Leaf treatments	2	513.55	256.78	20.91	**
Period after inoculation	2	10,450.26	5,225.13	425.50	**
Interaction	4	1,536.73	384.18	30.01	**
Error	32	393.02	12.28		
Total	44	12,920.18			

1/ Mean of 10 discs.

** Significant at 1% level.

Table A3. Number of hyphae growing from sample discs from upper and lower leaves of Virginia gold at various periods after intercellular flooding with conidial suspensions of two isolates of Verticillium dahliae.

Replicate	Leaf Position: Lower						Leaf Position: Upper						Total
	Isolate: V3			Isolate: V58			Isolate: V3			Isolate: V58			
	Period after inoculation (hours)												
	4	24	120	4	24	120	4	24	120	4	24	120	
1	52.7[1/]	14.4	2.4	39.6	5.7	2.2	12.3	0.7	0.1	5.0	0.9	0.2	136.2
2	61.8	8.3	3.5	36.5	7.7	2.2	6.7	0.6	0.7	3.6	0.7	0.1	132.4
3	54.4	9.2	5.6	29.2	10.3	3.8	11.3	0.1	0.2	2.1	0.4	0.2	126.8
4	60.9	8.5	3.2	30.6	8.2	2.1	8.6	0.5	0.6	2.7	0.5	0.4	126.8
Total	229.8	40.4	14.7	135.9	31.9	10.3	38.9	1.9	1.6	13.4	2.5	0.9	522.2

(Continued)

Table A3. Continued.

Source	Analysis of Variance				
	df	SS	MS	F	
Replications	3	15.3	5.1	0.9	
Isolates (I)	1	365.2	365.2	64.1	**
Position (P)	1	3,396.9	3,396.9	595.9	**
Duration (D)	2	5,654.1	2,827.1	496.0	**
I x P	1	139.0	139.0	24.4	**
I x D	2	530.2	265.1	46.5	**
D x P	2	3,061.1	1,530.6	268.5	**
D x I x P	2	161.1	80.6	14.1	**
Error	33	188.0	5.7		
Total	47	13,510.9			

1/ Mean of 10 discs.

** Significant at 1% level.

Table A4. Number of hyphae growing from sample discs from upper and lower leaves of McNairs 121 tobacco at various periods after intercellular flooding with conidial suspensions of two isolates of Verticillium dahliae.

Replicate	Leaf Position: Lower, Isolate: V3, Period after inoculation (hours): 4	24	120	Lower, V58: 4	24	120	Upper, V3: 4	24	120	Upper, V58: 4	24	120	Total
1	55.4[1/]	22.8	2.1	23.5	2.6	0.3	3.9	1.7	0.6	2.1	0.7	0.3	116.0
2	41.4	15.1	4.2	17.2	8.2	1.1	5.7	0.5	0.6	0.8	1.5	0.2	96.5
3	45.6	8.7	5.3	26.2	7.2	1.3	1.1	1.1	0.7	3.2	1.0	0.2	101.6
4	42.2	10.6	2.3	21.7	3.4	1.0	1.8	1.2	0.6	1.7	0.4	0	86.9
Total	184.6	57.2	13.9	88.6	21.4	3.7	12.7	4.5	2.5	7.8	3.6	0.7	401.0

(Continued)

Table A4. Continued.

Source	Analysis of Variance				
	df	SS	MS	F	
Replications	3	36.7	12.2	1.4	
Isolates (I)	1	464.8	464.8	51.8	**
Position (P)	1	2,377.2	2,377.2	264.7	**
Duration (D)	2	2,530.5	1,265.3	140.9	**
I x P	1	377.5	377.5	42.0	**
I x D	2	262.1	131.0	14.6	**
D x P	2	1,943.7	971.9	108.2	**
D x I x P	2	224.2	112.1	12.5	**
Error	33	296.2	9.0		
Total	47	8,512.9			

1/ Mean of 10 discs.

** Significant at 1% level.

Table A5. Growth score of Verticillium dahliae from test discs of tobacco leaves surrounded by lamina circles.

Replicate	Control (test disc alone)	Surrounded by 'old' lamina circle	Surrounded by 'young' lamina circle
1	40[1/]	11	1
2	40	1	0
3	40	0	0
4	40	1	0
5	38	0	0
6	40	1	0
7	40	0	0
8	40	0	2
Total	318	14	3
Mean per disc	4.97	0.22	0.05

Analysis of Variance

Source	df	SS	MS	F	
Treatments	2	7,989.96	3,994.98	78.33	**
Error	21	107.00	5.10		
Total	23	8,096.96			

1/ Total of 8 discs scored on a 0 to 5 basis.

** Significant at 1% level.

Table A6. Growth score and length of hyphae of Verticillium dahliae from test discs of tobacco leaves with and without lamina circles and cover slips.

(i) Growth Score

Replicate	Treatments				
	A[1]	B	C	D	E
1	25[2]	20	2	28	23
2	30	18	3	29	25
3	30	20	0	21	30
4	30	20	0	24	30
Total	115	78	5	102	108
Mean per disc	4.79	3.25	0.21	4.25	4.50

Analysis of Variance

Source	df	SS	MS	F	
Treatments	4	2,027.3	506.8	70.7	**
Error	15	107.5	7.2		
Total	19	2,134.8			

(Continued)

Table A6. Continued.

(ii) Length of hyphae

Replicate	Treatments				
	A	B	C	D	E
1	64.0[3/]	68.2	5.7	80.4	80.2
2	98.4	139.8	8.8	87.0	69.6
3	105.5	69.2	0	43.4	99.9
4	116.1	72.6	0	67.0	97.6
Total	384.0	349.8	14.5	277.8	347.1
Mean per disc	16.0	14.6	0.6	11.6	14.5

Analysis of Variance

Source	df	SS	MS	F	
Treatments	4	22,635.5	5,658.9	12.0	**
Error	15	7,029.9	468.7		
Total	19	29,656.4			

(Continued)

Table A6. Continued.

1/ Treatments were A. Control, test disc without lamina circle but with cover slip; B. Lamina circles without cover slips; C. Lamina circles with cover slips; D. Autoclaved lamina circles without cover slips; E. Autoclaved lamina circles with cover slips.

2/ Total of 6 discs scored on a 0 to 5 basis.

3/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at the 1% level.

Table A7. Bioassay of cytokinin-like activity of xylem exudate from infected and uninfected tobacco plants.

	VGUN	VGIN	MNUN	MNIN
	22.3[1/]	11.7	17.7	19.6
	19.8	14.8	17.6	19.6
	19.8	13.1	16.5	22.2
	19.4	16.0	17.5	20.9
	19.4	18.0	19.9	20.1
	19.4	20.0	17.7	20.0
	25.1	9.3	17.0	18.7
	22.4	10.7	22.3	23.5
	25.1	11.9	16.5	21.0
	28.1	9.3	22.3	19.8
	28.1	8.3	16.5	17.5
	28.1	8.3	24.9	20.9
	22.4	9.8	15.6	16.0
	24.6	11.1	22.1	20.9
	22.4	9.8	19.8	20.9
Total	364.4	182.1	283.9	301.6
Mean	23.1	12.1	18.9	20.1

(Continued)

Table A7. Continued.

Source of variation	Analysis of Variance			
	df	SS	MS	F
Xylem saps	3	964.40	321.46	37.16 **
Error	56	484.15	8.65	
Total	59	1,448.55		

1/ Estimated ng of kinetin equivalents per ml of xylem exudate.

** Significant at the 1% level.

Table A8. Bioassay of cytokinin-like activity of extracts from infected and uninfected tobacco plant roots.

VGUN	VGIN	MNUN	MNIN
14.3[1/]	12.5	9.3	14.0
12.0	8.4	10.4	12.0
14.3	12.6	7.9	12.0
15.8	8.4	7.9	11.2
14.1	8.4	8.9	11.2
10.2	7.9	12.6	8.9
14.8	9.9	11.3	18.7
10.5	6.3	9.3	17.5
12.7	8.9	9.3	18.7
	5.7	7.5	10.0
	12.5	11.5	17.5

(Continued)

Table A8. Continued.

	VGUN	VGIN	MNUN	MNIN
		7.9	11.5	9.5
		7.1	10.4	9.2
		7.1		17.5
		7.9		17.5
Total	118.7	131.5	127.8	205.4
Mean	13.2	8.8	9.8	13.7

Analysis of Variance[2/]

Source	df	SS	MS	F	
Root extracts	3	242.3	80.8	12.4	**
Error	48	330.7	6.9		
Total	51				

(Continued)

Table A8. Continued.

	VGUN-VGIN	VGUN-MNUN	VGUN-MNIN	VGIN-MNUN	VGIN-MNIN	MNUN-MNIN
Difference between means 2/	4.43	3.36	0.50	1.07	4.93	3.87
L.S.D. (.05)	2.22	2.29	2.46	2.00	1.93	2.00
L.S.D. (.01)	2.96	3.05	3.28	2.67	2.57	2.67
	**	**			**	**

1/ Estimated ng of kinetin equivalents per gm dry weight of root tissue.

2/ As some samples were missing, the data were analyzed and the means compared using the methods described by Steele and Torrie (1960).

** Significant at the 1% level.

Table A9. Growth score and length of hyphae of Verticillium dahliae from test discs of tobacco leaves in infected and uninfected tobacco xylem exudates.

(i) Growth score

Replicate	Xylem exudates				
	Water	VGUN	VGIN	MNUN	MNIN
1	24[1/]	23	24	25	25
2	25	24	24	24	24
3	25	25	24	25	25
4	24	24	25	24	25
5	25	24	23	25	25
6	25	25	25	24	23
Total	148	145	145	147	147
Mean per disc	4.9	4.8	4.8	4.9	4.9

Analysis of Variance

Source	df	SS	MS	F
Replications	5	1.2	0.2	0.4
Exudates	4	1.2	0.3	0.6
Error	20	10.8	0.5	
Total	29	13.2		

(Continued)

Table A9. Continued.

(ii) Length of hyphae

Replicate	Xylem exudates				
	Water	VGUN	VGIN	MNUN	MNIN
1	71.9[2/]	88.5	78.1	72.9	88.8
2	65.5	72.0	77.9	59.2	81.6
3	76.2	61.6	74.8	56.7	61.2
4	79.9	68.1	90.8	58.3	79.0
5	85.6	70.7	60.2	62.3	66.9
6	77.7	76.6	76.1	62.8	73.1
Total	456.8	437.5	457.9	377.1	450.6
Mean per disc	15.2	14.6	15.3	12.6	15.0

Analysis of Variance

Source	df	SS	MS	F
Replications	5	600.0	120.0	2.0
Exudates	4	766.0	191.5	3.1 *
Error	20	1,230.6	61.5	
Total	29	2,596.6		

1/ Total of 5 discs scored on a 0 to 5 basis. The experiment was arranged in randomized blocks.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

* Significant at the 5% level.

Table A10. Germination of conidia of Verticillium dahliae in hanging drops of infected and uninfected tobacco xylem exudates.

	Exudates			
Field	VGUN	VGIN	MNUN	MNIN
1	91[1/]	88	92	92
2	90	93	95	93
3	92	92	95	95
4	85	92	90	89
5	88	98	88	93
Total	446	463	460	462
Mean	89.2	92.6	92.0	92.4

Analysis of Variance

Source	df	SS	MS	F
Exudates	3	37.8	12.6	1.5
Error	16	139.2	8.7	
Total	19	177.0		

1/ Percentage germination in five fields after 18 hours. Approximately 40 conidia were counted per field.

Table A11. Growth score and length of hyphae of Verticillium dahliae from test discs of tobacco leaves in various nutrient solutions.

(i) Growth score

Replicate	Nutrient Solutions				
	Water	PD broth	Czapek broth	V8 juice	Sunflower Xylem exudate
1	22[1/]	16	22	23	24
2	24	13	24	23	25
3	25	15	24	17	23
4	24	15	25	22	25
5	25	13	25	21	25
6	24	16	23	21	22
Total	144	88	143	127	144
Mean per disc	4.8	2.9	4.8	4.2	4.8

(Continued)

Table A11. Continued.

Source	Analysis of Variance			
	df	SS	MS	F
Replications	5	6	1.2	0.5
Nutrients	4	388	97.0	39.6 **
Error	20	49	2.4	
Total	29	443		

(ii) Length of hyphae

Replicate	Water	PD broth	Czapek broth	V8 juice	Sunflower Xylem exudate
1	46.7[2/]	25.5	46.2	41.9	53.9
2	54.0	21.0	55.5	42.4	51.2
3	53.5	23.8	58.9	34.1	48.8
4	52.0	27.1	52.6	40.8	51.5
5	57.3	19.5	54.8	44.7	50.9
6	55.6	24.8	59.6	36.9	50.1
Total	319.1	141.7	327.6	240.8	306.4
Mean per disc	10.1	4.7	10.9	8.0	10.0

(Continued)

Table A11. Continued.

Source	Analysis of Variance			
	df	SS	MS	F
Replications	5	25.5	5.1	0.4
Nutrients	4	4,054.3	1,013.6	69.4 **
Error	20	292.0	14.6	
Total	29	4,371.8		

1/ Total of 5 discs scored on a 0 to 5 basis. The trial was arranged in randomized blocks.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at the 1% level.

Table A12. Germination of conidia of Verticillium dahliae in hanging drops of various nutrient solutions.

Field	Nutrient Solutions			
	Water	PD broth	Czapek broth	V8 juice
1	79[1/]	71	82	88
2	79	85	84	86
3	82	79	85	88
4	77	72	91	88
5	85	84	87	92
Total	402	391	429	442
Mean	80.4	78.2	85.8	88.4

Source	Analysis of Variance			
	df	SS	MS	F
Solutions	3	333.2	111.1	6.4 **
Error	16	276.0	17.3	
Total	19	609.2		

1/ Percentage germination in five fields after 18 hours. Approximately 40 conidia counted per field.

** Significant at 1% level.

Table A13. Width of Verticillium dahliae germ-tubes in hanging drops of various nutrient solutions.

Germ tube	Nutrient Solutions			
	Water	PD broth	Czapek broth	V8 juice
1	12.8[1/]	18.2	25.8	24.6
2	11.5	18.4	21.1	22.9
3	15.1	23.4	21.0	21.2
4	15.7	20.7	23.6	20.7
5	13.3	21.4	20.9	23.9
6	13.4	22.7	22.5	23.2
7	12.7	22.2	23.7	23.5
8	12.6	20.3	22.4	23.5
9	13.3	21.2	20.7	21.8
10	12.3	23.3	22.9	23.3
Total	132.7	211.8	224.6	228.5
Mean width	0.93[2/]	1.48	1.57	1.60

(Continued)

Table A13. Continued.

Source	Analysis of Variance			
	df	SS	MS	F
Nutrients	3	608.4	202.8	89.0 **
Error	36	82.2	2.3	
Total	39	690.6		

1/ Mean of 5 measurements taken along each of 10 germ-tubes. Figures were divisions on filar micrometer.

2/ Width in microns (1 division of filar micrometer = 0.7 μ).

** Significant at the 1% level.

Table A14. Growth score and length of hyphae of Verticillium dahliae from test discs of tobacco leaves in various solutions.

(i) Growth score

Replicate	Solutions						
	Water	Alcohol (0.05%)	Benomyl (2 p.p.m.)	Buffer	Kinetin in buffer (20 p.p.m.)	Benzimidazole (120 p.p.m.)	Nickel sulphate (49 p.p.m.)
1	20[1/]	0	0	2	7	8	15
2	21	0	1	4	9	9	18
3	22	0	1	0	8	8	21
4	23	0	1	3	5	12	16
5	19	0	2	5	9	9	17
Total	105	0	5	14	38	46	87
Mean per disc	4.2	0	0.2	0.6	1.5	1.8	3.5

(Continued)

Table A14. Continued.

Source	Analysis of Variance			
	df	SS	MS	F
Solutions	6	1,988.6	331.4	132.6 **
Error	28	70.0	2.5	
Total	34	2,058.6		

(ii) Length of hyphae

Replicate	Water	Alcohol (0.5%)	Benomyl (2 p.p.m.)	Buffer	Kinetin in buffer (20 p.p.m.)	Benzimidazole (120 p.p.m.)	Nickel sulphate (49 p.p.m.)
1	49.2[2/]	0	0	5.0	22.7	22.2	26.3
2	50.0	0	0.2	6.9	27.4	22.1	30.1
3	56.4	0	1.2	0	15.8	17.4	42.3

(Continued)

Table A14. Continued.

Replicate	Water	Alcohol (0.5%)	Benomyl (2 p.p.m.)	Buffer	Kinetin in buffer (20 p.p.m.)	Benzimidazole (120 p.p.m.)	Nickel sulphate (49 p.p.m.)
4	60.5	0	0.3	14.8	13.3	26.2	29.5
5	50.1	0	0.4	7.7	18.2	18.7	33.6
Total	266.2	0	2.1	34.4	97.4	106.6	161.8
Mean per disc	0.84	0	0.01	0.11	0.30	0.33	0.51

Analysis of Variance

Source	df	SS	MS	F	
Solutions	6	11,047.6	1,841.3	95.7	**
Error	28	538.7	19.2		
Total	34	11,586.3			

1/ Total of 5 discs scored on a 0 to 5 basis.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at the 1% level.

Table A15. Growth scores and length of hyphae of Verticillium dahliae from test discs of tobacco leaves in various solutions.

(i) Growth score

Replicate	Solutions					
	Water	Benomyl (2 p.p.m.)	Benomyl (0.2 p.p.m.)	Alcohol (0.1%)	Kinetin in water (2 p.p.m.)	Kinetin in water (20 p.p.m.)
1	19[1/]	2	11	22	25	25
2	22	0	7	25	24	25
3	23	3	14	23	23	22
4	20	3	14	24	25	25
5	21	1	14	22	24	25
Total	105	9	60	116	121	122
Mean per disc	4.2	0.4	2.4	4.6	4.8	4.9

(Continued)

Table A15. Continued.

Source	Analysis of Variance			
	df	SS	MS	F
Solutions	5	2,067.8	413.6	138.8 **
Error	24	71.6	3.0	
Total	29	2,139.4		

(ii) Length of hyphae

Replicate	Solutions					
	Water	Benomyl (2 p.p.m.)	Benomyl (0.2 p.p.m.)	Alcohol (0.1%)	Kinetin in water (2 p.p.m.)	Kinetin in water (20 p.p.m.)
1	51.7[2/]	0.5	24.8	71.0	52.5	50.1
2	50.0	0	25.2	73.1	53.7	63.3
3	57.5	1.0	34.2	85.8	49.9	72.6
4	62.9	1.0	34.6	88.5	71.7	76.2
5	63.0	0.7	31.7	71.8	68.1	64.1

(Continued)

Table A15. Continued.

Replicate	Water	Benomyl (2 p.p.m.)	Benomyl (0.2 p.p.m.)	Alcohol (0.1%)	Kinetin in water (2 p.p.m.)	Kinetin in water (20 p.p.m.)
Total	285.1	3.2	150.5	390.2	295.9	326.3
Mean per disc	11.4	0.1	6.0	15.6	11.8	13.1

Analysis of Variance

Source	df	SS	MS	F	
Solutions	5	19,846.0	3,969.2	71.7	**
Error	24	1,328.8	55.4		
Total	29	21,174.8			

1/ Total of 5 discs scored on a 0 to 5 basis.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at 1% level.

Table A15. Continued.

Replicate	Water	Benomyl (2 p.p.m.)	Benomyl (0.2 p.p.m.)	Alcohol (0.1%)	Kinetin in water (2 p.p.m.)	Kinetin in water (20 p.p.m.)
Total	285.1	3.2	150.5	390.2	295.9	326.3
Mean per disc	11.4	0.1	6.0	15.6	11.8	13.1

Analysis of Variance

Source	df	SS	MS	F	
Solutions	5	19,846.0	3,969.2	71.7	**
Error	24	1,328.8	55.4		
Total	29	21,174.8			

1/ Total of 5 discs scored on a 0 to 5 basis.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at 1% level.

Table A16. Growth score and length of hyphae of Verticillium dahliae from test discs of tobacco leaves in various solutions.

(i) Growth score

Replicate	Solutions			
	Water	Methyl cellulose	Glucose	Galacturonic acid
1	25[1/]	23	23	13
2	25	25	25	9
3	24	24	20	15
4	24	25	22	8
5	20	22	25	15
Total	118	119	115	60
Mean per disc	4.7	4.8	4.6	2.4

Analysis of Variance

Source	df	SS	MS	F	
Solutions	3	494.8	164.9	30.7	**
Error	16	86.0	5.4		
Total	19	580.8			

(Continued)

Table A16. Continued.

(ii) Length of hyphae

Replicate	Solutions			
	Water	Methyl cellulose	Glucose	Galacturonic acid
1	72.9[2/]	55.9	67.4	35.1
2	64.1	78.5	71.1	25.9
3	71.3	69.5	49.5	44.0
4	71.1	78.7	69.7	27.2
5	51.8	65.2	66.7	45.2
Total	331.2	347.8	324.4	177.4
Mean per disc	13.3	13.9	13.0	7.1

Analysis of Variance

Source	df	SS	MS	F
Solutions	3	3,758.4	1,252.8	15.3 **
Error	16	1,311.4	82.0	
Total	19	5,069.9		

(Continued)

Table A16. Continued.

1/ Total of 5 discs scored on a 0 to 5 basis.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at 1% level.

Table A17. Growth score and length of hyphae of Verticillium dahliae from test discs of tobacco leaves in various respiratory inhibitors.

(i) Growth score

Replicate	Respiratory Inhibitors		
	2-4 DNP	Thiourea	Potassium fluoride
1	25[1/]	25	25
2	24	23	25
3	25	22	25
4	24	23	25
5	25	15	24
6	25	17	23
7	25	17	25
8	25	17	25
Total	198	159	197
Mean per disc	4.9	4.0	4.9

Analysis of Variance

Source	df	SS	MS	F	
Inhibitor	2	104.3	52.2	8.9	**
Error	21	123.5	5.9		
Total	23	227.8			

(Continued)

Table A17. Continued.

(ii) Length of hyphae

Replicate	Respiratory Inhibitors		
	2-4 DNP	Thiourea	Potassium fluoride
1	83.6[2/]	75.7	82.7
2	89.1	61.1	85.1
3	82.2	64.8	86.2
4	82.3	65.4	90.7
5	80.2	36.9	61.5
6	83.9	52.1	54.4
7	84.3	66.6	68.8
8	78.2	64.2	59.2
Total	663.8	486.8	588.6
Mean per disc	16.6	12.2	14.7

Analysis of Variance

Source	df	SS	MS	F	
Inhibitor	2	1,972.8	986.4	8.5	**
Error	21	2,435.1	116.0		
Total	23	4,407.9			

1/ Total of 5 discs scored on a 0 to 5 basis.

2/ Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at 1% level.

Table A18. Growth score of Verticillium dahliae from test discs of tobacco leaves in uninfected Virginia gold and McNairs 121 tobacco xylem exudates.

Replicate	Solutions																	
	Water						VGUN						MNUN					
	Oxygen tension																	
	Control	0	5	10	15	20	Control	0	5	10	15	20	Control	0	5	10	15	20
1	4.0[1/]	0.2	2.2	2.6	4.2	4.0	3.8	0	3.8	0.8	4.2	4.2	3.8	0	3.4	2.6	3.2	4.6
2	4.6	0	2.6	3.0	3.8	4.8	5.0	0.2	2.6	4.0	4.6	4.4	3.6	0	3.8	1.6	2.2	5.0
3	3.6	0	3.6	2.4	4.8	2.2	4.2	0	3.0	4.0	4.6	4.2	4.2	0	4.2	5.0	3.4	5.0
Total	12.2	0.2	8.4	8.0	12.8	11.0	13.0	0.2	9.4	8.8	13.4	12.8	11.6	0	11.4	9.2	8.8	14.6
Mean	4.1	0.1	2.8	2.7	4.3	3.7	4.3	0.1	3.1	2.7	4.5	4.3	3.9	0	3.8	3.1	2.7	4.9

(Continued)

Table A18. Continued.

Source	Analysis of Variance			
	df	SS	MS	F
Replicates	2	1.3	0.7	1.0
Treatments	(17)	(119.7)	(7.0)	(10.8) **
Solutions	2	0.7	0.4	0.5
Oxygen	5	111.2	22.2	34.2 **
Interaction	10	7.8	0.8	1.2
Error	34	22.2	0.7	
Total	53	143.2		

1/ Mean score on a 0 to 5 basis of five test discs in each treatment combination.

** Significant at 1% level.

Table A19. Length of Verticillium dahliae from test discs of tobacco leaves in uninfected Virginia gold and McNairs 121 tobacco xylem exudates.

	Solutions															
	Water						VGUN						MNUN			
Replicate	Control	0	5	10	15	20	Control	0	5	10	15	20	Control	0	5	10	15	20
1	10.4 ± 1/	0.6	4.8	6.2	9.0	6.2	10.2	0	7.4	1.9	8.8	5.6	11.1	0	7.0	6.5	9.6	7.4
2	16.4	0	9.8	7.5	10.8	13.0	13.0	0.1	7.5	13.5	13.5	11.7	9.0	0	8.7	3.3	6.2	16.9
3	12.3	0	9.3	7.2	13.6	6.5	12.0	0	7.9	6.4	11.5	10.7	12.2	0	9.1	11.3	9.4	11.9
Total	39.1	0.6	23.9	20.9	33.4	25.7	35.2	0.1	22.8	21.8	34.2	28.0	32.3	0	24.8	21.1	25.2	36.2
Mean	13.0	0.2	8.0	7.0	11.1	8.6	11.7	0.1	7.6	7.3	11.4	9.3	10.8	0	8.3	7.0	8.4	12.1

(Continued)

Table A19. Continued.

Source	Analysis of Variance				
	df	SS	MS	F	
Replicates	2	72.7	36.3	6.1	**
Treatments	(17)	833.7	49.0	8.3	**
Solutions	2	0.5	0.2	<1	
Oxygen	5	788.2	157.6	26.5	**
Interaction	10	45.0	4.5	<1	
Error	34	202.1	5.9		
Total	53	1,108.4			

1/ Mean length of longest hyphae from five test discs in each treatment combination. Figures are divisions on lanometer (1 divn = 0.079 mm).

** Significant at 1% level.