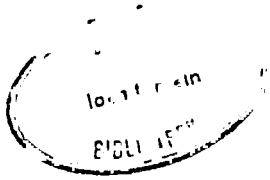

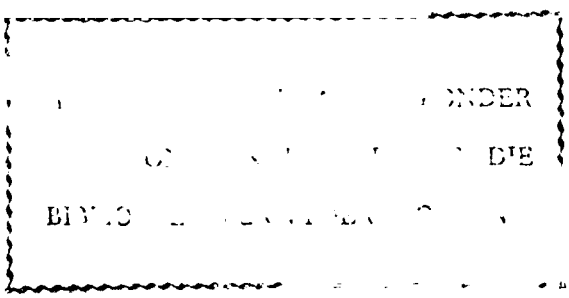

STUDIES ON THE SURVIVAL OF *VERTICILLIUM DAHLIAE* IN SOIL

by

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Thesis

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"Man does not know the total roster of micro-organisms in a single tiny lump of soil, let alone their complex interactions with each other and with their physical and chemical environment."

Baker & Cook, 1974

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PREFACE

I wish to express my sincerest appreciation and thanks to the following persons and instances:

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I certify that this thesis hereby submitted to the University of the Orange Free State for the degree of Doctor in Philosophy has not been previously submitted for a degree at any other University.

December, 1979

Schalk Willem Baard

CHAPTER 1

INTRODUCTION

The expansion of cotton production in South Africa has been accompanied by an increase in Verticillium wilt caused by *Verticillium dahliae* Kleb. The disease was found to be especially devastating on the heavier soils of the lower Orange River irrigation area and near Mkuze in Natal.

Verticillium dahliae is a plant pathogen of considerable economic importance, wide host range, and virtually world-wide distribution (Hall & Ly, 1972). The importance of vascular wilt diseases caused by this and other *Verticillium* spp. is stressed by the fact that an international *Verticillium* symposium was held at Wye College, University of London for the first time in September, 1971.

Isaac (1967) and Schnathorst (1973) reviewed the literature covering the controversy regarding the classification of *V. dahliae* and *V. albo-atrum* Reinke & Berth. Many taxonomists are of the opinion that the name "*V. albo-atrum*" should be used to include *V. dahliae*. Others consider them to be two different species. Klebahn (1913) distinguished between the two spp. in his original description of *V. dahliae* on the grounds that *V. dahliae* formed microsclerotia (MS) while *V. albo-atrum* did not. The ability of *V. dahliae* to form MS in pure culture was sometimes lost (Bewley, 1922; Rudolph, 1931), however, Schnathorst (1973)

could find no evidence that microsclerotial-forming Verticillia could be obtained from dark mycelial forms (V. albo-atrum).

It is clear that a distinction should be made between V. albo-atrum and V. dahliae on the grounds of their morphology, carbohydrate utilization, nitrogen utilization, pH requirements, response to temperature, and host range (Isaac, 1967; Schnathorst, 1973). According to Schnathorst (1973) no dark mycelial forms of Verticillium have been isolated directly from cotton in the United States of America.

The characteristics of V. dahliae have been described by Hawksworth & Talboys (1970) as follows: "Cultures growing rapidly on potato-dextrose agar (PDA) and malt agar (MA) at 23°C; the prostrate hyphae which are first produced are hyaline. Mycelium becoming flocculose and white, rather more densely compacted on PDA than MA; hyaline, whitish to cream in reverse after one week, later becoming black with the formation of microsclerotia. Hyaline sectors arise very frequently in the generally white colonies. Conidiophores abundant, more or less erect, hyaline, verticillately branched, 3 - 4 phialides arising at each node, phialides sometimes secondarily branched. Phialides variable in size, mainly 16-35 x 1-2,5 µ. Conidia arise singly at the apices of the phialides, ellipsoidal to irregularly sub-cylindrical, hyaline, mainly simple but occasionally 1-septate, 2,5-8 x 1,4-3,2 µ in var. dahliae; 5-12,5 x 1,6-3,4 µ in var. longisporum. Dark brown resting mycelium only formed in association with microsclerotia. Chlamydospores absent. Microsclerotia arising centrally in cultures, dark brown to black, torulose and botryoidal, consisting of swollen almost globular cells. Each microsclerotium arises from a single hypha by repeated budding. Microsclerotia very variable in shape, elongate to irregularly spherical; very variable in size, 15-50 (-100) µ diam."

Verticillium dahliae survives in the soil mainly as microsclerotia (Schreiber & Green, 1962) and is able to persist for many years in field soil in the absence of known host plants (Wilhelm, 1955). Microsclerotia are produced abundantly in tissues of host plants killed by this pathogen. Evans, Snyder & Wilhelm (1966) have shown that a single cotton plant growing in the field may return upward of 250 000 MS to the soil.

On account of its effective survival by means of MS (Schreiber & Green, 1962) and its extreme wide host range (Engelhard, 1957), V. dahliae is difficult to eradicate from cultivated soils. Efforts to control Verticillium wilt have included cultural practices, such as crop rotation, wilt-tolerant cotton cultivars, soil floo= ding, controlled irrigation and nitrogen applications, deep ploughing, fumigants, and systemic fungicides (DeVay, Forrester, Garber & Butterfield, 1974). However, these measures were relative= ly ineffective when soil propagules of V. dahliae were dense enough to cause near 100% prevalence of the disease (DeVay et al., 1974). The inoculum density of V. dahliae at the time a field is rotated to a non-susceptible crop might explain the "success" or "failure" of crop rotation (Benson & Ashworth, 1976). Because of the rela= tively low attrition rate of MS (Huisman & Ashworth, 1976) a pop= ulation in fields with 30 - 60 MS per gram soil would take several years (Benson & Ashworth, 1976) to reach the very low level of less than 3,5 MS/g soil to cause less than 100% disease (Ashworth, McCutcheon & George, 1972).

Ranney (1973) stated that intensification of research activities with regard to Verticillium wilt should be a major goal in cotton disease research. One of the major needs for continuous progress towards wilt control as pointed out by Ranney (1973) was studies on

the survival of the pathogen.

The difficulties encountered in the control of Verticillium wilt as expounded above, compel us to consider ways and means by which survival propagules may be reduced while cotton fields are devoid of host crops. Any information on factors controlling survival of MS is potentially of value in controlling diseases caused by the fungus.

The present study concerns the survival of MS in the field under natural and laboratory conditions. Factors affecting antagonist numbers in the soil were also investigated in order to consider the possibility of biological control. Linderman (1970) stated that an environment which favours the increase of organisms antagonistic to pathogens in soil is a desirable goal.

CHAPTER 2

REVIEW OF THE LITERATURE

Verticillium dahliae Klebahn was first described by Klebahn (1913) as a pathogen of dahlias. Since then Verticillium wilt, caused by V. dahliae, was reported to occur in all the major cotton growing areas of the world (Bell, 1973). The pathogen is also able to attack a wide range of plant species from different families (Engelhard, 1957) including commonly occurring weeds (Woolliams, 1966; Evans,1971).

Disease symptoms

V. dahliae isolates from cotton range in virulence from those causing defoliation and death to those which incite only mild chlorosis (Howell, 1973). The following general description of disease symptoms was taken from "The Yearbook of Agriculture" (Presley, 1953): ".....plants may be attacked at any stage of development. The cotyledons of infected cotton plants become yellowish and quickly dry out. Young plants with 3 - 5 true leaves suffer considerable stunting. The leaves appear darker green than those of a normal plant and become somewhat crinkled between the veins. The amount of stunting apparently depends on the stage of development of the plant when it becomes infected. The outstanding symptom is the chlorotic areas on the leaf margins and between the principle veins, which make it look mottled. In older plants the symptoms usually occur in the lower leaves first. They spread to the middle and upper leaves

of the plant later in the season. The clorotic areas gradually become larger and paler. Severely affected plants shed all the leaves and most of the bolls. Older plants may nevertheless survive the entire season and sometimes send up sprouts from the base of the plant."

Fungus penetration and development

Fungus penetration and development has been reviewed by Garber (1973).

Verticillium dahliae is able to survive in soil for extended periods (Nelson, 1950; Wilhelm, 1955; Schreiber & Green, 1962; Martinson & Horner, 1962). Classified as a soil-invading or root-inhabiting fungus, sensu Garrett (1956), infective propagules of V. dahliae remain quiescent in the soil until they are stimulated to germinate by nutrients (Schreiber & Green, 1963; Emmatty & Green, 1969). Root exudates from hosts very often supply such nutrients (Schreiber & Green, 1963). Garber & Housten (1966) described the process of infection as follows: Upon germination the fungus is capable of penetrating young, uninjured cotton seed= lings anywhere from the root cap to the hypcotyl within a matter of 24 h from the time of inoculation. The cortical tissue did not seem to offer much resistance to the further penetration of the fungus, but when it reached the primary vascular tissues there was a noticeable retardation of fungus growth.
Once the pathogen reached the xylem vessels, spores started to form, presumably by budding. This happened within 3 days

following inoculation. Conidia moved freely with the sap stream from one vessel to another and reached the top of the plant within a very short time. The fungus invaded the smallest vessels of the leaf blade, but did not invade the leaf parenchyma. Vessels invaded by the fungus invariably discoloured brown.

Garrett (1970) stated that in a suitably humid atmosphere, a vascular wilt fungus would emerge on the outside of the stem and the leaf petioles and sporulate thereupon when death of the plant was approaching, though not before. Garrett (1970) concluded that the mature parenchymatous tissues surrounding the vascular tracts are resistant to infection and that this resistance broke down only with the advent of disease-induced senescence.

Evans, Snyder & Wilhelm (1966) studied the course of invasion and sporulation of V. dahliae within and upon the cotton plant. They found that early invasion of cotton seedlings was followed by complete defoliation within 8 weeks from the time the first symptoms became evident. Defoliated plants, left in the field, became invaded by V. dahliae and MS were formed within the roots and basal 7,5 - 10 cm of the stem. The stems of very young plants were sometimes covered with conidiophores along the basal 5 - 10 cm, indicating that the fungus grew from the vascular tract through the cortex.

Older plants which became infected during the summer, did not usually succumb to the pathogen and Evans et al. (1966) did not find MS within these plants. When these plants were investigated during fall and winter, however, numerous MS were detected within their stems and roots.

Decaying leaves collected in the field during the growing season were found to contain MS in the petioles, vascular tissues, and mesophyll (Evans et al. 1966). The same authors estimated that a single cotton plant may return upward of 250 000 MS to the soil.

The nature of the infective propagule

Verticillium dahliae produces mycelium, thin-walled conidia, and microsclerotia on and within moribund cotton tissues (Evans et al. 1966). The presence of long-lived, hyaline chlamydospore-like cells which retained viability in white variants of V. dahliae have also been reported (Schnathorst, 1965; Tolmsoff, 1973).

In earlier attempts to determine the longevity and nature of the infective propagules, investigators made use of indicator plants. Schreiber & Green (1962) investigated the comparative survival of mycelium, conidia, and MS of V. dahliae in moist soil. After 82 weeks 0,5 mg of MS / g of soil continued to cause 100% infection, whereas 1, 3, or 7 mg of conidia plus mycelium / g of soil caused no infection after 14 weeks.

Green (1969) compared the inoculum potential and survival of conidia and MS in soil and found that 50 000 conidia as compared to 100 MS were needed per g soil to cause 100% infection in tomatoes. The inoculum potential of conidia decreased from 100% to zero after 3 weeks, while there was no reduction in the inoculum potential of MS after 7 weeks.

Green (1969) also studied the survival of conidia and MS by direct soil assay over a 14 - week period. Conidia declined from 100 000 / g to 5 000 / g soil within 4 weeks. The fungus, however, was still detected in soil after 14 weeks when conidia were used as the inoculum. This suggests either limited colonization of soil organic debris or conversion of conidia or mycelial fragments to more resistant propagules (Green, 1969). Microsclerotia decreased by about 20% from the original inoculum level of 5 000 propagules / g soil during the 14 - week period (Green, 1969).

Various other workers also reported that conidia and mycelia do not survive in soil for extended periods (Wilhelm, 1951; Isaac, 1946; 1953; 1956; Green, 1960). Powelson (1970), however, reported that potato plants became infected when planted in soil infested 6 months earlier with conidia of the microsclerotial strain of Verticillium.

The improvement of direct soil assay techniques led to further studies on the behaviour of the pathogen in the soil. By the use of such a technique, Menzies & Griebel (1967) have shown that after incorporation of MS into the soil,

the population increased 2 - 3 times beyond the initial inoculum concentration within the first two weeks, and then declined slowly. They also indicated that the popula= tion can remain 20% higher after 2 or 3 months.

Green & Papavizas (1968) reported that nutrients (glucose and sucrose) when added to the soil, caused propagules of V. dahliae to increase, but the population declined rapidly thereafter, indicating that there was production of spores or other propagules not as persistent in soil as the original MS. It took approximately 42 days for the increased popula= tion to decline to the level with which the soil was origi= nally infested. No increase was found in the controls.

Farley, Wilhelm & Snyder (1971) found that MS were capable of repeated germination and sporulation in soil repeatedly dried and remoistened. Their evidence suggests that under field conditions, MS may germinate and sporulate several times in the vicinity of organic matter or the rhizosphere of non-host plants and still have the capacity for growth and infection when contacted by host roots.

Emmatty & Green (1969) reported the formation of secondary microsclerotia from germinating MS, and suggested that these propagules accounted for the population increases observed by other workers.

Evans & Gleeson (1973) produced evidence that MS did not germinate to form conidia as an intermediate stage in the

infection process. The results of Evans & Gleeson (1973) and Evans, McKeen & Gleeson (1974) suggest that the MS itself serve as the sole means of survival of the pathogen and that infection is by germinating microsclerotia rather than by conidia.

The life cycle of the pathogen

According to Tolmsoff (1973) who reviewed the literature, the probable life cycle of V. dahliae is as follows:

"1. Stimulation of MS germination by host root exudates, especially at flowering time. The ploidy of germ cells is not known, but eventually haploids have to be produced for infection.

2. Production of large, possibly polytenic, haploid conidia that germinate rapidly and infect roots.

3. Invasion of the vascular system by the penetrating haploid hyphae.

4. Proliferation of the haploid within the vascular system, primarily by autoconidiation; conidia are carried and distri= buted by the xylem fluids.

5. Killing of host tissues, particularly leaf tissues, by the haploid.

6. Secondary invasion of dead host tissues by haploid mycelia; production of more conidia by verticillate conidiophores.

7. Transition from haploid to diploid state with enlarged hyphae and swollen conidia to produce black resting structures (MS) in the dead plant tissues.

8. Aging and internal production of haploid variants within

the MS; changes in dormancy probably occur with aging.
9. Release of aged MS from decomposed plant residues; random germination of less dormant MS cells, leaving more dormant propagules to be stimulated into germination by root exudates of the hosts."

Control

Maximum control of Verticillium wilt can be accomplished by combinations of the use of chemicals, cultural practices, and tolerant cultivars (Minton, 1973).

Chemical control

The chemical control of Verticillium diseases in field crops is aimed at (i) reducing the quantity of inoculum in post-harvest plant debris, (ii) reducing the soil inoculum by fumigation, and (iii) in the growing crop, limiting fungal growth by systemic compounds (Pegg, 1974). Various soil fumigants have been tried against Verticillium wilt of cotton with variable results. Where wilt has been controlled by soil fumigation, yield increases have not always offset the cost of chemicals and their application (Pegg, 1974).

Systemic fungitoxic chemicals have also been tried. Amongst these, benomyl and thiabendazole gave excellent control of wilt in the greenhouse, but gave fair to poor control of the disease in the field (Minton, 1973). The mutagenic effect of some systemic fungicides (e.g. benomyl) with the

possibility of resistant strains arising, should also be taken into account when their long-term use is considered (Pegg, 1974). Their selective anti-fungal properties might also have a detrimental effect on the micro-flora and fauna of the soil (Pegg, 1974).

Minton (1973) has concluded that all the chemicals tested have not given the desired control of wilt and that the returns from increased yield have not exceeded the cost of treatment.

Crop rotation

In a review by Powelson (1970) it was stated that the "dauermycelium" (DM) type of Verticillium (= V. albo-atrum) can be controlled effectively by crop rotation, but not the microsclerotial type (= V. dahliae).

Ranney (1973) reviewed the subject and argued that experimental proof exists that short term (5 year) rotations do have a benficial effect on Verticillium wilt of cotton. Rotating cotton with sweet clover, lucern, or lespedeza every 2 or 3 years resulted in marked reductions in the incidence of Verticillium wilt. Rotations with other crops, however less spectacular, also resulted in a reduction in wilt incidence (Hinkle & Fulton, 1963). Huisman & Ashworth (1976) expressed the opinion that short term rotations are of little value in the control of Verticillium wilt. Their results indicate that inoculum buildup from

very low populations (1 - 5 MS / g soil) to 30 - 40 MS / g soil can take place within one to two years. These values are over ten times the level (3,5 MS / g soil) needed to cause 100% infection in a cotton crop (Ashworth et al. 1972). Huisman & Ashworth (1976) indicated that although attrition does occur during nonsuscept culture, the rate is so slow that the Verticillium population level was still high enough after 6 years of non-host cropping to cause 100% infection in cotton. Butterfield, DeVay & Garber (1978) reported significant reductions in populations of V. dahliae following one-year rotations with ryegrass and paddy rice. Disease symptoms of cotton following paddy rice or ryegrass were reduced while lint yield increased.

Production practices

Ranney (1973) drew the following conclusions on cotton production practices for the control of wilt: Excessive nitrogen induces vegetative growth and late maturation and thus often increases disease incidence and yield loss. Too high or too low rates of potassium cause higher disease incidence and yield loss. The application of minor elements decreased wilt incidence and increased yield under some conditions, but not in others. A balanced nutrient supply of either minor or major elements, is recommended to reduce disease incidence and yield loss.

Irrigation is of paramount importance. Where cotton is irrigated, timing, and frequency of water application

influences Verticillium wilt occurrence and severity. Lighter applications, more frequently applied, increased disease incidence. Irrigation also reduces soil temperature which again causes higher wilt incidence and severity.

Increased plant populations increase yields and reduce the incidence of wilt.

The spread of infected plant material to disease-free fields, should be prevented. Plant residues should be shredded as finely as possible and incorporated into the soil as soon as possible after harvest. In drier areas one or two post-harvest irrigations might be necessary to induce faster microbial breakdown of stalks. Deep plowing, and particularly when the soil is completely inverted, is an effective way of reducing disease loss.

Many weeds are hosts of V. dahliae and should be eradicated to reduce pathogen populations. However, weed control by cultivation should be executed with care. Research has shown that root pruning in the presence of the pathogen is an excellent method of inoculating cotton with Verticillium wilt. It has also been indicated that as the depth of cultivation increased, the incidence of Verticillium wilt increased.

Planting should be done at the optimum time for rapid seed germination and seedling growth. Planting in beds has the effect of warming up the soil sooner, which is beneficial to

stand establishment as well as wilt control.

Resistance

The literature on the nature of disease resistance in cotton has been reviewed by Bell (1973) who came to the following conclusions: (i) Immunity to the most virulent strains of *V. dahliae* is absent from all *Gossypium* spp. (ii) High levels of tolerance are found only in cultivars of *G. barbadense* which show few if any wilt symptoms under field conditions. (iii) Attempts to transfer the *G. barbadense* level of resistance to *G. hirsutum* have been unsuccessful. (iv) By selection and hybridization some wilt tolerant cultivars have been developed which, however, may still be severely damaged under adverse conditions of climate or management.

Bell (1973) has also investigated factors such as the effect of the host, the effect of the pathogen and the environment on disease resistance in cotton and concluded that the level of tolerance displayed by the cotton plant is eventually influenced by all these factors.

Ranney (1973) states that while changes in cultural practices have been of benefit, it is the improvement in cultivars that has minimized disease loss.

The effect of antagonists on plant pathogens in soil

"Plant pathogens are necessarily adapted to their biotic and

abiotic surroundings and may not be easily subdued or extin= guished by manipulation of the environment. They exploit every advantage, no matter how small, often in amazingly re= sourceful ways. This versatality is an obvious strength of the pathogen and a potential difficulty in biological control" (Baker & Cook, 1974).

V. dahliae depends mainly on its MS for survival in soil in absence of host plants and is therefore capable of bridging adverse conditions until host plants become available again (Wilhelm, 1955). Sclerotium-forming pathogens should be controllable by biological means during the host-free period (Baker & Cook, 1974). However, it might be necessary to impose a change in the microbiological balance in the soil to achieve such a purpose (Baker & Cook, 1974).

The soil microflora are more or less inactive because of a lack of suitable nutrients in the soil (Garrett, 1956; Lingappa & Lockwood, 1964; Clark, 1965; Menzies, 1965; Patrick & Toussoun, 1965; Ko & Lockwood, 1967; Steiner & Lockwood, 1969). When nutrients are in short supply, the pathogens that persist in an inactive state between intervals of association with host plants are therefore not under unu= sual stress (Menzies, 1965). However, when nutrients are added to the soil to stimulate the general microbial activity, such pathogens are probably exposed to greatly intensified adverse factors, particularly antibiosis and predation (Menzies, 1965).

The effectiveness of antagonists in biological control requires that the population density of antagonists in an active state be sufficiently high (Patrick & Toussoun, 1965). It is also desirable in biological control to activate resident antagonists whenever possible (Baker & Cook, 1974).

Antagonists in soil may be stimulated by addition of organic amendments selectively favourable to them, by continued monoculture, by reinforcing the antagonists by addition of more of the specifically active ones, or by selective treatment with chemicals or steam to reduce micro-organisms that inhibit the antagonists or to weaken the pathogen and make it more vulnarable to antagonists (Baker & Cook, 1974). Mineral nutrients (Dimock, 1965) and selective chemicals (Papavizas, 1973) have been shown to stimulate the development of a different type of microflora in soil which had substantial antagonism to troublesome root pathogens.

Since a good deal of inhibition of pathogens results from weakening of survival structures by an active general soil microflora, factors such as addition of plant remains, manure, inorganic fertilizers, and alteration of pH, which increase the total number of micro-organisms, are useful in biological control (Baker & Cook, 1974)

The role of antagonists in the deterioration of sclerotia in soil

Sclerotia present a sizeable surface area for attack by micro-organisms. They are known to leak nutrients and support a

significant surface flora (Baker & Cook, 1974). The antago=nistic surface flora may stimulate nutrient leakage directly or by the osmotic effect of rapid removal of leachates from the surface of the parasite (Ko & Lockwood, 1967; Griffin, 1972). The higher the activity of the micro-organisms on the surface of the sclerotia, the more energy will be expended by it with the result that endolysis will ensue (Baker & Cook, 1974).

Many organisms claimed to be parasites of sclerotia have been isolated. These include Trichoderma viride on Sclerotinia trifoliorum and S. sclerotiorum; Gliocladium roseum and Coniothyrium minitans on S. trifoliorum; C. minitans on S. sclerotiorum; Penicillium frequentans on S. borealis; Mucor hyemalis on Claviceps pupurea; Trichoderma harzianum on Sclerotium rolfsii; and T. hamatum on Sclerotium delphinii (Henis & Chet, 1975).

Soil physical factors as well as biological factors are impli=cated in the deterioration of survival propagules of fungi in soil. Smith (1972a,b,c) has claimed that dried sclerotia of a number of fungi including Sclerotium cepivorum and S. rolfsii rot more quickly than non air-dried ones when placed in moist soil. He ascribed this decay to the intense microbial activity resulting from leakage by the dried sclerotia. However, Coley-Smith (1959) found that a large percentage of air-dried sclerotia of S. cepivorum survived in soil for 4 years. Dried sclerotia of Sclerotium delphinii rotted in moist soil whereas those of S. cepivorum, Botrytis cinerea, and B. tulipae did not (Coley-Smith, Ghaffar & Javed, 1974).

Drying and remoistening of the soil cause a decline in numbers of viable sclerotia of Sclerotinia sclerotiorum (Adams, 1975). The survival of S. sclerotiorum is hampered by wet summers and high temperatures (Halkilahti, quoted by Adams, 1975). Schmidt and Williams & Wester (Adams, 1975) reported that sclerotia survived better in cropped than in uncropped soil. Soil moisture also had an influence.

The microsclerotia of V. dahliae contain melanin in their cell walls which render them highly resistant to biological breakdown in soil. Melanins, located in cell walls, have the ability to inhibit the activity of cell-wall-lysing enzymes (Kuo & Alexander, 1967; Bull, 1970). However, biodegradation of pigmented fungal propagules in soil does occur (Old, 1977b).

One mechanism by which biodegradation of resistant propagules can take place, is through damage of the resistant cell walls. This phenomenon has been termed "perforation lysis" and the subject has recently been reviewed by Old & Wong (1976) and Old (1977b).

According to Old & Wong (1976) perforation lysis is caused by a component of the soil microbiota and has been found in spores incubated in soils from various localities (Old, 1977b). The agent responsible for such perforations seems to be widely distributed in soils.

Perforation lysis has been observed in a number of fungi including Cochliobolus sativus, Thielaviopsis basicola, (Clough &

Patrick, 1972), Alternaria tenius, Curvularia protuberata, Stemphylium dendriticum, Cladosporium spp., and Stachybotrys atra (Old & Wong, 1976).

Reisinger (Old 1977b) showed that bacteria colonized the lumina of conidia of Helminthosporium speciferum after they were damaged by mites. Naiki & Ui (1975) could not demonstrate perforations in the sclerotial cell walls of Rhizoctonia solani, but bacteria were detected in the amorphous layer, the cell wall matrix, and the empty sclerotial cells. Soil fungi also invaded the empty cells.

Old (1967) showed that conidia of C. sativus became perforated by holes allowing invasion of the spores by a wide range of soil micro-organisms. The non-melanized wall components were completely digested, leaving an empty, perforated spore shell (Old & Robertson, 1970; Wong & Old, 1974).

Old & Patrick (1976) studied perforations and lysis of conidia of C. sativus and chlamydospores of T. basicola in natural soil and suggested that holes up to 0,5 µm diameter were caused by direct penetration of the spore wall by bacteria. The larger holes were caused by soil amoebae (Old, 1977a,b).

The role of antagonists in the control of Verticillium wilt

Various fungal spp. have been reported to reduce the incidence of Verticillium wilt when they were applied to soil or cotton roots. They include: Aspergillus terreus, A. melleus,

Penicillium funiculosum, Cephalosporium spp., Chaetomium spp., Gliocladium spp., Botryodiplodia spp., Phoma spp., Colletotrichum gossypii, Stachybotrys spp., Myrothecium spp., Podospora spp., and Blastomyces luteus (Bell, 1973; Brinkerhoff, 1973).

Reports from Russia (Brinkerhoff, 1973) indicate that 50 to 70% control of Verticillium wilt was obtained by incorporating cultures of actinomycetes on cottonseed cake into the soil.

The effect of Trichoderma spp. on Verticillium wilt is not clear. Trichoderma viride has been found to enhance the development of wilt (Bell, 1973). However, Marupov (1974) reported that spore preparations of T. viride suppressed development of V. dahliae in soil. Compost containing Trichoderma spp. increased the development of micro-organisms antagonistic to V. dahliae when added to the soil with the result that wilt decreased and yields increased (Egamov, 1974). When trichodermin - 2, which was prepared from T. viride, was spread on the soil, disease incidence was reduced 2 to 4 times and the effect was retained for several years (Tillaev, 1977).

The effect of soil moisture and temperature on the survival of V. dahliae

According to Nadakavukaren (1960) MS of V. dahliae did not survive well in air-dry or flooded soils at temperatures from 5 to 40°C. Temperature, however, appeared to be more critical than moisture. Survival of MS in soil at 50% or 75% water-

holding capacity was 12 days to 6 months at 30°C, 3 to 35 days at 40°C, and more than 6 months at 5 to 15°C. Nadakavukaren (1960) could not detect viable MS in air-dry and 25% field capacity soil treatments after 4 months, while in flooded soil Verticillium could not be recovered after one month. In soil kept at 25 and 30°C detectable survival was best at 50 and 75% field capacity.

Schreiber & Green (1962) found that MS survived in soil at 50% water-holding capacity and at 25°C for more than 82 weeks.

Menzies (1962) reported that MS did not survive for more than 6 weeks in flooded soil. He attributed their death to anaerobic fermentation and demonstrated that MS were also killed in soil kept at 15% moisture under nitrogen gas for 3 weeks. when such soil was amended with lucern meal or sucrose, sclerotia were killed in 5 days.

In rotation studies in the field, Butterfield (1975) could not detect an appreciable reduction in viability of MS in soil flooded for 6 weeks. He admitted, however, that his method of flooding did not keep the soil continuously flooded, but rather nearer to field capacity. In a later study (Butterfield, DeVay & Garber, 1978) it was found that a significant reduction in viability of MS of V. dahliae occurred when cotton was grown in rotation with paddy rice which was flooded for 15 weeks. The water level within the plots was maintained at a depth of 15 - 30 cm.

The effect of soil amendments on survival and disease incidence

The survival and multiplication of soil-borne plant pathogens as affected by plant tissue amendments has been reviewed by Lewis & Papavizas (1975).

Wilhelm (1951) found that crop residues as well as amendments high in nitrogen, such as fish meal and blood meal, were effective in reducing the incidence of Verticillium wilt of tomato. Green & Papavizas (1968) point out, however, that such amendments could have an effect on the host rather than the *Verticillium* population. In the studies by Green & Papavizas (1968) in which propagule counts were made after incubation with soil amendments, it was found that lucern and oat residues caused a reduction of 50 - 60% in propagule numbers. Glucose, sucrose, and ribose also caused significant reductions in *Verticillium* populations.

Although amendment decomposition is an important factor in the complex ecological environment affecting survival of a soil-borne plant pathogen, this subject has received little attention during recent years (Lewis & Papavizas, 1975).

Jordan *et al.* (1972) investigated the effect of chitin, laminarin, wheat straw, and clover as soil amendments on the incidence of Verticillium wilt of strawberry. The results indicated that disease severity as well as the population of *V. dahliae* decreased significantly after soils were amended with laminarin and chitin at a rate of 0,2% (m/m).

Young *et al.* (Brinkerhoff, 1973) recorded reduced disease and increased yields after incorporation of lucern meal plus ammonium nitrate into the soil. This treatment sharply de= creased the disease incidence below that achieved with inor= ganic fertilizers rich in nitrogen. Menzies (1962) found a reduction in MS populations of *V. dahliae* in soil amended with lucern residues or sucrose and incubated under anaerobic conditions. Menzies (1962) also postulated that a diffusible fungicidal compound might be implicated.

The effect of volatile substances extracted from lucern hay on the survival of *V. dahliae* was investigated by Gilbert & Griebel (1969), who found that higher concentrations of the volatiles eliminated *V. dahliae* from soil.

The effect of soil pH and acidifying substances on disease incidence and survival of fungal sclerotia

Henis & Chet (1975) stated that the manipulation of soil pH as a means of control of plant pathogens is possible under the following conditions: (1) the pathogen must be capable of growing and inciting disease only at a relatively narrow pH range; (2) the host plant must be capable of growing at a pH range which is not suitable for the pathogen; (3) the buffering capacity of the environment may not be too great. Soil pH may also affect disease severity by changing the disease susceptibility of the host or by favouring the activity of the antagonistic microflora. Addition of sulphur to soil has been successfully used to control diseases caused by actino=

mycetes by lowering soil pH to 5,2 - 5,5 (Henis & Chet, 1975).

It has been proved that various diseases can be controlled by changing the soil pH from alkaline to acid conditions. Jackson (1940) reported that reduced damping-off of conifers occurred in soils with lower pH values. Taubenhaus & Ezekiel (1937) reported that Phymatotrichum omnivorum caused more disease, spread faster, and overwintered better in alkaline than in acid soils. Lyda (1973) attributed this phenomenon to the inability of low pH soil to retain CO_2 which is a pre= requisite for sclerotium formation by P. omnivorum. Black root rot of tobacco caused by Thielaviopsis basicola was also controlled by acidifying the soil (Doran, 1931). When the inoculum level does not exceed certain limits, Gaeumannomyces graminis is of minor importance on acid soils (Garrett, 1970). In culture T. viride proved to be parasitic on Armillaria mellea only at pH 3,4 - 5,1 (Aytoun, 1953) and Schüepp & Frei (1969) found that the fungistatic effect of the soil against T. koningii increased with increasing pH. Weindling & Fawcett (1936) obtained control of damping-off of citrus seedlings by acidifying the soil, but control was only effective in unsterilized soil.

Although Wilhelm (1950) supplied evidence that Verticillium wilt could be found in soils with pH values as low as 4,5, Verticillium wilt is now regarded as a disease prevalent on alkaline soils (Baker & Cook, 1974). Reports of control by acid soil conditions have also appeared in the literature as early as 1928 (Haenseler, 1928). Guba (1934) obtained

control of eggplant wilt caused by V. albo-atrum in green-house experiments by acidifying soil to below pH 5 with aluminium sulphate. When sulphur was used for acidification, disease, although slight, was still observed at pH 4 - 4,2. In Verticillium-infested soils, eggplants yielded 32% more marketable fruits on acid plots (Martin, 1931). Liming of the soil increased disease incidence and severity of Verticillium wilt of eggplant (Haenseler, 1928; Martin, 1931) and tomato (Jones & Woltz, 1972), while Chester (1942) stated that Verticillium wilt of cotton was restricted to highly alkaline soil.

The older literature frequently refers to the soil sterilizing effect of aluminium sulphate which was used to reduce soil pH (Line, 1926; Wiant, 1929; Steinmann, 1930). However, Line (1926) doubted the view that aluminium salts were toxic to micro-organisms. Sykes (1965) also stated that aluminium salts were not very toxic to micro-organisms and only became toxic at concentrations of 12% or higher. Weindling & Fawcett (1936) proved that the control of damping-off of citrus obtained by the application of aluminium sulphate or acid peat moss to change the soil reaction to pH 4, was effective in unsterilized soil only. The disease was not controlled in sterilized soil of the same acidity in the absence of Trichoderma spp. Weindling & Fawcett (1936) suggested that the change in soil pH favoured the development of organisms antagonistic to Corticium solani (= Thanatephorus cucumerus).

Leszczenko (1928) investigated the effect of soil solutions

on the germination of some pathogens and concluded that no injurious action on germination of fungal spores, including those of V. albo-atrum, was found to be exerted by the aluminium ion in soil solutions. Van Wyk & Baard (1971) recorded 23% germination of conidia of V. dahliae on soil acidified to pH 4,5 by incorporating aluminium sulphate. At pH 3,8 no germination was observed. Isaac (1967) and Malca et al (1966) proved that Verticillium was tolerant to fairly low pH values in pure culture.

Evidence that soluble aluminium (Al) is toxic to micro-organisms can also be found in the literature. Ko & Hora (1972) reported that germination of ascospores of Neurospora tetrasperma was completely inhibited in solutions containing 0,65 ppm Al at pH 4,8. Moreover, they concluded that the effect of aluminium in the soil was fungicidal and not fungistatic. Johnson, according to Orellana, Foy & Fleming (1975) showed that Al inhibited growth of V. albo-atrum in nutrient culture. Orellana et al. (1975) studied the effect of soluble Al on growth and pathogenicity of V. dahliae and found that growth was inhibited on agar media containing 8 ppm Al. The pathogen was characterized by hyaline, apparently unpigmented mycelia and few, if any microsclerotia. This Al-sensitivity was related to the toxicity of soluble Al in the culture substrate at pH 4,7 or below. Disease symptoms on sunflower plants were increased when the soil pH was increased from pH 4,4 - 5,4.

Although it was stated that the pH of the soil did not appear to have any pronounced effects on survival of sclerotia of

most fungi (Coley-Smith & Cook, 1971), some notable exceptions to this generalization do exist. Helicobasidium pupureum survives well in alkaline soils, but not in acid soils. The decreased survival of sclerotia in acid soils is associated with a high incidence of spontaneous germination (Valder, 1958). Similarly, Phymatotrichum omnivorum does not persist in acid soils (Lyda, 1973). Sclerotium rolfsii, on the other hand, is sensitive to high dosages of ammonia and Henis & Chet (1967) concluded that the direct toxic effect of ammonia on S. rolfsii was a function of high pH and time.

Structure and germination of microsclerotia of V. dahliae

Verticillium dahliae exists in soil as microsclerotia, either as free units or embedded in decaying plant tissue (Evans et al. 1966; Ashworth et al. 1972). Evans et al. (1966) also esta= blished that V. dahliae lost its viability fairly quickly in natural soil. However, various workers claimed that V. dahliae may maintain a high inoculum potential in soil for many years (Wilhelm, 1955; Martinson & Horner, 1962; Schreiber & Green, 1962). Schreiber & Green (1962) have indicated that the survival of V. dahliae in mineral soil in the absence of host plants is dependent on continued viability of microsclerotia.

The MS of V. dahliae are rindless and consist of poorly organized aggregations of three kinds of cells: thick-walled, melanized, vacuolate cells with mitochondria and other cytoplasmic inclusions; thin-walled, hyaline or lightly pigmented cells in close contact with the thick-walled cells; and short thin-walled hyphae

intertwined in the sclerotial mass (Nadakavukaren, 1962; 1963; Schnathorst, 1965). It has been thought for some time that both hyaline and hyphal cells can germinate. It has been claimed that the melanized cells have a protective or storage function and that they can act as a source of substrate for germinating hyaline cells (Gordee & Porter, 1961; Nadakavukaren, 1962; Schnathorst, 1965; Isaac & McGarvie, 1966). Later studies on ultrastructure suggest, however, that the hyaline cells are non-functional while the heavily-pigmented cells are capable of germination (Brown & Wyllie, 1970). The latter have organized cytoplasm, including a single nucleus and mitochondria,and are interconnected via septal pores. Germination may take place through these septal pores into degenerate hyaline cells and intracellular hyphae may extend through several cells. The prior claim of germination of hyaline cells may therefore be due to observation of germ hyphae that had grown from adjacent pigmented cells (Brown & Wyllie, 1970). Whether this can explain the observed germination of isolated hyaline cells and lack of germination of isolated pigmented cells is still open to question (Coley-Smith & Cook, 1971).

The MS of V. dahliae are ideally suited to sustain the fungus in the soil for long periods in the absence of a host (Schreiber & Green, 1963) while conidia and mycelia do not persist in soil for extended periods (Schreiber & Green, 1962; Menzies & Griebel, 1967).

Isaac & McGarvie (1966) found that resting bodies of V. dahliae did not germinate without soaking in water, and may therefore

be considered as dormant. The dormancy, however, is not related to maturity, in that there was no evidence of a "rest" period as an essential prerequisite for germination. Green, according to Pegg (1972), has shown that MS of V. dahliae have no inherent dormancy nor do they require exogenous nutrients for germination. Schreiber & Green (1962; 1963) point out, however, that all the cells of a single MS would not germinate under any single set of environmental conditions.

Tolmsoff (1973) reported that germination of air-dried MS of V. dahliae was highly asynchronous, suggesting dormancy. After being washed and plated on agar media, the majority of viable MS germinated in 24 to 48 hours, but some required up to 24 days for germination.

When soils amended with MS of V. dahliae were moistened, a temporary increase in propagule counts was found (Menzies & Griebel, 1967; Farley et al. 1971). The propagules responsible for these increases were conidia which formed from germinated MS. When the soils were dried and remoistened nine times consecutively, increases in propagule counts were found after each remoistening (Farley et al. 1971). This indicates a wide range in the degree of dormancy among the cells of individual MS (Tolmsoff, 1973).

Menzies & Griebel (1967) were of the opinion that the MS of V. dahliae are not fully dormant structures that remain quiescent until stimulated to germinate by host roots. They offered proof that MS are able to germinate and, apparently, sporulate

over an extended period of time in uncropped soil.

According to Powelson (1970) Carlstrom assayed MS in infested soils under field conditions over a 5-year period and found seasonal sporulation cycles.

Schreiber & Green (1963) demonstrated that MS are subject to soil fungistasis. In their experiments 11,5% of the MS germinated when in contact with soil while 91,7% germinated in the control. Emmatty & Green (1969) found 17% germination in soil and 96% in the control. However, root exudates of host and non-host plants (Schreiber & Green, 1963), various sugars and amino acids (Emmatty & Green, 1969), and volatiles from plant residues (Gilbert & Griebel, 1969) were able to nullify the inhibitory effect of soil fungistasis on V. dahliae.

The problem of determining the viability of V. dahliae in soil

Various workers have published methods for assaying populations of V. dahliae in soil. The number of articles concerned with such assays emphasizes not only the importance of this widespread plant pathogen, but also the difficulties encountered in estimating the abundance of its propagules. Susceptible indicator plants were used by various investigators to determine the disease producing potential of Verticillium-infested soils (Wilhelm, 1950; 1955; Isaac, 1957; Schreiber & Green, 1962). However, Garrett (1970) stressed that systemic diseases such as Verticillium wilt can be incited by a single pathogen propagule. In soils with a high population of

V. dahliae it is therefore conceiveable that the widespread root system of cotton would become infected by a large number of propagules which would not be reflected in the number of diseased plants. This fact has been shown up clearly by Evans et al. (1974) who demonstrated that multiple infections by MS occurred on the roots of Datura stramonium which does not become systemically infected by V. dahliae. This property of D. stramonium has been used by Evans et al. (1974) to bioassay soils for Verticillium populations.

An accurate quantitative assay for soil-borne populations is necessary in studies on the survival of V. dahliae. The slow growth characteristics of the pathogen and its susceptibility to inhibition by volunteer (unwanted) fungi in pure culture, limits the usefulness of traditional soil-dilution techniques in the absence of selective isolation media.

Several papers describing methods and media for estimating the numbers of V. dahliae in soil, have been published. Nadakavukaren (1960) described an ethanol-streptomycin agar medium (ESA) which was useful for dilution-counting of propagules in soils artificially infested with large numbers of the pathogen. Ausher, Katan & Ovadia (1975) found it necessary to improve Nadakavukaren's medium. They added sucrose and pentachloronitrobenzene (PCNB) to ESA and incubated their cultures at 18°C.

Menzies & Griebel (1967) developed a soil-extract agar containing antibiotics, for the isolation of V. dahliae. However, Farley, Wilhelm & Snyder (1971) stated that this medium could be improved by the addition of polygalacturonic acid as suggested by Green & Papavizas (1968) and PCNB. Polygalacturonic acid

enhanced the identification of V. dahliae by stimulating micro= sclerotial development and pigmentation of the colonies (Green & Papavizas, 1968). PCNB prevented certain fast growing fungi from obscuring colonies of V. dahliae on the dilution plates (Farley et al. 1971).

Jordan (1971) found Nadakavukaren's (1960) and Menzies & Griebel's (1967) media unsatisfactory in his studies. Consequently Jordan (1971) developed a sorbose agar medium with which he isolated V. dahliae from strawberry soils.

Ashworth et al. (1972) were able to recover very low numbers of V. dahliae from naturally infested soils by wet-sieving and plating residues on cellophane overlying sugarless agar. Further improvements of procedures and substrates were later published. A pectate agar medium was developed to replace the cellophane-sugarless agar (Huisman & Ashworth, 1974a).

Evans, Snyder & Wilhelm (1966) made use of a flotation tech= nique which was later improved by Evans et al. (1967), to collect and concentrate the MS from soil and to remove clay and other particles from the soil suspension. The fraction containing the MS was then plated on dilute PDA containing antibiotics and PCNB.

Huisman & Ashworth (1972) employed a sucrose flotation tech= nique which enabled them to use much larger soil samples than Evans et al. (1967). Another flotation method in which cesium chloride was used, was described by Ben-Yephet & Pinkas (1976).

Harrison & Livingston (1966) and DeVay *et al.* (1974) have described the use of an "Anderson" air sampler for distributing soil on suitable agar sustrates for assays of *Verticillium*. Butterfield & DeVay (1977) used the "Anderson" sampler in conjunction with a modification of Huisman & Ashworth's (1974a) pectate agar to isolate *V. dahliae* from air-dried soils.

Apart from the various techniques, agar medium composition, and chemicals from different sources, some other factors such as soil type, prior storage treatments, dry or wet plating, and milling procedures also influence the apparent numbers of viable propagules which can be recovered from soil (Ashworth, Harper & Andris, 1974; Evans *et al.* 1974; Butterfield, 1975; Butterfield & DeVay, 1977). Butterfield & DeVay (1977) concluded that reductions of more than 75% in propagule counts were observed when sodium polygalacturonate from different sources was used in the agar medium.

CHAPTER 3

MATERIALS AND METHODS - GENERAL

3.1 The pathogen

Verticillium dahliae was isolated from diseased cotton plants from a cotton field at the Agricultural College at Glen, near Bloemfontein, and was used throughout in this study. Stock cultures were kept on potato-dextrose-agar (PDA) medium.

3.2 Soils

Glen soil: Soil was collected from Glen, air-dried, sieved through a 1 mm sieve, and stored until used. The soil was a clay loam containing 50% fine sand, 20% clay, 28% silt, and 3% coarse sand (analysis by the Soil Science Department, Glen). The initial pH determined in distilled water, varied between 8 and 8,6. Field capacity was determined in a pressure plate apparatus at 1/3 Bar and was found to be 23,6%.

George soil: Soil of a naturally low pH of 4,5 was collected from a pine forest in the George area. The soil was sandy and very light in texture. Field capacity determined as above, was 12,1%.

3.3 Preparation of inoculum

Initially the method described by van Wyk (1969) was used to prepare inoculum of V. dahliae. The fungus was cultured on

Czapek's gelatin medium at 20°C for one month. On this medium it formed a dark sclerotial mat which could be easily lifted from the liquified medium. However, in later experiments a different method was used to prepare inoculum. V. dahliae was grown on coarse sand containing 3% maize meal for one month. The sclerotia were collected by shaking in sterile distilled water and decanting onto 125 /um and 36 /um sieves. The particles which passed through the 125 /um sieve but were retained by the 36 /um sieve were used as inoculum. The microsclerotia were washed and freeze-dried before use. In some experiments, infected cotton stems, ground to pass a 1 mm sieve, were used as inoculum.

3.4 Soil pH - measurement and adjustment

The pH of the soil was measured by suspending 5 g of soil in 25 ml distilled water. The suspension was allowed to stand for 3 h with intermittent shaking after which measurements were made with a "Metrohm" model E 396 B pH meter.

The pH of Glen soil was lowered by adding various predetermined amounts of $Al_2(SO_4)_3.18H_2O$ to the soil (Wiant, 1929; Chapman, 1965). In some experiments, predetermined amounts of sulphur or H_2SO_4 were used to compare their effect with that of aluminium sulphate. In experiments where the acidified soil had to be restored to its original pH level, $Ca(OH)_2$ was added in predetermined amounts.

3.5 Techniques for the isolation of *V. dahliae* from soil

Attempts were made to isolate *V. dahliae* from soil by various means. Originally (Chapters 4 & 5) ethanol streptomycin agar (ESA) (Nadakavukaren & Horner, 1959; Nadakavukaren, 1960) was used, but its limitations soon became clear: isolations from acid soil showed a rapid reduction in viable propagules of the pathogen and as the dilution factor was reduced, volunteer (unwanted) fungi which developed on the agar plates, inhibited or obscured *Verticillium* colonies.

In some experiments (Chapter 7) a flotation method (Huisman & Ashworth, 1972) and a bioassay method (Evans *et al.* 1974) were used.

Indicator plants were of limited use because they do not give estimates of actual numbers of propagules present in the soil. Moreover, in some experiments, the soil was subjected to drastic changes in pH which could not be tolerated by some indicator plants. A suitable medium which would support sufficient growth of *V. dahliae* in competition with volunteer fungi on isolation plates, had to be found.

Techniques and isolation media described in the literature, were tested in preliminary experiments. Most of these media were fairly successful when isolations were made from high pH soils, but volunteer fungi were cumbersome in isolations from low pH soils.

The soil-washing technique in conjunction with plating on pectate agar (Huisman & Ashworth, 1974a) seemed to hold great promise, but in the present studies the pectate agar proved unsatisfactory. Apart from the fact that the medium is cumbersome to prepare, various fungi resembling V. dahliae developed on the medium. When these fungi were subsequently isolated and plated on PDA, they were found to be anything but Verticillium. The apparent reason for the failure to isolate V. dahliae on pectate agar only became clear after the finding of Butterfield & DeVay (1977) that chemicals from different sources, when used in the medium, had an effect on the numbers of propagules isolated.

Preliminary experiments indicated that the exposure of MS to acid soil for prolonged periods, had a detrimental effect on the pathogen's ability to form MS on isolation media. Frequently MS were totally absent and identification was dependent on the formation of conidiophores and conidia by V. dahliae in culture.

The agar medium (VIA), developed in this laboratory, and eventually used in most of the present experiments consisted of 2 g sucrose; 1,5 g KH_2PO_4; 4 g K_2HPO_4; 2 g Na-polygalacturonate; 0,05 g commercial "Terrachlor" (75% wettable PCNB); 1 ml "Tergitol NPX"; 10 g agar (Oxoid No 3); 100 ml sugarless Czapek-Dox medium; 0,05 g each of streptomycin-sulphate, chlortetracyclin, and chloramphenicol; distilled water to make 1 L medium.

The agar medium was sterilized at 121°C for 15 min before the antibiotics, suspended in 5 ml 95% ethyl alcohol, were added. After sterilization the medium was poured into 9 cm diameter petri dishes, 15 ml per dish, and allowed to set and dry for 3 days at room temperature. Drying was necessary to absorb excess moisture when the dilutions were made.

Before microsclerotia-containing soil could be plated out on the medium described above, the fraction containing the MS had to be purified from the bulk of unwanted material. In this way the MS were concentrated 3- to 8-fold and some of the unwanted fungal propagules removed (Huisman & Ashworth, 1974b). The method used was that described by Huisman & Ashworth (1974a). Two g soil from each treatment were suspended in 200 ml distilled water containing 1% "Calgon" water softener and c. 0,01% "Tergitol NPX" and blended for 30 seconds in an "Atomix MSE" blendor. The soil suspension was then washed through 125 and 36 µm sieves. A stationary sieving device supplied with a sieving head of plexiglass with 3 spray nozzles and sieve pan with outlet, was used. The soil was washed by spraying for 20 min, the 36 µm sieve containing the MS was dipped in 0,05% NaOCl for 15 sec and again washed for another 10 minutes. The residues on the 36 µm sieve were then collected and suspended in 100 ml distilled water. The soil was then kept in suspension by magnetic stirring while 25 ml of the suspension was spread over 25 petri dishes containing VIA; each plate receiving 1 ml of the soil suspension.

The plates were incubated at room temperature for 14 days before counts of developing Verticillium colonies were made under a dissecting microscope. When required, the compound microscope was used to verify identifications.

3.6 Soil preparation before dilution plating on VIA

Treated soils were air-dried at room temperature for 48 h and ground in a mortar to pass through a 0,5 mm sieve. Since grinding affects the number of propagules isolated (Ashworth et al. 1974), grinding was kept to a minimum. The soil was then stored at 4°C in a refrigerator until plating could be done.

CHAPTER 4

FIELD STUDIES ON SURVIVAL OF, FUNGI ASSOCIATED WITH, AND ANTAGONISM AGAINST V. DAHLIAE

The survival of individual MS of V. dahliae was studied by incorporating MS grown on glass fibre disks into the soil. The response of soil fungi in the vicinity of cotton roots upon release of spores of V. dahliae was measured, and antagonism of some of these fungi to the pathogen was determined.

4.1 MATERIALS AND METHODS

4.1.1 Survival

Glass fibre disks of c. 90 mm diameter were heat sterilized and placed on well-hardened PDA in petri dishes. Small blocks of agar containing growth of V. dahliae from stock cultures, were then placed aseptically on the glass fibre disks and the cultures incubated in the dark for about one month after which the fibre disks contained masses of black MS. The disks with the MS were then lifted from the agar medium and buried in the soil at a depth of 10 cm.

Apart from weeding the soil was only disturbed once to bury the fibre disks and again when the disks were removed. Soil moisture was recorded from adjacent locations at weekly intervals by drying the soil in an oven at 105°C until the mass remained constant. Soil temperature was recorded at 10 cm depth with a "Lambrecht" soil thermograph.

At one-month intervals one fibre disk containing MS was dug from the soil and the MS were picked off the disk under a dissecting microscope and transferred to media in petri dishes. The media used were PDA and ESA. Each medium received 100 MS and the cultures were incubated at room temperature until the colonies of V. dahliae could be identified and counted. Bacteria, actinomycetes, and fungi developing from MS plated on PDA were also recorded from the third month.

4.1.2 The distribution in time of V. dahliae and associated fungi in the vicinity of cotton roots in a wilt-infested soil

Cotton (cultivar Cape Acala 4/42) was planted under irrigation during October of each year for two consecutive seasons. The site was at Glen where the soil was naturally infested with V. dahliae. Isolation of the fungi was started in November of each year.

4.1.2.1 Collection of soil samples and isolation techniques

Plants were collected in the field, excess soil shaken from the roots, and placed into plastic bags for transportation to the laboratory.

4.1.2.1.1
Dilution plates: Air-dried cotton roots were shaken up in 495 ml sterile distilled water until 5 g rhizosphere soil was shaken from the roots and suspended in the water. The suspension was shaken in a "Griffen" wrist-arm shaker to break up the soil particles and serial dilutions to 10^{-5} were made from the continuous=

ly agitated soil suspensions. All dilutions were made in sterile distilled water and 1 ml of the final dilution was pipetted into each of 5 petri dishes. The suspension was then swirled with sucrose yeast extract agar (Jooste, 1963) kept at 45°C. The plates were incubated at 25°C and the fungi were subsequently counted and identified.

4.1.2.1.2

Soil plates: The soil plate method (Warcup, 1950) was modified as follows: 0,002 g soil was scraped off the roots and distributed into petri dishes. Ten ml of Martin's peptone-dextrose-agar medium plus rose bengal and streptomycin (Johnson et al. 1959) cooled to c. 45°C, was poured into each petri dish and the dishes were swirled to break up and distribute the soil particles. The cultures were incubated at 25°C and inspected twice daily with a dissecting microscope, for two weeks. Developing colonies were cut out and transferred to Zcapek Dox agar medium in petri dishes for subsequent identification.

4.1.2.1.3

Profile plates: Profile plates (Andersen & Huber, 1965) were used to monitor the actively growing fungi in the vicinity of cotton roots. The plates were wrapped in aluminium foil, steam sterilized (110°C for 20 min), and allowed to cool. The holes in the plates were aseptically filled with sterile corn meal agar (CMA). Excess solidified agar was removed from the plates with a sterile spatula, and the holes were covered with autoclaved PVC tape. The plates were then again wrapped in aluminium foil and transferred to the field.

In the field a profile was prepared by driving a sharpened steel plate into the soil at right angles to the surface and about

5 cm from the stem of the cotton plant. The steel plate was inserted by driving it into the ground with a mallet. Once in the ground the soil was removed from one side of the plate with a spade until the whole plate was exposed. The plate was then removed with the least disturbance to the soil profile. A sterile dissecting needle was used to punch small holes in the tape directly above the agar-filled holes in the profile plate. The plate was immediately placed firmly against the vertical flat surface of the soil profile so that the top row of horizontal holes was c. 25 mm below ground level and the centre row of vertical holes was directly in line with the stem of the cotton plant. The soil removed to expose the profile, was packed firmly against the back of the plate, and aluminium foil was placed over the top of the plate to exclude soil and water. The profile plates were exposed in the soil for 5 days before removal. Upon return to the laboratory, the tape was removed from the plate, and each agar plug was transferred to the centre of a petri dish containing CMA. The plates were incubated at room temperature and the fungi were identified (Barnett, 1955; Clements & Shear, 1957; Gilman, 1945). Fungi which had not formed spores after one month were designated as sterile fungi.

4.1.3 Anatagonism of soil fungi to V. dahliae

Isaac's method (Johnson et al. 1959) was used to test the fungi for antagonism to V. dahliae in pure culture. Petri dishes containing PDA were inoculated about 3 cm apart with plugs of actively growing cultures of potential antagonists and of V. dahliae. The cultures were incubated at 25°C for 10 days before antagonism was recorded.

4.2 RESULTS

4.2.1 Survival of *V. dahliae* on glass fibre in soil

The MS of *V. dahliae* survived in the soil for 24 months which was the total period of investigation (Table 1). A semi-log linear regression line (Fig. 1) was fitted to the data obtained from plating out on ESA and by means of this it could be predicted that individual MS would be able to survive in soil for 43 months under the conditions of this experiment.

ESA, being selective for *V. dahliae* (Nadakavukaren, 1960), proved to be better than PDA for recording the survival of the individual MS. On PDA consistently lower numbers of viable MS were recorded except for three instances when very low numbers were recorded on both media, viz. after 12, 21, and 22 months of incubation. PDA, being a non-selective medium, allowed the development of large numbers of bacteria, actinomycetes, and fungi which grew from the MS plated on this medium.

Both seasons were relatively dry and soil moisture levels below 10% prevailed from the start of the experiment in November until the end of January the following year, and again from October until the end of February the next year. Moisture levels exceeding 20% (*c.* field capacity) were rarely found. During April of the first season it was unusually wet and a mean moisture content of approximately 20% was recorded (Fig. 2).

The temperature of the soil (Fig. 3) at a depth of approximately 10 cm also varied considerably from a minimum in winter of 1°C

Table 1 - Survival of microsclerotia of *V. dahliae* on glass fibre disks in soil

Incubation period in months	Percentage survival determined on		Numbers of organisms developing from MS plated on PDA[a,b]		
	ESA	PDA	Bacteria	Actinomycetes	Fungi
1 (Dec.)	78	30	Not recorded		
2	55	20	"	"	
3	54	32	12	8	43
4	15	4	30	16	43
5	76	29	42	5	43
6	92	29	45	10	27
7	64	7	58	0	54
8	53	47	19	25	5
9	52	49	26	7	11
10	10	3	26	19	61
11	46	35	44	10	12
12	3	7	24	40	29
13 (Dec.)	46	14	20	37	27
14	27	12	24	20	32
15	20	2	19	16	61
16	40	22	19	19	23
17	38	17	11	10	60
18	39	27	28	23	21
19	5	1	17	16	60
20	5	0	8	13	72
21	3	8	20	7	61
22	2	3	22	7	55
23	1	0	30	9	40
24	1	0	34	46	17

a: More than one organism sometimes developed from a single MS.

b: Counts were only made on PDA because ESA suppressed development of bacteria and actinomycetes and of many fungi.

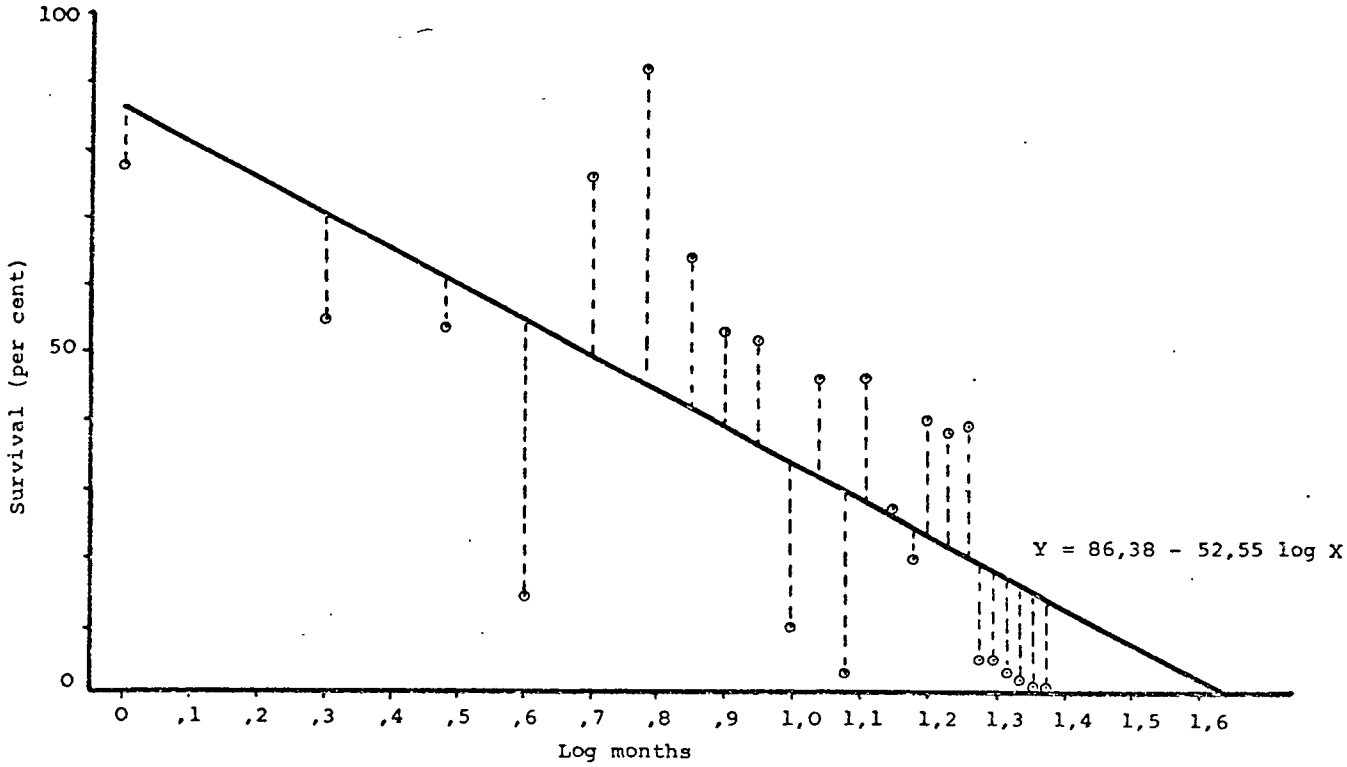


Fig. 1 - Calculated regression of survival on period of V. dahliae in soil

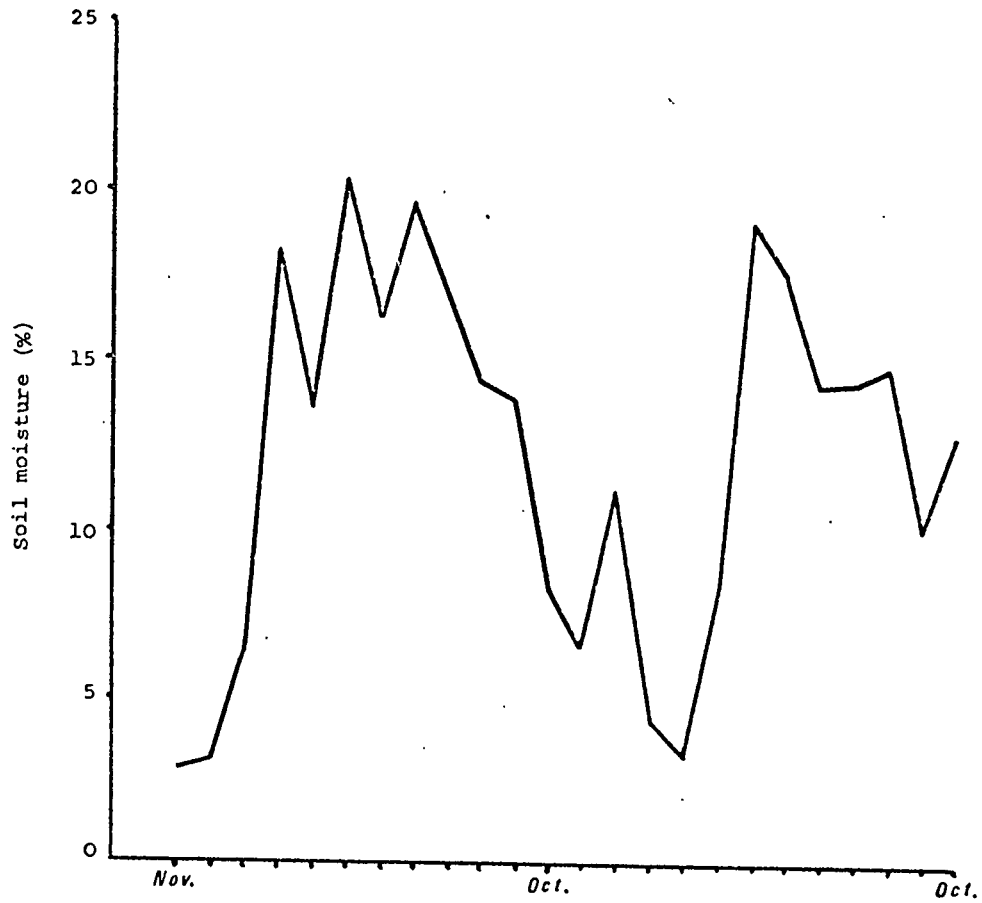


Fig. 2 - Monthly mean soil moisture
(Field soil; Glen)

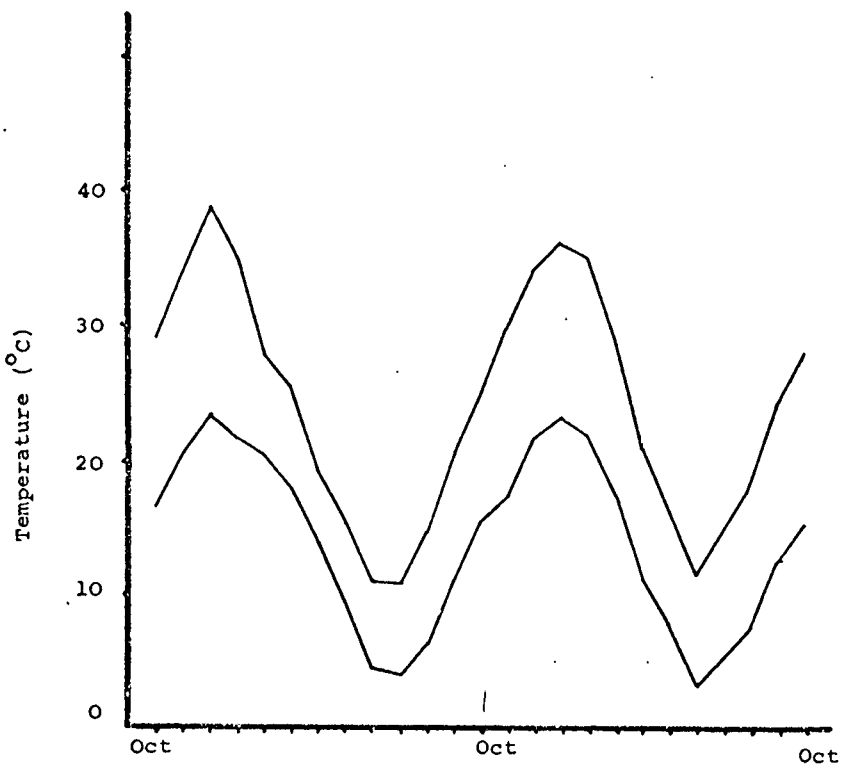


Fig. 3 - Monthly mean minimum and maximum temperatures (Field soil; Glen)

to a maximum in summer of 44°C.

4.2.2 Verticillium dahliae and associated fungi in the root zone of cotton

The numbers of V. dahliae and other fungi associated with the roots of cotton planted in a Verticillium-infested soil, were recorded at monthly intervals from November to July over two consecutive seasons (Tables 2, 3, and 4).

4.2.2.1 Verticillium dahliae

The pathogen started to appear on cultures during January of the first season (Table 3) and appeared regularly on the plates after February (Tables 2 & 3). The appearance of the pathogen on the isolation plates co-incided with the dying-off of infected plants and was regularly isolated after the plants were killed by frost in April. The largest numbers of the pathogen were isolated with the dilution plate technique while only a single colony appeared on the profile plates.

4.2.2.2 Fungi other than V. dahliae (associated fungi)

The largest numbers of associated fungi were isolated with the soil plates (Table 3) while the dilution plates yielded the lowest numbers (Table 2). The soil plates yielded 22, the dilution plates 19, and the profile plates 12 identified genera. Fusarium spp. were most frequently isolated and consisted of 36,6% of all isolates. However, on the profile plates 83,4% were Fusaria.

Table 2 - Numbers of fungi isolated by dilution plates at monthly intervals over two seasons[a]

	First season										Second season									
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jne.	Jly.	Tot.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jne.	Jly.	Tot.
Acremonium	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	2
Acrostalagmus	2	0	55	1	6	1	2	0	0	67	0	2	0	5	2	0	1	0	0	10
Alternaria	4	0	0	0	0	3	0	0	1	8	0	1	0	0	1	1	0	2	0	5
Ascochyta	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Aspergillus	1	12	0	0	4	1	2	0	0	20	2	0	3	4	0	2	1	1	1	14
Blastomyces	0	0	0	0	2	0	0	0	0	2	1	0	1	1	0	0	0	0	0	3
Cephalosporium	14	2	1	1	2	0	2	0	0	22	1	1	11	1	0	0	0	0	0	14
Chaetomium	7	0	0	1	0	0	0	2	0	10	0	0	2	0	0	0	1	0	0	3
Cladosporium	0	0	0	1	1	4	0	0	0	6	0	0	0	0	0	2	0	0	0	2
Fusarium	15	14	2	1	35	2	8	6	7	90	24	34	5	13	20	4	10	7	5	122
Non sporulating	13	19	1	0	8	1	3	3	4	52	0	2	1	1	1	4	2	4	2	17
Penicillium	43	0	3	39	12	0	4	0	0	101	9	5	7	32	22	5	0	0	0	80
Phoma	8	2	0	2	2	11	1	3	9	38	3	0	1	1	0	18	6	6	2	37
Pyrenochaeta	1	0	12	0	0	30	0	0	0	43	0	0	0	0	0	0	8	0	0	8
Rhizopus	0	0	0	0	1	0	0	0	0	1	0	6	0	0	0	0	0	0	0	6
Sclerotium	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2
Stachybotrys	0	0	1	0	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Thielavia	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Trichoderma	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	2
V. dahliae	0	0	0	9	1	1	33	14	18	76	0	0	0	1	9	12	10	22	25	59
Unidentified	5	1	0	4	2	0	3	2	1	18	0	14	0	9	1	1	1	1	0	27
Tot.	113	52	75	60	78	54	59	30	40	561	40	67	32	69	57	50	40	43	35	433

a: Each figure represents the total of 5 plates.

Table 3 - Numbers of fungi isolated by soil plates at monthly intervals over two seasons[a]

	First season										Second season									
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jne.	Jly.	Tot.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jne.	Jly.	Tot.
Acremonium	3	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	2	0	3	6
Acrostalagmus	0	0	3	0	1	5	1	1	4	15	3	1	3	6	2	3	0	2	4	24
Alternaria	10	7	0	3	16	4	8	6	20	74	7	1	1	8	20	10	8	9	16	80
Ascochyta	24	3	1	2	0	8	1	0	10	49	0	10	1	1	0	0	6	4	1	23
Aspergillus	5	3	6	0	7	3	4	0	4	32	7	6	2	8	8	3	12	6	8	60
Blastomyces	37	5	10	4	0	2	7	0	0	65	7	7	5	5	2	3	8	0	2	39
Candida	0	0	0	0	0	0	1	1	0	2	1	0	0	0	3	0	1	0	1	6
Cephalosporium	0	0	3	0	0	1	1	3	1	9	5	7	3	1	1	2	1	2	0	22
Chaetomium	0	2	11	2	3	5	4	1	15	43	3	2	12	0	5	2	10	2	0	36
Cladosporium	0	1	0	1	6	3	4	1	10	26	5	2	1	5	6	1	2	5	0	27
Fusarium	33	79	51	8	27	27	6	8	31	270	45	40	47	28	50	12	16	17	12	267
Non sporulating	20	7	22	4	9	22	3	12	21	120	8	30	25	25	5	5	4	9	12	123
Penicillium	8	12	14	6	1	15	1	5	6	68	58	5	10	4	5	4	9	5	10	110
Phoma	0	4	16	8	15	137	9	25	50	264	28	0	2	8	96	12	28	17	33	224
Pyrenochaeta	0	0	2	0	1	0	0	2	17	22	0	7	0	0	4	4	0	2	0	17
Rhizoctonia	0	2	0	0	0	0	0	0	0	2	0	0	0	7	0	0	3	0	1	11
Rhizopus	4	0	3	5	2	1	0	0	0	15	0	0	1	1	0	1	4	1	0	8
Stachybotrys	0	0	0	0	0	6	0	0	4	10	0	9	4	6	0	0	2	3	0	24
Sphaeronema	0	0	0	0	0	0	0	1	11	12	0	0	0	0	0	0	1	0	9	10
Thielavia	8	1	3	4	0	4	7	0	8	35	8	7	10	2	8	2	0	7	6	50
Torula	0	1	1	0	0	0	0	0	1	3	0	0	2	0	1	0	2	1	1	7
Trichoderma	0	0	1	0	0	2	0	0	1	4	1	0	4	1	2	0	0	2	1	11
V. dahliae	0	0	3	0	0	0	1	0	14	18	0	0	0	1	1	0	4	1	9	16
Unidentified	6	13	39	18	46	58	10	32	97	319	17	31	33	31	8	9	21	18	23	191
Tot.	158	140	189	65	134	303	68	98	325	1480	204	165	166	148	227	73	144	113	152	1392

a: Each figure represents the total of 3 plates.

Table 4 - Numbers of fungi isolated by profile plates at monthly intervals over two seasons[a]

	First season										Second season									
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jne.	Jly.	Tot.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jne.	Jly.	Tot.
Alternaria	2	2	2	0	0	1	0	1	0	8	1	0	2	1	1	0	0	1	0	6
Aspergillus	0	1	0	0	0	0	3	0	0	4	0	0	0	0	1	2	0	0	0	3
Blastomyces	0	4	0	1	0	2	0	0	0	7	0	0	1	1	0	3	0	0	0	5
Cladosporium	0	0	0	1	2	2	1	0	2	8	1	0	1	1	0	2	0	2	1	8
Fusarium	129	109	138	82	128	55	16	1	1	659	112	112	120	118	55	63	21	1	2	624
Non sporulating	8	7	1	12	3	6	3	2	3	47	2	4	1	6	7	2	1	2	2	27
Penicillium	0	0	2	1	2	1	0	0	0	6	1	0	2	0	1	1	0	0	0	5
Phoma	0	0	0	1	2	0	0	0	0	3	0	0	0	1	0	4	0	0	0	5
Rhizopus	0	1	3	0	0	1	1	0	0	6	3	1	1	1	0	1	1	0	0	8
Sclerotium	0	0	0	0	2	0	0	0	0	2	0	0	0	1	1	1	0	0	0	3
Stachybotrys	0	0	0	1	1	1	0	0	0	3	0	0	0	1	1	0	0	0	0	2
Torula	0	9	1	0	7	1	0	0	0	18	0	1	0	0	1	3	0	0	0	5
V. dahliae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Unidentified	0	4	1	10	13	5	0	16	0	49	2	0	0	1	8	4	0	0	0	15
Tot.	139	139	148	109	160	75	24	20	6	820	122	118	128	132	97	86	23	6	5	717

a: Each figure represents the total number of fungi recovered by two plates.

Heavily sporing fungi were dominant on dilution and soil plates. The largest numbers of unidentified fungi, consisting of lesser known genera, and the non-sporulating types, were obtained from soil plates. The profile plates yielded slightly more unidentified fungi than the dilution plates.

4.2.3 Antagonism of root-zone fungi to *V. dahliae* in culture

The antagonism against *V. dahliae* of 103 fungal isolates from the root zone of cotton was tested (Table 5). In cases where more than one isolate of a specific genus were tested, the isolates differed from each other in culture, and were considered as separate spp. A relatively large number (56%) of the test fungi proved to be antagonistic to the pathogen in culture. Although only a few isolates of *Cephalosporium*, *Sclerotium*, *Stachybotrys*, *Torula*, and *Trichoderma* were available, all of them proved to be antagonistic to *V. dahliae*. Many isolates of *Aspergillus*, *Penicillium*, *Phoma*, and unidentified fungi were antagonistic also.

Table 5 - Antagonism of root-zone fungi on *V. dahliae* in vitro

Genera	No of isolates investigated[a]	Antagonistic isolates
Acremonium	1	0
Acrostalagmus	1	0
Aschochyta	1	0
Aspergillus	15	8
Cephalosporium	1	1
Chaetomium	3	0
Fusarium	6	3
Penicillium	20	16
Phoma	5	4
Rhizopus	1	0
Sclerotium	2	2
Stachybotrys	1	1
Thielavia	1	0
Torula	1	1
Trichoderma	2	2
Unidentified[b]	42	20

a: Where more than one isolate from the same genus were used, they were regarded as different spp.

b: Unidentified fungi included sterile fungi.

4.3 DISCUSSION

4.3.1 Survival on glass fibre in soil

Although the literature deals extensively with the survival of V. dahliae in soil, this problem still seemed to offer controversy and was, therefore, investigated in this study.

Evans et al. (1966) reported a rapid decline of viable MS in cultivated soil and concluded that records on the longevity of V. dahliae (Nelson, 1950; Wilhelm, 1955) might be erroneous because the fungus can maintain itself on weeds and certain non-hosts (Martinson & Horner, 1962; Wilhelm & Taylor, 1965; Lacey & Horner, 1966).

In the present study the soil was kept free of weeds to exclude the possibility of V. dahliae surviving on the weeds. It was established that the pathogen survived in the soil for the whole period of investigation and by fitting a regression curve to the results, it was possible to predict that the fungus would last in the soil for 43 months under the conditions of this experiment (Fig. 1).

Soil factors such as temperature and moisture could have played an important role in the decline of the pathogen in the soil. Nadakavukaren (1960) found that the survival of V. dahliae was poor at temperatures above 25°C. As soil temperatures increased from 25 to 40°C the number of viable MS declined sharply. At 40°C survival was limited and only in air-dry soil did MS sur= vive for more than 5 days.

In the present studies soil temperature varied considerably (Fig. 3). During Nov., Dec., Jan., and Feb. of the first and Dec., Jan., and Feb. of the second season minimum and maximum temperatures exceeded 20 and 30°C, respectively. However, the decline of the MS were not steeper during these months than during the colder months (Table 1). It would appear, therefore, that the viability of MS is not affected to a large extent by fluctuating temperatures encountered under field conditions.

Nadakavukaren (1960) found no clear-cut effect of soil moisture on the viability of MS. There was, however, a sharp reduction in the MS population in flooded soils, while survival was best at moderate moisture levels. In the present study there was no flooding and very dry conditions seldom prevailed for long enough to cause an appreciable reduction in the number of viable propagules (Fig. 2).

Considerable variation in numbers of viable propagules was found between the samplings on both ESA and PDA (Table 1). ESA, being selective for V. dahliae (Nadakavukaren, 1960), yielded higher numbers of the pathogen because it inhibited the development of organisms recovered from the soil with the MS. Since PDA allowed the development of various other organisms capable of suppressing the growth of V. dahliae, lower numbers of the pathogen were isolated on PDA. This is indicative of the close association of antagonists with the MS of V. dahliae in the soil.

The variation which was found between samplings was not caused by soil temperature and moisture. The decline in numbers of

viable MS observed on some of the disks during the earlier stages of the experiment, might have been the result of the antagonistic activities of micro-organisms from specific micro-habitats.

Micro-organisms are not evenly distributed throughout the soil, but exist in a more or less inactive state in specific micro-habitats until stimulated towards renewed activity when new substrates become available (Warcup, 1967; Alexander, 1971). Sclerotia are such substrates (Baker & Cook, 1974), and by stimulating their antagonists by their excretions, much like plant roots stimulate plant pathogens (Dickenson & Coley-Smith, 1970; Gilbert & Linderman, 1971; Baker & Cook, 1974), may contribute towards their own elimination. It is conceivable that the MS of a single glass fibre disk placed in the vicinity of highly effective antagonists may be eliminated in this way while others escaped this fate because the soil in the immediate vicinity of these disks was free of antagonists lethal to V. dahliae.

4.3.2 The occurrence of V. dahliae and associated fungi in the root zone of cotton

It is believed that V. dahliae is inactive as a saprophyte (Wilhelm, 1951; Isaac, 1953) and do not abound in the soil except when, after the plants are killed, MS are being formed on the surface of degenerate roots (Evans et al. 1967).

Three isolation methods (Tables 2, 3, & 4) were used to isolate V. dahliae and associated fungi from the roots of cotton. The three different methods were used to obtain a wider spectrum of fungi present in the soil than would have been possible

if only a single method was used. The soil and dilution plates sampled the fungi in the rhizosphere while the profile plates sampled the actively growing fungi in the vicinity of the roots.

4.3.2.1
Verticillium dahliae

The pathogen appeared on soil plates only during the first season in January, but was isolated regularly, especially by the dilution plate technique, after February of both seasons. Dilu= tion plates yielded 135 colonies of V. dahliae during the two seasons while soil plates yielded only 34. Since soil plates are considered to be superior to dilution plates for the isolation of the rarer and more slow growing fungi from the soil (Warcup, 1950), the larger numbers of V. dahliae on dilution plates, were unexpected. The appearance of abundant colonies of V. dah= liae on the plates after some plants started dying off, and especially after frost set in in April, is in accordance with the view that the pathogen is seldomly found outside the host plant during the growing season (Isaac, 1953), but starts sporu= lating on moribund tissues after the plants become senescent (Sewell, 1959).

The occurrence of V. dahliae on the profile plates, which was designed for the isolation of actively growing fungi (Andersen & Huber, 1965), was a surprise in view of the fact that the pathogen is considered to be inactive in the soil (Wilhelm, 1951; Isaac, 1953). V. dahliae is, however, capable of germination and limited growth through the soil (Farley et al. 1971) and could have reached the plates by this means. Another possibility is that the pathogen could have been transported towards the

plate by soil fauna.

4.3.2.2
Associated fungi

Waksman (1945) stated that enrichment of a soil with propagules of a plant pathogen resulted in the development of larger numbers of antagonists in the soil. The release of spores of V. dahliae into the soil after sporulation on moribund roots, results in a natural enrichment of the soil with the pathogen (Tables 2 & 3) and should, according to Waksman's finding, be followed by an increase of its antagonists. To investigate the possibility that some of the fungi which are antagonists of V. dahliae, might increase after spore release by the pathogen, the fungi which which occurred in the root zone of the plants were isolated and counted.

The composition and numbers of fungi varied considerably over the period of investigation. Some fungi were isolated only occasionally; others regularly. During the warmer months more fungi were isolated than during the winter. The profile plates (Table 4) in particular, yielded much less fungi during winter than in summer, indicating that the activity of the fungi in the soil is greatly impaired during the winter.

The observed variation in numbers of isolated fungi made it impossible to determine any tendencies which might have occurred, but no obvious increase after the release of propagules of V. dahliae occurred with the majority of the fungi which were isolated in larger numbers.

4.3.3 Antagonism of some associated fungi to *V. dahliae*

Cultures of the more regularly isolated genera were tested *in vitro* for their antagonistic effect agains *V. dahliae*. It was found that 56% of the fungi tested were antagonistic to the pathogen (Table 5). This figure can be considered high, since Luke (1952) found 17% of his isolates antagonistic to *Pythium arrhenomanes* and Williams & Kaufman (1962) found 6 to 19% antagonistic to *Fusarium roseum*. However, increases in antagonists after the pathogen became freely available in the soil in February, were rare.

CHAPTER 5

FACTORS AFFECTING ANTAGONISTS OF *V. DAHLIAE* IN SOIL

(a) Bacterial and actinomycetous antagonists

Conditions which might have an effect on numbers of bacterial and actinomycetous antagonists were investigated with the purpose to increase their numbers and consider their use in biological control of *V. dahliae*. The factors investigated were: soil pH, temperature and aeration in high and low pH soils, and combinations of fertilizers and *Verticillium* propagule amendments in high and low pH soils.

(b) Fungal antagonists

The effect of reduced soil pH on the numbers of fungi antagonistic to *V. dahliae*, total soil fungi, and *Trichoderma* spp. was investigated.

5.1 MATERIALS AND METHODS

5.1.1 Bacterial and actinomycetous antagonists

5.1.1.1
Soil pH: The pH of Glen soil was modified by the addition of various predetermined amounts of aluminium sulphate to give high (*c.* pH 8), medium (*c.* pH 6), and low (*c.* pH 4,5) values.

5.1.1.2
Temperature: Soils with high and low pH values were incubated at temperatures of 25, 30, and 35°C to determine the optimum

temperature for the development of antagonists.

5.1.1.3
Aeration: Aeration was improved by loosening the soil with vermiculite. The following soil : vermiculite ratios were used: 100 : 0 (low), 75 : 25 (medium), and 50 : 50 (high). soils with high and low pH values were used in the experiment.

5.1.1.4
Fertilizers: Fertilizers [N, 50 ppm (NH_4NO_3); P, 25 ppm ($NaH_2PO_4.2H_2O$); and K, 15 ppm (KCl)] were added alone or in combination as follows: N, P, K, N+P, N+K, P+K, and N+P+K. These experiments were done with naturally alkaline or acidified soils, half of which received Verticillium propagules while the other half served as controls. Each fertilizer experiment was done individually with a separate batch of microsclerotial inoculum prepared for each.

5.1.1.5
Verticillium propagules: Microsclerotia were obtained from Czapek's gelatin cultures (van Wyk, 1969). The sclerotial mat was lifted from the liquified medium after one month of growth at 20°C and macerated in sterile distilled water in an "Atomix" homogenizer. The homogenate was centrifuged at 1 000 X g to collect the MS and the supernatant discarded. The homogenate was macerated and centrifuged 6 more times to remove as much as possible of the nutrients which might have adhered to the MS. After the MS were washed, they were resuspended in sterile distilled water, added to the soil and thoroughly mixed. Each treatment was replicated three times.

5.1.1.6
Culture conditions: Unless otherwise specified, the cultures were incubated at 25°C in the dark, and the soil moisture

was kept at 20% (m/m) of the air-dry mass of the soil.

5.1.1.7
Assay of bacterial and actinomycetous antagonists: The numbers of antagonists were determined by the triple-agar-layer tech= nique (Herr, 1959) after 4 and 8 weeks of incubation. The first layer consisted of 10 ml of 1,5% water agar and was allowed to harden for one day before the second layer was added. The second layer, 5 ml of 1% water agar contained the soil suspension.

Soil dilutions were made in 1% carboxy-methyl-cellulose (CMC 12 HP) solution in distilled water (Kaufman & Williams, 1965) buffered at pH 7 (60% M/15 Na_2HPO_4, 40% M/15 KH_2PO_4). It was found essential to buffer the solution because coagulation, which interfered with the dispersal of the soil particles in the solution, occurred with acidified soils.

The first 1:10 dilution was shaken on a "Griffin" wrist-arm shaker for 10 min while each subsequent dilution was shaken for two minutes. The final dilution was in 1% water agar kept liquid in a water bath at c. 45°C. The flasks with agar were then swirled and a set of seven to nine plates made from each.

The plates were incubated for two days at 25°C after which the third agar layer containing propagules of V. dahliae, was added. The MS recovered from Czapek's gelatin medium were washed as described above and suspended in Czapek's agar medium at c. 45°C. Seven ml of the Czapek's agar-macerated-fungus suspension were pipetted into each plate. The plates were incubated at room temperature in the dark for 7 to 10 days and the numbers of antagonists recorded.

5.1.2 Fungal antagonists

Glen soil was air-dried and devided into two batches, one of which received 4% aluminium sulphate to change the soil reaction to *c.* pH 4,5. The reaction of the untreated soil was *c.* pH 8. Both the natural and acidified soils were again halved and one half of each treatment was amended with *Verticillium* propagules prepared as described on p. 64. Aliquots (50 g) of the treated and untreated soils were added to individual 250 ml erlenmeyer flasks. The moisture content was adjusted to 20% of the air-dry mass of the soil. The flasks were stored at 25°C for 4 and 8 weeks after which numbers of fungal antagonists of *V. dahliae* were determined.

The flasks were weighed once every week during the storage period and the soil moisture replenished by adding sterile distilled water. At each sampling date, soil from three erlenmeyer flasks from each treatment were used to determine the numbers of antagonists.

Total numbers of fungi and *Trichoderma* spp. in particular, from natural and acidified soils were determined by dilution plating on peptone-dextrose-agar medium plus rose bengal and streptomycin (Johnson *et al.* 1959).

5.1.2.1
Assay of fungal antagonists: Numbers of fungal antagonists were determined as follows (Williams & Kaufman, 1962):
Each soil sample was air-dried and the clumps broken up. Five g soil were then suspended in 50 ml 1% CMC buffered at pH 7. Appropriate dilutions were made and 1 ml of the final

dilution was swirled with 10 ml of OAES agar (Schmithenner & Williams, 1958) per plate. After 4 days of incubation at 25°C, the fungus colonies were marked on the bottom of the plate. A second layer of agar containing the assay fungus was then added. The inoculum was prepared by mixing three-day-old cultures of V. dahliae (50ml of liquid Czapek-dox medium in 250 ml erlenmeyer flasks) in a blendor for one min. Seven ml of the homogenate, mixed 1:1 with warm (± 45°C) Czapek-dox agar, were pipetted onto the soil suspension layer. Special care had to be taken when this layer was added to prevent the dispersal of spores of the developing fungus colonies on the plates. After 2 days of incubation at 25°C, the colonies pro=ducing inhibition zones were counted.

5.2 RESULTS

5.2.1 Factors affecting bacterial and actinomycetous antagonists in soil

5.2.1.1 Soil pH

The pH of the soil had a profound effect on numbers of antagonists isolated from the soil (Fig. 4 & Table 6). Soils with high and medium pH levels yielded significantly more antagonists after both 4 and 8 weeks of incubation than low pH soils. At both time levels, the differences between numbers from high and medium pH soils were not statistically significant. Figure 4 shows that the largest numbers of antagonists occurred in medium pH soil after 4 weeks of incubation, while medium pH soil yielded intermediate numbers after 8 weeks of incubation. This difference in tendency was responsible for a significant pH X time interaction. Significantly more antagonists were isolated from both high and medium pH soils after 4 weeks than after 8 weeks of incubation. The differences between antagonists from low pH soils after 4 and 8 weeks of incubation were not statistically significant.

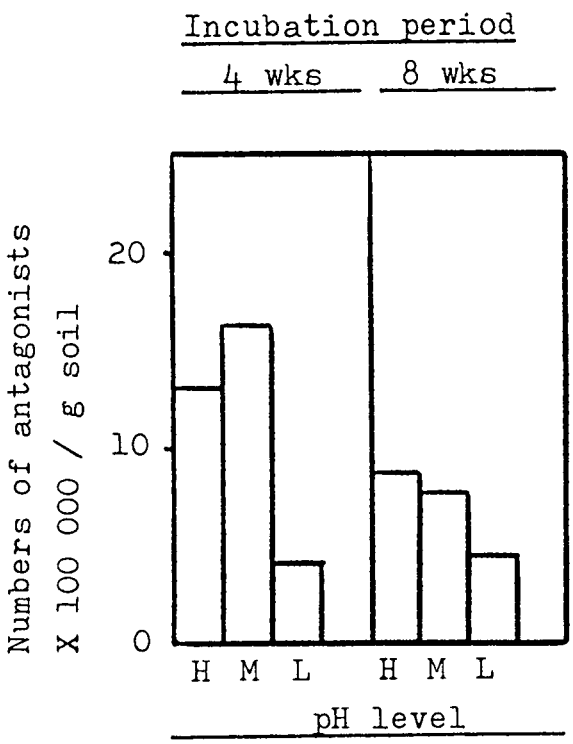


Fig 4 - Numbers of bacterial and actinomycetous antagonists isolated from high (pH 8), medium (pH 6), and low (pH 4,5) pH soils after 4 and 8 weeks of incubation at 25°C.

Table 6 - Mean numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from soil at different pH levels after incubation at 25°C for 4 and 8 weeks[a]

pH	Incubation period		Mean
Approximately	4 wks	8 wks	
8 (High)	3,72	3,10	3,41
6 (Medium)	4,11	2,92	3,52
4,5 (low)	2,26	2,82	2,54
Mean	3,36	2,95	

a: Mean number per plate of 7 plates per treatment; each treatment was replicated 3 times.

Critical values: $S_{\bar{x}} = 0,1204$

(Tukey) $Q_{0,05}\ (2;12) = 0,3708$ (rows)

$Q_{0,05}\ (3;12) = 0,4539$ (columns)

5.2.1.2

Temperature and soil pH

After 4 weeks of incubation, temperature did not have a significant effect on numbers of antagonists in high and low pH soils (Fig. 5 & Table 7). However, the pH X temperature interaction was significant. The significant interaction occurred because the tendency of high pH soil to yield the lowest numbers of antagonists at 30°C, was not repeated in acid soil. However, pH had a significant effect on antagonist numbers within all three temperature levels.

After 8 weeks of incubation (Fig. 5 & Table 8) the largest numbers of antagonists occurred in high pH soil incubated at 35°C. High pH soil at 30°C yielded intermediate numbers of antagonists while in low pH soil it yielded the lowest. This change in tendency resulted in a significant pH X temperature interaction.

Significantly more antagonists were isolated from alkaline than acid soil within all three temperature levels.

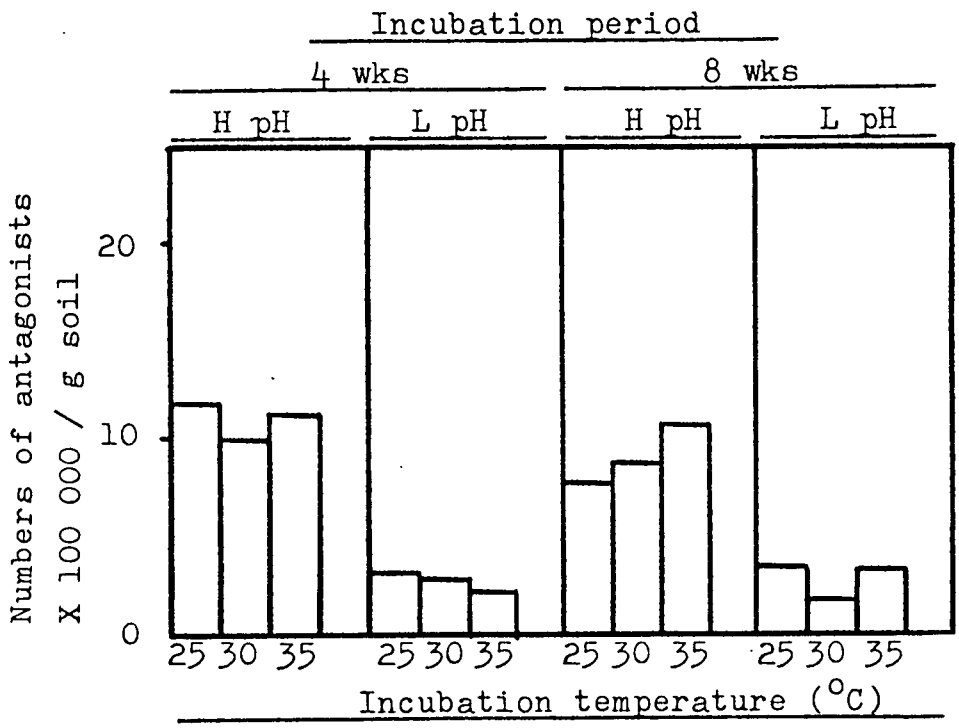


Fig. 5 - The effect of temperature on numbers of bacterial and actinomycetous antagonists of V. dahliae in high and low pH soils after incubation periods of 4 and 8 weeks.

Table 7 - Mean numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils incubated for 4 weeks at different temperature levels[a]

Temperature	pH		Mean
°C	High	Low	
25	3,5605	2,0243	2,7924
30	3,3929	1,9281	2,6605
35	3,4805	1,7258	2,6032
Mean	3,4780	1,8928	

a: See Table 6.

Critical values: $S_{\bar{x}} = 0,1132$

(Tukey) $Q_{0,05}$ (2;12) = 0,3487 (rows)

(3;12) = 0,4268 (columns)

Table 8 - Mean numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils incubated for 8 weeks at different temperature levels[a]

Temperature	pH		Mean
°C	High	Low	
25	2,9386	2,0467	2,4927
30	3,1129	1,5929	2,3529
35	3,4176	2,0029	2,7103
Mean	3,1564	1,8808	

a: See Table 6.

Critical values: $S_{\bar{x}} = 0,0574$

(Tukey) $Q_{0,05}$ (2;12) = 0,1768 (rows)

(3;12) = 0,2164 (columns)

5.2.1.3
Aeration and soil pH

After 4 weeks of incubation the largest numbers of antagonists were isolated from highly aerated, high pH soil (Fig. 6 & Table 9). However, the differences in numbers from medium and highly aerated, high pH soils were not statistically significant.

After 8 weeks of incubation the effect of aeration in high pH soil was not significant (Fig. 6 & Table 10) and considerably less antagonists were isolated than after 4 weeks of incubation.

Medium aerated, low pH soil yielded the lowest numbers of antagonists after both 4 and 8 weeks of incubation. The differences were statistically significant for both incubation periods.

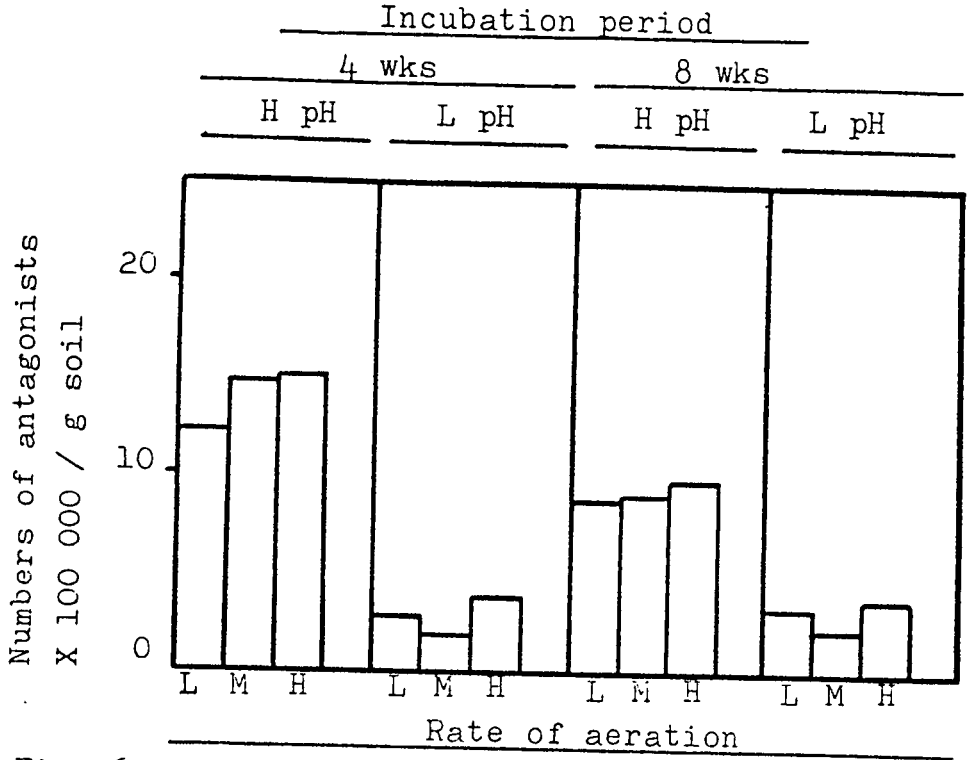


Fig. 6 - The effect of aeration on numbers of bacterial and actinomycetous antagonists of V. dahliae in high and low pH soils after incubation periods of 4 and 8 weeks at 25°C.
M = medium; H= high; L = low.

Table 9 - Mean numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils incubated for 4 weeks at different levels of aeration[a]

Aeration[b]	pH		Mean
	High	Low	
Low	3,6233	1,9300	2,7767
Medium	3,9433	1,6567	2,8000
High	4,0133	2,1367	3,0750

a: See Table 6.

b: Improved aeration was obtained by adding vermiculite to the soil in the following v:v ratios: Low, none; Medium, 25%; and High, 50%.

Critical values: $S_{\bar{x}} = 0,07$

(Tukey) $Q_{0,05}$ (2;12) = 0,2156 (rows)

(3;12) = 0,2639 (columns)

Table 10 - Mean numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils incubated for 8 weeks at different levels of aeration[a]

Aeration[b]	pH		Mean
	High	Low	
Low	3,1238	2,0905	2,6072
Medium	3,1371	1,7348	2,4360
High	3,2657	2,1514	2,7086
Mean	3,1755	1,9922	

a: See Table 6.

b: See Table 9.

Critical values: $S_{\bar{x}} = 0,0927$

(Tukey) $Q_{0,05}$ (2;12) = 0,2855 (rows)

(3;12) = 0,3495 (columns)

5.2.1.4
Fertilizers, *Verticillium* propagules, and pH

5.2.1.4.1
Nitrogen

After 4 weeks of incubation (Table 11 & Fig. 7) the *Verticillium* X pH (Table 11.1) and the *Verticillium* X N (Table 11.2) interactions were significant. The main factors, viz. *Verticillium*, pH, and N were also statistically significant. An analysis of the pH X *Verticillium* interaction (Table 11.1) indicates that significantly more antagonists were isolated from soil at the higher pH value than from soil at low pH. Although an increase in numbers of antagonists occurred in both high and low pH soils after the incorporation of *Verticillium* propagules, the differences were only signifi= cant for high pH soil. With N amended, antagonist numbers were suppressed in both *Verticillium*-amended and non-amended soils (Table 11.2). However, the differences were significant only for the *Verticillium*-amended soil.

After 8 weeks of incubation (Fig. 7 & Table 12), only the *Verticillium* X pH interaction (Table 12.1) was statistically significant apart from the single factors *Verticillium* and pH which also were significant. The suppressing effect of N on numbers of antagonists which was significant after 4 weeks of incubation, was not evident after 8 weeks of incubation.

Analysis of the *Verticillium* X pH interaction (Table 12.1) indicates that significantly more antagonists were present

in soil of both pH levels when Verticillium propagules were present. The antagonists from low pH soils were also significantly less than those from high pH soils whether Verticillium was present or not.

After 8 weeks of incubation two treatments from low pH soil (i, amended with N and Verticillium; ii, amended with Verti= cillium only) yielded exceptionally high numbers of antagonists compared to both high and low pH controls (Fig. 7). This tendency was not observed after 4 weeks of incubation.

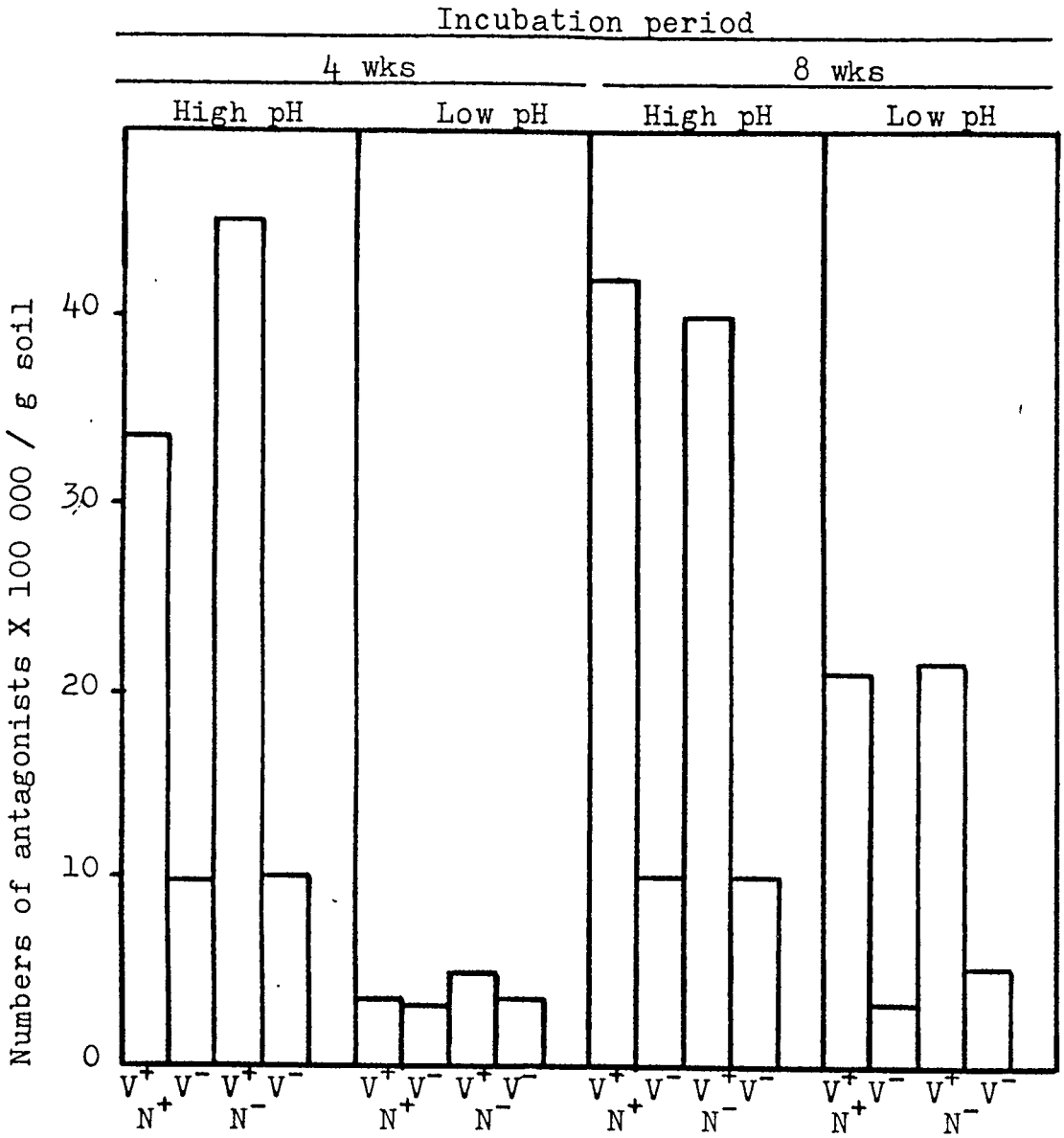


Fig. 7 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soil with Verticillium propagules and N incorporated.
V^+ = Verticillium amended; V^- = Verticillium absent;
N^+ = Nitrogen amended; N^- = Nitrogen absent.

Table 11 - The effect of N on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 4 weeks of incubation[a]

Verticillium	pH	N		Mean	Mean for pH	
		Present	Absent		High	Low
Present	High	5,86	6,76	6,31	4,79	2,12
	Low	2,02	2,35	2,21		
	Mean	3,97	4,55	4,26		
Absent	High	3,25	3,30	3,27		
	Low	1,98	2,07	2,02		
	Mean	2,61	2,68	2,65		
Mean		3,29	3,62			

a: Mean number/plate of 9 plates/treatment; each treatment was replicated 3 times.

Table 11.1 - Analysis of the pH X *Verticillium* interaction for 4 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	6,3165	2,2169	4,0996*
Absent	3,2754	2,0228	1,2526*
Difference	2,0411*	0,1941ns	

Table 11.2 - Analysis of the Verticillium X N interaction for 4 weeks of incubation

Verticillium	N		Difference
	Present	Absent	
Present	3,9680	4,5654	0,5974*
Absent	2.6141	2,6840	0,0699ns
Difference	1,3539*	1,8814*	

Critical values for Tables 11.1 & 11.2: $S_{\bar{x}}$ = 0,1202; (Tukey) $Q_{0,05}$ = 0,3606; ns = non-significant; * = significant.

Table 12 - The effect of N on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	N		Mean	Mean for pH	
		Present	Absent		High	Low
Present	High	6,49	6,26	6,37	4,85	3,48
	Low	4,67	4,73	4,70		
	Mean	5,58	5,49	5,53		
Absent	High	3,39	3,28	3,33		
	Low	2,10	2,44	2,27		
	Mean	2,74	2,86	2,80		
Mean		4,16	4,17			

a: See Table 11.

Table 12.1 - Analysis of the *Verticillium* X pH interaction for 8 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	6,3790	4,6974	1,6816*
Absent	3,3367	2,2704	1,0663*
Difference	3,0423*	2,4270*	

Critical values: $S_{\bar{x}} = 1,1000$

(Tukey) $Q_{0,05} = 0,3000$

* = significant.

5.2.1.4.2
Phosphate

After 4 weeks of incubation (Fig. 8 & Table 13) the Verticillium X pH interaction was significant. The results of the analysis of the interaction (Table 13.1) indicate that at both pH levels the incorporation of Verticillium propagules caused an increase in numbers of antagonists and that high pH soils yielded higher numbers of antagonists irrespective of the level of Verticillium.

The single factors Verticillium and pH were significant, but P did not have a significant effect. However, the majority of the treatments showed a slight increase in numbers of antagonists after the addition of P to the soil.

After 8 weeks of incubation (Fig. 8 & Table 14) the Verticil= lium X pH (Table 14.1) and the P X pH (Table 14.2) interactions were significant. Antagonist numbers were higher in soils of both pH levels when Verticillium propagules were incorporated and lower numbers of antagonists occurred in low pH soil irrespective of the level of Verticillium (Table 14.1).

Table 14.2 indicate that P caused an increase in numbers of antagonists in high pH soil, but not in soil with reduced pH. The effect of pH was independent of the level of P.

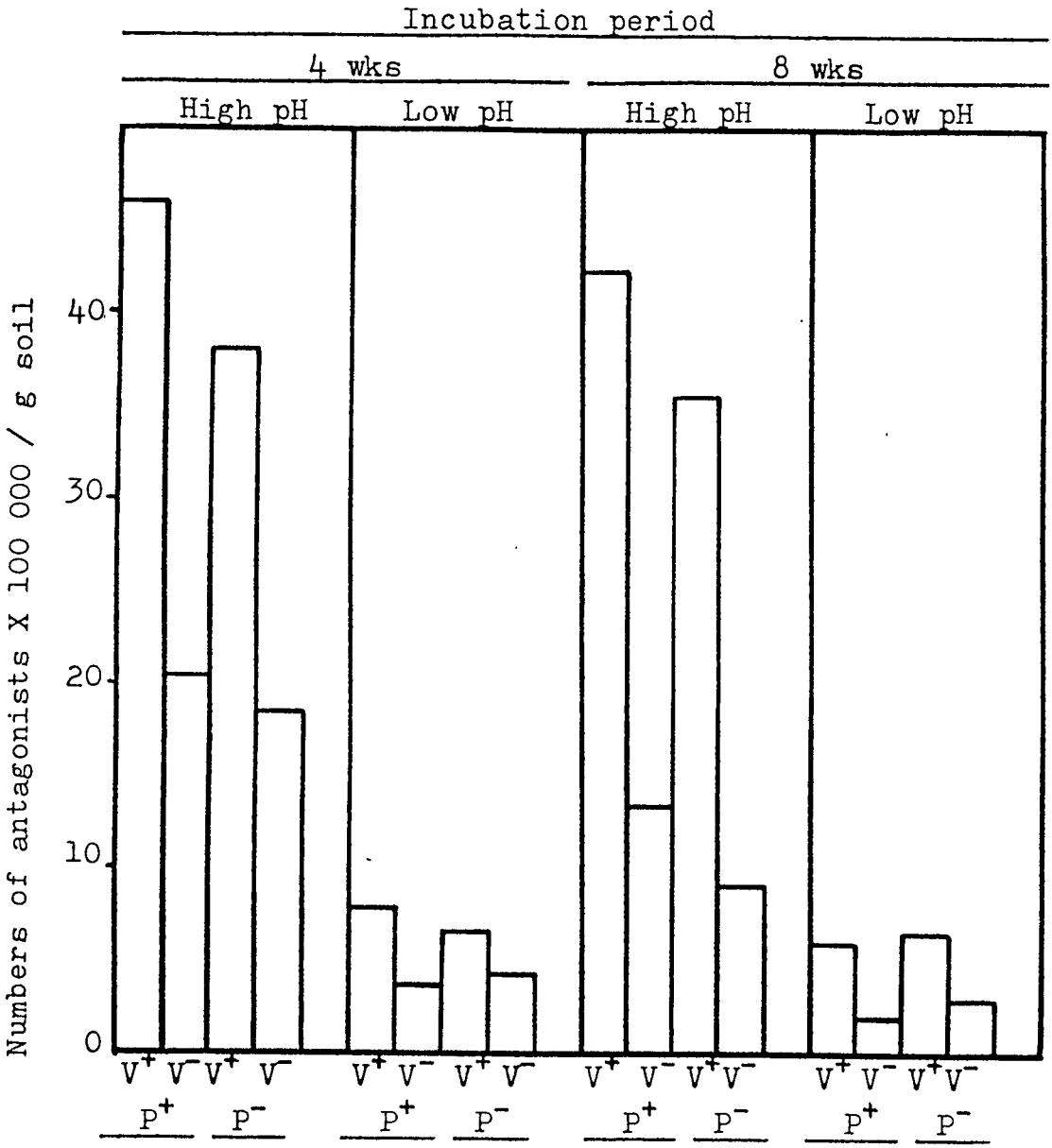


Fig. 8 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soil with Verticillium propagules and P incorporated.
V^+ = Verticillium amended; V^- = Verticillium absent;
P^+ = Phosphate amended; P^- = Phosphate absent.

Table 13 - The effect of P on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 4 weeks of incubation[a]

Verticillium	pH	P		Mean	Mean for pH	
		Present	Absent		High	Low
	High	6,85	6,23	6,54	5,52	2,47
Present	Low	2,92	2,69	2,80		
	Mean	4,88	4,46	4,67		
	High	4,64	4,39	4,51		
Absent	Low	2,10	2,20	2,15		
	Mean	3,37	3,29	3,33		
Mean		4,12	3,87			

a: See Table 11.

Table 13.1 - Analysis of the Verticillium X pH interaction for 4 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	6,5372	2,8011	3,7361*
Absent	4,5161	2,1530	2,3631*
Difference	2,0211*	0,6481*	

Critical values: $S_{\bar{x}} = 0,1401$

(Tukey) $Q_{0,05} = 0,4203$

* = significant.

Table 14 - The effect of P on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	P		Mean	Mean for pH	
		Present	Absent		High	Low
	High	6,57	6,04	6,30	4,77	2,18
Present	Low	2,58	2,66	2,62		
	Mean	4,57	4,35	4,46		
	High	3,36	3,14	3,25		
Absent	Low	1,61	1,89	1,75		
	Mean	2,48	2,51	2,50		
Mean		3,53	3,43			

a: See Table 11.

Table 14.1 - Analysis of the Verticillium X pH interaction for 8 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	6,3063	2,6230	3,6833*
Absent	3,2483	1,7509	1,4974*
Difference	3,0580*	0,8721*	

Table 14.2 - Analysis of the P X pH interaction for 8 weeks of incubation

pH	P		Difference
	Present	Absent	
High	4,9631	4,5915	0,3716*
Low	2,0956	2,2783	0,1827ns

Critical values for Tables 14.1 & 14.2: $S_{\bar{x}} = 0,1062$

(Tukey) $Q_{0,05} = 0,3186$

* = significant.

ns = non-significant.

5.2.1.4.3
Potassium

Potassium apparently had a slight depressing effect on numbers of antagonists isolated from high pH soil after 4 weeks of incubation (Fig. 9 & Table 15). However, the differences were not statistically significant. The main effects Verticillium and pH were statistically significant as well as the Verticillium X pH interaction (Table 15.1).

The yield of antagonists from high pH soil was significantly higher than that from low pH soil. The effect of amending the soil with Verticillium popagules was to cause an increase in numbers of antagonists. The differences were statisti= cally significant, however, for soil of high pH only.

After 8 weeks of incubation (Table 16 & Fig. 9) only the two main factors Verticillium and pH had a significant effect on numbers of antagonists. Verticillium propagules added to the soil, stimulated an overall increase in antagonist numbers while low soil pH caused a decrease in numbers.

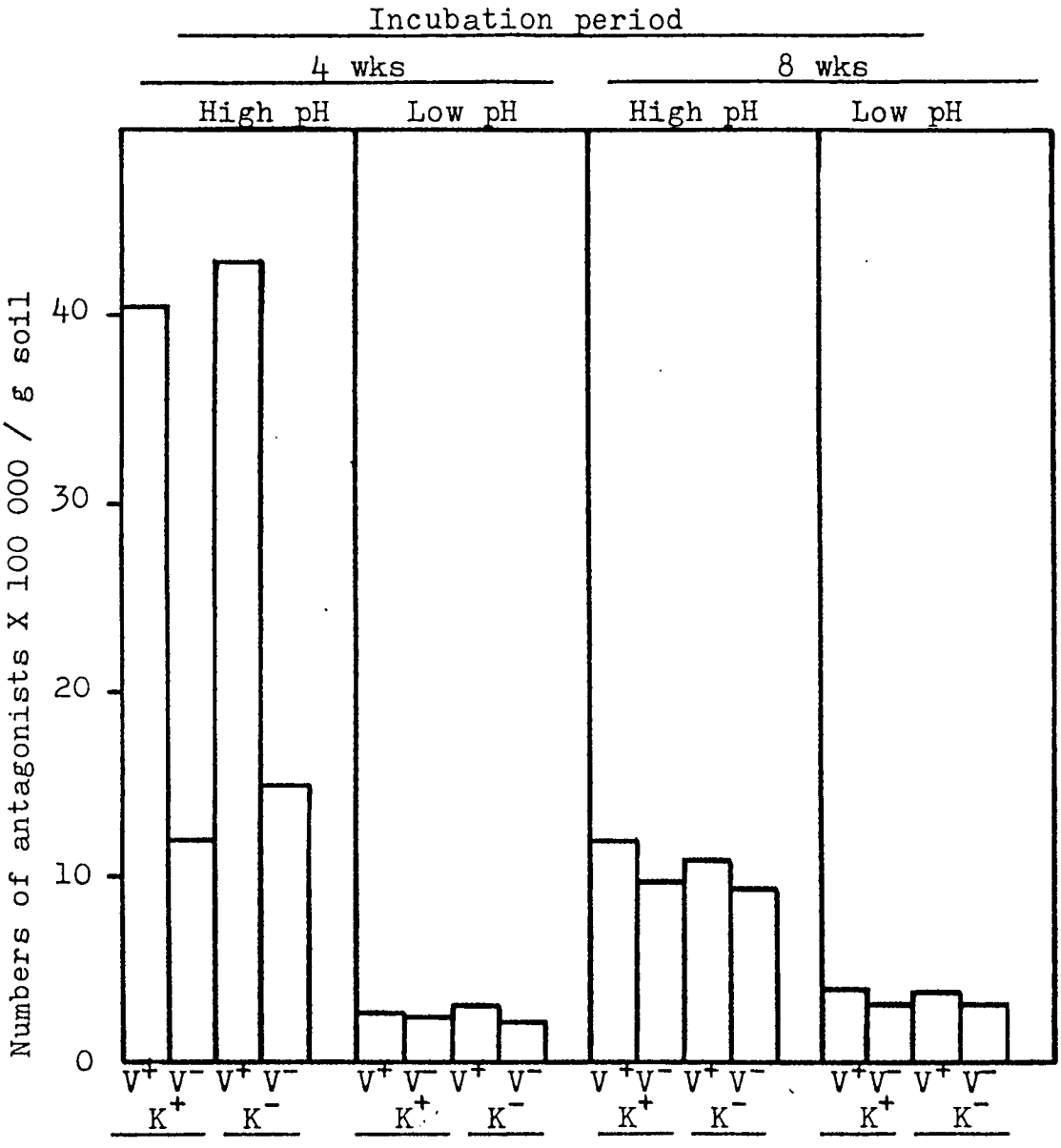


Fig. 9 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soil with Verticillium propagules and K incorporated.
V^+ = Verticillium amended; V^- = Verticillium absent;
K^+ = Potassium amended; K^- = Potassium absent.

Table 15 - The effect of K on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soil after 4 weeks of incubation[a]

Verticillium	pH	K		Mean	Mean for pH	
		Present	Absent		High	Low
	High	6,42	6,59	6,50	5,12	1,85
Present	Low	1,86	1,97	1,91		
	Mean	4,14	4,28	4,21		
	High	3,54	3,95	3,74		
Absent	Low	1,83	1,74	1,78		
	Mean	2,68	2,84	2,76		
Mean		3,41	3,56			

a: See Table 11.

Table 15.1 - Analysis for the Verticillium X pH interaction for 4 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	6,5072	1,9119	4,5953*
Absent	3,7433	1,7848	1,9585*
Difference	2,7639*	0,1271ns	

Critical values: $S_{\bar{x}} = 0,0913$

(Tukey) $Q_{0,05} = 0,2739$

* = significant.

ns = non-significant.

Table 16 - The effect of K on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	K		Mean	Mean for pH	
		Present	Absent		High	Low
	High	3,59	3,43	3,51	3,35	2,05
Present	Low	2,19	1,99	2,09		
	Mean	2,89	2,71	2,80		
	High	3,24	3,17	3,20		
Absent	Low	2,01	2,01	2,01		
	Mean	2,62	2,59	2,60		
Mean		2,76	2,65			

a: See Table 11.

5.2.1.4.4
Nitrogen plus phosphate (NP)

In this experiment in which the effect of N + P on numbers of antagonists after 4 weeks of incubation was investigated, the three-factor interaction of Verticillium X pH X NP was significant at the 5% level (Fig. 10 & Table 17). The interaction was analysed according to the method of Steel & Torrie (1960, p. 207 - 209) (Tables 17.1 -17.3).

The Verticillium X pH interaction was first examined (Table 17.1) because this interaction was also significant according to the analysis of variance. The data indicate that the interaction was the result of the different effect which Verticillium propagules had on the numbers of antagonists at the different pH levels: In high pH soil Verticillium propagules caused an increase in numbers of antagonists, but not in low pH soil. In fact, a slight decrease was observed. The effect of pH was the same for both levels of Verticillium, i.e. a suppression of antagonists in soil with reduced pH.

Because the three-factor interaction was significant, a further analysis was made and because only the Verticillium X pH interaction of the remaining possibilities was significant, the three-factor interaction implied that the Verticillium X pH interaction differs with the levels of NP (Tables 17.2 & 17.3). It is apparent from these analyses that NP caused the significant interaction. In the presence of NP, with Verticillium propagules incorporated, increased numbers of antagonists occurred, but only in high pH soil.

After 8 weeks of incubation (Fig 10 & Table 18) all the main factors were statistically significant as well as the Verticillium X pH (Table 18.1) and the Verticillium X NP (Table 18.2) interactions. More antagonists were present in soils amended with Verticillium propagules than in soils without propagules irrespective of the pH level of the soil. Contrary to previous experience within this series in which various fertilizers were incorporated into the soil, low pH soil yielded significantly more antagonists than high pH soil when Verticillium propagules were also incorporated.

Analysis of the Verticillium X NP interaction (Table 18.2) indicated that significantly more antagonists were present in Verticillium-amended than in non-amended soils irrespective of the fertilizer level. However, NP-amended soils yielded more antagonists than NP-non-amended soils, but only when Verticillium propagules were present.

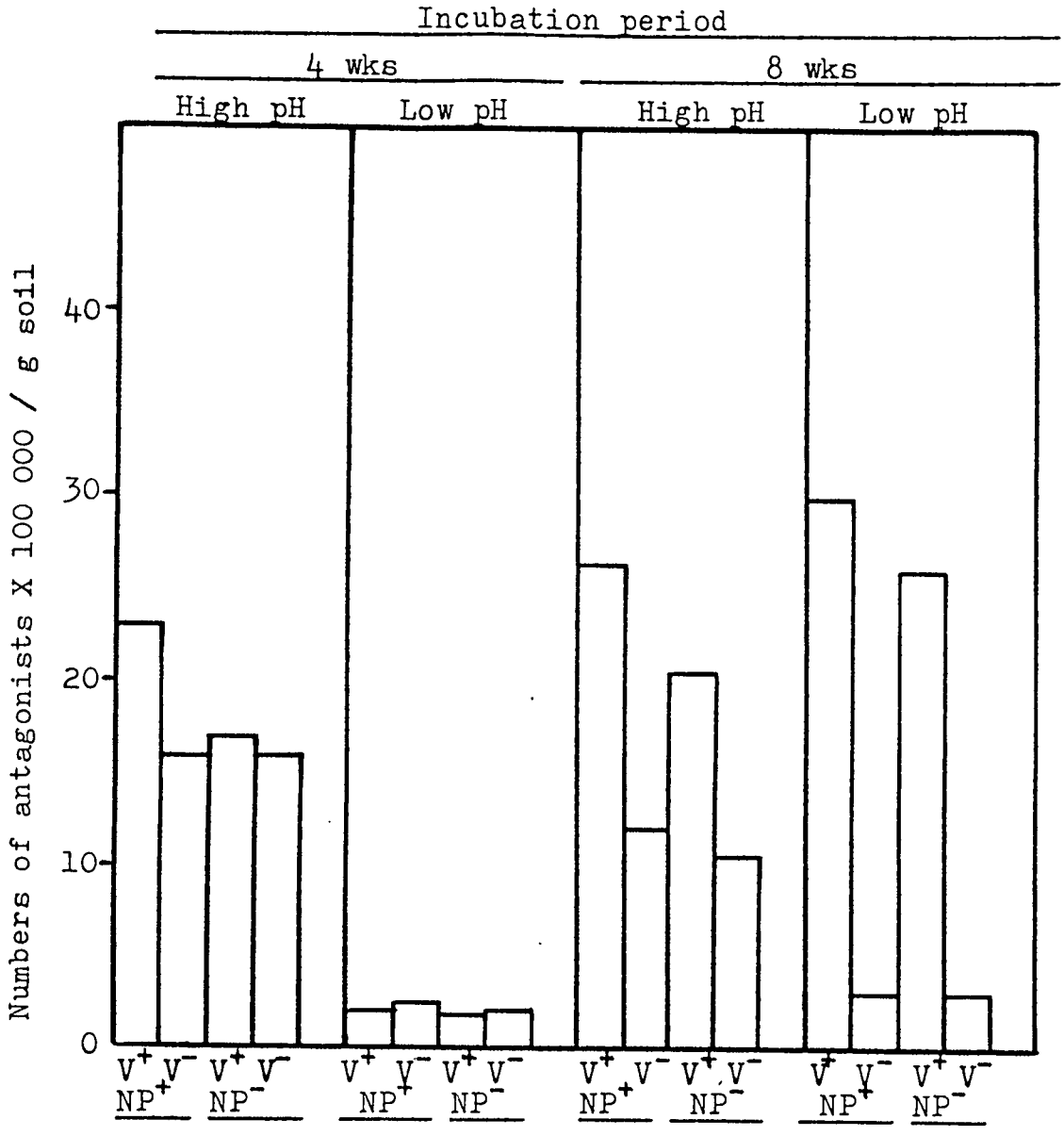


Fig. 10 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soil with Verticillium propagules and N+P incorporated.
V^+ = Verticillium amended; V^- = Verticillium absent;
NP^+ = N and P amended; NP^- = N and P absent.

Table 17 - The effect of N+P on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists in high and low pH soils after 4 weeks of incubation[a]

Verticillium	pH	N+P		Mean	Mean for pH	
		Present	Absent		High	Low
Present	High	4,87	4,24	4,55	4,32	1,68
	Low	1,65	1,59	1,62		
	Mean	3,26	2,91	3,08		
Absent	High	4,09	4,10	4,09		
	Low	1,82	1,66	1,74		
	Mean	2,95	2,88	2,91		
Mean		3,10	2,89			

a: See Table 11.

Table 17.1 - Analysis of the Verticillium X pH interaction for 4 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	246,19	87,39	158,80
Absent	221,18	94,12	127,06

SS of pH within Verticillium present = 233,49*
SS of pH within Verticillium absent = 149,48*
SS of Verticillium within high pH = 5,79*
SS of Verticillium within low pH = 0,42ns

Table 17.2 - Analysis of the Verticillium X pH interaction for N+P present (4 weeks)

pH	N+P Present		Difference
	Verticillium present	Verticillium absent	
High	131,62	110,43	21,19
Low	44,58	49,21	4,63

SS for Verticillium within high pH for N+P added = 8,32*
SS for Verticillium within low pH for N+P added = 0,40ns

Table 17.3 - Analysis of the *Verticillium* X pH interaction for N+P absent (4 weeks)

pH	N+P absent		Difference
	Verticillium present	*Verticillium* absent	
High	114,57	110,75	3,82
Low	42,81	44,91	2,10

SS for *Verticillium* within high pH for N+P absent = $0,27^{ns}$
SS for *Verticillium* within low pH for N+P absent = $0,80^{ns}$
* = significant.
ns = non-significant.

Table 18 - The effect of N+P on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	N+P		Mean	Mean for pH	
		Present	Absent		High	Low
Present	High	5,21	4,61	4,91	4,19	3,66
	Low	5,55	5,19	5,37		
	Mean	5,38	4,90	5,14		
Absent	High	3,59	3,36	3,47		
	Low	1,93	1,97	1,95		
	Mean	2,76	2,66	2,71		
Mean		4,07	3,78			

a: See Table 11.

Table 18.1 - Analysis of the Verticillium X pH interaction for 8 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	4,9072	5,3694	0,4622*
Absent	3,4794	1,9515	1,5279*
Difference	1,4278*	3,4179*	

Table 18.2 - Analysis of the Verticillium X NP interaction for 8 weeks of incubation

Verticillium	N+P		Difference
	Present	Absent	
Present	5,3781	4,8985	0,4796*
Absent	2,7626	2,6683	0,0943ns
Difference	2,6155*	2,2302*	

Critical values for Tables 18.1 & 18.2: $S_{\bar{x}} = 0,08165$

(Tukey) $Q_{0,05} = 0,2450$

* = significant.

ns = non-significant.

5.2.1.4.5
Nitrogen and potassium (NK)

Although somewhat more antagonists were present in high pH soils amended with N and K after 4 weeks of incubation (Fig.11 & Table 19), analysis of the results indicated that the differences were not statistically significant. The main factors Verticillium and pH both had significant effects and the Verticillium X pH interaction (Table 19.1) was also significant. The analysis indicate that low soil pH caused a significant reduction in numbers of antago= nists irrespective of the level of Verticillium. Also, more antagonists were isolated from soil amended with Verticillium propagules , but only from high pH soils.

After 8 weeks of incubation (Fig. 11 & Table 20), N and K still did not affect the numbers of antagonists significantly. However, significantly more antagonists were present in high pH soil and in soil amended with Verticillium propagules.

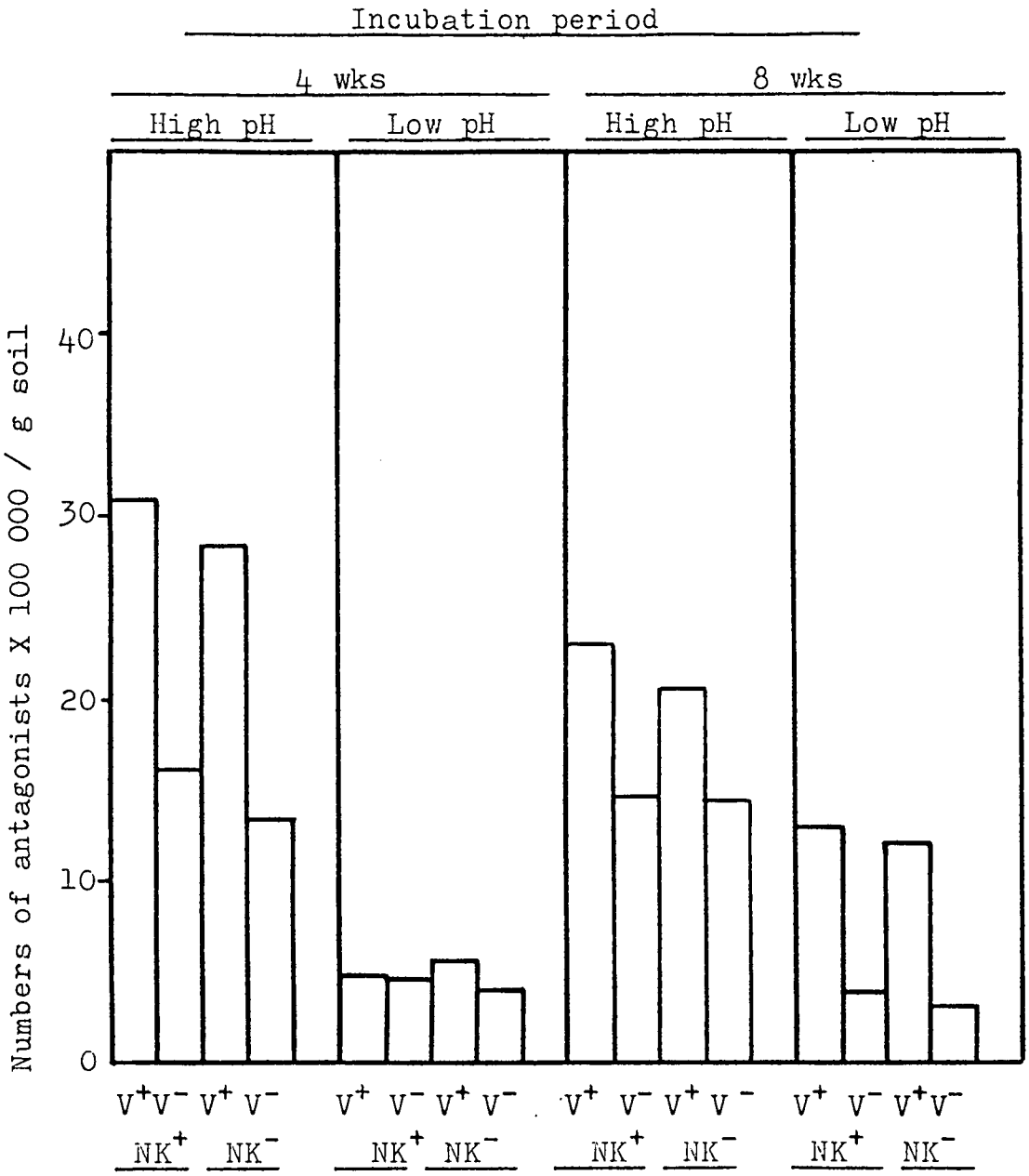


Fig. 11 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soil with Verticillium propagules and N+K incorporated.
V^+ = Verticillium amended; V^- = Verticillium absent;
NK^+ = N and K amended; NK^- = N and K absent.

Table 19 - The effect of N+K on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 4 weeks of incubation[a]

Verticillium	pH	N+K		Mean	Mean for pH	
		Present	Absent		High	Low
Present	High	5,62	5,39	5,50	4,72	2,35
	Low	2,34	2,55	2,44		
	Mean	3,98	3,97	3,97		
Absent	High	4,11	3,76	3,93		
	Low	2,32	2,20	2,26		
	Mean	3,21	2,98	3,09		
Mean		3,59	3,47			

a: See Table 11.

Table 19.1 - Analysis of the Verticillium X pH interaction for 4 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	5,5048	2,4444	3,0604*
Absent	3,9393	2,2561	1,6832*
Difference	1,5655*	0,1883ns	

Critical values: $S_{\bar{x}}$ = 0,0794 (Tukey) $Q_{0,05}$ = 0,2382

* = significant.
ns = non-significant.

Table 20 - The effect of N+P on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	N+K		Mean	Mean for pH	
		Present	Absent		High	Low
Present	High	4,99	4,62	4,80	4,34	2,81
	Low	3,69	3,52	3,60		
	Mean	4,34	4,07	4,20		
Absent	High	3,87	3,88	3,87		
	Low	2,14	1,91	2,02		
	Mean	3,00	2,89	2,95		
Mean		3,67	3,48			

a: See Table 11.

5.2.1.4.6
Phosphate and potassium (PK)

After 4 weeks of incubation (Fig. 12 & Table 21) the pH X Verticillium interaction (Table 21.1) as well as the main factors pH and Verticillium were statistically significant. The results indicate that PK applied to the soil, caused an increase in numbers of antagonists when Verticillium propagules were absent, but not when Verticillium was incorporated. The addition of Verticillium to the soil caused an increase in antagonists only in absence of PK. High pH soils yielded significantly more antagonists than low pH soils.

After 8 weeks of incubation (Fig 12 & Table 22) the pH X PK interaction was significant (Table 22.1). PK added to the soil caused an increase in numbers of antagonists in high pH soil, but not in low pH soil. High pH soil yielded larger numbers of antagonists than low pH soil, irrespective of the fertilizer level. The main factor Verticillium did not have a significant effect on numbers of antagonists in this experiment.

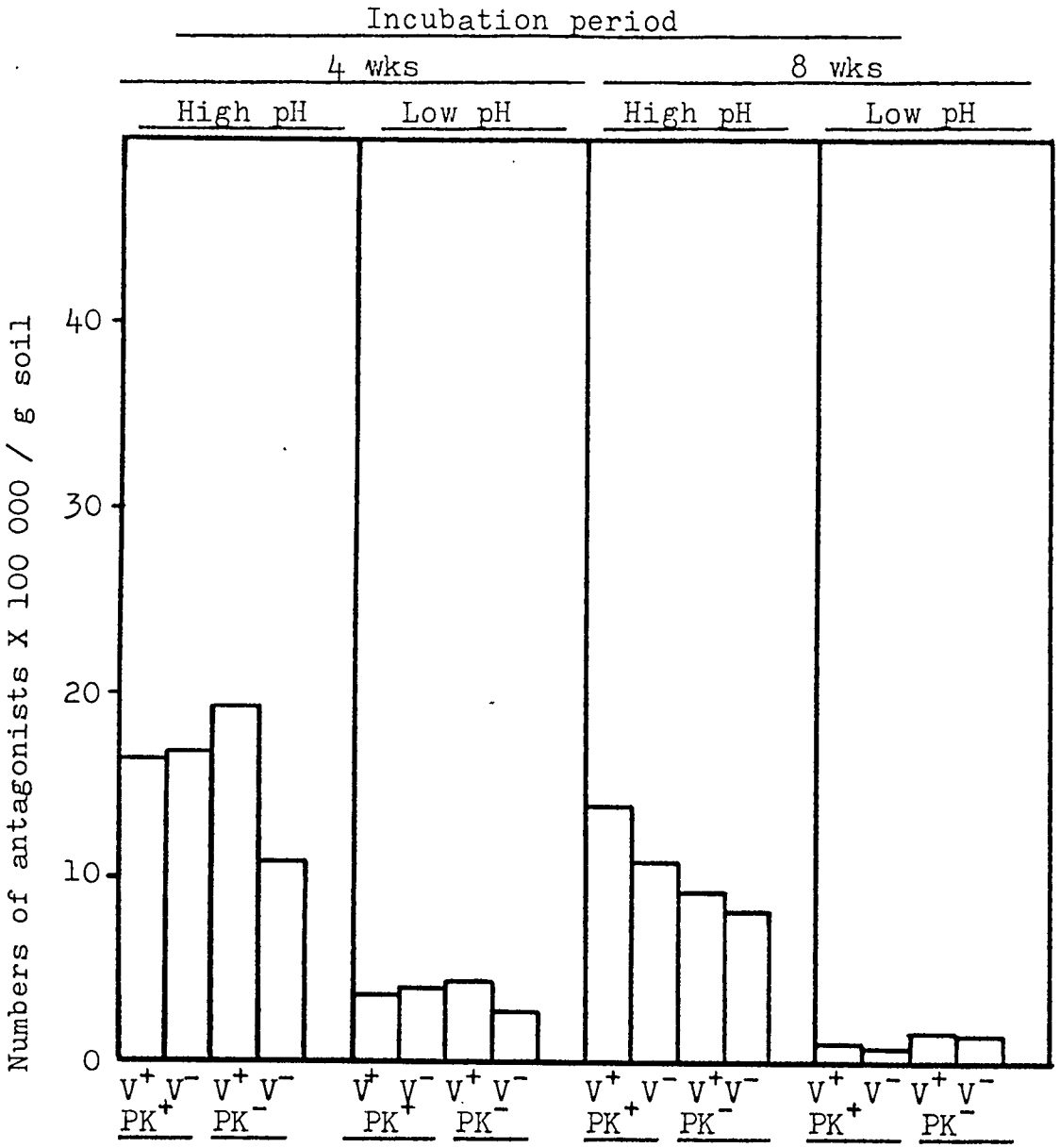


Fig. 12 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soil with Verticillium propagules and P+K incorporated.
V^+ = Verticillium amended; V^- = Verticillium absent; PK^+ = P and K amended; PK^- = P and K absent.

Table 21 - The effect of P+K on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 4 weeks of incubation[a]

Verticillium	pH	P+K		Mean	Mean for pH	
		Present	Absent		High	Low
	High	4,13	4,46	4,29	4,05	2,09
Present	Low	2,11	2,22	2,16		
	Mean	3,12	3,34	3,23		
	High	4,18	3,44	3,81		
Absent	Low	2,20	1,84	2,02		
	Mean	3,19	2,64	2,91		
Mean		3,15	2,99			

a: See Table 11.

Table 21.1 - Analysis of the Verticillium X PK interaction for 4 weeks of incubation

Verticillium	P+K		Difference
	Present	Absent	
Present	3,1196	3,3415	$0,2219^{ns}$
Absent	3,1931	2,6424	$0,5507^{*}$
Difference	$0,0735^{ns}$	$0,6991^{*}$	

Critical values: $S_{\bar{x}} = 0,1347$

(Tukey) $Q_{0,05} = 0,4014$

* = significant.

ns = non-significant.

Table 22 - The effect of P+K on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	P+K		Mean	Mean for pH	
		Present	Absent		High	Low
	High	3,84	3,19	3,51	3,36	1,41
Present	Low	1,37	1,54	1,45		
	Mean	2,60	2,36	2,48		
	High	3,43	3,00	3,21		
Absent	Low	1,28	1,48	1,38		
	Mean	2,35	2,24	2,29		
Mean		2,48	2,30			

a: See Table 11.

Table 22.1 - Analysis of the pH X PK interaction for 4 weeks of incubation

pH	P+K		Difference
	Present	Absent	
High	3,6343	3,0937	0,5406*
Low	1,3222	1,5091	0,1869ns
Difference	2,3121*	1,5847*	

Critical values: $S_{\bar{x}} = 0,0892$

(Tukey) $Q_{0,05} = 0,2676$

* = significant.

ns = non-significant.

5.2.1.4.7
Nitrogen , phosphate, and potassium (NPK)

The three-factor interaction NPK X pH X Verticillium was significant after 4 weeks of incubation (Fig. 13 & Table 23). Its analyses (Steel & Torrie, 1960, p. 207 - 209) appear in Tables 23.1 - 23.9.

Regarding the three-factor interaction the analyses clarify the following: (i) The effect of soil pH was constant within all treatments: A reduction in pH caused a reduction in numbers of antagonists irrespective of the level of Verticillium or the level of fertilizer. (ii) The tendency of Verticillium propagules to stimulate antagonist development was evident in high pH soil and also when fertilizer was incorporated. (iii) The effect of fertilizer was more com= plicated and possibly caused the significance of the three-factor interaction. The following deductions could be made from the analyses: (a) In presence of Verticillium propagules an increase in numbers of antagonists occurred at both pH levels. (b) In absence of Verticillium propagules NPK caused a reduction in numbers of antagonists, but only in high pH soil.

After 8 weeks of incubation (Fig. 13 & Table 24) the three-factor interaction was no longer significant. However, the interactions Verticillium X pH (Table 24.1) and pH X NPK (Table 24.2) were significant. The results indi= cate that low pH soils suppressed numbers of antagonists irrespective of the level of Verticillium propagules and

that the addition of Verticillium to the soil, caused a significant increase in numbers of antagonists in high pH soil only. The effect of NPK (Table 24.2) was non-significant in high pH soil, but in low pH soil NPK had a suppressing effect on numbers of antagonists. The same tendency was observed in high pH soil after 4 weeks of incubation. Low pH suppressed the numbers of antagonists irrespective of the levels of any of the other factors.

General observation

In all these experiments on the effect of soil pH on numbers of antagonists, lower numbers were observed in low pH soil. However, it was most striking that although the experiments were designed to isolate bacterial and actinomycetous antagonists, acidified soils yielded mainly fungal antagonists. To test this observation, a further experiment was included to investigate the effect of reduced soil pH on total numbers of fungi, numbers of Trichoderma spp., and numbers of fungal antagonists in general.

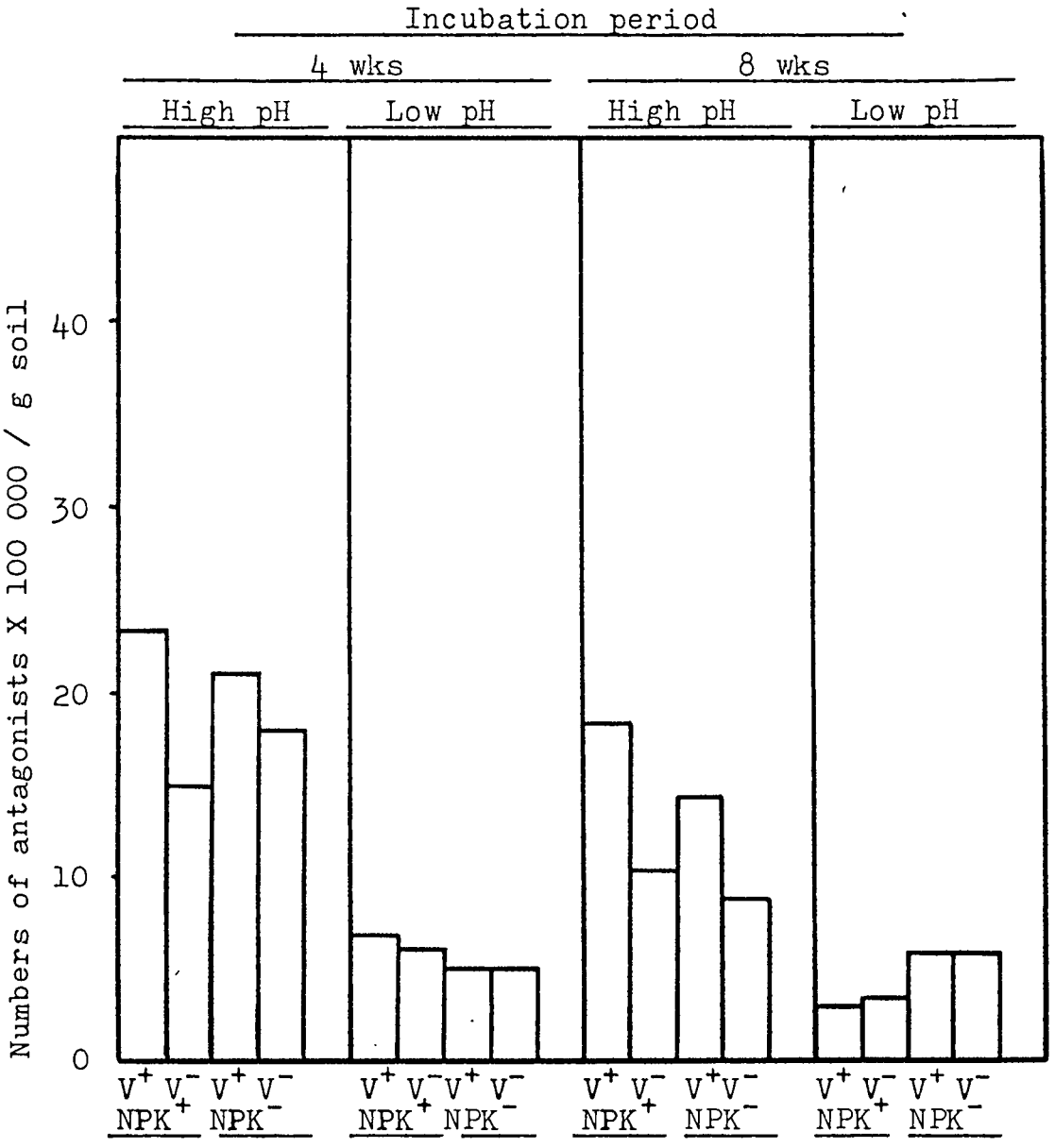


Fig. 13 - Numbers of bacterial and actinomycetous antagonists isolated from high and low pH soils with Verticillium propagules, N, P, and K incorporated.
V^+ = Verticillium amended; NPK^+ = N, P, and K amended.

Table 23 - The effect of NPK on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 4 weeks of incubation[a]

Verticillium	pH	N+P+K		Mean	Mean for pH	
		Present	absent		High	Low
Present	High	4,93	4,67	4,80	4,47	2,54
	Low	2,74	2,40	2,57		
	Mean	3,83	3,53	3,68		
Absent	High	3,96	4,34	4,15		
	Low	2,62	2,41	2,51		
	Mean	3,29	3,37	3,33		
Mean		3,56	3,45			

a: See Table 11.

Table 23.1 - Analysis of the Verticillium X pH interaction for 4 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	259,14	138,81	120,33
Absent	224,15	135,89	88,26
Difference	34,99	2,92	

SS of pH within Verticillium present = 134,07*
SS of pH within Verticillium absent = 72.13*
SS of Verticillium within high pH = 11,33*
SS of Verticillium within low pH = 0,08ns

Table 23.2 - Analysis of the pH X NPK interaction for 4 weeks of incubation

pH	NPK		Difference
	Present	Absent	
High	239,96	243,33	3,37
Low	144,93	129,77	15,16
Difference	95,03	113,56	

SS of NPK within pH high = 0,105ns
SS of NPK within pH low = 2,128ns
SS of pH within NPK present = 83,617*
SS of pH within NPK absent = 119,406*

Table 23.3 - Analysis of the *Verticillium* X NPK interaction for 4 weeks of incubation

Verticillium	NPK		Difference
	Present	Absent	
Present	207,16	190,79	16,37
Absent	177,73	182,31	4,58
Difference	29,43	8,48	

SS of NPK within *Verticillium* present = $2,481^{ns}$
SS of NPK within Verticillium absent = $0,194^{ns}$
SS of *Verticillium* within NPK present = $8,019^{*}$
SS of *Verticillium* within NPK absent = $0,665^{ns}$

Table 23.4 - Analysis of the *Verticillium* X pH interaction for NPK present (4weeks)

pH	NPK		Difference
	Verticillium present	*Verticillium* absent	
High	133,05	106,91	26,14
Low	74,11	70,82	3,29
Difference	58,94	36,09	

SS : *Verticillium* within high pH for NPK present = $12,65^{*}$
SS : *Verticillium* within low pH for NPK present = $0,20^{ns}$
SS : pH within *Verticillium* present for NPK present = $64,33^{*}$
SS : pH within *Verticillium* absent for NPK present = 24.12^{*}

Table 23.5 - Analysis of the *Verticillium* X pH interaction for NPK absent (4 weeks)

pH	NPK		Difference
	Verticillium present	*Verticillium* absent	
High	126,09	117,24	8,85
Low	64,70	65,07	0,37
Difference	61,39	52,17	

SS: *Verticillium* within high pH for NPK absent = $1,45^{*}$
SS: *Verticillium* within low pH for NPK absent = $0,002^{ns}$
SS: pH within *Verticillium* present for NPK absent = $69,79^{*}$
SS: pH within *Verticillium* absent for NPK absent = $50,40^{*}$

Table 23.6 - Analysis of the pH X NPK interaction for *Verticillium* present (4 weeks)

NPK	*Verticillium* present		Difference
	High pH	Low pH	
Present	133,05	74,11	58,94
Absent	126,09	64,70	61,39
Difference	6,96	9,41	

SS: pH within NPK present for *Verticillium* present = 64,33*
SS: pH within NPK absent for *Verticillium* present = 69,79*
SS: NPK within high pH for *Verticillium* present = 0,88*
SS: NPK within low pH for *Verticillium* present = 1,64*

Table 23.7 - Analysis for the pH X NPK interaction for *Verticillium* absent (4 weeks)

NPK	*Verticillium* absent		Difference
	High pH	Low pH	
Present	106,91	70,82	36,09
Absent	117,24	65,07	52,17
Difference	10,33	5,75	

SS: pH within NPK present for *Verticillium* absent = 24,12*
SS: pH within NPK absent for *Verticillium* absent = 50,40*
SS: NPK within high pH for *Verticillium* absent = 1,98*
SS: NPK within low pH for *Verticillium* absent = 0,61ns

Table 23.8 - Analysis of the *Verticillium* X NPK interaction for high pH (4 weeks)

Verticillium	High pH		Difference
	NPK present	NPK absent	
Present	133,05	126,09	6,96
Absent	106,91	117,24	10,33
Difference	26,14	8,85	

SS: NPK within *Verticillium* present for high pH = 0,90*
SS: NPK within *Verticillium* absent for high pH = 1,98*
SS: *Verticillium* within NPK present for high pH = 12,65*
SS: *Verticillium* within NPK absent for low pH = 1,45*

Table 23.9 - Analysis of the *Verticillium* X NPK interaction for low pH (4 weeks)

Verticillium	Low pH		Difference
	NPK present	NPK absent	
Present	74,11	64,70	9,41
Absent	70,82	65,07	5,75
Difference	3,29	0,37	

SS: NPK within *Verticillium* present for low pH = $1,64^{*}$
SS: NPK within *Verticillium* absent for low pH = $0,61^{ns}$
SS: *Verticillium* within NPK present for low pH = $0,20^{ns}$
SS: *Verticillium* within NPK absent for low pH = $0,002^{ns}$

* = significant.
ns = non-significant.

Table 24 - The effect of NPK on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from high and low pH soils after 8 weeks of incubation[a]

Verticillium	pH	N+P+K		Mean	Mean for pH	
		Present	Absent		High	Low
	High	4,36	3,88	4,12	3,67	2,26
Present	Low	1,92	2,55	2,23		
	Mean	3,14	3,21	3,17		
	High	3,34	3,09	3,21		
Absent	Low	2,04	2,52	2,28		
	Mean	2,62	2,80	2,28		
Mean		2,91	3,01			

a: See Table 11.

Table 24.1 - Analysis of the Verticillium X pH interaction for 8 weeks of incubation

Verticillium	pH		Difference
	High	Low	
Present	4,1220	2,2365	1,8855*
Absent	3,2154	2,2765	0,9389*
Difference	0,9066*	0,0400ns	

Table 24.2 - Analysis of the pH X NPK interaction for 8 weeks of incubation

pH	N+P+K		Difference
	Present	Absent	
High	3,8496	3,4876	0,3620ns
Low	1,9804	2,5326	0,5522*
Difference	1,8692*	0,9550*	

Critical values for Tables 24.1 & 24.2: $S_{\bar{x}} = 0,1326$

(Tukey) $Q_{0,05} = 0,3978$

* = significant.

ns = non-significant.

5.2.2 Factors affecting fungal antagonists in soil

The numbers of fungi antagonistic to *V. dahliae* present in naturally alkaline and acidified soils were determined (Fig. 14 & Table 25).

The pH X time interaction (Table 25.1) as well as the main factors pH and time were statistically significant. The analysis indicates that significantly more antagonists were isolated from acid than from alkaline soil. The acid soil yielded significantly less antagonists after 8 weeks of incubation than after 4 weeks of incubation. Comparatively very few antagonists were isolated from alkaline soil.

The incorporation of *Verticillium* caused an apparent increase in fungal antagonists (Fig.14), but this tendency was not statistically significant.

Table 26 contains the results of total numbers of fungi isolated from alkaline and acidified soils. It is obvious that acidified soil favoured the development of fungi in general and also that of *Trichoderma* spp.

Remark

The technique (Williams & Kaufman, 1962) employed to study fungal antagonists (p.67) proved to be very cumbersome and if great care was not exercised in applying the assay-fungus-agar layer, spreading of antagonist colonies occurred, which

led to erroneous results. Because of this and other impracticalities in the technique which made quick and accurate execution of routine experiments difficult, further studies on fungal antagonists were abandoned.

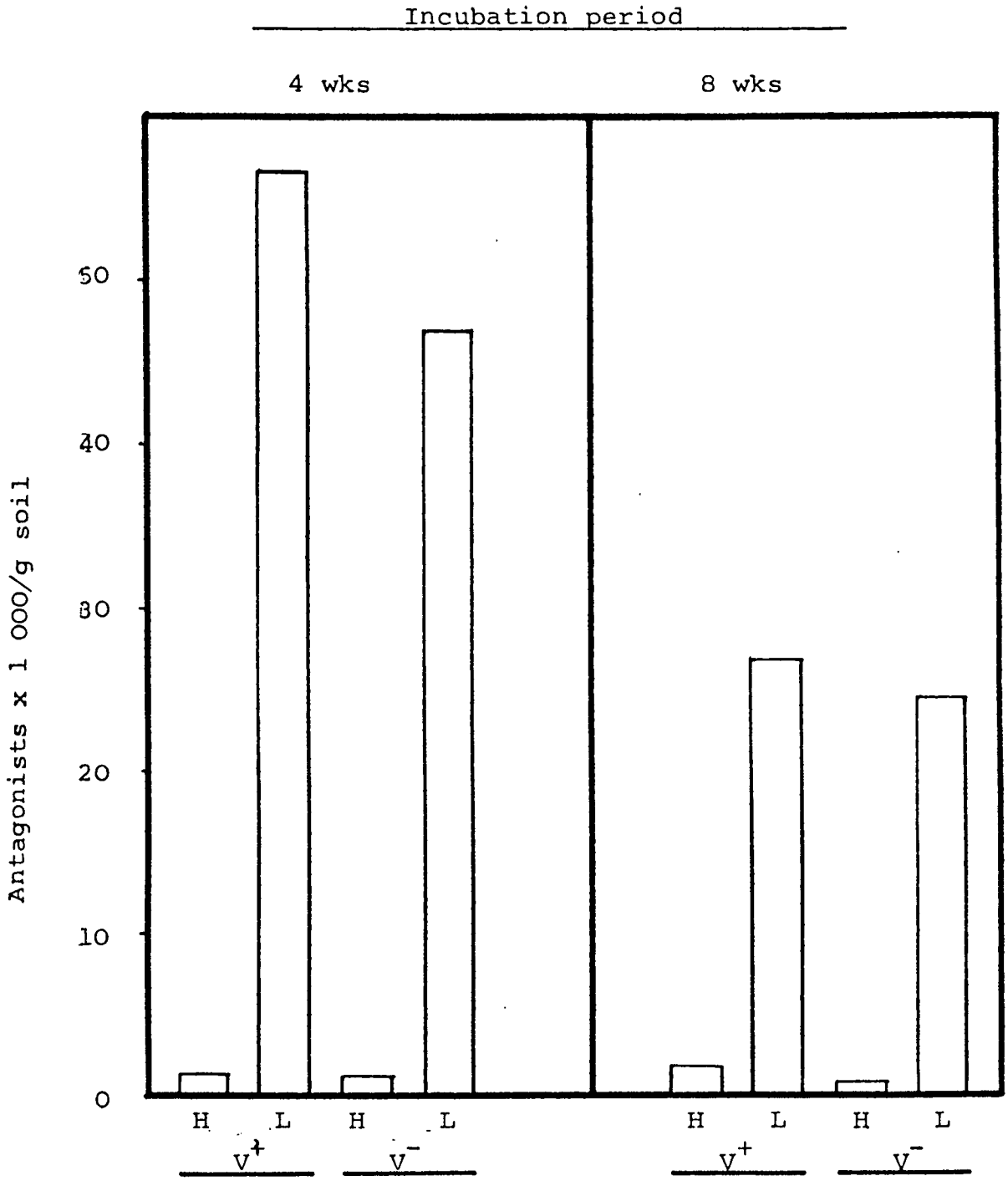


Fig. 14- Mean numbers of fungal antagonists isolated from high and low pH soils amended with Verticillium propagules.

Legend: H = High pH; L = Low pH; V^+ = Verticillium amended.

Table 25 - Numbers ($\sqrt{n+1}$ transformed data) of fungal antagonists isolated from high and low pH soils, amended or non-amended with *Verticillium* propagules, after incubation for 4 and 8 weeks[a]

Verticillium	pH	Incubation period		Mean	Mean for pH	
		4 wks	8 wks		High	Low
	High	1,106	1,152	1,129	1,106	2,882
Present	Low	3,477	2,484	2,980		
	Mean	2,291	1,818	2,054		
	High	1,091	1,076	1,083		
Absent	Low	3,184	2,383	2,783		
	Mean	2,137	1,729	1,933		
Mean		2,214	1,773			

a: See Table 11.

Table 25.1 - Analysis of the pH X Time interaction

pH	Incubation period		Difference
	4 wks	8 wks	
High	1,10	1,11	0,01^{ns}
Low	3,33	2,43	0,90*
Difference	2,23*	1,32*	

Critical values: $S_{\bar{x}}$ = 0,0781 (Tukey) $Q_{0,05}$ = 0,23

* = significant.
ns = non-significant.

Table 26 - Total numbers of fungi and *Trichoderma* spp. isolated from high and low pH soils[a]

Soil pH	Mean no. of fungi X 10 000 / g soil	
	Total fungi	*Trichoderma* spp.
4,5	96,5	1,2
8,0	43,4	0,0

a: Means of 3 replicates.

5.3 DISCUSSION

5.3.1 Factors affecting antagonists of *V. dahliae* in soil

No evidence could be found in the literature that the MS of *V. dahliae* can be destroyed by antagonistic soil micro-organisms. However, evidence exists that such resting structures of other fungi can be destroyed in the soil through the action of antagonistic micro-organisms. For instance, sclerotia of *Sclerotium delphinii* and *Sclerotinia sclerotiorum* are colonized by *Penicillium* and *Trichoderma* spp. (Ferguson, 1953), and *Coniothyrium minitans* actively parasitize and destroy sclerotia of *Sclerotinia trifoliorum* (Tribe, 1957). Clark (1942) also found that the activity of the soil microflora appeared to be essential for the destruction of the sclerotia of *Phymatotrichum omnivorum* in soil. It was stressed by Patrick & Toussoun (1965), however, that the effectiveness of antagonists in biological control requires that the population density of antagonists in an active state be sufficiently high.

It was found (Tables 2 & 3) that the fungi in the root zone of cotton, of which many proved to be antagonistic to *V. dahliae in vitro* (Table 5), did not appreciably increase after the release of MS from moribund cotton roots under natural conditions. It was, therefore, considered necessary to investigate the possibility of increasing bacterial and actinomycetous antagonists in soil by the addition of *Verticillium*

propagules and fertilizers, and by modifying environmental conditions.

5.3.1.1
Soil pH

It is known that soil pH influences the microbial population of the soil (Waksman, 1922; Sadasivan, 1965) and in many instances diseases, including Verticillium wilt (p. 25), have been controlled by acidifying the soil (Haenseler, 1928; Martin, 1931; Guba, 1934).

Soil pH had a profound effect on numbers of antagonists isolated from the soil (Fig. 4 - 14). Alkaline soil constantly yielded many more bacterial and actinomycetous antagonists of V. dahliae than the acidified soil. However, the present results indicated that different types of antagonists were isolated from soils of different pH. From alkaline soils hardly any fungal antagonists were isolated, but the antagonists from acidified soils consisted mainly of fungi. This indicates that a shift from bacterial and actinomycetous antagonists in alkaline to fungal antagonists in acidified soils occurred.

The effect of soil pH on fungal antagonists in particular, was also studied (Fig. 14). It was found that fungal antagonists and soil fungi in general (Table 26), were more abundant in acidified than in alkaline soils. Verticillium-amended acid soil yielded 56 700 antagonists per g soil while alkaline soil yielded only 1 300.

This finding is in agreement with the concept that alkaline soils support bacteria and actinomycetes while fungi prevail in acid soils (Alexander, 1961), a principle which also holds true for antagonists (Baker & Cook, 1974).

5.3.1.2
Temperature

The effect of temperature on numbers of antagonists was not clear cut. Although the largest numbers of antagonists were isolated after 4 weeks of incubation at 25°C, the differences were not statistically significant. After 8 weeks of incubation, significantly more antagonists were found in high pH soil incubated at 35°C than in the same soil incubated at 30 or 25°C. However, in low pH soil significantly more antagonists were present after incubation at 25°C than after incubation at 30 or 35°C. This tendency indicates that bacterial and actinomycetous antagonists prevailing in high pH soil were favoured by high temperature (35°C) while fungal antagonists prevailing in low pH soil, were favoured by lower temperature (25°C).

5.3.1.3
Aeration

Aeration of the soil by the incorporation of vermiculite, similarly had little effect on bacterial and actinomycetous antagonists (Fig. 6). After 4 weeks of incubation, medium and highly aerated soils of high pH yielded the highest numbers of antagonists, but this tendency was not repeated after 8 weeks of incubation.

5.3.1.4
Verticillium propagules

The total numbers of antagonists were increased by the incorpo= ration of MS into the soil in all the experiments (Fig. 7 - 13). This finding is in agreement with the concept that enrichment of a soil with propagules of a plant pathogen, causes an in= crease of antagonists of that pathogen (Waksman, 1945; Lockwood, 1968).

In alkaline soil the tendency for bacterial and actinomycetous antagonists to increase after incorporation of MS was consis= tent, although not always significantly so, for all the experi= ments. In acidified soil, significantly more antagonists were isolated less frequently than from natural soil (Tables 12.1, 13.1, 14.1, 18.1, 20) and it was more evident after 8 weeks of incubation than after 4 weeks of incubation. The difference in reaction of antagonists from high and low pH soils could possibly contributed to the different organisms prevalent in natural versus acidified soil. More fungal anta= gonists (Fig. 14) were also recovered from acidified soil after the incorporation of MS to the soil. However, the differences were not statistically significant. These results indicate that fungal antagonists which were more prevalent in acidified soil, did not respond to the same extend as bacterial and actinomycetous antagonists, in natural soil, upon the incorporation of Verticillium propagules.

The increase of antagonists after the introduction of a pathogen into a soil, can sometimes effect the elimination of that

same pathogen from the soil (Vojinović; Gerlagh; Smiley; quoted by Baker & Cook, 1974) and this could be the reason why severe Verticillium wilt is sometimes followed by a re= duction in disease incidence after some years of continuous cropping (Anon., 1939).

5.3.1.5
Fertilizers

Increases in numbers of soil micro-organisms after the incorpo= ration of fertilizers have been reported (Waksman, 1922; Guillemat & Montegût, 1960; Kaufman & Williams, 1964; 1965; Sadasivan, 1965; Bagyaraj & Rangaswami, 1967). In the present study total numbers of antagonists isolated from fertilizer-amended soils, tended to be higher than those from non-amended soils. Ten out of 14 experiments (Tables 11 - 24) yielded higher total numbers of antagonists after fertilizer application. In eight of these the effect of fertilizer was statistically significant either as a main factor or in one or more interac= tions (Tables 11, 14, 17, 18, 21, 22, 23, 24).

After 4 weeks of incubation, N caused the total numbers of anta= gonists to decrease significantly (Fig, 7); a tendency which, however, did not last until the eighth week of incubation. The suppressive effect of N on antagonist numbers, although transitory, is not readily explained and is in contrast with results obtained with soil micro-organisms in general. Nitrogen usually causes increases in numbers of soil micro-organisms (Waksman, 1922; Guillemat & Montegût, 1960; Kaufman & Williams, 1964; 1965; Bagyaraj & Rangaswami, 1967). However,

fungal antagonists were also suppressed by N fertilization of maize soils (Kaufman & Williams, 1965).

Potassium alone (Fig. 9) did not have a significant effect on antagonist numbers. After 4 weeks of incubation a non-significant decrease in antagonist numbers occurred which was transitory. In combination with N (Fig. 11), K had no significant effect on antagonists either.

Phosphate, supplied as the only fertilizer (Fig. 8), caused increases in antagonist numbers in high pH soil which was statistically significant at the eight-week incubation level. In combination with N (Fig. 10), K (Fig. 12), and N + K (Fig.13) phosphate also caused significant increases in antagonist numbers.

The present results, therefore, indicate that antagonist numbers can be increased in soil by the incorporation of fertilizers and the indications are that phosphate is of particular impor= tance.

CHAPTER 6

FACTORS AFFECTING ANTAGONISTS RELATED TO THE SURVIVAL OF V. DAHLIAE IN SOIL

These experiments were designed to determine whether antagonist numbers, as affected by various treatments, could be related to the survival of the pathogen in the soil.

6.1 MATERIALS AND METHODS

6.1.1 The effect of soil reaction on numbers of antagonists and survival of V. dahliae in soil

In this experiment the soil was treated as described on p. 66. Results on the viability of V. dahliae as well as the numbers of bacterial and actinomycetous antagonists present in the soil were taken after 2, 4, 8, 16, and 32 weeks of incubation. Each treatment was replicated three times. Antagonist numbers were determined by the method previously described (p. 65) while numbers of V. dahliae surviving the treatments were determined by dilution plating on ESA (Nadakavukaren, 1960).

The numbers of MS added to the soil at the start of the experiment were determined as follows: Three erlenmeyer flasks with soil to which Verticillium propagules from Czapek's gelatin cultures were added, were emptied on glass plates. The soil was air-dried for 48 h and serially plated out on ESA. The numbers of MS of V. dahliae developing into colonies on ESA were counted and the numbers per g soil calculated. The origi-

nal number of MS added to the soil in this experiment was cal= culated at 19 300 / g soil.

Carbon dioxide release from the soil was measured during this investigation to get an indication of the activity of micro-organisms in natural and acidified soils. A direct method employing ascarite (sodium-hydroxide-asbestos) (Kolthoff & Sandell, 1958) was used.

6.1.2 The effect of sulphur and gypsum on numbers of antagonists and survival of *V. dahliae* in soil

Air-dried Glen soil was sieved and devided into two lots. Gypsum was added at a rate of 26 000 kg/ha (0,65 g/100 g soil) to one lot while the other received sulphur at a rate of 5 500 Kg/ha (0,13 g/100 g soil) (Mahmoud *et al.* 1969). Each lot was thoroughly mixed with a "Kenwood" food mixer.

Verticillium propagules from Czapek's gelatin medium were washed free of nutrients (p. 65) and added in suspension to half of each of the gypsum and sulphur treated soils. Control treat= ments received *Verticillium* propagules only. Aliquots (50 g) of the differently treated soils were added to individual 250 ml erlenmeyer flasks. The moisture content was adjusted to 20% of the air-dry mass of the soil and the cultures were stored at 25°C for 4 and 8 weeks. Numbers of antagonists were recorded by the method described on p. 64 while numbers of *V. dahliae* surviving in the soil were determined by dilution plating on ESA (Nadakavukaren, 1960). Numbers of MS added to the soil were determined as described above (p. 123). The number of

MS added to the soil in this experiment were c. 64 000/g soil. Each experiment was replicated 3 times.

6.2 RESULTS

6.2.1 The effect of soil pH on numbers of bacterial and actinomycetous antagonists related to survival of V. dahliae

The numbers of antagonists from alkaline and acidified soils amended and non-amended with Verticillium were isolated over a 32-week period (Fig. 15 & Table 27).

These results confirmed the previously found tendency of alkaline soil to yield larger numbers of antagonists than acidified soil. In both soil types it was again observed that the incorporation of Verticillium caused the numbers of antagonists to increase. However, the increase was more pronounced in alkaline than in acidified soil (Fig. 15). In alkaline soils, both with and without propagules, the numbers of antagonists increased rapidly, attaining a maximum at 4 weeks after which the numbers declined steadily. In acid soils the numbers decreased during the first 8 weeks and then increased until 16 weeks to a level higher than that observed after 2 weeks. Thereafter the numbers declined to such an extent that no antagonists could be recovered after 32 weeks.

Statistical analyses of the results showed that the three-factor interaction (Verticillium X pH X time) was significant. In view of the fact that the Verticillium X time interaction was non-significant, the three-factor interaction implied that the time X pH interaction differed at the different time levels. Soil pH was responsible for the three-factor interaction (Tables 27.1 - 27.7): The incorporation of Verticillium to the soil significantly affected the numbers of antagonists isolated from alkaline soil at all time levels up to 16 weeks. At the 32-week level,

the difference between antagonist numbers isolated from Verticillium-free and Verticillium-amended soils was no longer statistically significant. However, higher numbers were still obtained from Verticillium-amended soils. The differences observed between acidified Verticillium-amended and non-amended soils were non-significant.

Both pH and time had significant effects on the numbers of Verticillium propagules which could be recovered from the soil (Fig. 16 & Table 28). At the start of the experiment 19 300 propagules were incorporated per g soil. In acid soil these declined to 208/g during the first two weeks, and after 4 weeks of incubation no viable propagules could be isolated. In alkaline soil a very sharp decline occurred during the first 4 weeks when 93,4% of the spores were killed, but during the following 28 weeks only 3,7% were killed.

The CO_2 released by high and low pH soil amended and non-amended with Verticillium propagules was measured (Table 29).

During the first two days considerably more CO_2 was released by acidified soil than by alkaline soil. During the 3 to 7-day period it was still slightly higher, but after that it remained much the same for all the treatments. The incorporation of Verticillium had an effect in high pH soil only in that it stimulated CO_2 release during the first two days after the soil was moistened.

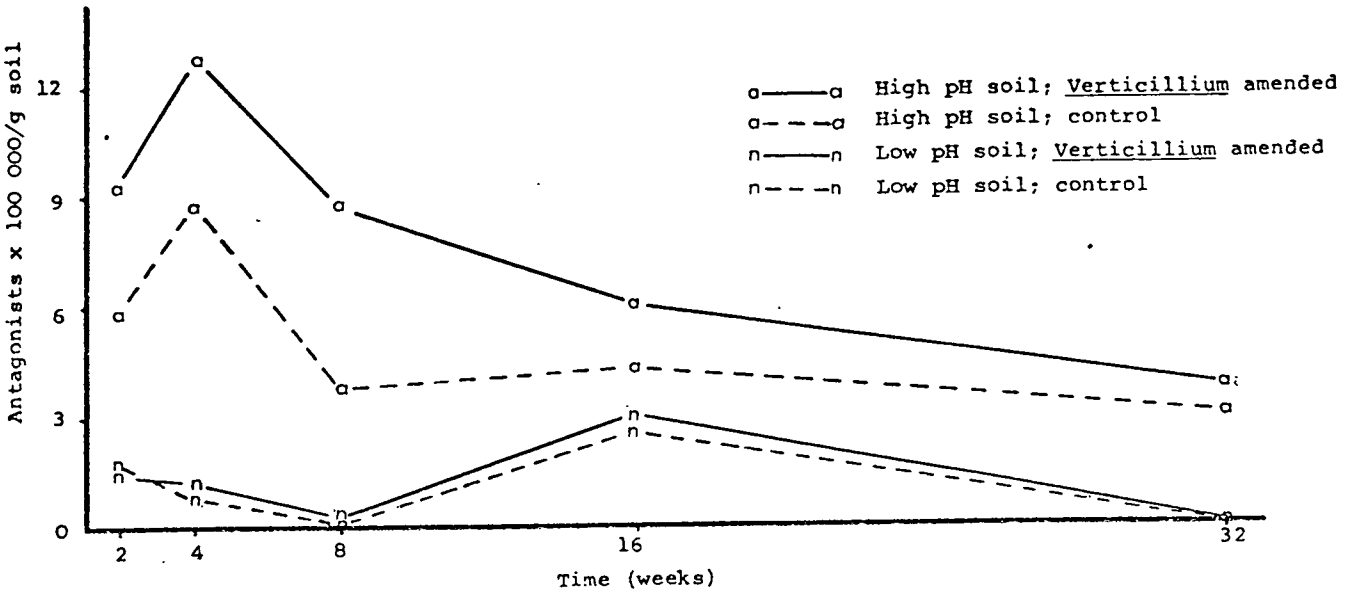


Fig.15 - Numbers of bacterial and actinomycetous antagonists isolated over a period of 32 weeks

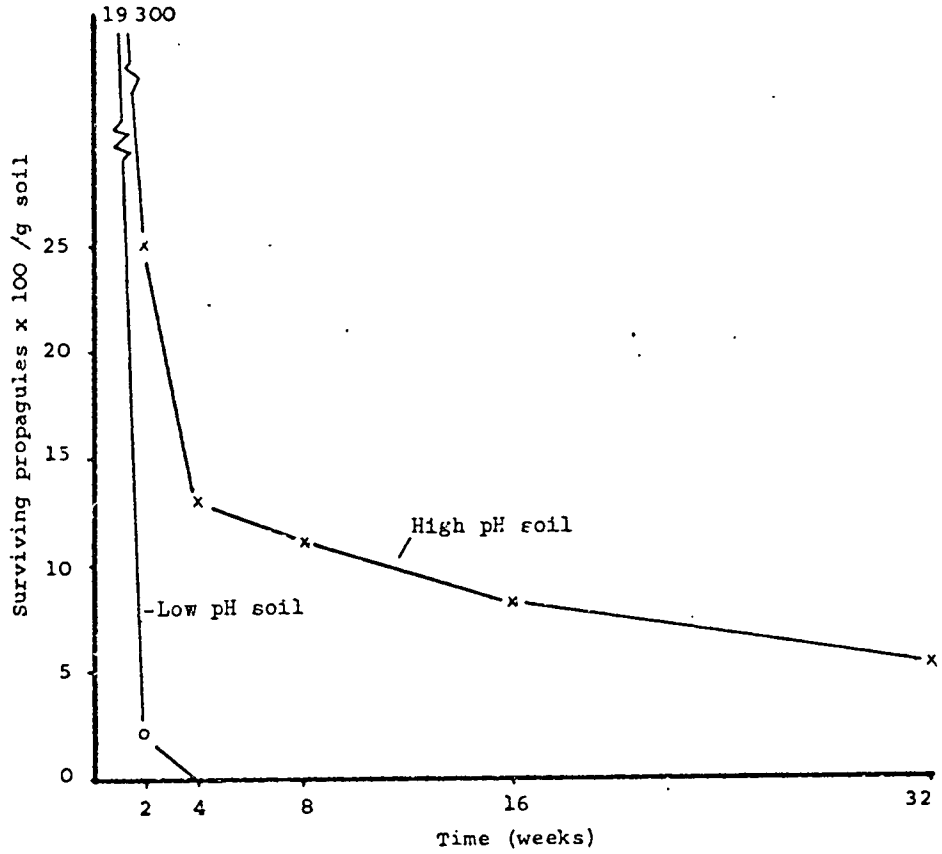


Fig. 16 Survival of V. dahliae in alkaline and acid soils determined over a period of 32 weeks

Table 27 - The effect of soil pH on numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists in Verticillium-amended and non-amended soils over a period of 32 weeks[a]

Verticillium	pH	Incubation period in weeks					Mean	Mean for pH	
		2	4	8	16	32		High	Low
	High	4,86	5,69	4,74	3,96	3,20	4,49	4,08	1,79
Present	Low	2,08	1,94	1,21	2,85	1,00	1,82		
	Mean	3,47	3,82	2,98	3,41	2,10	3,15		
	High	3,88	5,00	3,20	3,37	2,86	3,66		
Absent	Low	2,28	1,68	1,15	2,67	1,00	1,76		
	Mean	3,08	3,34	2,18	3,02	1,93	2,71		
Mean		3,28	3,58	2,58	3,21	2,02			

a: See Table 11.

Table 27.1 - Analysis of the pH X time interaction

Time (wks.)	pH		Difference
	High	Low	
2	236,09	117,74	118,35
4	288,83	97,78	191,05
8	214,36	63,89	150,47
16	197,88	148,97	48,91
32	163,71	54,00	109,71

SS of pH within : 2 weeks = 129,68*
4 " = 337,96*
8 " = 209,64*
16 " = 22,15*
32 " = 111,45*

Table 27.2 - Analysis of the Verticillium X pH interaction

Verticillium	pH		Difference
	High	Low	
Present	606,19	245,45	360,74
Absent	494,68	236,93	275.75

SS of pH within: Verticillium present = 481,98*
Verticillium absent = 281,62*

Table 27.3 - Analysis of the Verticillium X time interaction

Time (wks.)	Verticillium Present	Verticillium Absent	Difference
2	187,42	166,41	21,01
4	206,13	180,48	25,65
8	160,77	117,48	43,29
16	183,96	162,89	21,07
32	113,36	104,35	9,01

SS of Verticillium within: 2 weeks = 4,09*
4 weeks = 6,09*
8 weeks =17,35*
16 weeks = 4,11*
32 weeks = 0,75ns

Table 27.4 - Analysis of the pH X time interaction for Verticillium present

Time (wks.)	Verticillium present High pH	Low pH	Difference
2	131,23	56,19	75,04
4	153,63	52,50	101,13
8	127,98	32,79	95,19
16	106,99	76,97	30,02
32	86,36	27,00	59,36

SS for pH within: 2 weeks for Verticillium present = 104,28*
4 " " " " = 189,39*
8 " " " " = 167,80*
16 " " " " = 16,69*
32 " " " " = 65,25*

Table 27.5 - Analysis of the pH X time interaction for *Verticillium* absent

Time (wks.)	*Verticillium* absent		Difference
	High pH	Low pH	
2	104,86	61,55	43,31
4	135,20	45,28	89,92
8	86,38	31,10	55,28
16	90,89	72,00	18,89
32	77,35	27,00	50,35

SS for pH within: 2 weeks for *Verticillium* absent = 34,74*
4 " " " " = 149,73*
8 " " " " = 56,59*
16 " " " " = 6,61*
32 " " " " = 46,95*

Table 27.6 - Analysis of the *Verticillium* X time interaction for high pH

Time (wks.)	High pH		Difference
	Verticillium Present	*Verticillium* Absent	
2	131,23	104,86	26,37
4	153,63	135,20	18,43
8	127,98	86,38	41,60
16	106,99	90,89	16,10
32	86,36	77,35	9,10

SS for *Verticillium* within: 2 weeks for high pH = 12,88*
4 " " " " = 6,29*
8 " " " " = 32,05*
16 " " " " = 4,80*
32 " " " " = 1,53ns

Table 27.7 - Analysis of the *Verticillium* X time interaction for low pH

Time (wks.)	Low pH		Difference
	Verticillium Present	*Verticillium* Absent	
2	59,19	61,55	5,36
4	52,50	45,28	7,22
8	32,79	31,10	1,69
16	76,97	72,00	4,97
32	27,00	27,00	0,00

SS for *Verticillium* within: 2 weeks for low pH = ns
4 " " " " = ns
8 " " " " = ns
16 " " " " = ns
32 " " " " = ns

* = significant.
ns = non-significant.

Table 28 - The effect of soil pH on numbers ($\sqrt{n+1}$ transformed data) of viable propagules of *V. dahliae* recovered from soil over a period of 32 weeks[a]

pH	Incubation period in weeks					Mean
	2	4	8	16	32	
High	1,78	1,47	1,42	1,32	1,21	1,44
Low	1,09	1,00	1,00	1,00	1,00	1,02

a: Mean number / plate of 8 plates / treatment; each treatment was replicated 3 times.

Critical values: $S_{\bar{x}} = 0,057$
(Tukey) $Q_{0,05} = 0,24$

Table 29 - The amount of CO_2 released by high and low pH soils amended and non-amended with *Verticillium* propagules[a]

Incubation intervals (days)	High pH soil		Low pH soil	
	Verticillium present	*Verticillium* absent	*Verticillium* present	*Verticillium* absent
0-2	9,7	1,8	34,2	35,0
3-7	0,8	1,4	3,5	2,4
8-11	0,5	1,6	0,7	1,3
12-14	1,3	0,3	1,4	1,8
15-22	1,5	1,3	0,4	0,5
23-30	1,0	0,3	0,6	0,4
31-37	0,2	0,5	0,3	0,4
38-43	0,9	0,8	0,7	0,7
44-51	0,4	0,4	0,5	0,4

a: Each figure represents the mean of 3 replicates and gives the amount of CO_2 in mg CO_2/50 g soil.

6.2.2 The effect of gypsum and sulphur on numbers of bacterial and actinomyceous antagonists and the survival of *V. dahliae* in soil

The survival of *Verticillium* as well as the numbers of antagonists present in soils treated with sulphur and gypsum, was studied (Fig. 17 & 18 and Table 30 & 31).

6.2.2.1 Survival of *V. dahliae* (Fig 17 & Table 30)

The two-factor interaction was very closely significant and warranted further analyses. The effect of time proved to be highly significant while treatment effects were non-significant. In the *Verticillium* treatment a progressive and significant decrease in numbers of viable propagules was observed over a period of 8 weeks. To a certain extent this tendency was repeated in both the *Verticillium*-gypsum and the *Verticillium*-sulphur treatments. In the *Verticillium*-gypsum treatment a significant decline occurred during the first 4 weeks, but from 4 to 8 weeks the decline, although apparent, was not significant. This tendency was reversed in the *Verticillium*-sulphur treatment. In this case a negligible decline occurred during the first four weeks, but from 4 to 8 weeks, the number of viable propagules were reduced significantly.

A comparison of treatment effects within time indicated that within the control series (0 weeks) the *Verticillium*-sulphur treatment yielded significantly less propagules than either the *Verticillium* and *Verticillium*-gypsum treatments. In all

other treatments within a specific time level, the results obtained from the different treatments were not significantly different from that of the controls (Verticillium-0-weeks, Verticillium-4-weeks, and Verticillium-8-weeks).

6.2.2.2
Numbers of antagonists (Fig.18 & Table 31)

No significant interactions were found, but the main factors had significant effects. The incorporation of Verticillium caused significant increases in numbers of antagonists in all treatment combinations while the incubation period, when corresponding treatment combinations were compared, had a significant effect only within the Verticillium-amended controls. Although the differences were not significant, it is apparent that treatments incubated for 8 weeks yielded less antagonists than corresponding treatments incubated for 4 weeks.

Results obtained from treatments within a specific incubation period and with the same Verticillium status did not differ significantly from each other, but all treatments yielded significantly less antagonists after 8 weeks than the four-week control. It was observed that the decrease in numbers of antagonists after amendment with sulphur was near enough the same as that of the eight-week control, and the gypsum treatment yielded slightly more antagonists than the eight-week control. In absence of Verticillium the gypsum treatment also yielded slightly more antagonists than the corresponding controls for the eight-week incubation period.

Within the four-week incubation series, lower numbers of antagonists were isolated from all treatments than from their corresponding controls irrespective of the Verticillium level. This tendency was also repeated for the sulphur treatment without Verticillium after 8 weeks of incubation.

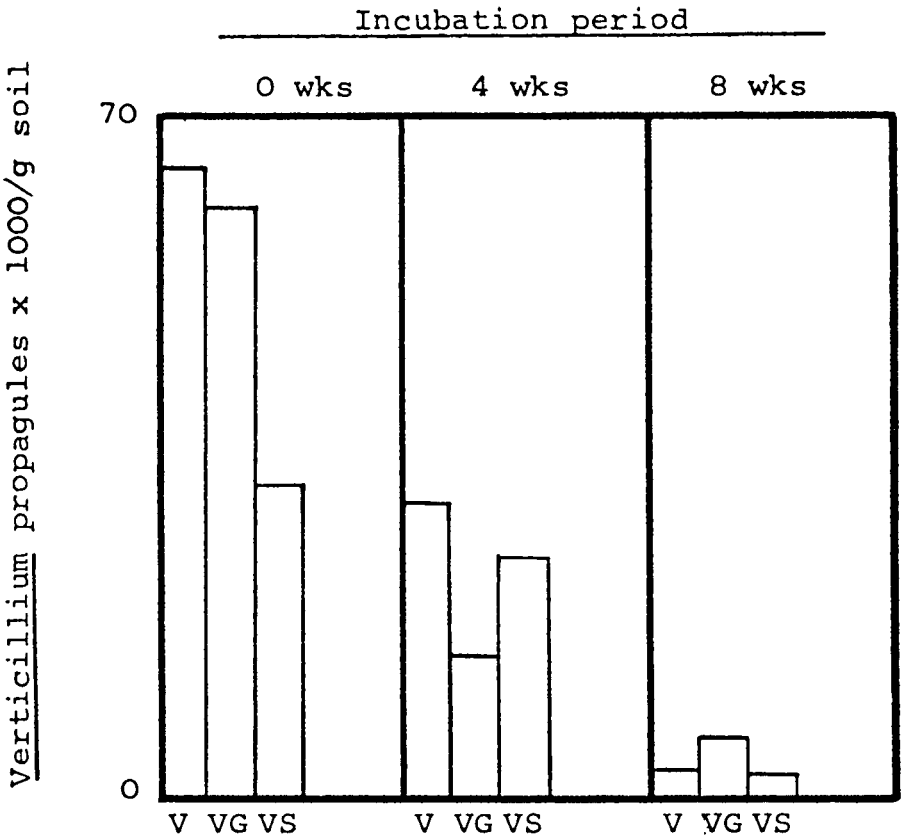


Fig. 17 Mean numbers of Verticillium propagules isolated from soil treated with gypsum or sulphur after incubation for various periods.
Legend: V = Verticillium propagules only; VG = Verticillium + Gypsum; VS = Verticillium + sulphur

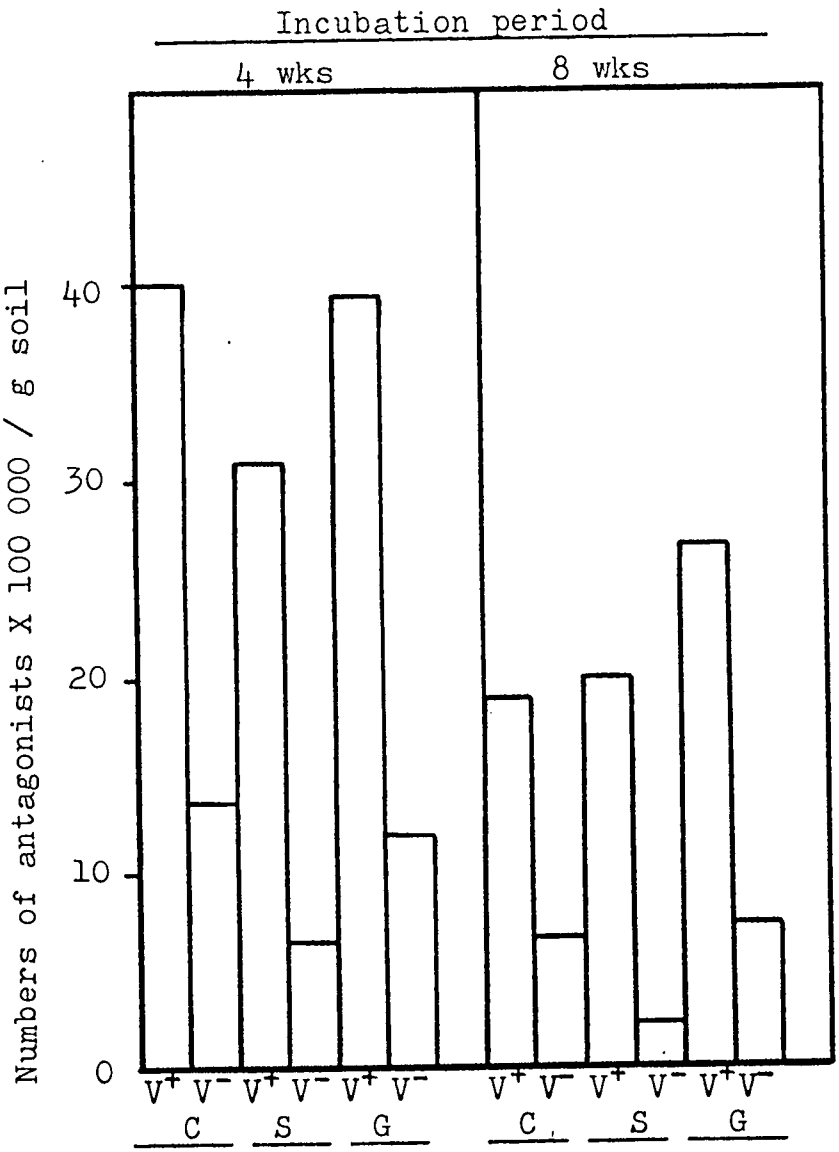


Fig. 18 - Numbers of bacterial and actinomycetous antagonists isolated from soil amended with Verticillium propagules, gypsum, or sulphur after incubation for 4 and 8 weeks. C = control; S = sulphur; G = gypsum; V^+ = Verticillium present; V^- = Verticillium absent.

Table 30 - Mean numbers ($\sqrt{n+1}$ transformed data) of *Verticillium* propagules recovered from soil treated with gypsum or sulphur and incubated for 4 and 8 weeks

Incubation period (wks)	Treatments[a]	Propagules[b]	Mean pH
0	V	8,04	7,98
	VG	7,81	7,63
	VS	5,73	7,82
4	V	5,25	7,94
	VG	3,81	7,63
	VS	5,01	7,53
8	V	1,93	7,94
	VG	2,56	7,62
	VS	1,82	7,01

a: V = *Verticillium*; VG = *Verticillium* + Gypsum; VS = *Verticillium* + Sulphur.

b: Mean of 3 replicates / treatment; 10 samples / replicate.

Critical values: $S_{\bar{x}} = 0,5568$
(Tukey) $Q_{0,05} = 2,01$

Table 31 - Mean numbers ($\sqrt{n+1}$ transformed data) of bacterial and actinomycetous antagonists isolated from soils amended with *Verticillium* propagules, gypsum, or sulphur, and incubated for 4 and 8 weeks

Incubation period (wks)	Treatments	*Verticillium*	Antagonists[a]
4	Control	Present	6,39
		Absent	3,79
	Sulphur	Present	5,57
		Absent	2,66
	Gypsum	Present	6,33
		Absent	3,55
8	Control	Present	4,43
		Absent	2,70
	Sulphur	Present	4,53
		Absent	1,74
	Gypsum	Present	5,24
		Absent	2,84

a: Means of 3 replicates / treatment; 9 samples / replicate.

Critical values: $S_{\bar{x}} = 0,2236$; (Tukey) $Q_{0,05} = 1,14$

6.3 DISCUSSION

6.3.1 The effect of soil pH on survival of *V. dahliae* and antagonist numbers

The present studies have shown that bacterial and actinomycetous antagonists are dominant in alkaline soils and fungi are more prevalent in acidified soils, but the survival of MS of the pathogen under these conditions still needed investigation.

Various diseases, including Verticillium wilt of various hosts, can be reduced or controlled by planting in acid soils (p. 25 - 29). The survival of MS. however, have not been studied.

The rate of attrition was very fast during the first four weeks of incubation in alkaline soil (Fig. 16). Thereafter, however, the attrition rate slowed down considerably and after 32 weeks in soil approximately 600 MS / g soil still survived. This finding is indicative of the great variability in resistance of individual MS to factors responsible for their decline and could be related to germination of MS in soil and the effect of antagonists on germlings. This aspect will be discussed in Chapter 8.

In soil of which the pH was reduced to *c.* pH 4,5 by the addition of aluminium sulphate, the attrition rate of MS was much faster than in alkaline soil (Fig. 16). After 2 weeks of incubation only 208 MS / g soil could be determined, and after 4 weeks, none appeared on the isolation plates. The technique employed for the recovery of MS (Nadakavukaren, 1960) was, however,

not particularly suited for the recovery of low numbers of MS from soil. A fast decline of MS numbers in acid soils and further reductions in the dilution factor resulted in the development of numerous volunteer fungi on the dilution plates which either inhibited or obscured Verticillium colonies. Later experiments (Fig. 19 - 21) support the finding that acidified soil causes a steeper decline in viability, but since those experiments were done with a selective medium (developed later on) which was much more effective as an isolation medium for V. dahliae, lower numbers of the pathogen could be detected over longer periods of survival. The present experiment did not give a clear picture of the period the MS could survive in acidified soil, but the fast reduction in viability is of major importance.

The fast attrition rate in acidified soil could have been caused by (a) pH itself, (b) the effect of pH on the solubility of toxic substaces in the soil, or (c) by the effect of pH on antagonists in the soil.

The direct effect of pH and its effect on the solubility of toxic substances in the soil will be discussed later. Presently, only the possible effect of antagonists will be discussed.

It was shown (Fig. 15) that antagonists increased during the first four weeks and thereafter declined. This decline continued for the whole period of investigation. Larger numbers of antagonists were present in Verticillium-amended alkaline soil than in non-amended soil. The acidified soil yielded considerably less antagonists than the alkaline soil, but this

could be expected since the technique (p. 65) was designed for the isolation of bacterial and acinomycetous antagonists and it has been established that acid soil mainly support the increase of fungal antagonists (Fig. 14). The increase in fungal antagonists could be responsible for the observed decline in viability of MS. It has, after all, been shown that various diseases, including Verticillium wilt, could be controlled by reducing the pH of the soil (p. 25 - 29) and Weindling & Fawcett (1936) and Shipton, Cook & Sitton (1973) have shown that antagonistic micro-organisms are sometimes implicated.

The increased volume of CO_2 released after acidifying the soil (Table 29) coincided with the increase in numbers of fungal antagonists and might have been an indication of their increased activity. However, increased CO_2 release was of short duration and need not necessarily be related to increased activity of fungal antagonists. Moreover, acidifying of the soil with aluminium sulphate will generate acidic substances which will oxidize carbonaceous substances and this can probably explain the initial increase in CO_2 concentration (Bolt & Bruggenwert, 1976).

A further indication that fungal antagonists were not responsible for the fast attrition of *V. dahliae* in acidified soil is supported by previous experiments which showed that fungal antagonists did not appreciably increase after the release or incorporation of *Verticillium* propagules into the soil. Furthermore, the very fact that the attrition rate was so fast in acidified soil, left the impression that antagonism was not solely, if at all, responsible for the attrition.

6.3.2 The effect of sulphur and gypsum on survival of *V. dahliae* and antagonist numbers

Mahmoud *et al.* (1960) reported enhanced microbial activity in alkaline soils of which the pH was reduced by the addition of sulphur or gypsum while Guba (1934) reported a reduction in disease incidence of Verticillium wilt of egg-plant after sulphur was incorporated into the soil.

The effect of sulphur and gypsum on antagonist numbers and survival of *V. dahliae* at the rates advised by Mahmoud *et al.* (1960), was investigated (Fig. 17 & 18), and it was found that sulphur had a suppressive effect on numbers of MS which could be reisolated immediately after it was incorporated into the soil. However, the inhibition was not evident after 4 and 8 weeks of incubation and can be disregarded. Gypsum had no significant effect on the viability of MS and the decline which was observed within the different treatments, was also evident within the controls.

The effect of sulphur on antagonists was also somewhat suppressive and it was more evident after 4 weeks of incubation than after 8 weeks. However, the effects of sulphur and gypsum seem to be negligible at the rates at which they were added to the soil in the present study. No significant increases in antagonists or decreases in MS were recorded.

CHAPTER 7

LABORATORY AND GREEN-HOUSE STUDIES ON SOME FACTORS AFFECTING THE SURVIVAL OF V. DAHLIAE IN SOIL

The following aspects were investigated:

(a) The effect of reduced pH on survival in ground cotton stems

This experiment was designed to determine (i) whether reduced soil pH had any effect on the survival of MS embedded in infected cotton residues, (ii) the effectiveness of the sucrose-flotation method (Huisman & Ashworth, 1972) for the recovery of MS from the soil, and (iii) whether infected cotton residues could be used as a source of inoculum for further experimentation.

(b) The effect of soil pH, varying soil moisture, and fertilizers on survival

Alternate drying and remoistening of the soil have been reported to affect the viability of the pathogen under laboratory conditions (Farley et al. 1971). An attempt was made to determine if drying and rewetting of the soil would cause a significant decline in pathogen populations in soils of different pH, amended with fertilizers, under green-house conditions.

(c) Different pH-reducing substances

An attempt was made to determine if pH-reducing substances devoid of Al salts would influence the survival of V. dahliae in soil,

and whether the influence of low pH is fungitoxic or fungistatic to the pathogen.

(d) Pentachloronitrobenzene (PCNB)

PCNB was added to the soil to determine whether PCNB-sensitive fungi such as T. viride (Kreutzer, 1963) were responsible for the decline of V. dahliae in acidified soils. It was also necessary to determine the effect of PCNB on the survival of V. dahliae itself.

(e) Varied soil types, soil reaction, and aluminium sulphate

The survival of V. dahliae in two different soils which varied in texture and pH, was determined. The effect on survival of reducing or increasing the pH artificially by incorporating aluminium sulphate and calcium hydroxide respectively, was also determined.

(f) Urea, soil moisture, and organic amendments

It is known that urea (Tsao & Zentmeyer, 1971), soil moisture (Nadakavukaren, 1960; Menzies, 1962; Butterfield et al. 1978), and organic soil amendments (Menzies, 1962; Brinkerhoff, 1973) can affect the survival of a variety of plant pathogens in the soil and was, therefore, investigated in this study.

7.1 MATERIALS AND METHODS

7.1.1 The effect of reduced soil pH on survival in ground cotton stems

Sieved, air-dried, Glen soil was amended with 4% aluminium sulphate to change the reaction to c. pH 4,5. The reaction of the control soil was c. pH 8. Infected cotton stems were ground to pass a 1 mm sieve and mixed with the soil at a rate of 2% by mass. Soil moisture was adjusted to 20% of the air-dry mass of the soil and the cultures were incubated at 25°C for three months. The erlenmeyer flasks containing 50 g aliquots from each treatment were weighed twice weekly and water was added when necessary. Each treatment was replicated 6 times.

After the incubation period expired, the soil was air-dried, ground to pass a 1 mm sieve, and counts of viable propagules were made by the sucrose flotation method (Huisman & Ashworth, 1972). One gram soil was drawn from each replicate, suspended in 70% sucrose solution, shaken up and centrifuged at 1 700 X g for 15 min. Residues remaining in the supernatant fluid were collected on No 90 Whatman filter paper (pore size, 5 µm) (Isaac, Fletcher & Harrison, 1971) and washed with sterile distilled water. The process of suspending the soil sample, centifugation, and washing was repeated three times to remove all organic material, including MS, from the soil (Huisman & Ashworth, 1972).

To determine the effectiveness of the flotation method to remove viable MS from the soil, the soil remaining in the

centrifuge tubes was collected on No 90 Whatman filter paper, washed, and air-dried. Both filter papers containing residues from the supernatant fluid and from the soil were then sepa= rately macerated for 2 min in a "Büler" high speed macerator. Serial dilutions were made in sterile distilled water and plated on VIA, each of 5 petri dishes receiving one ml from the appropriately diluted suspension. The cultures were incu= bated at room temperature for two weeks and numbers of viable propagules determined with the aid of a dissecting microscope.

7.1.2 Soil pH, varying soil moisture, and fertilizers

Sieved, air-dried, Glen soil was inoculated with infected cotton stems ground to pass a 1 mm sieve, at a rate of 2% (m/m). Soil pH was reduced by incorporating 4% (m/m) aluminium sulphate into the soil. Fertilizers, dissolved in distilled water, were added at the following rates: N, 150 ppm (NH_4NO_3); P, 50 ppm ($NaH_2PO_4 \cdot 2H_2O$).

The treated soils were then devided into 6 X 5L plastic pots without holes in the bottom; each pot receiving 3 000 g of treated soil. All treatments received 20% moisture on an air-dry basis, at the start of the experiment. The low-soil-moisture treatment was allowed to dry and was kept dry for the duration of the three-month incubation period. The soil of the high-soil-moisture treatment was covered with a 25 mm layer of "styrofoam" balls (3 - 5 mm diam.) to prevent water loss. Water was replenished every second day. The treatment in which the soil moisture was allowed to vary, was remoistened

every second week. During this period the water retained by the soil dropped to between 2,6 and 11,8%, with a mean of 8,2%, depending on weather conditions.

After three months in the green-house a 100 g soil sample was taken from each pot for examination in the laboratory. The remaining soil was spread on plastic sheets and air-dried. After drying, the soil from each pot was devided into equal lots and put into 2,5L plastic pots. One pot was planted with cotton while the other pot was planted with Datura stramonium. Soil moisture was then again adjusted to 20% for all treatments, the soil covered with "styrofoam" balls, and the pots placed in the green-house. Water was added every second day to all treatments.

Twenty days after seedling emergence the D. stramonium plants were removed from their pots. The roots were washed clean and 100 cm of roots from each pot were plated on ESA (Evans et al. (1974). This method of Evans et al. (1974) is a bio= assay method for the determination of populations of V. dahliae in soil.

The cotton plants were allowed to grow to maturity and disease based on symptom expression as well as Verticillium colonies which developed from stems and roots plated on ESA, were recorded.

The 100 g soil samples drawn from each pot from each treatment for analyis of numbers of viable MS were air-dried to kill mycelia and conidia. Two g soil from each sample were then washed through 125 and 36 µm sieves and plated out on VIA.

After two weeks of culturing at room temperture, colonies of *V. dahliae* were identified and counted.

7.1.3 Different pH-reducing substances

The pH of Glen soil was reduced to *c.* pH 4,5 by the addition of dusting sulphur, H_2SO_4, and $Al_2(SO_4)_3.18H_2O$. The quantities needed were predetermined and were as follows: Sulphur, 0,61%, sulphuric acid (98%), 1,86%, and aluminium sulphate, 4% (m/m). The soil was inoculated by adding 2% infected cotton stem meal after it was ground to pass a 1 mm sieve. The ingredients were well mixed into the soil before the soil was placed into 12 X 5L plastic pots. The controls received inoculum but no pH-reducing agents. Soil moisture was adjusted to 20% of the air-dry mass of the soil and replenished every second day if needed. The cultures were incubated in the green-house for three months.

When the incubation period expired, the soil was shaken from the pots and allowed to air-dry on plastic sheets. After drying, soil from 6 pots from each low pH treatment was mixed with 64 g $Ca(OH)_2$ to restore the pH to its original value. In the remainig 6 pots of each treatment the low pH values were retained. The controls did not receive calcium hydroxide. The soil moisture was restored to 20% of the air-dry mass of the soil and again incubated in the green-house for one month.

After this incubation period a 50 g soil sample was taken from each pot and allowed to air-dry. This soil sample was used to determine the viability of *V. dahliae* on VIA after the

soil was washed through 125 and 36 µm sieves (p. 39). In the remaining soil, seeds of D. stramonium were planted to determine the viability of V. dahliae by bioassay (Evans et al. 1974).

7.1.4 Pentachloronitrobenzene

Glen soil, air-dried and sieved, was infested with ground, infected cotton stems at a rate of 2% by mass and used in the following experiments:

7.1.4.1 The effect of PCNB on the survival of V. dahliae

Aluminium sulphate (4%, m/m) was mixed with the soil to acidify it. The controls were high and low pH soils without PCNB. PCNB (commercial "Terrachlor", 75% wettable powder) was added to the soil at rates of 20, 1,0, 0,1, 0,05, and 0,01 g PCNB / Kg soil, respectively. Of each treatment 50 g soil was placed into each of 5 X 250 ml erlenmeyer flasks. Soil moisture was adjusted to 20% (m/m) on an air-dry basis and the cultures were incubated at 25°C for 3 months. Soil moisture was re= plenished weekly if necessary. After the incubation period had expired, the soil was air-dried, washed through 125 and 36 µm sieves and viability tested on VIA (p. 39).

7.1.4.2 The effect of PCNB on the survival of V. dahliae in soils with different pH values

Essentially the same experimental procedures as described above, were used in this experiment. Different pH values were obtained by adding respectively, 5, 4, 3, 2, and 1%

(m/m) aluminium sulphate to air-dried Glen soil. PCNB was added at 0,1 g / Kg soil. Each treatment was replicated 5 times.

7.1.5 Varied soil types, soil reactions, and aluminium sulphate

The pH of Glen soil (sieved and air-dried) was reduced to c. pH 4,5 by the addition of 4% (m/m) aluminium sulphate. George soil is a light, sandy soil with a natural pH of 4,5. The pH of this soil was increased to c. pH 8 by the addition of 0,5% (m/m) $Ca(OH)_2$. An ensuing treatment of George soil to investigate the effect of aluminium sulphate at pH 4,5, received 4% aluminium sulphate as well as 1% calcium hydroxide to maintain the pH at its original level. Unamended Glen (c. pH 8) and George (c. pH 4,5) soils served as controls.

Freeze-dried MS of propagule size 36 - 125 µm (p. 36) were added to the soil at a rate of 0,1 g MS / 50 g soil. Agar-plate dilutions indicated that each gram of soil received 2525 viable MS. The soil-inoculum mixtures were individually mixed by hand and placed into 250 ml glass beakers. Distilled water was added to adjust soil moisture to 20% of the air-dry mass of the soil. The beakers were covered with aluminium foil and incubated at 25°C in the dark. Soil moisture was replenished twice weekly if necessary. After 3 months of incubation the soil was air-dried and stored for 6 more weeks (Butterfield, 1975) after which it was washed and plated out on VIA (p. 39) to test the viability of MS.

7.1.6 Urea, soil moisture, and organic soil amendments

7.1.6.1 Urea

Natural Glen soil was amended with urea at the following rates: 0 (control), 0,025, 0,05, 0,075, 0,1, 0,25, and 0,5% (m/m, air-dried soil). Soil moisture was kept at 20% (m/m) and the incubation temperature was 25°C.

7.1.6.2 Moisture

Natural Glen soil, inoculated with MS of *V. dahliae*, was incubated at 25°C at the following moisture levels: air-dry, 10, 20, 30, 40, 50, and 60% moisture, based on the air-dry mass of the soil. Air-dried soil contained 3,6% moisture (oven dried at 105°C for 12 h). Field capacity of the soil as determined by the gravitational method described by Nadakavukaren (1960), was 36%. The cultures were weighed twice weekly and water added when necessary.

7.1.6.3 Organic soil amendments

Soyabean, lucern, wheat, groundnut, oat, and maize residues and oil-cake meal, crayfish shells, and soyabean pods were ground to pass through a 1 mm sieve and incorporated into natural Glen soil (1%, m/m). One additional treatment received 1% undamaged seeds of *Tagetes minuta*. *Tagetes minuta* seeds were included because Wilhelm (Baker & Cook, 1974) found that a cover crop of *T. minuta* in an olive grove infected with *V. albo-atrum*, reduced disease incidence.

Soil moisture was adjusted to 20% and maintained at this level throughout the incubation period.

The soil in all three experiments were inoculated with 0,1 g freeze-dried MS / 50 g soil. The cultures were incu= bated at 25°C for three months and survival was determined by washing and plating on VIA (p. 39-41) after it was stored for 6 more weeks (Butterfield, 1975). Each treatment was replicated 5 times.

7.2 RESULTS

7.2.1 The effect of reduced soil pH on survival in ground cotton stalks

After 3 months of incubation in natural and acidified soils, infected cotton residues were recovered from the soil by means of sucrose flotation. Both this fraction and the remaining soil were plated on VIA to determine the viability of the pathogen. The results are schematically presented in Fig. 19.

Six times as many MS survived in high pH soil than in acid soil after an incubation period of 3 months at 25°C and 20% moisture and 77% of the MS in the soil could be recovered by sucrose flotation.

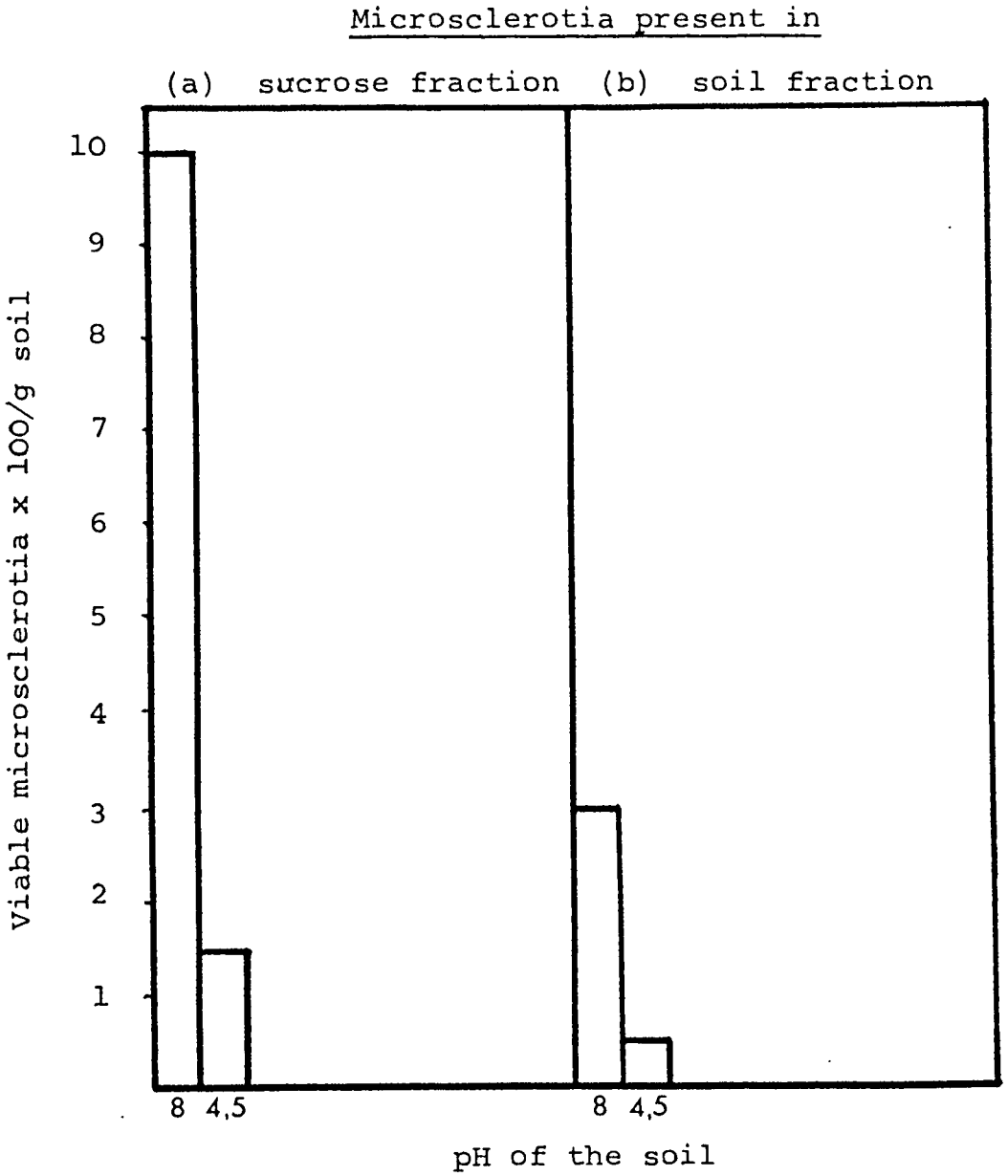


Fig. 19 Viable microsclerotia recovered from high and low pH soils by means of sucrose flotation (a) and those remaining in the soil (b) after sucrose flotation

7.2.2 The effect of soil pH, varying soil moisture, and nitrogen plus phosphate on survival

Cotton grew very poor on acidified soil, but did not get infected (Table 32). On high pH soil 55,5% of the plants became infected. Only the effect of pH was statistically significant.

Analysis of variance of the number of colonies of V. dahliae isolated from the roots of D. stramonium indicate that pH and moisture had significant effects on the survival of the pathogen while the interactions were all non-significant (Table 33). However, by applying Tukey's multiple comparison test (Winer, 1971), it was found that soil moisture did not exert a significant effect.

Table 34 represents the results obtained by direct plating on VIA. Analyses of the results (Tables 34.1 & 34.2) indicate that (i) the attrition rate of Verticillium propagules was significantly increased in acidified soil. (ii) The addition of N and P caused a reduction in Verticillium propagules in both high and low pH soils. However, analysis of the interactions within the different moisture treatments indicated that NP had a significant effect in air-dried soil only. (iii) In both high and low pH soils, moisture had no significant effect. However, within treatments with NP present, survival was significantly reduced in air-dry soil. With NP absent, significantly less propagules survived in high moisture soil than in air-dried soil. The differences between propagules recovered from low and varied and high and varied moisture regimes were non-significant.

Fig. 20 is a representation of the data in original figures, presented in Tables 33 and 34 to compare the two methods of isolation of V. dahliae as well as the effect of the different treatments on the pathogen.

Table 32 - The effect of soil pH, soil moisture, and fertilization on infection of cotton by V. dahliae[a]

Treatments			Plants infected out of 6
Moisture varied	High pH	NP^{+}[b]	4
		NP^{-}	3
	Low pH	NP^{+}	0
		NP^{-}	0
Moisture high	High pH	NP^{+}	3
		NP^{-}	2
	Low pH	NP^{+}	0
		NP^{-}	0
Moisture low	High pH	NP^{+}	4
		NP^{-}	4
	Low pH	NP^{+}	0
		NP^{-}	0

a: Statistical analysis for "dichotomous data" (Winer, 1971).
b: NP^{+} = fertilizers added; NP^{-} = fertilizers absent.

Table 33 - The effect of soil pH, soil moisture, and fertilization on the survival of V. dahliae as determined by the number of colonies / 100 cm of roots of D. stramonium[a]

Moisture	pH	N+P	Mean number of colonies recovered / 100 cm roots ($\sqrt{n+1}$ transformed data)
Varied	High	Present	6,32
		Absent	6,30
	Low	Present	1,49
		Absent	1,50
High	High	Present	5,93
		Absent	6,08
	Low	Present	1,00
		Absent	1,00
Low	High	Present	5,84
		Absent	6,17
	Low	Present	1,00
		Absemt	1,00

a: Evans et al. (1974). Critical values: $S_{\bar{x}} = 0,1353$
(Tukey) $Q_{0,05} = 0,46$

Table 34 - The effect of soil pH, soil moisture, and fertilization on the survival of V. dahliae in soil as determined by direct plating on VIA

		Mean no of colonies / treatment ($\sqrt{n+1}$ transformed data)[a]			
		N+P		Mean for pH	
Moisture	pH	Present	Absent	High	Low
	High	5,37	5,24	5,41	1,84
Varied	Low	1,88	2,19		
	Mean	3,63	3,72		
	High	5,64	5,72		
High	Low	1,44	1,61		
	Mean	3,54	3,67		
	High	4,59	5,82		
Low	Low	1,24	2,65		
	Mean	2,92	4,24		
Mean		3,36	3,87		

a: Means of 6 replications / treatment.

Table 34.1 - Analysis of the NP X moisture interaction

Moisture	N+P		Difference
	Present	Absent	
Varied	3,6225	3,7167	$0,0942^{ns}$
High	3,5392	3,6642	$0,1250^{ns}$
Low	2,9142	4,2383	$1,3244^{*}$

Table 34.2 - Analysis of the pH X moisture interaction

Moisture	pH		Difference
	High	Low	
Varied	5,3042	2,0350	3,2692*
High	5,6767	1,5267	4,1500*
Low	5,2042	1,9408	3,2634*

Critical values: $S_{\bar{x}} = 0,1609$

(Tukey) $Q_{0,05} = 0,5471$

* = significant.

ns = non-significant.

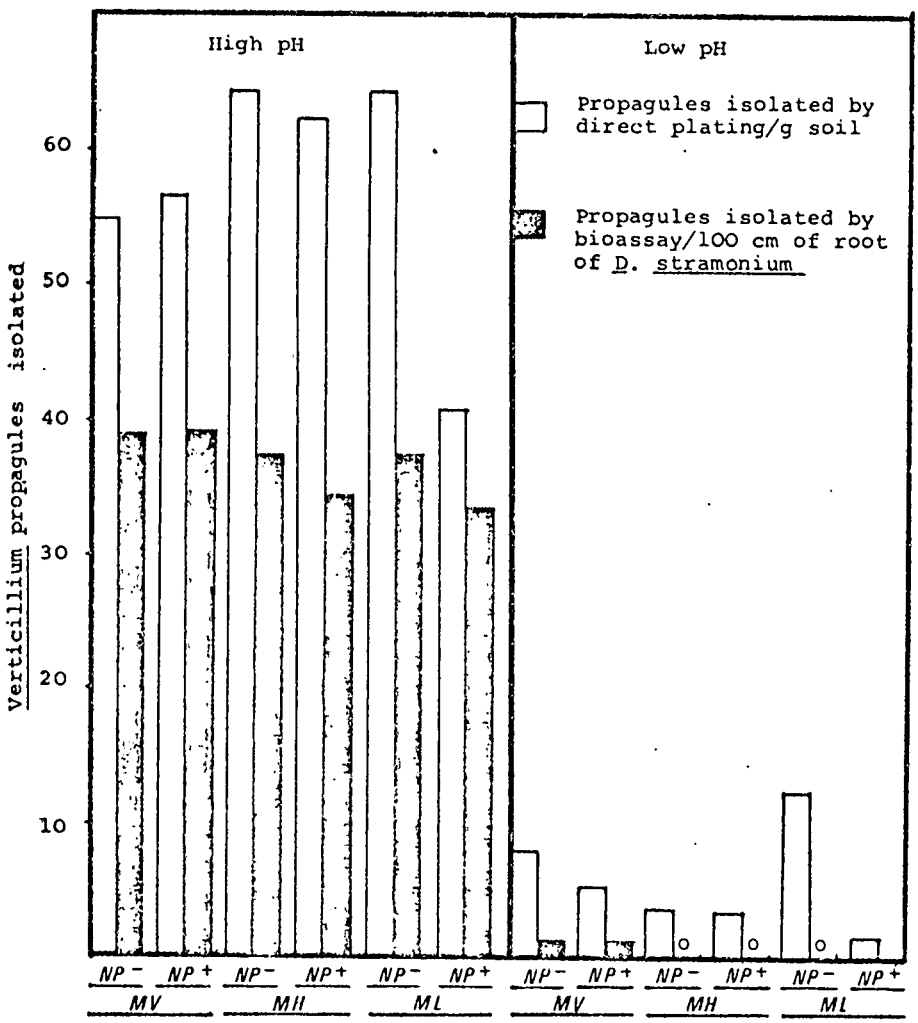


Fig. 20 Mean numbers of V. dahliae propagules isolated from variously treated high and low pH soil by direct plating and bioassay (Evans et al.1974).

Legend: NP^- = No fertilization; NP^+ = Fertilizer added; MV = Moisture varied; MH = Moisture high; ML = Moisture low.

7.2.3 The effect of different pH reducing substances on survival

Verticillium dahliae did not survive well in soils of which the pH have been reduced, irrespective of whether sulphur, sulphuric acid, or aluminium sulphate have been used to induce acid conditions (Table 35).

By direct plating of the soil on VIA, more V. dahliae propagules were recovered per g soil than from 100 cm of roots of D. stramonium (Compare Table 35 and Fig. 21). Some propagules were recovered from acidified soil, but their numbers were considerably less than those of the controls which received no pH-reducing agents (Fig. 21 & Table 36).

Low pH treatments reduced the numbers of viable propagules significantly, irrespective of which acidifying agent was used (Table 36). The apparent decline of viable propagules was significantly counteracted by amendment with calcium hydroxide in the case of the aluminium sulphate treatment.

The sulphur treatment yielded slightly more viable propagules after calcium hydroxide amendment, but the difference was not statistically significant. The sulphuric acid treatment on the other hand, yielded significantly less viable propagules after calcium hydroxide treatment.

Table 35 - The effect of pH-reducing agents on the survival of *V. dahliae* as determined by colony counts on the roots of *D. stramonium*[a]

Treatment	Mean numbers[b] of colonies of *V. dahliae* / 100 cm of roots of *D. stramonium*			
	pH reduced	Final pH	pH reduced for three months and restored with $Ca(OH)_2$	Final pH
Sulphur	0,00	4,0-4,5	0,50	7,5-7,8
H_2SO_4	0,86	4,7-5,3	0,16	7,9-8,0
$Al_2(SO_4)_3.18H_2O$	0,00	4,6-5,6	0,00	7,9-8,1
Control[c]	7,70	7,1-8,1	7,30	7,5-7,8

a: Evans *et al.* (1974).
b: Means of 6 replicates / treatment.
c: High, unaltered pH values.

Table 36 - The effect of pH-reducing agents on the survival of *V. dahliae* as determined by direct plating on VIA

Acidifier	Mean no of colonies[a] of *V. dahliae* X 2 / g soil[b]			Final pH
	pH reduced	Final pH	pH reduced for 3 months and restored with $Ca(OH)_2$	
Sulphur	1,122[c] y	3,5-4,5	1,523[c] y ns[f]	7,5-7,8
H_2SO_4	2,928 z	4,7-5,3	2,033 y *	7,9-8,0
$Al_2(SO_4)_3.18H_2O$	1,000 y	4,6-5,6	1,957 y *	7,9-8,1
Control[e]	4,743 w	7,1-8,1	4,700 z ns	7,5-7,8

a: $\sqrt{n+1}$ transformed data. b: Means of 6 replicates / treatment. c: Data followed by the same letter do not differ significantly from each other. d: ns = non-significant; * = significant. e: High, unaltered pH values. f: Differences within treatments.

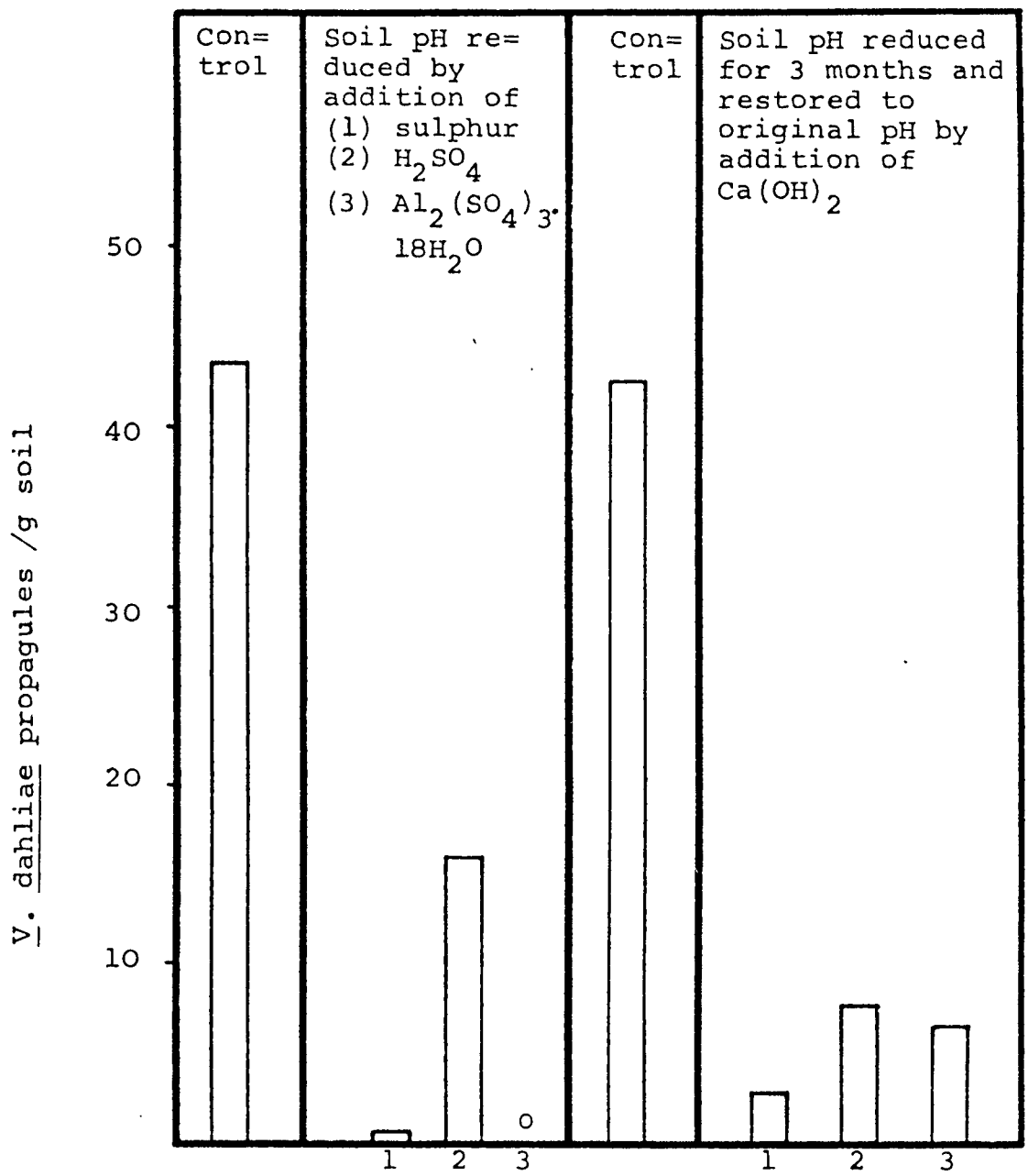


Fig. 21 Mean numbers of propagules of V. dahliae isolated from soil after reducing the pH of the soil by various reducing agents.

7.2.4 The effect of PCNB on survival in acidified soil

In acidified soil at a single pH value (Fig. 22) different concentrations of PCNB did not have a significant effect on the survival of *V. dahliae*. In acidified soils of different pH values (Fig. 23 & Table 37) obtained by the addition of aluminium sulphate, less *Verticillium* propagules were recovered as the pH was reduced progressively. The PCNB X pH interaction was significant in spite of the fact that the PCNB treatment was not significant. It is noteworthy that larger numbers of *Verticillium* propagules survived in soil of higher pH values in presence of PCNB than in absence of PCNB. This tendency was reversed in soils of lower pH values (4% and 5% aluminium sulphate) and is probably responsible for the significant interaction.

Table 37 - The effect of various concentrations of aluminium sulphate (giving different pH levels) and aluminium sulphate plus PCNB on the survival of *V. dahliae*[a]

Aluminium sulphate added (%)	Surviving *Verticillium* propagules[b]		pH range
	PCNB absent	PCNB present[c]	
0	6,702	7,592	6,9-7,7
1	5,813	6,501	6,5-7,3
2	5,637	5,898	6,3-6,7
3	4,538	4,144	5,7-6,2
4	3,136	3,023	4,9-5,0
5	2,585	2,421	4,4-4,5

a: $\sqrt{n+1}$ transformed data. b: Means of 5 replicates / treatment. c: 0,1 g PCNB / Kg soil.
Critical values: Within each PCNB level, between treatments: $S_{\bar{x}} = 0,249$; (Tukey) $Q_{0,05} = 1,05$. Within treatments, between PCNB levels: $S_{\bar{x}} = 0,249$; (Tukey) $Q_{0,05} = 0,71$.

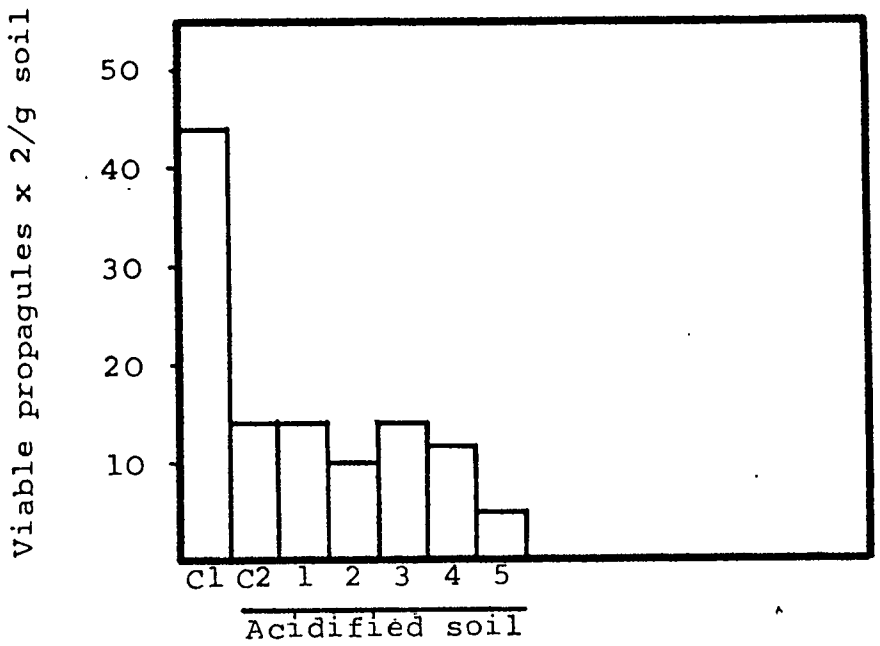


Fig. 22 The effect of PCNB on the survival of V. dahliae in aluminium sulphate amended soils. Legend: C1 = high pH control; C2 = low pH control; 1 = 0,01 g/kg; 2 = 0,05 g/kg; 3 = 0,1 g/kg; 4 = 1 g/kg; 5 = 20 g/kg PCNB.

Within the low pH treatment the differences were not statistically significant.

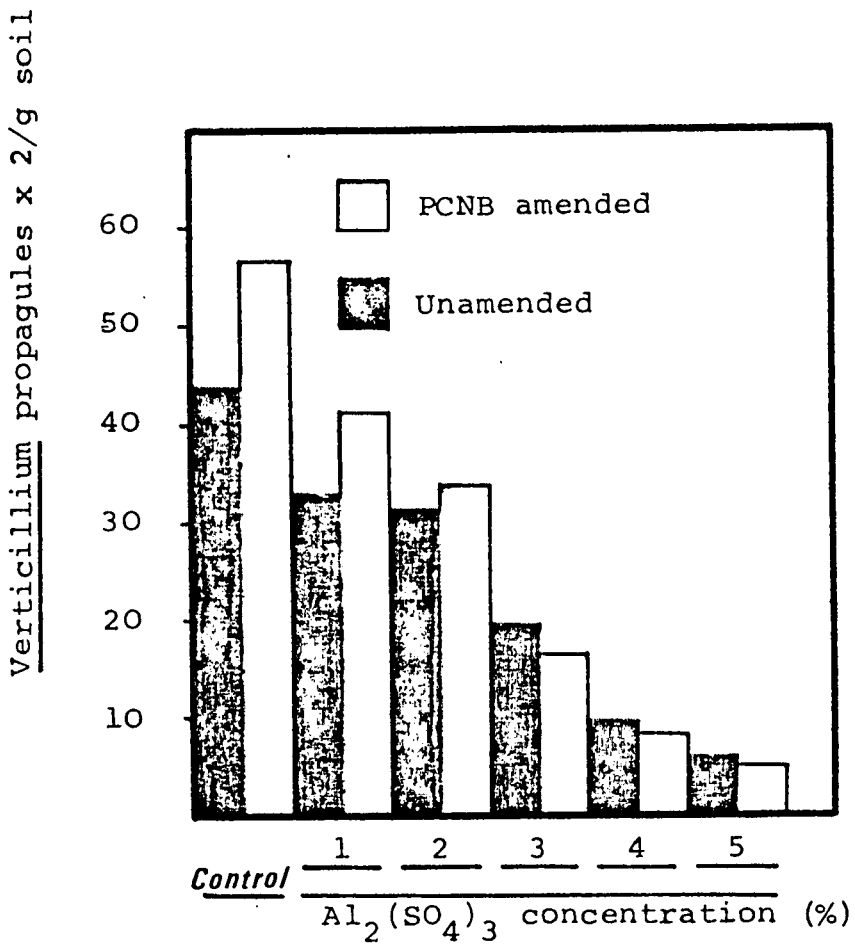


Fig.23 The influence of various concentrations aluminium sulphate giving different pH levels and aluminium sulphate + PCNB on the survival of V. dahliae in soil.

7.2.5 The effect of varied soil types, soil pH, and aluminium sulphate on survival

Acid soil, whether natural or artificially created, did not support survival of *V. dahliae* as well as soils of high pH (Fig. 24 & Table 38). All the treatments received 2525 viable MS per g soil at the start of the experiment. After 3 months of incubation in acid soils the viable population of MS was reduced by 90 - 92%. There was no statistically significant differences between results from low pH soils which received Al or not. Chemical analyses of the experimental soils indicated that none of the constituents investigated, could be held responsible for the decline of viability of *V. dahliae*.

After three months in the alkaline clay soil from Glen, 77% of the MS incorporated into the soil, were not viable any more. In the sandy soil from George (initial pH 4,5) of which the pH was increased to pH 8, more MS (25%) were reisolated than was added to the soil initially.

Microsclerotia from acid soils which germinated on VIA formed small colonies which sometimes remained hyaline throughout the two weeks of observation in culture. Some colonies formed MS, but they were less intense in colour than those from alkaline soils. Conidia were readily formed and could be used as an additional means of identification.

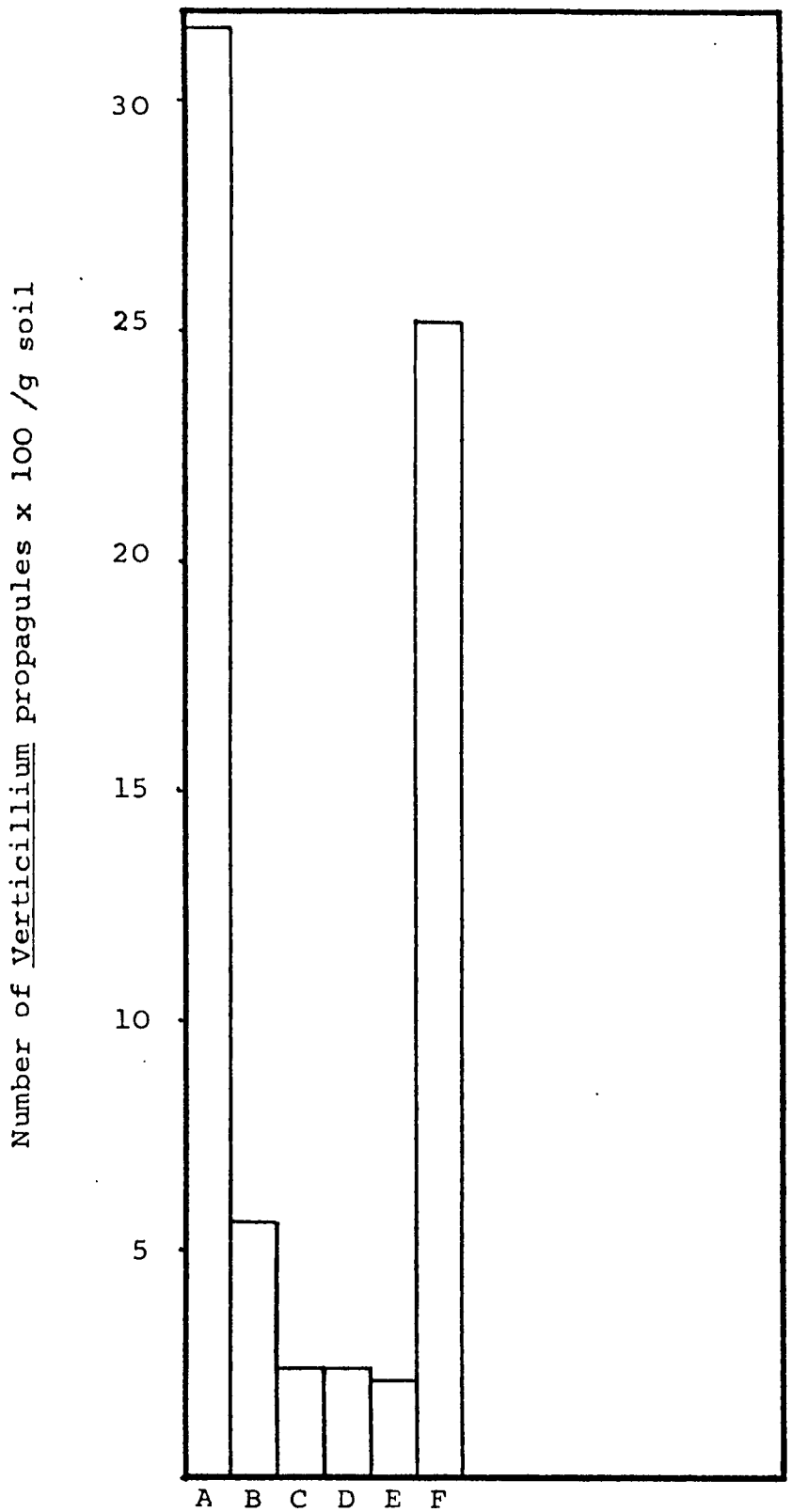


Fig. 24 The influence of varied soil types, soil reactions and aluminium sulphate on the survival of V. dahliae.
Legend: A = George soil + $Ca(OH)_2$, pH 8; B = Glen soil, pH 8; C = George soil, pH 4,5; D = George soil + $Al_2(SO_4)_3 \cdot 18H_2O$ + $Ca(OH)_2$, pH 4,5; E = Glen soil + $Al_2(SO_4)_3 \cdot 18H_2O$, pH 4,5; F = Number of MS added at the start of the experiment.

Table 38 - The relationship between survival of V. dahliae and concentration of soil constituents with different treatments

Treatments	pH	Survival[a]	Soil constituents (ppm)[b]									
			NO_3-N	NH_4-N	P	K	Na	Cu	Mn	Fe	Zn	Al
George soil	4,5	11,01x	37	20	12	99	74	2,00	5,0	290	3,0	0,18
George soil + 0,5% $Ca(OH)_2$	8.0	39,73y	92	44	17	90	73	2,75	5,0	320	2,0	0,00
George soil + 1% $Ca(OH)_2$ + 4% $Al_2(SO_4)_3$	4,5	10,84x	11	33	13	94	95	2,00	5,1	210	1,8	49,50
Glen soil	8,0	16,73z	128	6	113	341	255	8,00	45,0	118	8,5	0,27
Glen soil + 4% $Al_2(SO_4)_3$	4,5	10,21x	89	11	128	359	277	5,00	40,0	80	3,5	46,67

a: Transformed ($\sqrt{n+1}$) data of mean numbers of viable MS / 0,5 g soil; 5 replicates / treatment. Means followed by the same letter do not differ significantly from each other.

b: Analysis by the South African Cooperative Citrus Exchange Limited.

Critical values: $S_{\bar{x}} = 0,439$

(Tukey) $Q_{0,05}\ (5;20) = 2,32$

7.2.6 The effect of urea, soil moisture, and organic amendments on survival

7.2.6.1 Urea

In soil *V. dahliae* survived concentrations of urea up to 0,1%, but did not survive 0,25% or higher (Table 39 & Fig. 25)

Table 39 - Mean numbers ($\sqrt{n+1}$ transformed data) of *Verticillium* propagules surviving treatments with various concentrations of urea in soil after incubation at 25°C and 20% moisture for three months

Urea amended to the soil (%)	Number of viable propagules / 0,5 g soil
0 (Control)	16,84x[b]
0,025	17,34x
0,05	15,53x
0,075	17,80x
0,1	15,99x
0,25	1,08y
0,5	1,16y

a: Means of 5 replicates / treatment.

b: Figures followed by the same letter do not differ significantly from each other.

Critical values: $S_{\bar{x}} = 0,62$

(Tukey) $Q_{0,05} = 2,77$

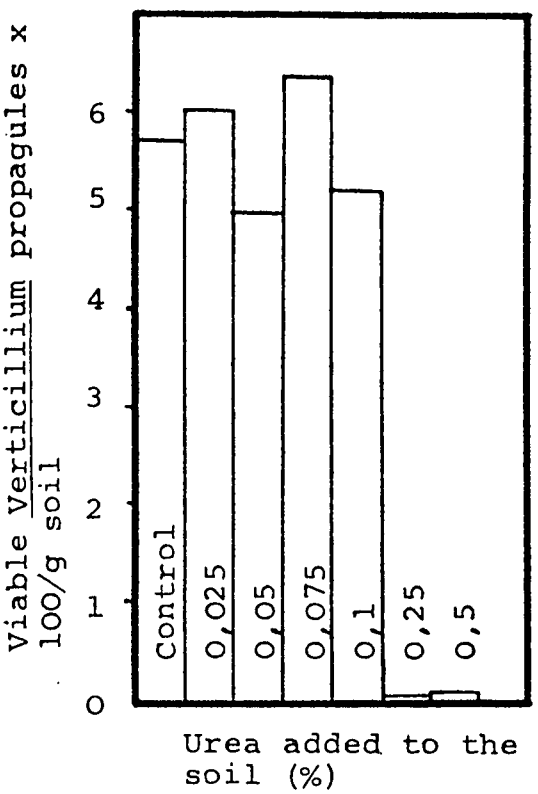


Fig. 25 The survival of V. dahliae in soil treated with urea and incubated at 25°C for 3 months

7.2.6.2 Soil moisture

The results (Table 40 & Fig. 26) indicate that *V. dahliae* survive best at 10 - 20% moisture in the soil. A significant reduction in viability was observed when the soil was held at 30 - 40% moisture, and a further decrease occurred in soils with 50 - 60% moisture and air-dry soil. Free water on top of the soil was found only with the 60% moisture treatment.

Table 40 - The effect of soil moisture on the survival of *V. dahliae* in soil incubated at 25°C for three months[a]

Moisture content[b]	Mean number of viable MS / 0,5 g soil[c]
Air-dry	9,026 z[d]
10	26,524 x
20	25,166 x
30	19,804 y
40	19,512 y
50	11,378 z
60	10,726 z

a: $\sqrt{n+1}$ transformed data.

b: Percentage of the air-dry mass of the soil.

c: Means of 5 replicates / treatment.

d: Figures followed by the same letter do not differ significantly from each other.

Critical values: $S_{\bar{x}} = 1,149$

(Tukey) $Q_{0,05} = 5,297$

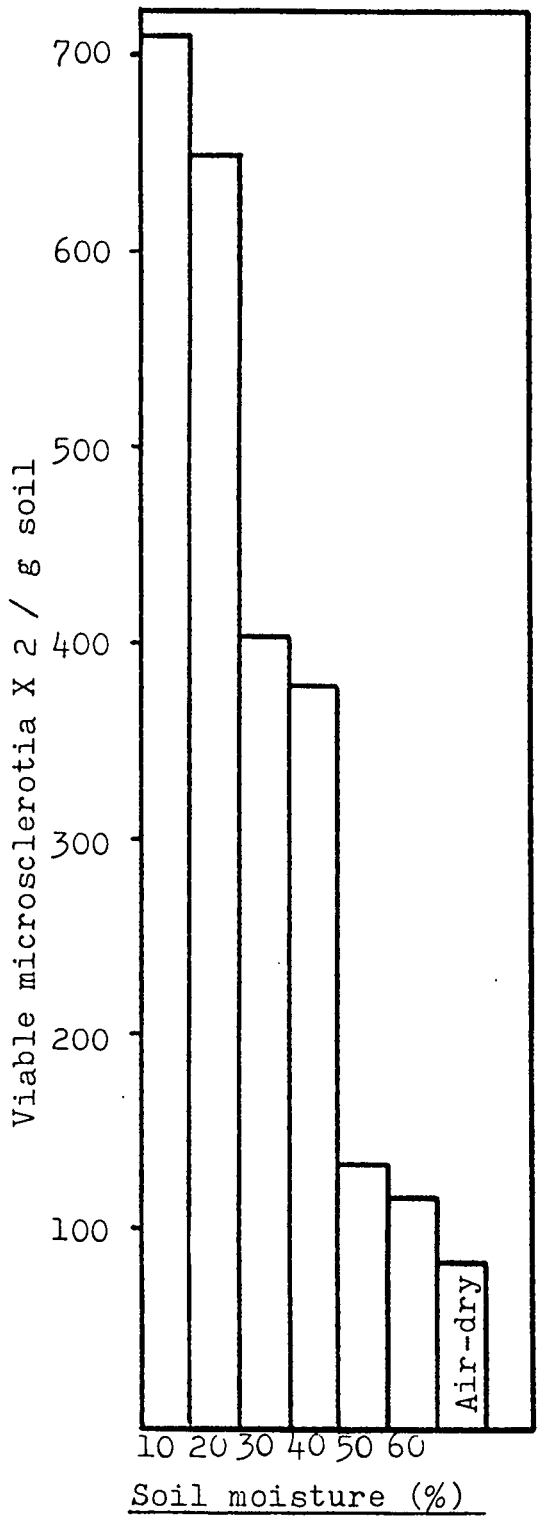


Fig. 26 - The effect of various levels of soil moisture on the survival of V. dahliae in soil incubated at 25°C for three months.

7.2.6.3 Organic soil amendments

The results (Table 41) indicate that only four treatments, viz., soyabean, maize, soyabean pods, and lucern caused a significant reduction in numbers of *V. dahliae* when the data were recorded immediately after the soil was air-dried (48 h). However, after the soil samples were stored air-dry for a further six weeks at 4°C, completely different results were obtained (Table 41 & Fig 27). Not only were considerably more propagules of *V. dahliae* isolated, but many more residues caused a significant reduction in propagule numbers. The residues varied in their effect on the pathogen with maize being the most effective population-density-reducing agent, followed by soyabean pods. Oats, groundnuts, oil-cake meal, and crayfish shells all had about the same effect. Wheat and lucern was less effective than those mentioned above, but still had a significant reducing effect on the population density.
The effect of germinating seeds of *T. minuta* and soyabean residues did not differ significantly from the control.

Table 41 - The effect of organic soil amendments on the survival of *V. dahliae* after 3 months of incubation at 25°C and 20% moisture

Amendment	Mean number of propagules / 0,5 g soil[b]		C:N ratio[c]
	Directly after air-drying	6 weeks after air-drying	
Control	5,25	13,47	-
soyabean	3,33	11,12	13,5
T. minuta	5,05	9,56	-
Lucern	3,48	8,79	12,8
Wheat	4,12	8,49	44,3
Oil-cake meal	5,00	6,71	5,0
Crayfish shell	4,91	6,73	4,0
Groundnuts	5,10	6,71	15,9
Oats	4,20	5,68	18,1
Soyabean pods	3,35	5,15	8,7
Maize	3,35	3,94	54,0
	$S_{\bar{x}} = 0,316$	$S_{\bar{x}} = 0,912$	
	(Tukey) $Q_{0,05} = 1,52$	(Tukey) $Q_{0,05} = 4,38$	

a: $\sqrt{n+1}$ transformed data.

b: Means of 5 replicates / treatment.

c: Walkley & Black, 1934.

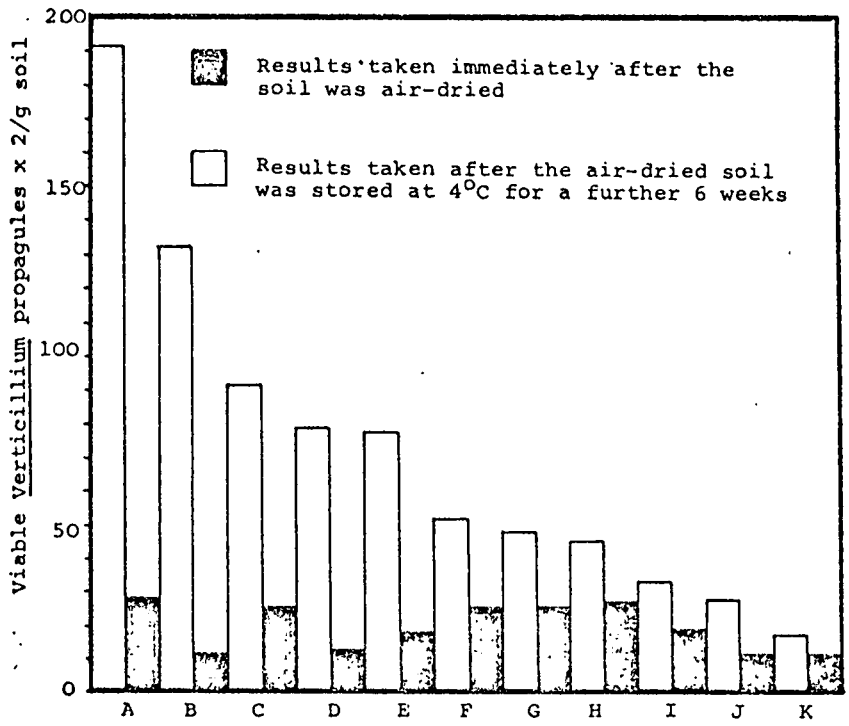


Fig. 27 The effect of soil amendments on the survival of V. dahliae after 3 months' incubation at 25°C. Legend: A = Control; B = Soyabean; C = Germinating seeds of Tagetes minuta; D = Lucern; E = Wheat; F = Oil-cake meal; G = Crayfish shell; H = Ground-nuts; I = Oats; J = Soyabean pods; K = Maize.

7.3 DISCUSSION

7.3.1 Comparison of techniques

For any study concerning the survival of a plant pathogen in soil a reliable technique for the estimation of propagule numbers is an essential prerequisite (Garrett, 1970).

The inhibitory effect of volunteer (unwanted) fungi on non-selective agar medium is well known and is illustrated in the present study (Table 1). Various selective media and isolation techniques which reduced, if not eliminated this problem, have been described (review p. 32 - 35). However, none of these techniques or media were previously used in studies with acid soils which support the development of fungi in general and also of fungal antagonists of V. dahliae (Tables 25 & 26).

Low numbers of V. dahliae could not be recovered from acidified soil by dilution plating on ESA (Nadakavukaren, 1960) (Fig. 16) and this was probably caused by inhibition or overgrowth by volunteer fungi on the isolation plates.

In the present study various techniques were used to recover V. dahliae from the soil. By means of the sucrose flotation method (Huisman & Ashworth, 1972) approximately 76% of the MS could be recovered from the soil. This finding is in corrobo= ration with the results of Huisman & Ashworth (1974b) who found that 70 % of the MS could be recovered from the soil by this method. However, this method was replaced by Huisman

& Ashworth (1974a) by a technique which involved washing of the soil over 125 and 36 µm sieves and plating on pectate agar. In the present studies preliminary experiments showed that pectate agar was not satisfactory, probably because chemicals from different sources were used (Butterfield & DeVay, 1977). Pectate agar was replaced by a selective medium (VIA) developed locally (p. 39). This medium proved to be very effective for the isolation of *V. dahliae* from both high and low pH soils. *Verticillium* propagules could still be recovered from low pH soils after incubation periods of three months (Fig. 19 - 24), whereas, with dilution plating on ESA (Nadakavukaren, 1960), no propagules could be recovered after 4 weeks of incubation (Fig. 16). Soil washing and plating on VIA also proved to be superior to the bioassay technique (Evans *et al.* 1974) (Fig. 20), which, because of the fact that the roots were washed before they were plated on selective medium, was less subjected to the effect of volunteer fungi on the plates.

7.3.2 Soil pH

Because of the fast attrition found in acidified soil (Fig. 16), much emphasis was placed on the effect of soil pH on survival to determine the mechanism of the detrimental effect of low pH on MS. In some of the experiments, ground cotton stems were used to represent natural inoculum and it was found that the MS, although embedded in cotton tissue, was still subject to the detrimental effect of reduced soil pH (Fig. 19).

The influence of soil pH was also investigated in combination with varying soil moisture and fertilization. Soil pH had a

significant effect on infection of both cotton and D. stramonium (Tables 32 & 33). Although the cotton plants grew badly in acidified soil, none became infected, while only a few infections occurred on the roots of D. stramonium. This finding is in corroboration with the results obtained by direct plating on VIA (Table 34 & Fig 20) and serves as further proof of the de= trimental effect of reduced soil pH on the survival of V. dahliae.

Aluminium sulphate was used in the present studies to reduce the pH of the soil. However, Orellana et al. (1975) reported that a sclerotium-bearing isolate of V. albo-atrum (= V. dahliae) from sunflower, did not tolerate more than 8 ppm Al in vitro. Johnson (Bell, 1973) found 50 to 100 ppm Al to inhibit growth of V. dahliae in nutrient culture.

It was determined (a) whether pH reducing substances devoid of aluminium salts also influenced the survival of V. dahliae in the soil and (b) if the effect of low pH is fungistatic or fungitoxic to the pathogen. When the reaction of the soil was reduced to c. pH 4,5 for a period of three months by the addition of sulphur, sulphuric acid, or aluminium sulphate, and then restored to its original pH (pH c. 8) very little infec= tion occurred on the roots of D. stramonium (Table 35).

By direct plating on VIA (Fig. 21) it was possible to prove that in the case of the sulphur and sulphuric acid treatments the reduction in Verticillium propagules was permanent, because restoration of the pH with the addition of calcium hydroxide did not result in higher yields of propagules (Table 36). Significant differences were, however, found within some treatments:

Restoration of the aluminium treated soil to its original pH caused a small, but significant, increase in numbers of viable propagules isolated, indicating that a fungistatic effect, however small, might have been operative in this case.

Aluminium added to the soil had more or less the same effect on the survival of V. dahliae as the other pH-reducing agents. Less propagules of the pathogen survived after treatment with pH-reducing agents than in the controls, whether the pH-reducing agents contained Al salts or not. Furthermore, restoration of soil pH to its original high level, could never restore the population of V. dahliae to its original level, indicating that the effect of pH was fungitoxic rather than fungistatic.

In an attempt to clarify the effect of soil pH and aluminium sulphate on the survival of V. dahliae, the fungus was incubated in a naturally acid soil with a very low Al content and in the same soil amended with 4% (m/m) aluminium sulphate. The results (Fig. 24 & Table 38) indicate that aluminium in the soil could not be held responsible for the rapid decline in viability of MS in acid or acidified soils. Soil of a naturally low pH containing 0,18 ppm soluble aluminium caused as much reduction in population counts as a soil artificially acidified with aluminium sulphate, which contained 46,67 ppm soluble aluminium. The soils were held at the same pH range, therefore, differences in solubility due to precipitation at different pH levels (Buckman & Brady, 1969) were prevented. This finding contrasts with that of Orellana et al. (1975) who reported that V. albo-atrum could not tolerate more than 8 ppm aluminium in culture media and that the aluminium salts in acid soils are responsible

for the fact that less wilt occurred on such soils. Their studies indicated that low pH *per se* had a negligible effect on growth and they attributed growth retardation of *V. dahliae* solely to the inhibitory effect of aluminium salts.

The present investigation differs in two important aspects with that of Orellana *et al.* (1975). Firstly, in the present study the survival of MS in absence of the host plant was investigated while Orellana *et al.* (1975) studied growth of the fungus in culture. It is possible that a comparison between growth and survival might be irrelevant. Secondly, although Orellana *et al.* (1975) described their fungus as the microsclerotial form of *V. albo-atrum* (= *V. dahliae*), they described it as having dark mycelia and thus differing from *V. dahliae*. It is, therefore, not clear which species they used in their studies. In the present study *V. dahliae*, forming MS and hyaline mycelia, was used. The possibility that two different species were investigated, thus exists.

The present study is in corroboration with previous reports that *V. dahliae* does not tolerate low pH soils(Haenseler, 1928; Martin, 1931; Guba, 1934; Chester, 1942; Jones & Woltz, 1972). However, this apparently is the first investigation which yields proof that, whatever the effect of soil pH on the plant might be (Henis & Chet, 1975), survival of MS is detrimentally affected by naturally acid or acidified soils. Moreover, the view that aluminium salts which become soluble in low pH soils are responsible for this intolerance at very low concentrations (Orellana *et al.* 1975), can not be supported.

7.3.3 The effect of varying soil moisture and nitrogen plus phosphate on survival in high and low pH soils

In high pH soils nitrogen plus phosphate (NP) caused a significant increase in antagonist numbers (Table 17.2) which may be implicated in the decline of MS of *V. dahliae* in the soil. These fertilizers were amended into soils of different pH values, held at high, medium, and low moisture levels, to determine if NP would enhance the decline of the MS.

The results (Table 34.1) indicate that less propagules survived in both high and low pH soils after amendment with N and P. The effect of NP did not vary significantly within the different pH regimes, but the moisture X NP interaction was significant; indicating that the effect of moisture was not the same within the different levels of NP. With NP present, survival was significantly reduced in air-dry soil, whereas, in soils without NP, survival was significantly better in air-dried soil than in high moisture soil. The latter effect was not as evident in high pH soil as in low pH soil and can be contributed to the exceptionally high level of survival in soil of low pH without fertilizer (Fig. 20). The reason for this phenomenon is unknown.

The fact that the survival of *V. dahliae* was detrimentally affected by the incorporation of NP into the soil could be attributed to the action of increased antagonists in response to NP amendment (Table 18.2). However, because the soil was air-dried, the activity of antagonists should be greatly reduced and it seems doubtful that this could be the reason for the lower numbers of *Verticillium* propagules recovered from the soil.

It was formerly reported that MS of V. dahliae germinated in the soil when air-dried soil was remoistened (Menzies & Griebel, 1967; Farley et al. 1971). With successive germinations, however, germination percentage and the number of germ tubes and conidia formed, decreased (Farley et al. 1971). A secon= dary effect established by Menzies & Griebel (1967) was a reduc= tion in drought resistance of the MS after repeated germination and they suggested that drying and remoistening of the soil might be put to practical use in the control of wilt. Smith (1972a,b,c) investigated the effect of drying on the survival of sclerotia of various sclerotium-forming fungi and found that drying of the soil over short periods caused the fungi to loose viability.

In the present study V. dahliae was subjected to natural drying out of the soil for two weeks whereupon it was remoistened. The experiment lasted for three months and at the end of this period numbers of viable propagules were determined by bioassay (Evans et al. 1974) and by direct plating on VIA, but no appre= ciable decline of V. dahliae was found in soils in which moisture was varied. However, a significant decline, detected by plating on VIA, was found in air-dried soil.

7.3.4 Pentachloronitrobenzene (PCNB)

Fungal antagonists (Fig. 14), soil fungi in general, and Trichoderma spp. (Table 26) are favoured by acidified soil. The effect of Trichoderma spp. on V. dahliae is not clear. Some research workers claim that it has a favourable effect on the pathogen while others claim that it has a detrimental

effect (reviewed p. 22). However, Trichoderma spp. are sensitive to PCNB (Kreutzer, 1963). In the present study it was attempted to determine if PCNB-sensitive fungi, including Trichoderma spp., were responsible for the fast decline of MS in acidified soil. It was found that the addition of PCNB to acidified soil did not favour the survival of the pathogen within the pH range (pH 4,5 - 5,0) in which the largest reduction of Verticillium propagules occurred (Table 37). This indicates that if the decline of V. dahliae in acidified soil is of biological origin, PCNB-sensitive fungi, such as Trichoderma spp., are not responsible for that decline.

PCNB seemed to have a beneficial effect on the survival of V. dahliae in soils of high pH values (Fig. 23). Whether this is the result of a stimulatory effect of PCNB on V. dahliae or an inhibitory effect on antagonists in high pH soil, is not clear. However, the fact that this tendency is reversed in soils of lower pH values, leaves the impression that the effect on survival of V. dahliae is indirect rather than direct and the possibility that antagonists are involved, cannot be disregarded.

7.3.5 Urea

High concentrations of urea seem to have a detrimental effect on the survival of some plant pathogens. Phytophthora cinnamomi was reduced in or eliminated from soil by the addition of 0,1% urea to the soil (Tsao & Zentmeyer, 1977). Popov et al. (1976) also reported that V. dahliae is killed by "higher" rates of urea incorporated into the soil.

In the present study it was found that urea caused an effective reduction in viability of V. dahliae when the soil was amended with 0,25% (m/m) urea. At 0,1% or less, urea had no effect.

7.3.6 Soil moisture

The controversy as to whether soil flooding reduced Verticillium populations in the soil (Nadakavukaren, 1960; Menzies, 1962), or not (Butterfield, 1975), and the development of a more reliable method for the isolation of V. dahliae, led to the present study where soil was flooded for a period of 12 weeks and a significant reduction in viability of MS registered. However, approximately 10% of the MS still survived the treatment. Considering that less than 5 propagules / g soil can cause 100% infection in cotton (Ashworth et al. 1972), it would mean that the treatment could only be effective if it was applied to soil with a natural population of less than 50 / g. Larger populations have been recorded in soil from Glen and Mkuze (Baard, unpublished). Longer periods of flooding may, however, be more effective (Butterfield et al. 1978).

The differences observed between the present results and those reported by Nadakavukaren (1960) and Menzies (1962) who reported loss of viability under flooded conditions, can be attributed to the more effective method of isolation employed in the present study.

The reduction in viability observed in air-dry soil is in corroboration with the results of Nadakavukaren (1960). The attrition rate is approximately the same as that in flooded soil over a period of three months. It would seem, therefore, that an air-drying period of three months is just as effective

as soil flooding in eliminating MS from the soil.

However, in a previous study (Fig. 20), executed in the green-house, it was found that attrition, although significant in air-dried soil, was not as steep as that found in the present experiment (Fig.26). The main differences between the two experiments were: In the green-house experiment the soil was moistened at the start of the experiment and allowed to air-dry, and ground, infected cotton stems were used as inoculum (p. 148). In the latter experiment, freeze-dried, laboratory grown MS were used and the soil was not moistened at the start of the experiment (p. 153). The differences between these results (Fig. 20 & Fig. 26) can possibly be contributed to (i) the possibility that MS germinated in the soil which was initially moistened and that population increases occurred (Menzies & Griebel, 1967; Farley et al. 1971) or (ii) that the prior treatments of the various inocula was responsible for the faster decline of MS in the laboratory experiment. A greater protection of MS might have been operative in the case where inoculum, lodged in cotton residues, was used than would be the case when naked MS, grown in the laboratory, were used. It could also be an artifact of the cleaning and collec=ting process employed in the laboratory (p. 37).

7.3.7 Organic soil amendments

Various soil amendments have been reported to reduce disease severity and/or reduce the inoculum potential of V. dahliae in soil (reviewed p. 24). Lucern meal in particular, had a

very beneficial effect on both disease incidence (Brinkerhoff, 1973) and the attrition of Verticillium propagules (Menzies, 1962) in soil.

In the present studies the effect of organic materials on the survival of V. dahliae in soil was investigated and it was found that residues of various crops caused a significant decrease in numbers of viable propagules. After three months of incubation in soil, soil amended with maize residues yielded 91% less MS than the controls. These results are in corroboration with results previously found in the field. In preliminary studies cotton was grown in a two-year rotation with maize or fallow. When maize followed cotton, only 20 MS could be recovered per 100 cm of root of D. stramonium one year after the cotton was harvested (Pauer & Baard, unpublished). When cotton followed cotton, 62 MS were recovered, and when fallow followed cotton, 44 were recovered. The mechanism by which maize residues reduce the viability of V. dahliae in soil is not known, and the attrition rate in the field is too slow to effectively reduce the population level to less than 3,5 MS / g soil (Ashworth et al. 1972) in a short term rotation. However, this positive effect of maize residues on the attrition rate of Verticillium propagules should be further studied and its exploitation investigated.

Other materials which were also very effective in reducing the population of V. dahliae, were soyabean pods, oats, groundnuts, crayfish shells, and oil-cake meal. Wheat and lucern were less effective and soyabean residues and germinating seeds of Tagetes minuta did not have significant effects (Fig. 27).

The attrition rate found with lucern meal in the present study was not as fast as that recorded by Menzies (1962), but the conditions under which the experiments were executed, were completely different. So were the methods used to recover viable MS from the soil. Differences in the two sets of results can thus be expected.

Crayfish shells and seeds of T. minuta were included in the experiment because (i) Jordan et al. (1972) reported significant decreases in Verticillium populations after the addition of chitin to the soil. Crayfish shells contain approximately 12 - 20% chitin (Mitchell, 1963) and de Swardt (1977) reported that crayfish shells added to the soil at the same rate as pure chitin, had approximately the same detrimental effect as chitin on the saprophytic survival of R. solani in soil. In the present study crayfish shells had a significant effect on the attrition rate of MS of V. dahliae in soil, but it was considerably less effective than some of the other treatments. (ii) Wilhelm (Baker & Cook, 1974) reported that a cover crop of T. minuta in an olive grove infected with V. albo-atrum, reduced disease incidence. However, T. minuta was declared a noxious weed and the experiment could not be completed. In the present study it was attempted to determine whether germinating seeds of T. minuta could have led to the decline of Verticillium propagules in the soil, but although an apparent decline occurred, the results did not differ significantly from that of the controls (Fig. 27).

The actual mechanism by which plant residues induce the attrition of Verticillium propagules, is not known. In the present

studies the C/N ratio did not seem to be of importance. This has also been found previously in studies concerning the effect of C/N ratios on the survival of V. dahliae (Green & Papavizas, 1968).

7.3.8 The effect of storage on numbers of MS recorded on isolation plates

The effect of storage seems to be a very important factor to be kept in mind when population counts of V. dahliae are made from soil. Butterfield (1975) reported that if air-dried MS were plated out on growth media after different storage periods, considerable variation in results occurred. There was an increase in the apparent numbers of propagules on the fourteenth day of drying and an equal decrease after 21 days. Consistent counts which were relatively high, were obtained only after 35 days of drying. Butterfield (1975) did not find this fluctuation in air-dried soil when it was assayed by a technique involving partial sterilization with NaOCl. Butterfield & DeVay (1977) suggested that washing of the soil removed fungistatic principles from the suspension which otherwise operates on the dilution plates. They also suggested that by air-drying the soil for at least 35 days, this fungistatic principle is eliminated from the soil.

In the present studies, soil treated with various amendments, was air-dried for 48 hours before samples were plated out on VIA after wet-sieving, involving the use of NaOCl for partial sterilization. The soil was then stored in a refrigirator at 4°C (to inhibit possible microbial activity) for a further

6 weeks after which plating was again done in the same way as before.

The results (Fig. 27) indicate the differences which was found at the two sampling dates. For each treatment, the numbers of viable Verticillium propagules isolated with the second plating were considerably higher than those of the first plating. This finding corroborates with the results reported by Butterfield (1975) in which partial sterilization with NaOCl was omitted. However, Butterfield (1975) suggested that the storage period would not have an effect on colony initiation by V. dahliae if NaOCl was used in the wet-sieving process because of the removal of a fungistatic principle from the soil suspension (Butterfield & DeVay, 1977). In this respect the present results are contrary to Butterfield's (1975) suggestion. NaOCl was used in the washing process in the present study. However, larger numbers were still recovered after 6 weeks of storage than after 48 hours of air-drying. In the experiment under discussion an additional treatment which Butterfield (1975) did not include in his studies, was storage at low temperature, which could be the cause of the improved colony initiation of MS on VIA after 6 weeks of storage. It appears, therefore, as if fungistasis operating on the MS in the isolation plates, is not as important as Butterfield & DeVay (1977) have suggested. It is suggested that the enhanced germination of MS after cold storage, is the result of a dormancy-breaking effect.

CHAPTER 8

STRUCTURE, GERMINATION, AND LYSIS OF MICROSCLEROTIA

This study was undertaken to investigate the behaviour and ultrastructural changes of the MS of *V. dahliae* undergoing microbial breakdown in soil.

8.1 MATERIALS AND METHODS

An isolate of *V. dahliae* from cotton was grown on PDA for seven days and on sand-maize meal for two months. The cultures from PDA were used to study young MS while sand-maize meal cultures were used in all other investigations.

MS from sand-maize meal cultures were harvested by shaking them with a quantity of sterile distilled water in a mechanical shaker and then passing the suspension through presterilized 75 µm and 45 µm sieves. Sieves with these particular pore sizes were used to ensure that MS of fairly uniform sizes were used in the study. Two litres of sterile distilled water were used to wash the MS while still on the sieves. After washing, the MS remaining on the 45 µm sieve were freeze-dried, and stored in a refrigerator until further use.

Sieved Glen soil was amended with aluminium sulphate to reduce the pH to *c.* pH 4,5.

Dried MS were resuspended in sterile distilled water and deposited evenly on 47 mm "Nuclepore" membrane filters, pore size 1,0 µm, by applying suction. A second membrane was placed

over the first which contained the MS and the margins sealed together with a ring of vacuum grease (Old & Patrick, 1976). The MS were thus contained in membrane packets which were placed in the soil in petri dishes. Soil moisture was adjusted to 40% of the air-dry mass of the soil and allowed to dry to 20% after which water was added twice weekly. The incubation temperature was 25°C.

Packets containing MS were removed from the soil after incubation periods of 36 h and 7, 15, 30, 60, and 90 days. The MS were removed from the membranes and fixed in 3% gluteraldehyde in 0,1 M phosphate buffer (pH 7) for at least three hours. They were washed once in the same buffer and fixed in 1% OsO_4 in buffer for two hours. The OsO_4 was then removed from the MS by washing in two changes of buffer. Dehydration was done in 50%, 70%, 95%, and two changes of absolute alcohol, each step taking 20 to 30 minutes. The material was then impregnated with Spurr's (1969) epoxy resin. The final impregnation was done under vacuum over night. Polymerization was at 70°C for 8 hours. Sections were cut with an "LKB III Ultratome" fitted with a diamond knife. Staining of the sections was done with 6% uranyl acetate in water for 20 minutes and lead citrate for 10 minutes. After staining, the sections were examined with a "Philips EM 300" transmission electron microscope (TEM) (Glauert, 1974).

Fixation and staining for scanning electron microscopy (SEM) was in 3% gluteraldehyde in 0,1 M phosphate buffer (pH 7) as described above. After dehydration the material was dried

under CO_2 in a "Polaron" critical point drier. Thereafter it was vacuum coated with gold: palladium (60 : 40) and scanning micrographs were taken with an "ISI 100" scanning electron microscope using 20-kV accelerating voltage (Glauert, 1974).

The viability of the MS were tested by picking up individual MS from the membranes and plating them on VIA in petri dishes. The cultures were incubated for two weeks at 25°C in the dark before germinated MS were counted.

Germination in the soil was investigated as follows: MS on the membranes were covered with a thin layer of water agar at 50°C and allowed to set. The agar layer containing the MS was then removed from the membrane, stained with rose bengal, and examined with the compound microscope to record the numbers of MS which germinated.

8.2 RESULTS

8.2.1 Germination of microsclerotia on membranes in soil

The apparent numbers of MS which germinated on the membranes in the soil appear in Fig. 28. After 36 h in the soil 18% MS in acid and 34% MS in alkaline soil had germinated. After 7 days the germination percentage rose to 56% in acid and 62% in alkaline soil. After 15 days 59% had germinated and after 30 days only 12% in alkaline soil. In acid soil the apparent germination declined faster from the seventh to the 15th day than in alkaline soil. From the fifteenth to the thirtieth day the decline was negligible in acid soil. However, while recording the results on viability (Fig. 29), it was found that volunteer fungi grew from the MS into the isolation medium. These fungi became apparent on isolation plates from MS incubated in low pH soil from the seventh day of incubation. Consequently, the hyphae observed, might not necessarily have been germ tubes of *V. dahliae*. It is impossible to distinguish between hyphae of volunteer fungi and germ tubes of *V. dahliae*. Volunteer fungi were seldom found on MS recovered from alkaline soil and the hyphae seen, probably were germ tubes of *V. dahliae* (Plate 1). After 15 days and again after 30 days only a single MS from high pH soil was found to be colonized.

After the seventh day of incubation in low pH soil, 12% MS were colonized by fungi and 83% of these fungi were identified as *Penicillium* spp.

It was observed that hyphae bearing conidiophores and conidia

resembling those of V. dahliae grew from MS after the fifteenth day of incubation. Their frequency was very low and appeared in not more than 1% of the MS investigated.

Because of fungal growth between the membranes recovered from acidified soil, no further data were recorded on germination percentage after the thirtieth day. The cultures were left to air-dry and again investigated after 60 days. The following observations were recorded:

(i) Fungal hyphae which appeared to be viable and attached to MS as well as lysed hyphae, were abundant.

(ii) A single structure resembling a viable conidiophore of V. dahliae with conidia attached and itself being attached to a MS, was recovered from acid soil (100 MS were investigated).

(iii) Unattached conidia resembling those of V. dahliae and appearing to be viable, were plentiful in acid soil.

(iv) Large numbers of conidia from unidentified fungi as well as large numbers of bacteria were regularly observed.

8.2.2 Viability of microsclerotia recovered from "Nuclepore" membranes

The viability of MS recovered from the membranes incubated in soil at 25°C and 20% moisture for 90 days was recorded by plating individual MS on VIA medium.

The results (Fig. 29 & Table 42) indicate a faster decline in viability in acidified than in alkaline soil. In alkaline soil 75% of the MS were still viable after 90 days of incubation while in the acidified soil only 21% survived. The pH X time

interaction was significant, but it was because attrition rate over time within high pH was not statistically significant while in low pH soil it was.

Table 42 - Viability of MS of *V. dahliae* after incubation on "Nuclepore" membranes in alkaline and acidified soils held at 25°C and 20% moisture for 90 days ($\sqrt{n+1}$ transformed data)

Soil pH	Incubation period (days)	Mean no of viable MS[a]
8	0	4,378
	7	4,356
	15	4,236
	30	4,042
	60	3,904
	90	3,826
4,5	0	4,260
	7	3,148
	15	2,806
	30	2,446
	60	2,106
	90	1,904

a: Mean of 5 replicates.

Critical values: $S_{\bar{x}} = 0,226$; (Tukey) $Q_{0,05} = 0,949$

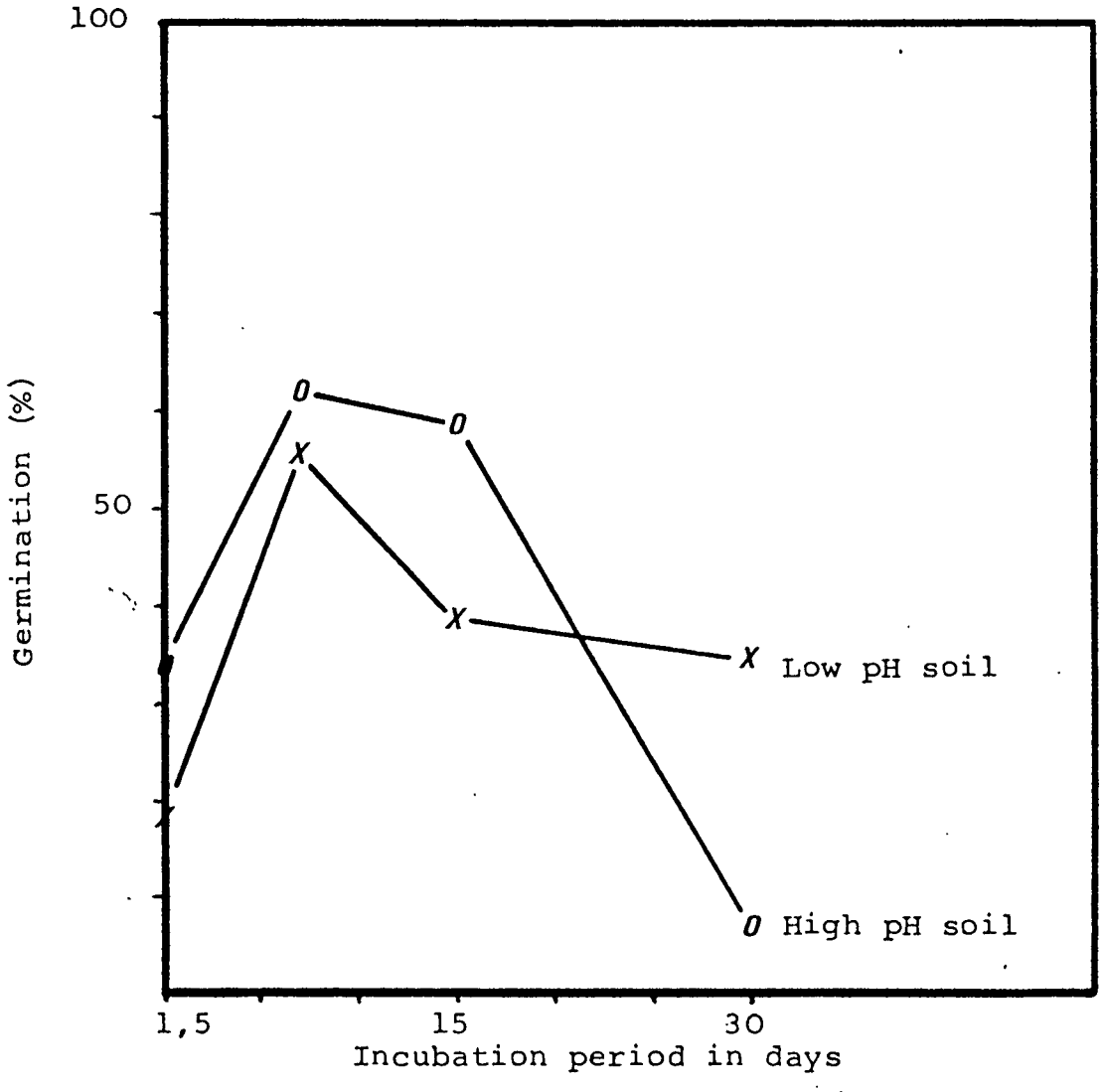


Fig. 28 Apparent germination of MS. of V. dahliae incubated on "Nuclepore" membranes in alkaline and acidified soils

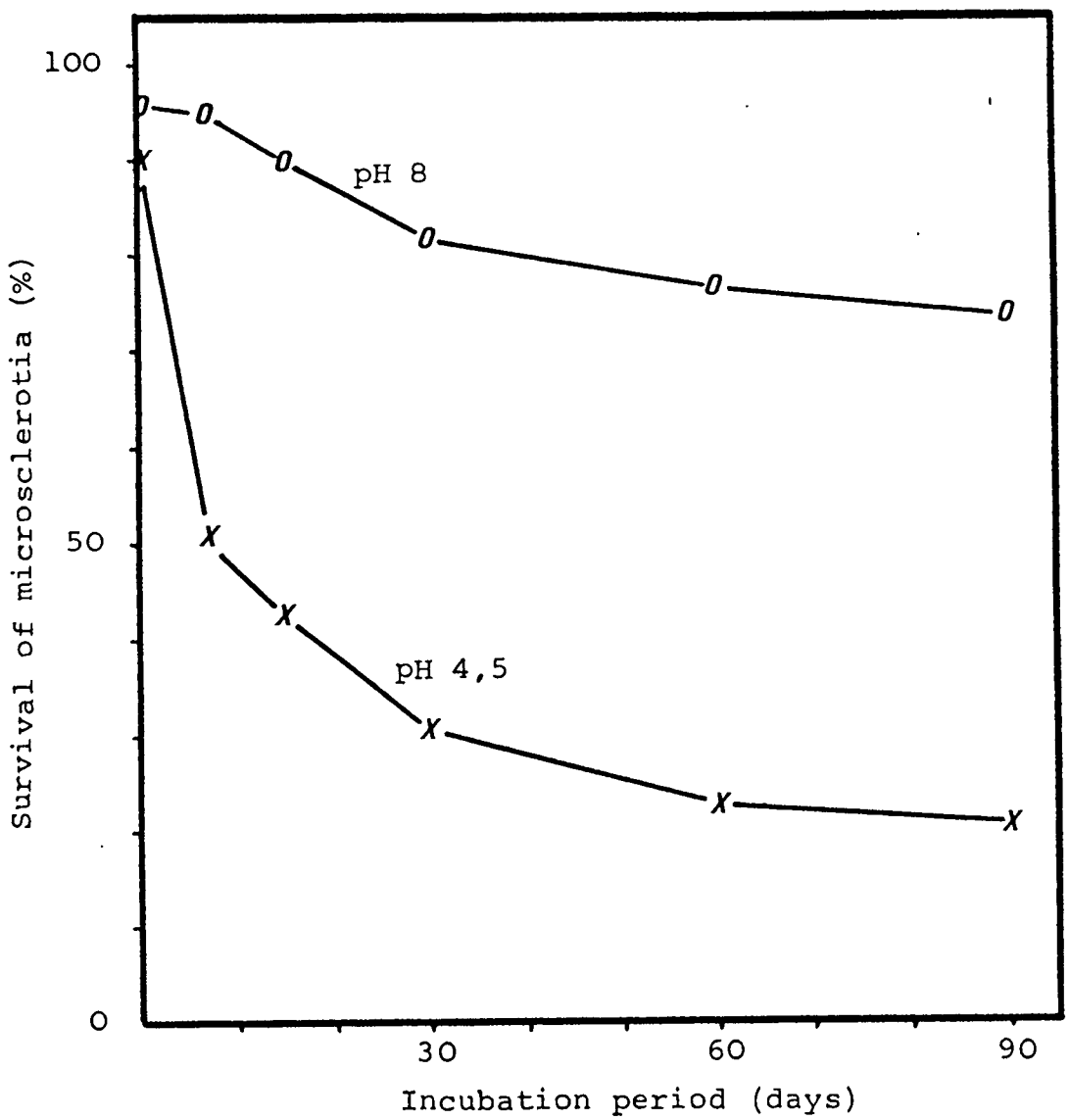


Fig. 29 Viability of microsclerotia of V. dahliae after incubation in soils of different pH levels for various periods

8.2.3 Structure and lysis of microsclerotia

Microsclerotia of V. dahliae are irregular in form and vary considerably in size (Plate 1). Each MS consists of a non-specific number of cells (Plate 2) which vary in size, cell contents, and cell-wall thickness. Plate 2 depicts a section of a MS recovered from high pH soil after an incubation period of 30 days. Many of the cells appear to be empty while others have cytoplasm compressed to the cell walls and large vacuoles are evident. This is best illustrated in Plate 8.

Plate 3 shows a section of a colony of V. dahliae grown on PDA for seven days. The variation in size and cell-wall thickness can easily be seen. Young sclerotial cells have thicker walls than the hyphae and appear as individual cells which later aggregate to form multicellular MS. The individual cells have a single nucleus surrounded by a nuclear envelope, relatively small lipid drops, and a comparatively simple cell wall (Plate 4). In cells older than seven days nuclei were rarely seen (Plates 5 - 7) and lipid drops and vacuoles became more evident. Triple layered cell walls (Plate 6) and secondary cell-wall thickening (Plate 7) were sometimes observed. Mitochondria (Plates 5 - 7) and the endoplasmic reticulum (Plate 5) were evident. Individual cells were regularly surrounded by melanin granules (Plates 5 - 7). One-year old cells from pure culture, have large vacuoles and the cytoplasm is highly electron dense (Plate 8). The cells appear to be dead. Septal pores interconnecting individual cells of a MS were often observed (Plate 9) and apparently these pores were open to the surface (Plate 22).

MS which were placed in soils of high and low pH values were quickly colonized by bacteria. Plate 10 shows bacteria present in the matrix surrounding the MS after 36 hours in the soil. Bacteria were often found penetrating through fissures or cracks within the cell walls of the MS (Plates 11 - 14). Some photomicrographs show bacteria of various shapes embedded in remains of microsclerotial cell walls which appear to have lysed (Plates 14 - 15). Others seem to have attached themselves to the cell wall with a consequent swelling or thickening of the cell walls (Plates 16 - 17).

Various types of bacteria seem to be involved in the colo= nization of the MS. Some bacteria are surrounded by a capsule (Plate 18) while in others (Plate 12) the capsule appear to be absent. Plate 18 shows that bacteria were sometimes peculiarly arranged in what appears to be a zoo= gloeal form. Bacteria which appear to resemble the helically lobed bacteria reported by Old & Wong (1972), were also found (Plate 19). Scanning electron photomicrographs also show bacteria embedded in the surface of the MS while the MS cell wall appear to be eroded in the immediate vicinity of the bacterium (Plate 21).

The surface of the MS were extensively colonized by fungi in low pH soils after 60 days of incubation (Plates 20, 22, 23). Conidia were formed by an unidentified fungus (Plate 20) while hyphae were sometimes seen apparently on the verge of entering a pore on the surface of the MS (Plate 23).

TEM studies showed that fungal colonization of the MS occurred in both soil types, but was more prevalent in low pH soil. Plate 24 is of a MS incubated in alkaline soil showing colonization by both bacteria and fungi. The relative prevalence of bacteria over fungal structures can be seen from this photomicrograph. Plates 25 and 26 are enlargements of fungal structures and helically lobed (?) bacteria found in Plate 24.

Fungal structures were most prevalent on the MS incubated in low pH soils for 30 days while bacteria were relatively few (Plate 27). Plate 28 shows a fungal hypha which appears to be at the point of penetrating a MS cell through a fissure in the cell wall. Fungal hyphae were sometimes found within MS cells of which the cell contents were completely disorganized (Plate 29). Plate 30 shows an unidentified structure of an unidentified fungus in close association with a microsclerotium.

The deterioration of melanin particles which occur when MS are incubated in soil is evident when Plates 31 and 32 are compared. Plate 31 is a photomicrograph of a MS recovered from high pH soil after 3 months of incubation, showing extensive deterioration of the electron-dense matrix between the cells. In Plate 32 which is an electronmicrograph of an one-year old MS kept dry in pure culture, the electron-dense matrix shows little sign of deterioration.

Plate 31 also contains some interesting structural phenomena which are shown enlarged in Plate 33. A second cell seems to have been formed within a primary cell. Bacteria are evident

between the cell walls and within the cell lumen (Plate 33). Other unusual detail was also found within the cells of certain MS. The contents of a cell recovered from low pH soil after 30 days of incubation (Plate 34) appear granular and completely disorganized while those of another cell incubated in high pH soil for 36 h (Plates 35 -36) also appear granular, but some organization, much like virus particles, exists.

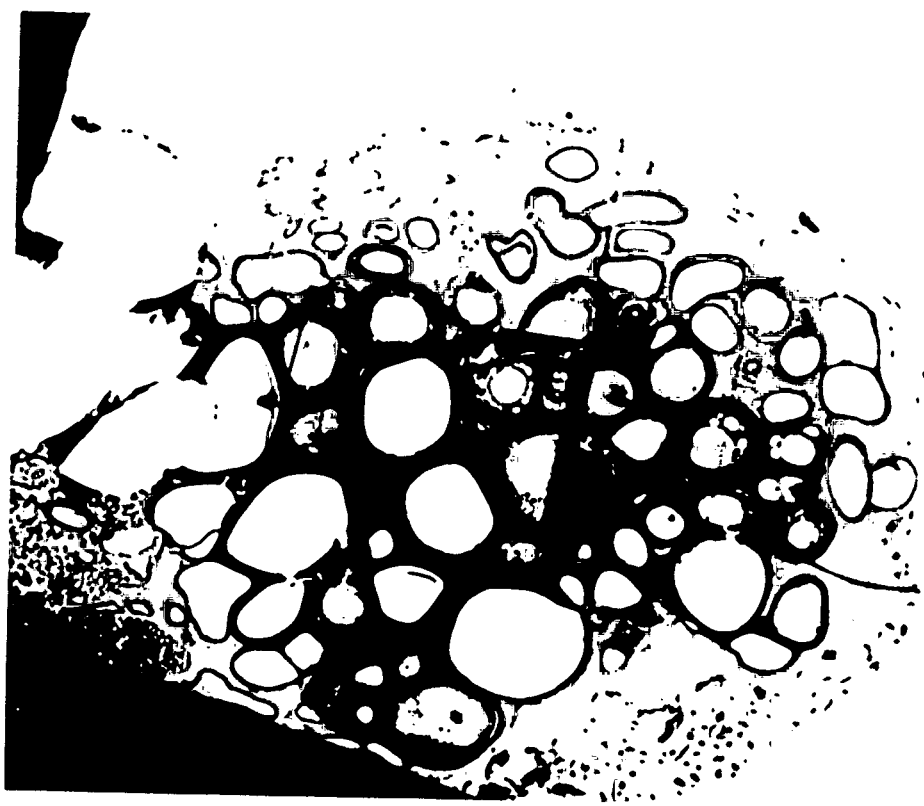
Plate 1 - Microsclerotia, one of which had germinated, which were recovered from high pH soil after 60 days of incubation. Note the difference in size and irregularity in form.

Plate 2 - A section through a MS. Cells vary considerably in size and cell-wall thickness. Many cells appear to be empty while cell contents can be observed within others. (From high pH soil; 30 days of incubation)

10 µm

1

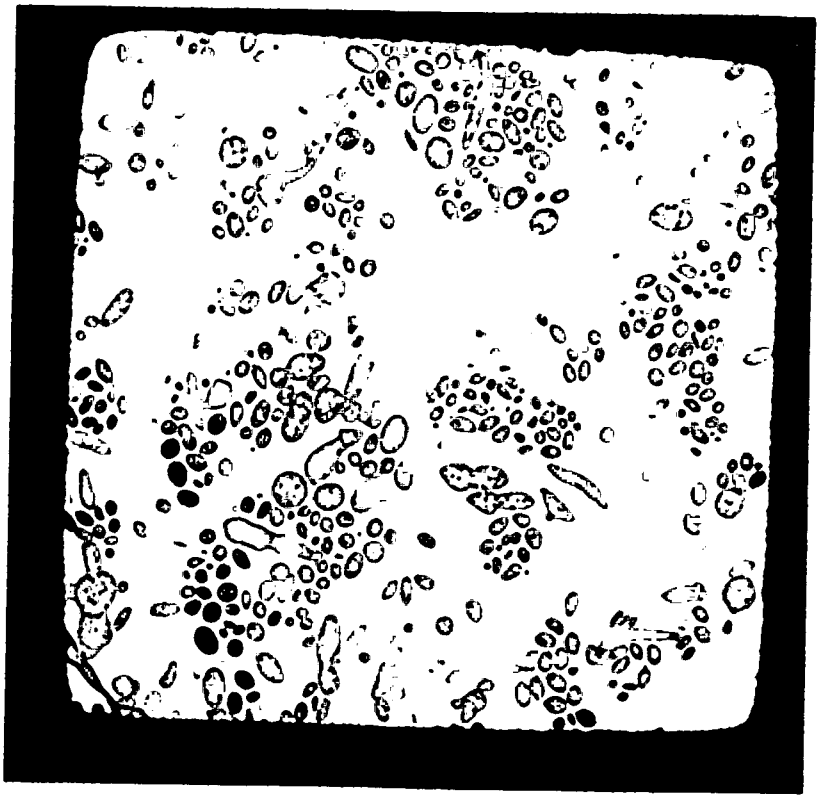
10 µm



2

Plate 3 - A section through a seven-day old colony of V. dahliae grown on PDA. Thin-walled hypahe as well as thicker-walled, young MS cells can be observed.

Plate 4 - A MS cell from a seven-day-old pure culture. N: Nucleus; CW: Cell wall; L: Lipid drop.



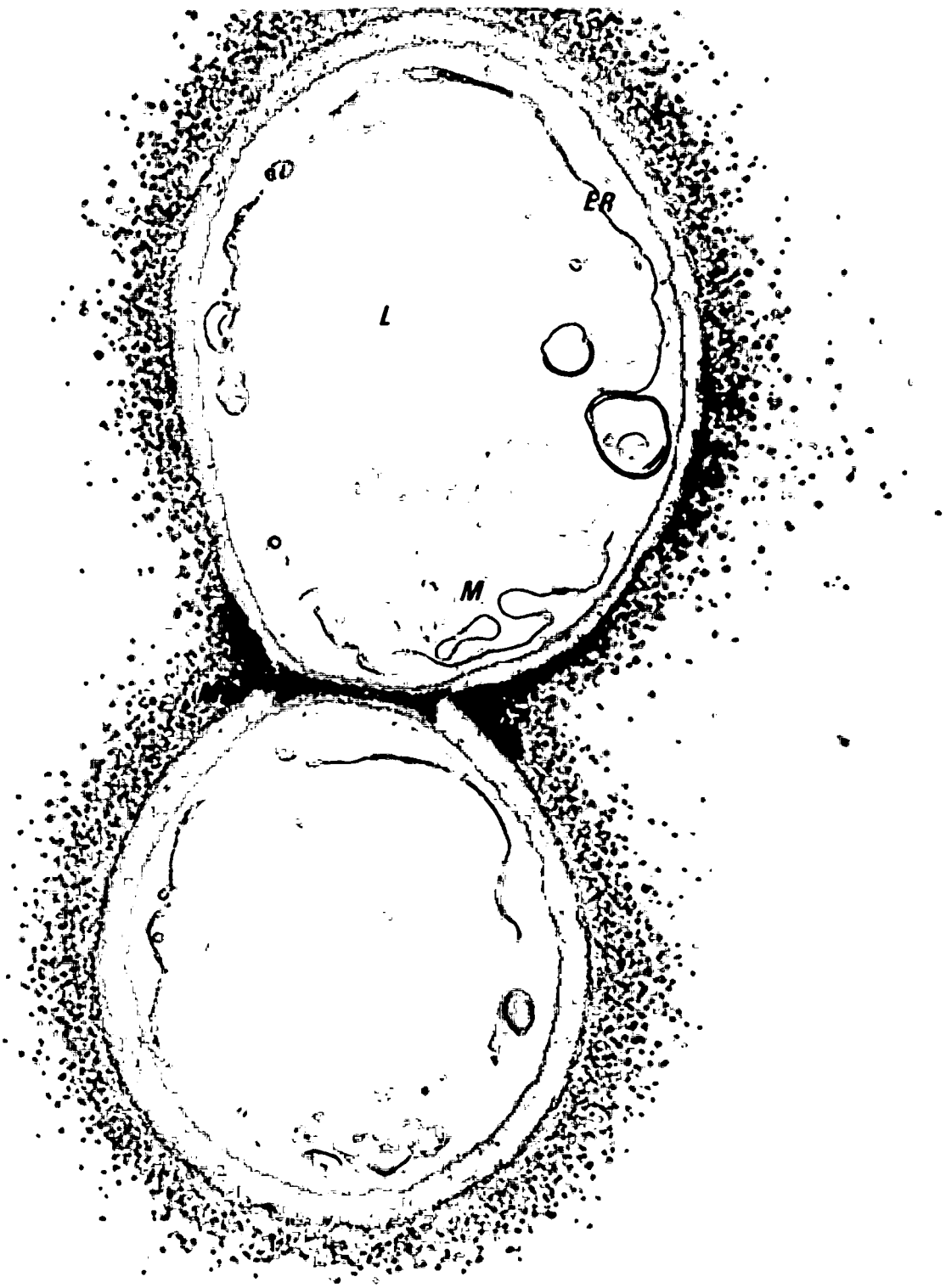
10 µm

3

1 µm

4

Plate 5 - Two-month-old MS cells from pure culture showing details of internal structure.
ER: Endoplasmic reticulum; M: Mitochondria;
L: Lipid; MG: Melanin granules.

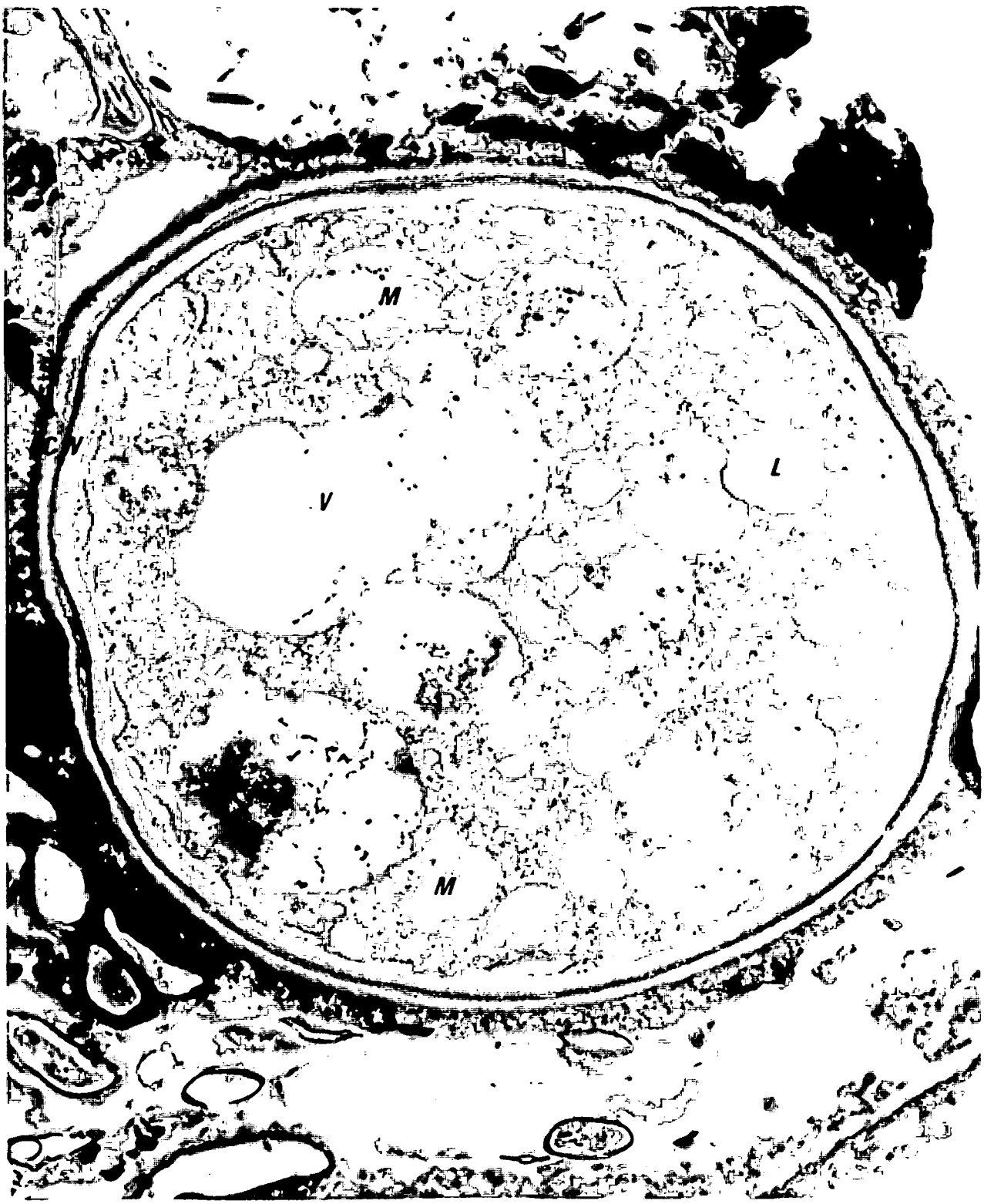


5

1 µm

Plate 6 - An intact, viable MS cell incubated in high pH soil for 60 days.

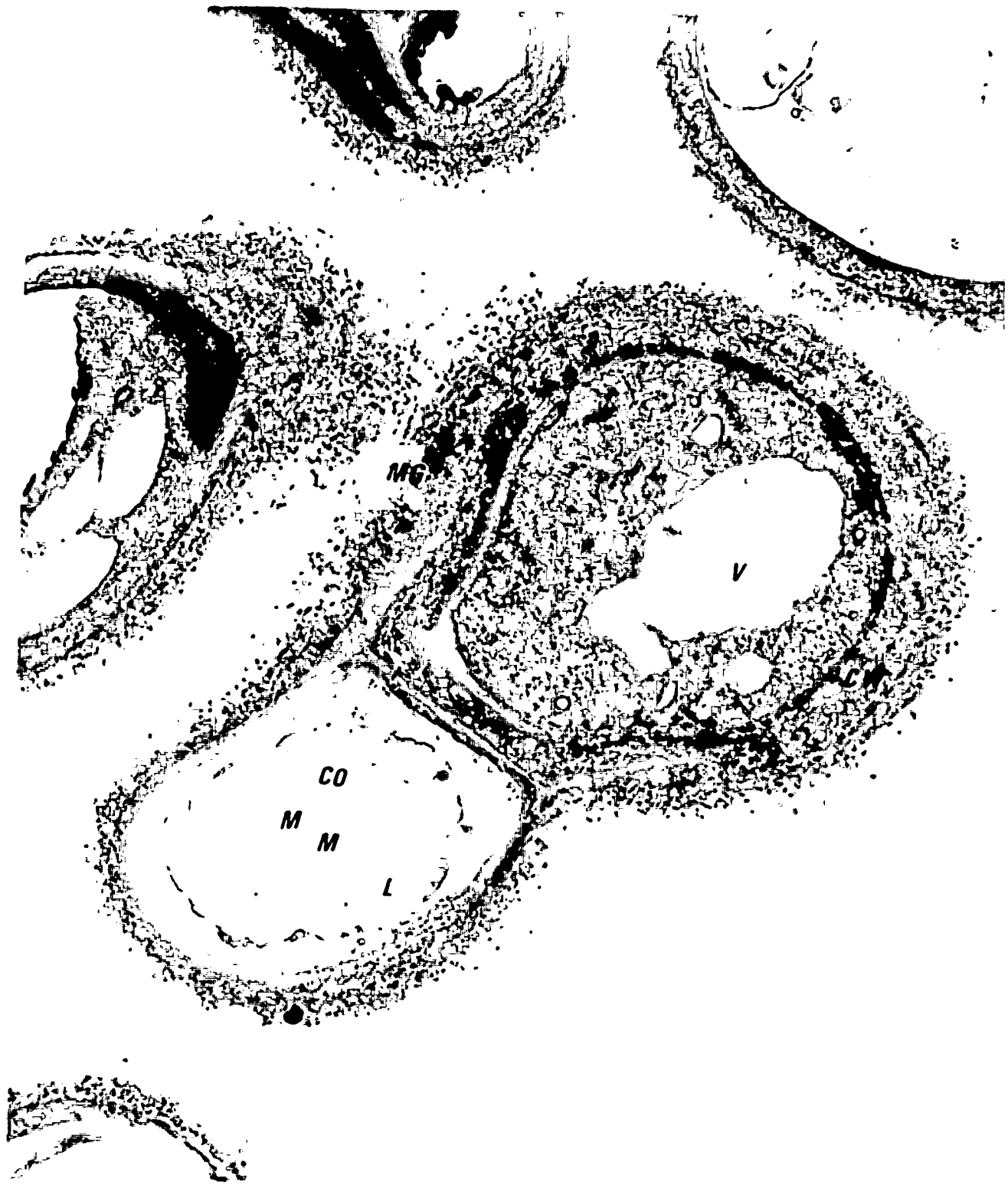
CW: Triple layered cell wall; M: mitochondria; V: Vacuoles; L: Lipid; X: Unidentified electron-dense structure.



6

1 µm

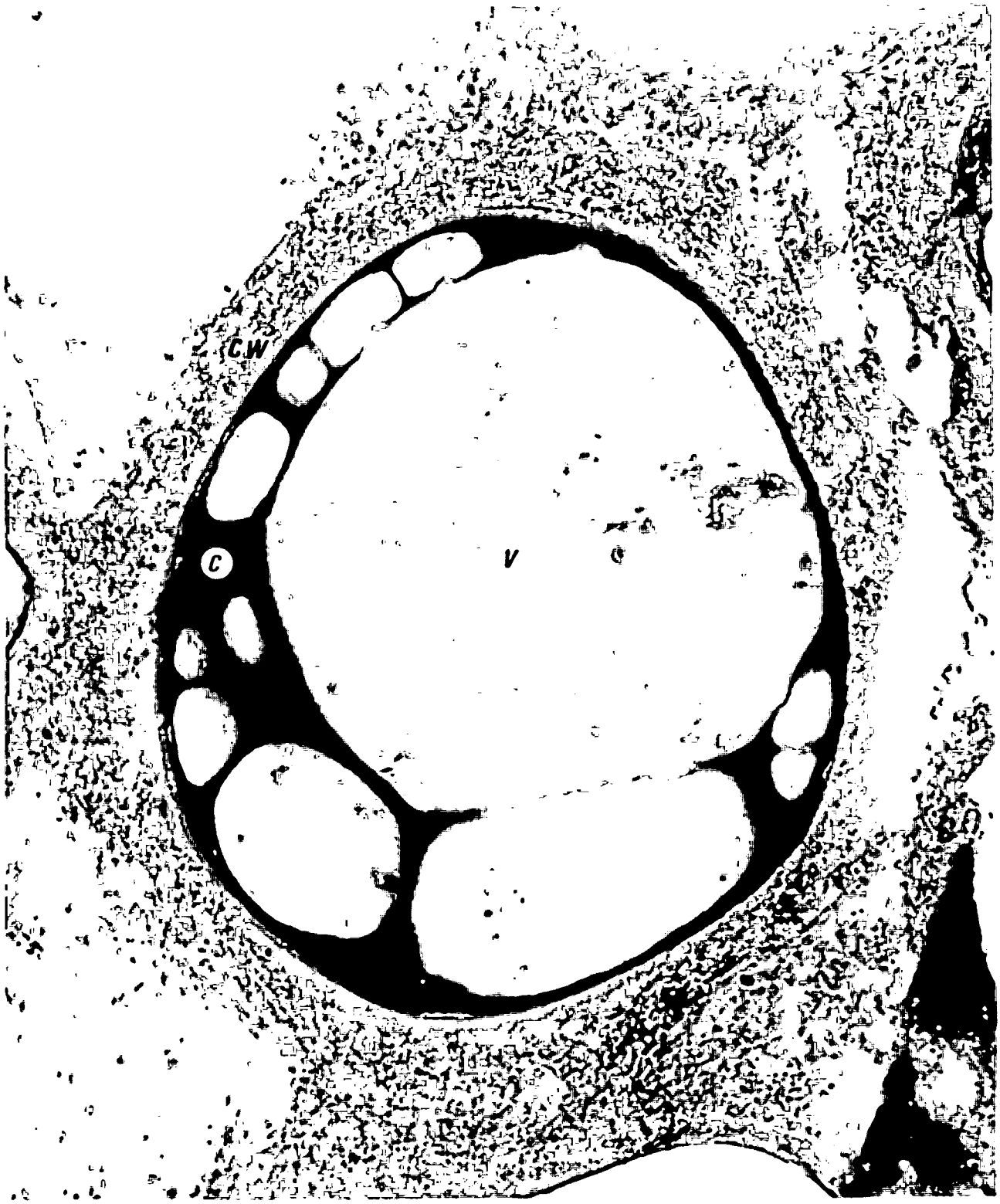
Plate 7 - A two-month-old MS cell from pure culture with cell walls differing in thickness.
CW: Cell wall with secondary thickening; CO: Cell of origin; undergoing plasmolysis; M: Mitochondria; V: vacuole.



7

Plate 8 - A one-year-old MS cell from pure culture, considered to be dead.

V: Vacuole; CW: Cell wall; C: Cytoplasm compressed to the cell wall.



8

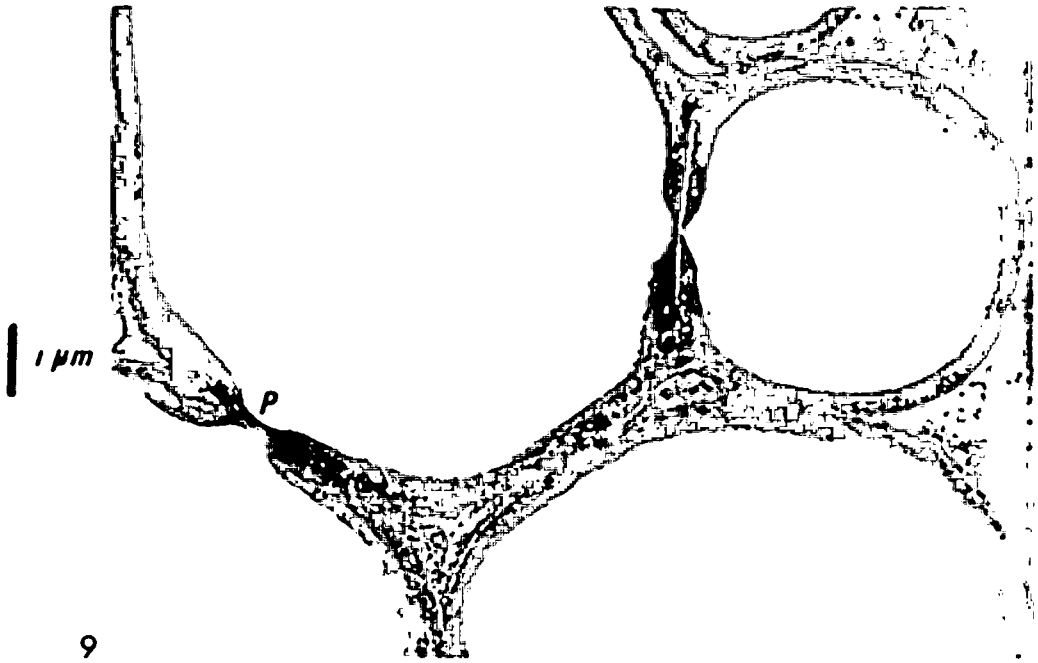
1 µm

Plate 9 - A MS cell showing detail of interconnecting pores (P) between cells.

Plate 10 - Bacteria (B) embedded in the microsclerotial matrix after 36 h in high pH soil.

Plate 11 - A bacterium (B) apparently entering a MS cell through a fracture (F) in the cell wall while some already are within the cell.

(From high pH soil incubated for two months)



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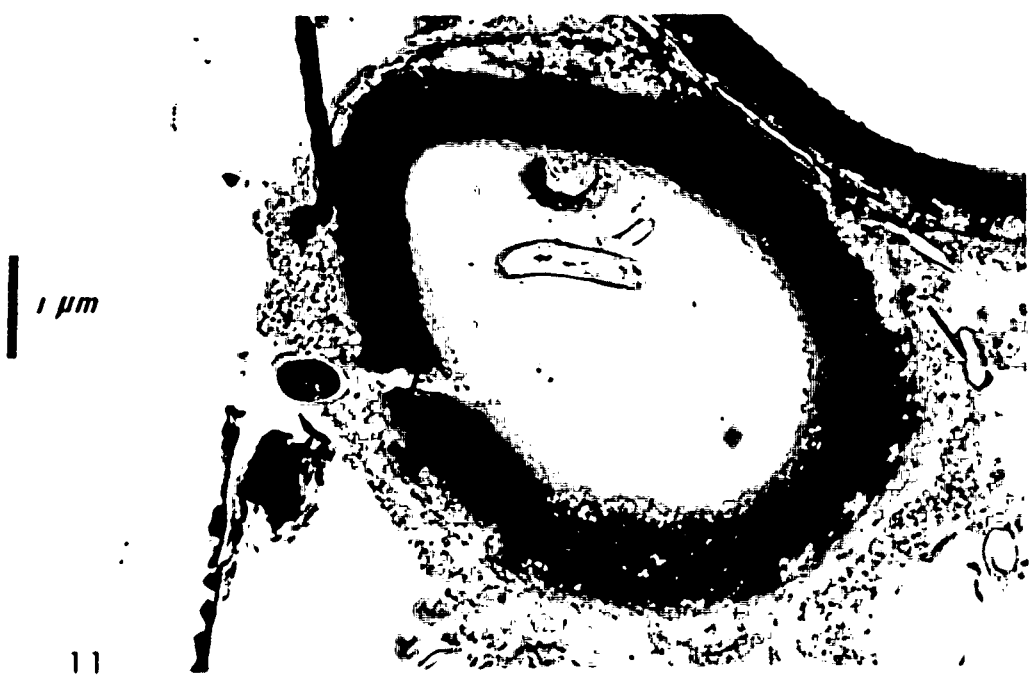
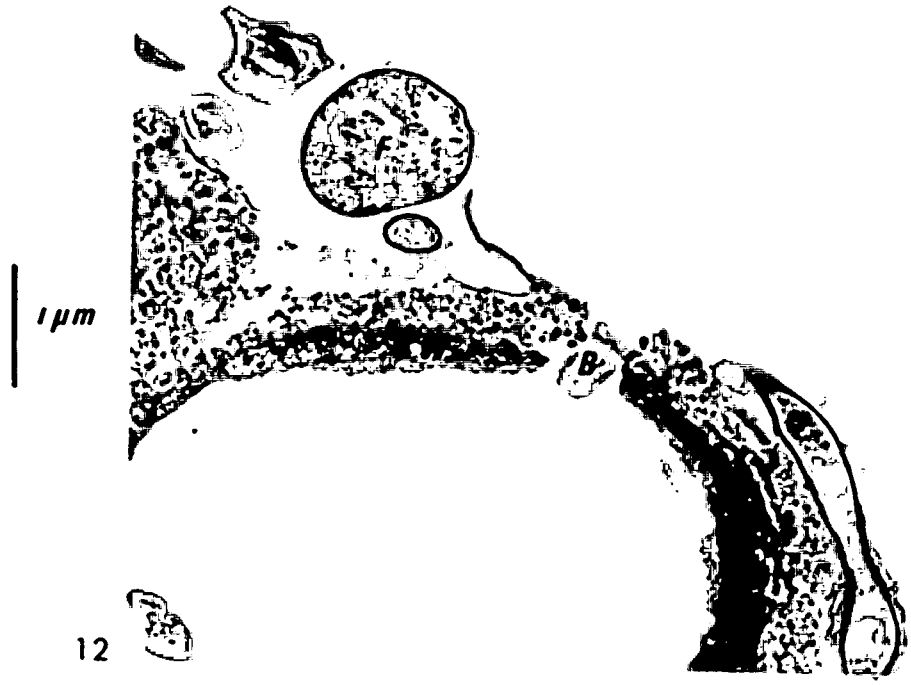


11

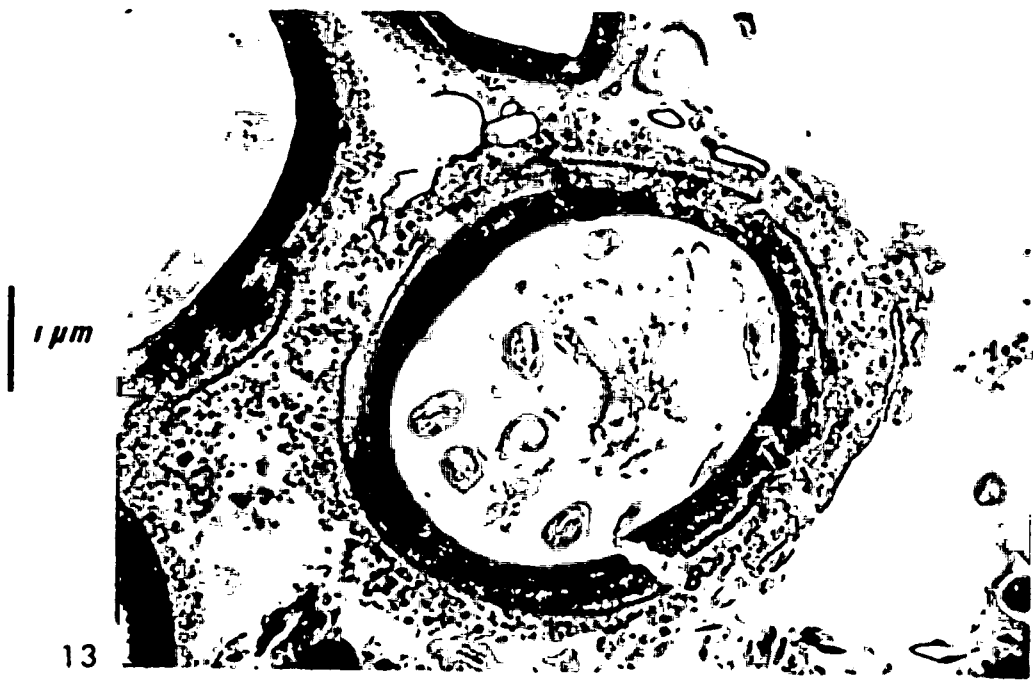
Plate 12 - A bacterium (B) shown penetrating through a gap in the cell wall (CW) of a MS cell.
F: Fungal structure.
(From high pH soil incubated for two months)

Plate 13 - A bacterium (B), irregular in form, apparently penetrating a MS cell through a fissure in the cell wall (CW).
(From high pH soil incubated for two months)

Plate 14 - Bacteria (B) inside a MS cell and entering through a fissure in the cell wall.
LCW: Possible cell wall lysis.
(From high pH soil incubated for two months)



12



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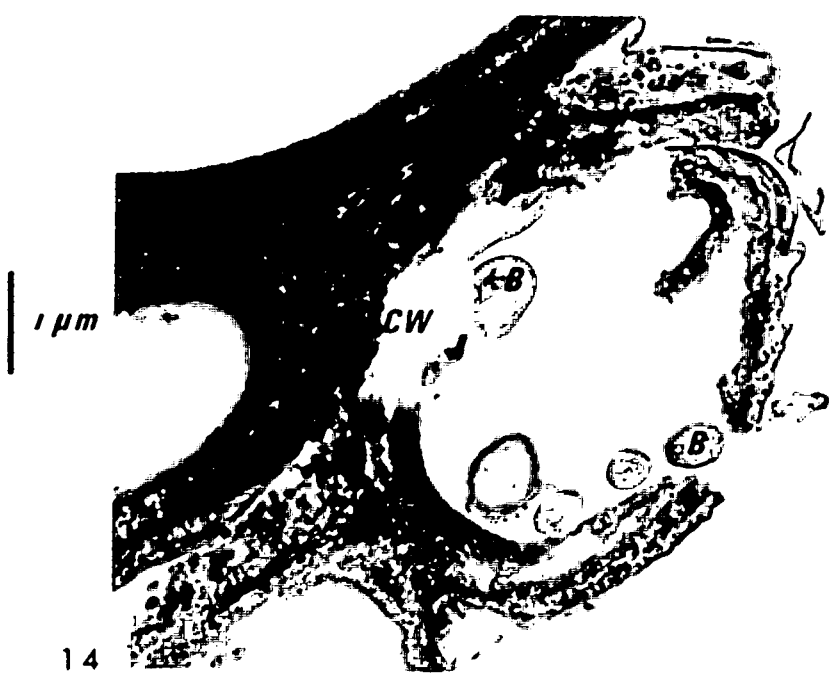


14

Plate 15 - A bacterium (B) embedded in the remnants of a MS cell wall (CW).

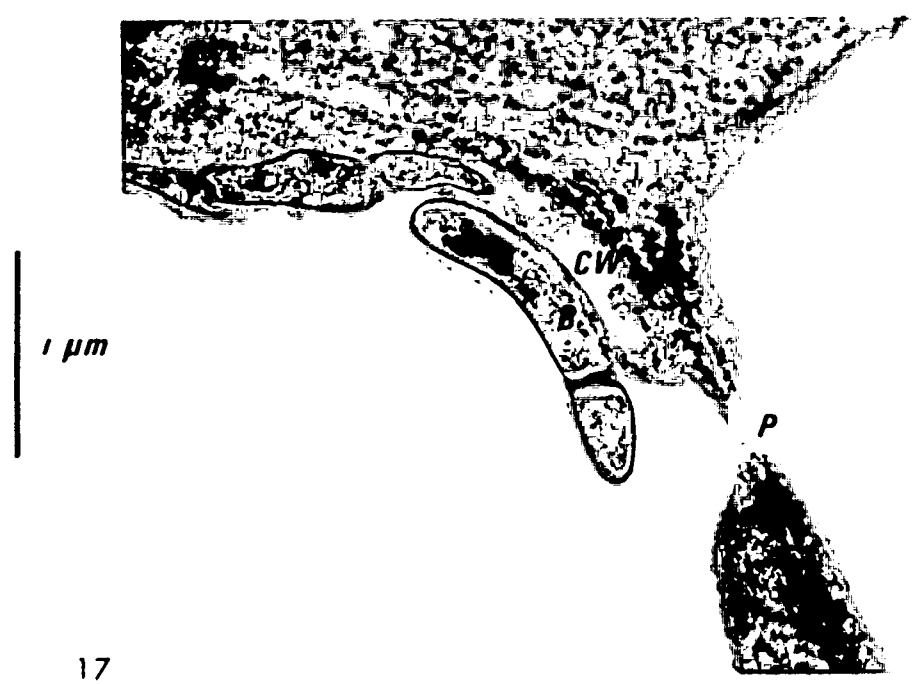
(From high pH soil incubated for two months)

Plates 16 & 17 - Two examples of bacteria (B) attached to MS cell walls (CW) which seem to have undergone some sort of swelling or thick= ening (S).

(From high pH soil incubated for two months)

15

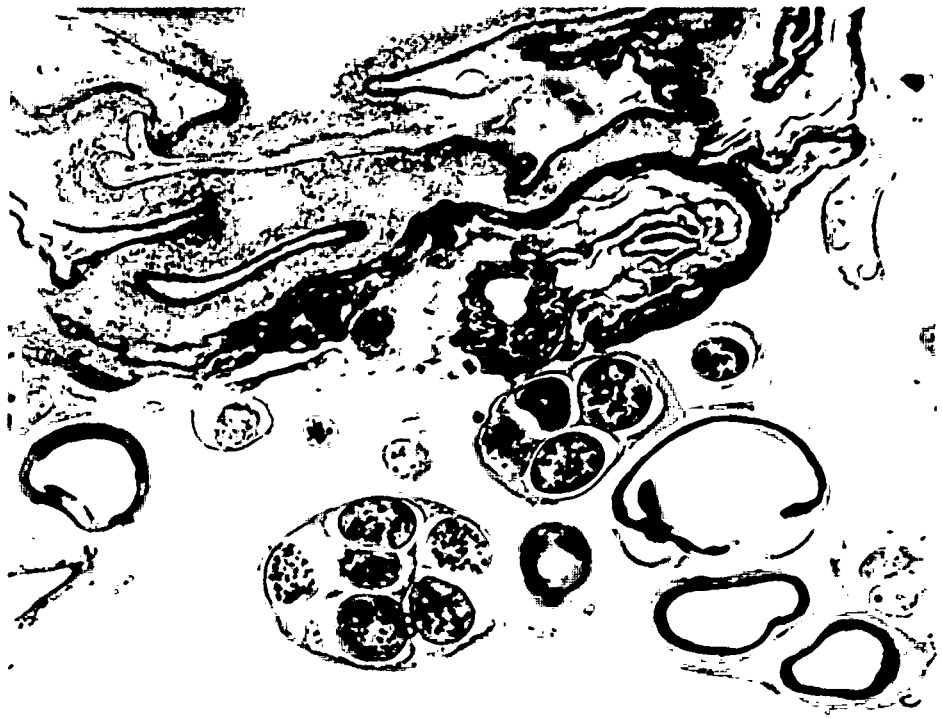
16



17

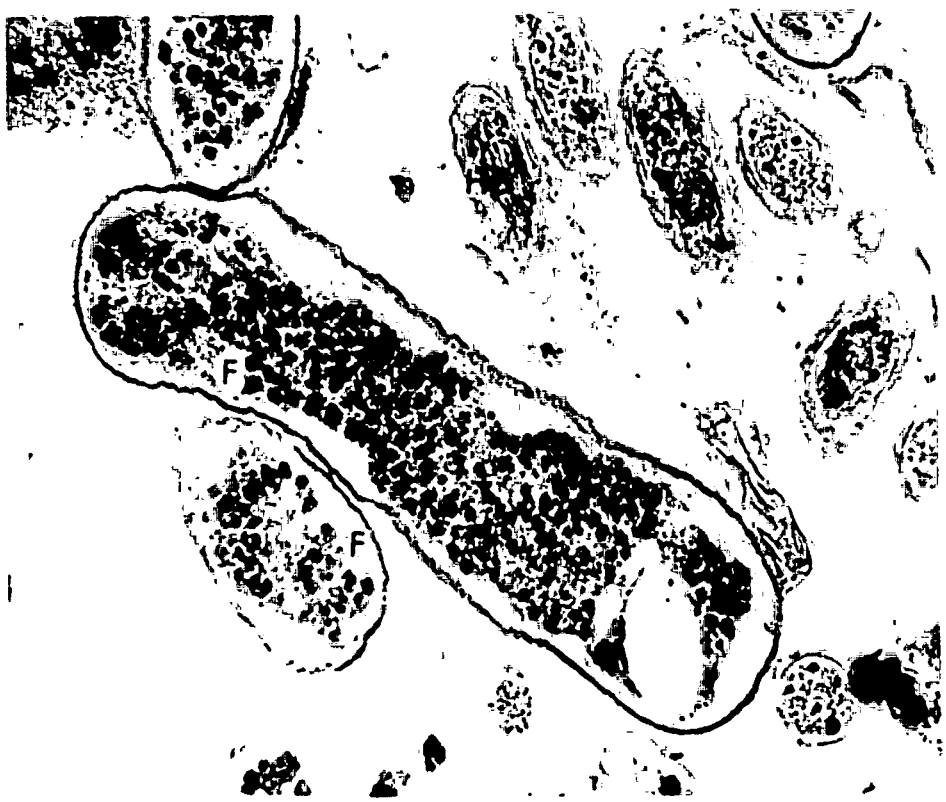
Plate 18 - Bacteria (B) with wall and capsule and arranged in what appears to be a zoogloeal mass.
(From high pH soil incubated for two months)

Plate 19 - Helically lobed (?) bacteria (HB) and fungal structures (F) found in the MS matrix.
(From high pH soil incubated for two months)



1 µm

18

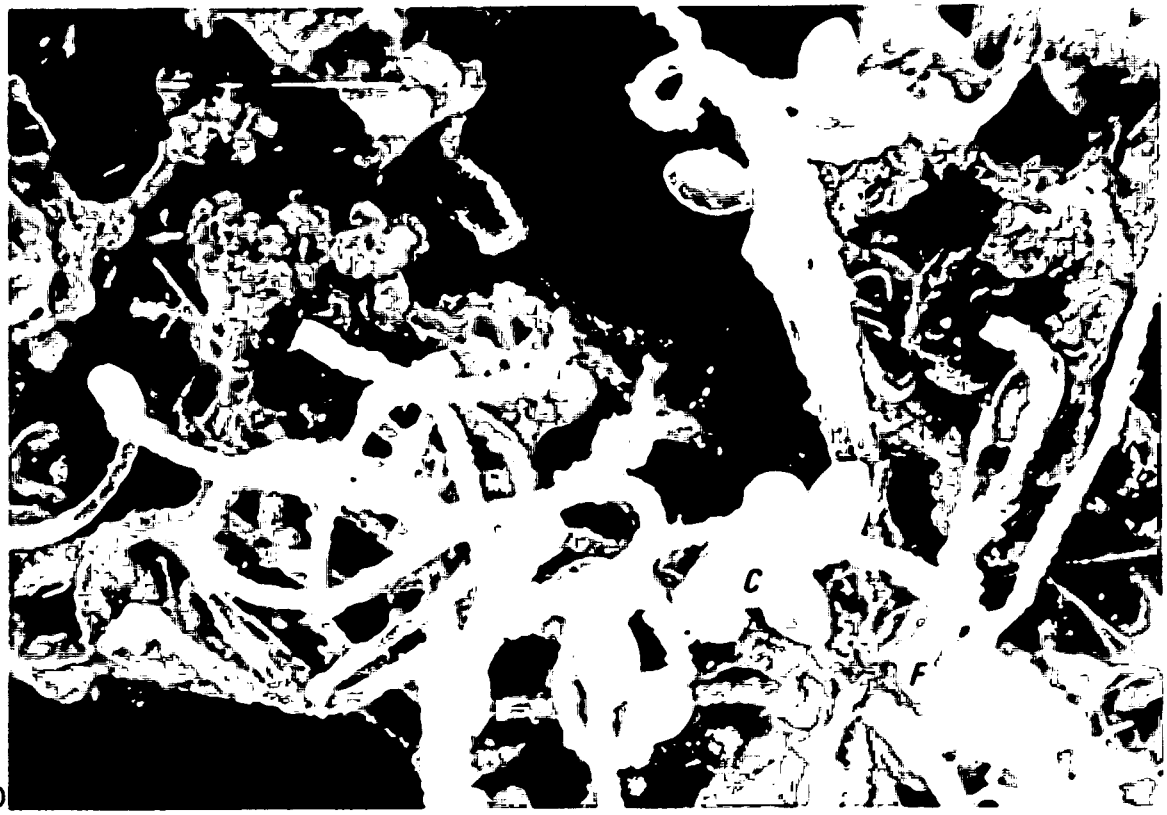


1 µm

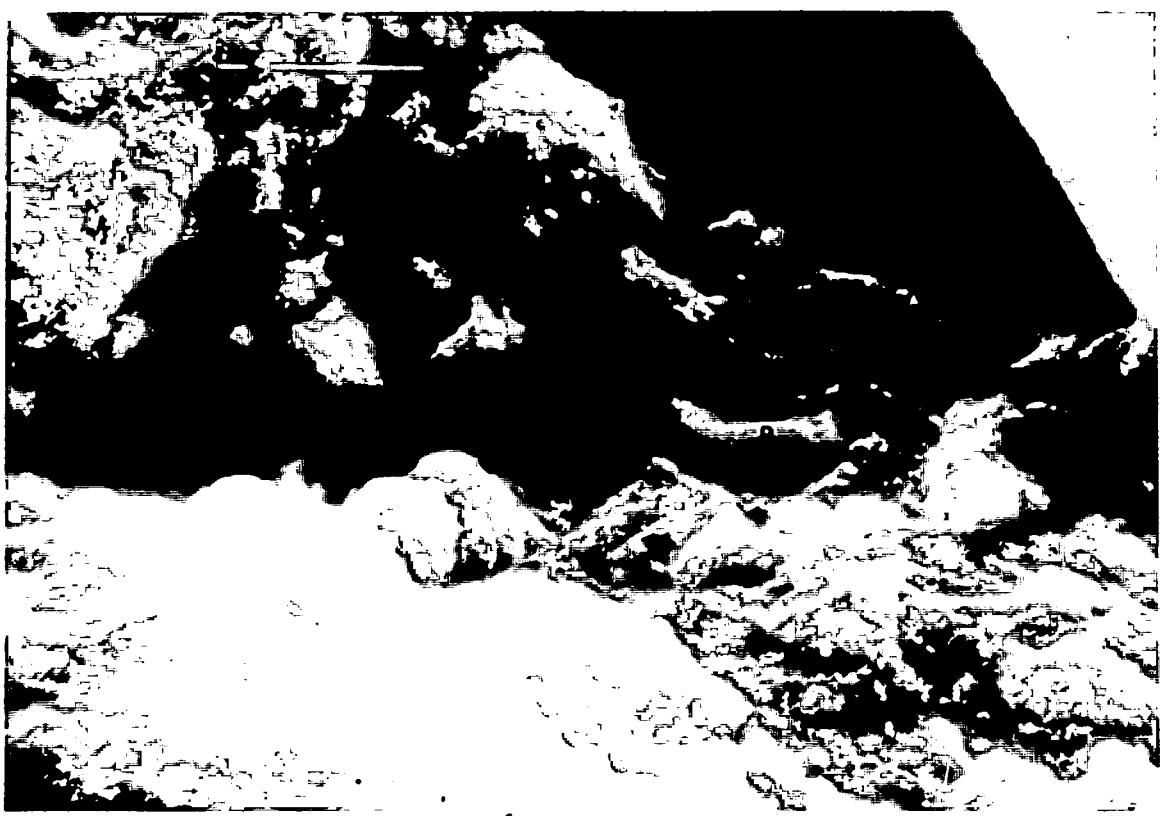
19

Plate 20 - Fungal growth (F) and conidia (C) on a MS recovered from low pH soil after 60 days of incubation.

Plate 21 - A bacterium (B) embedded within the surface of a MS. Lysis of the wall appear to have taken place in the immediate vicinity of the bacterium. (From high pH soil; 30 days of incubation)



20



21

Plate 22 - A MS recovered from low pH soil after 30 days of incubation. Hyphae of fungi colonizing the MS are evident as well as pores (P) open to the surface of the MS.

Plate 23 - A hypha (F) apparently at the point of entry into the MS through a pore on the surface. (Low pH soil; 30 days of incubation)

1 µm

22

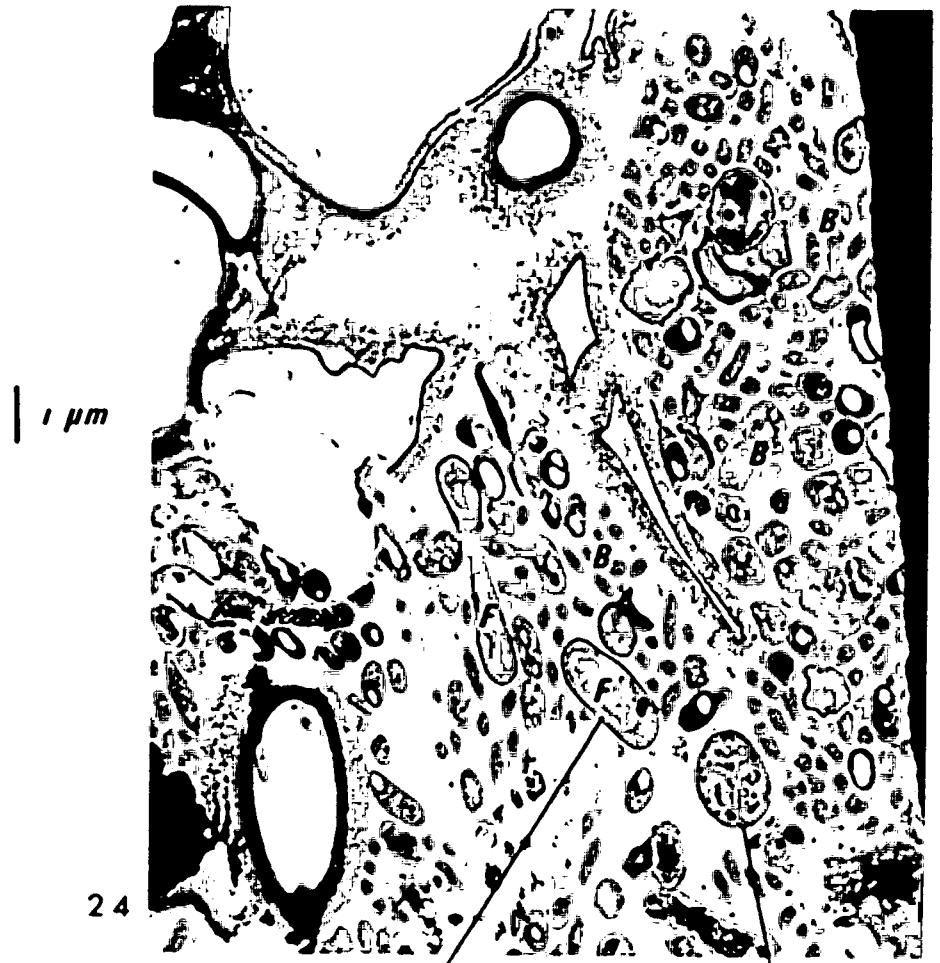
1 µm

23

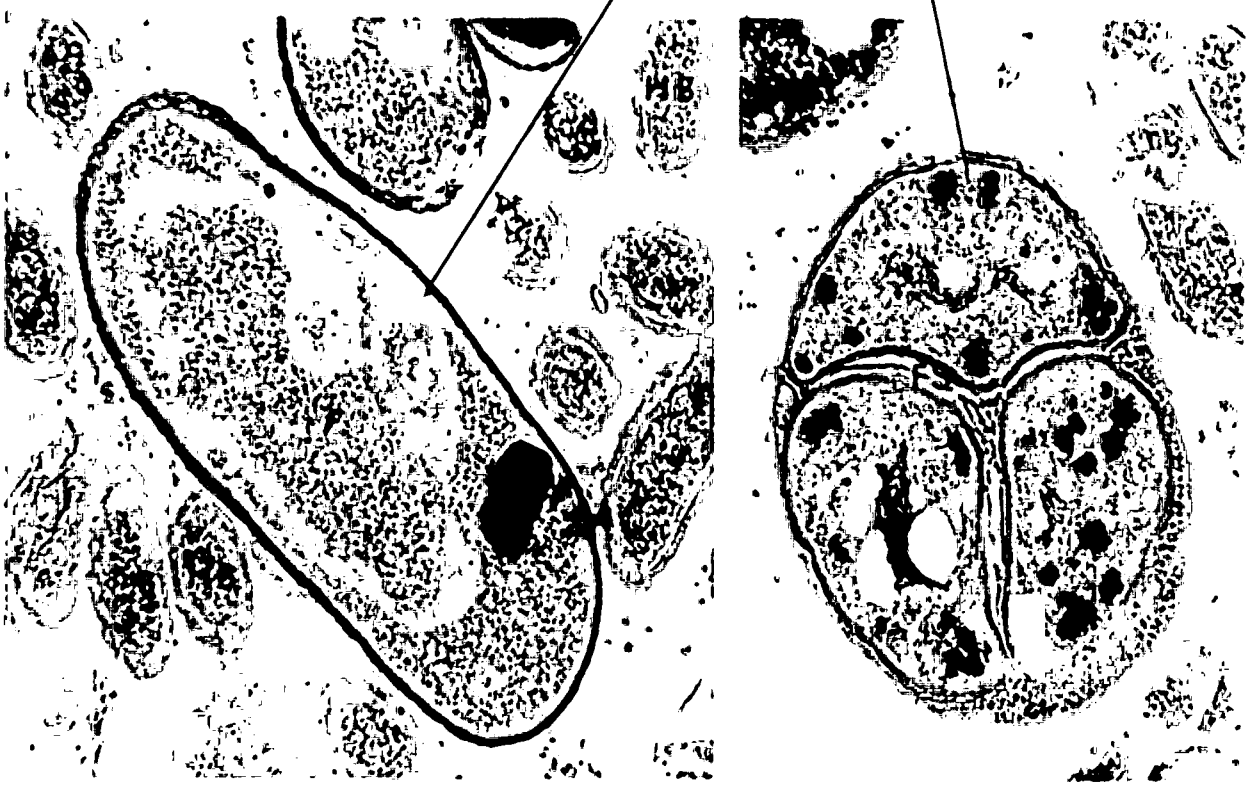
Plate 24 - A MS colonized by both bacteria and fungi. Note the relative prevalence of bacteria (B) in ratio to fungi (F). (High pH soil; 30 days of incubation)

Plate 25 - Enlargement of a section from Plate 24. F: Fungal structure; HB: Helically lobed (?) bacteria.

Plate 26 - A structure resembling a fungal spore (FS) found in the matrix of a MS.



24



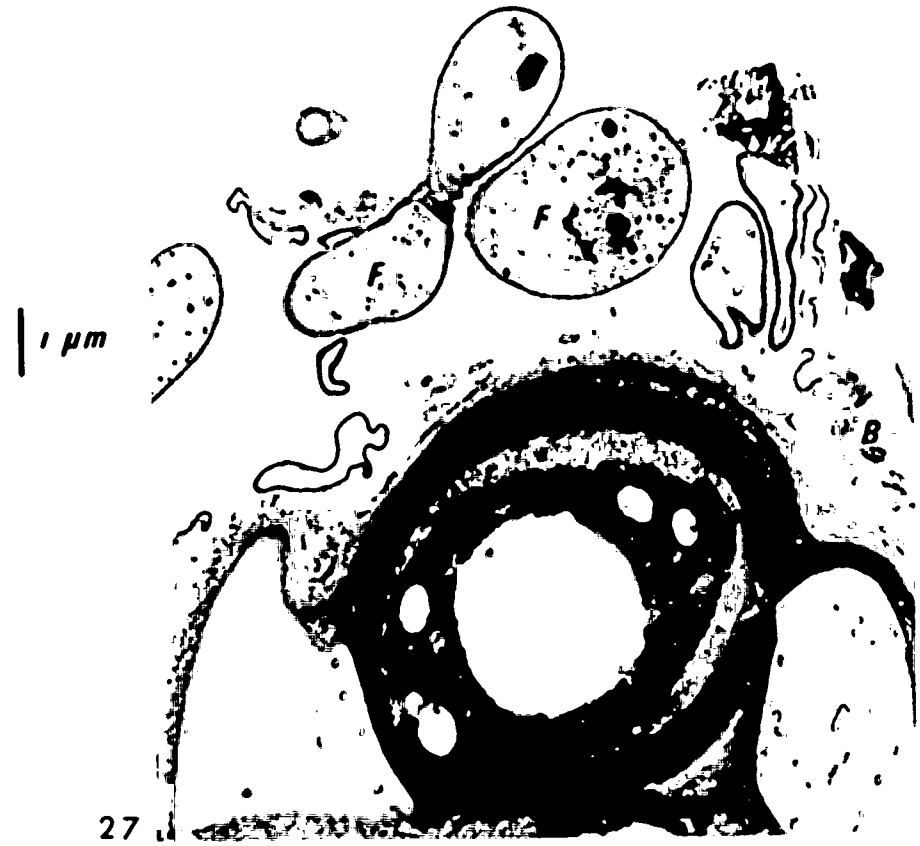
25

26

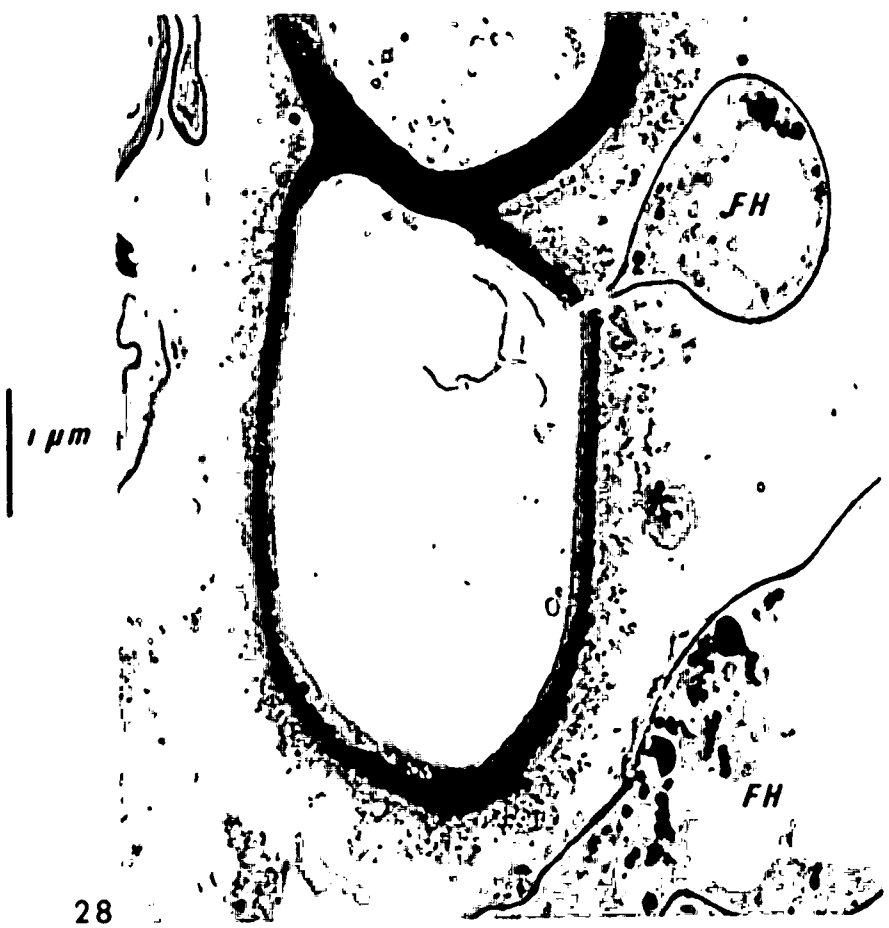
1 µm

Plate 27 - Fungi (F) and bacteria (B) in the matrix of a MS incubated in low pH soil for 30 days.

Plate 28 - A fungal hypha (FH) apparently penetrating through a fissure in a MS cell wall.
(From low pH soil incubated for 30 days)



27

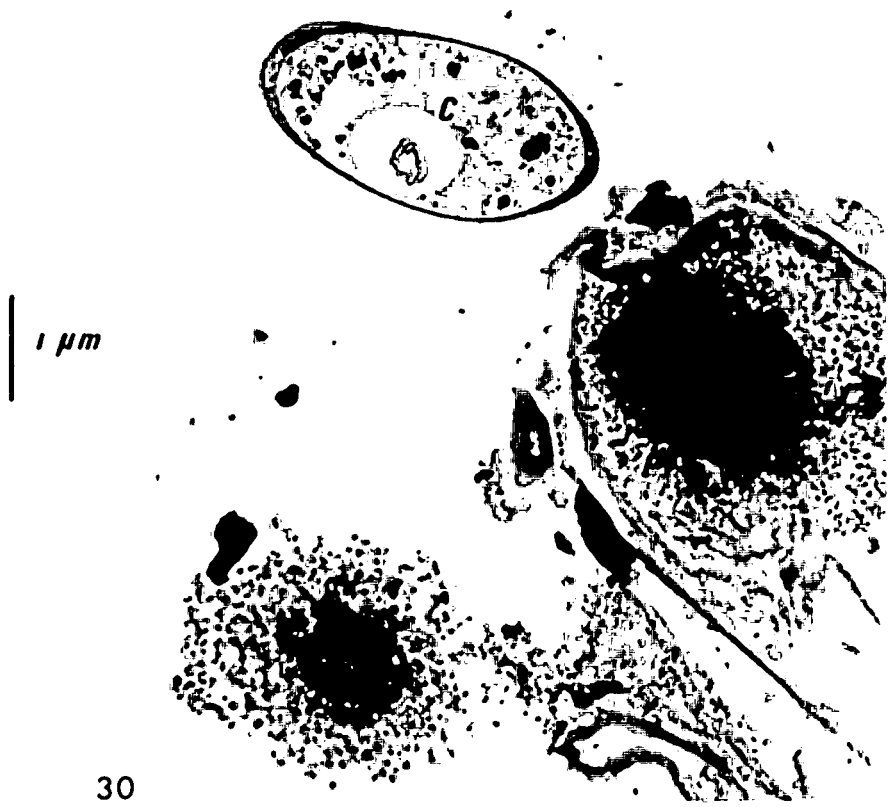


28

Plate 29 - A fungal hypha (FH) found within a MS cell.
(From low pH soil incubated for 30 days)

Plate 30 - Unidentified fungal structures (P) and (C)
found in close association with a MS.
(From low pH soil incubated for 30 days)

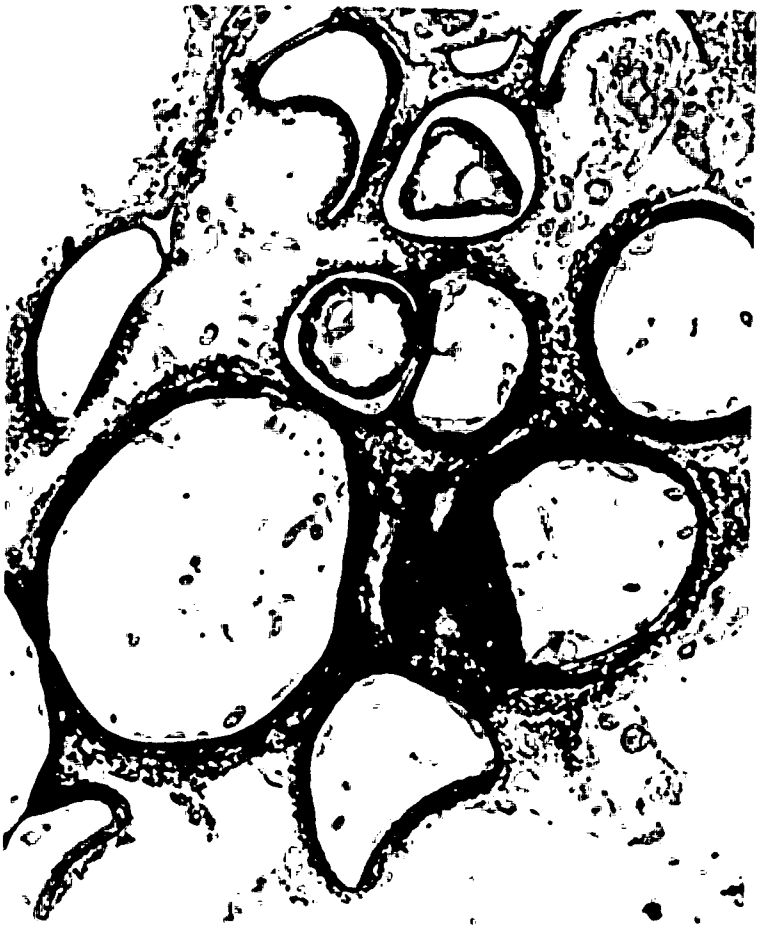
29



30

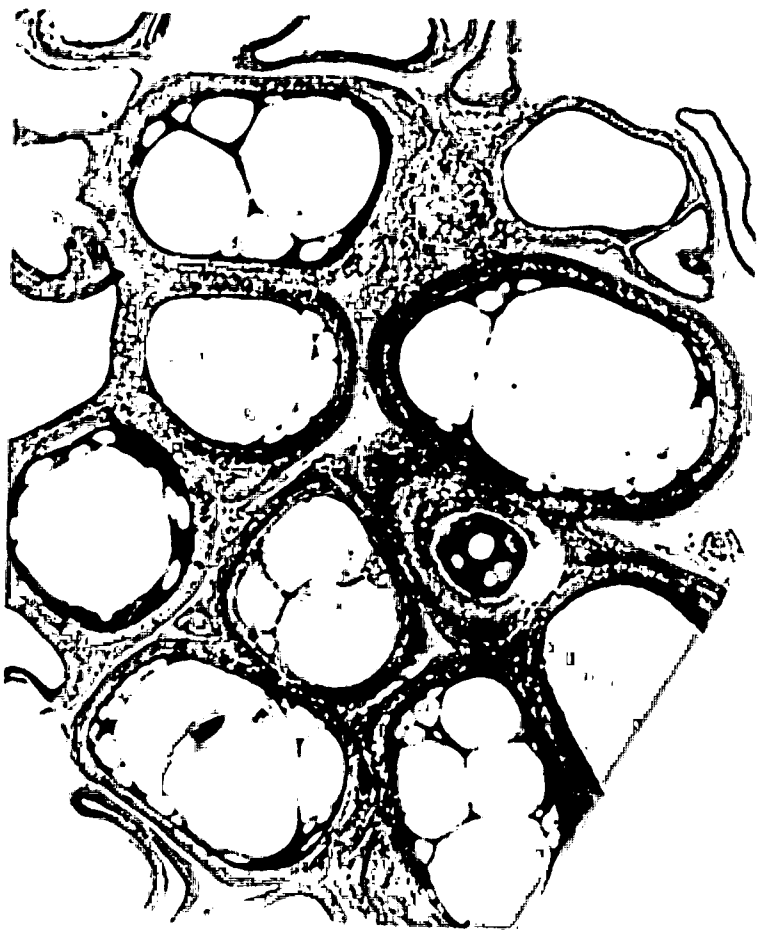
Plate 31 - A MS incubated in high pH soil for 90 days. Melanin granules between cells are loosely arranged, and appear to be degenerating. Abundant bacteria appear within and around the cells.

Plate 32 - A one-year-old MS stored dry in pure culture. Melanin granules appear more compact and homogeneous between cells. Thickening of cell walls is very prominent.



31

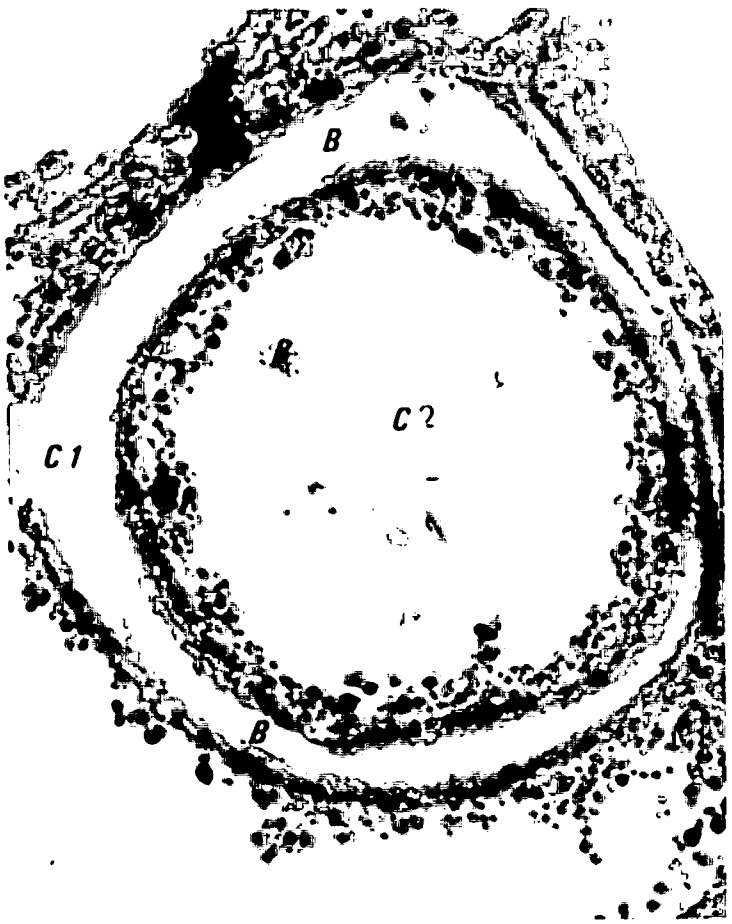
1 µm



32

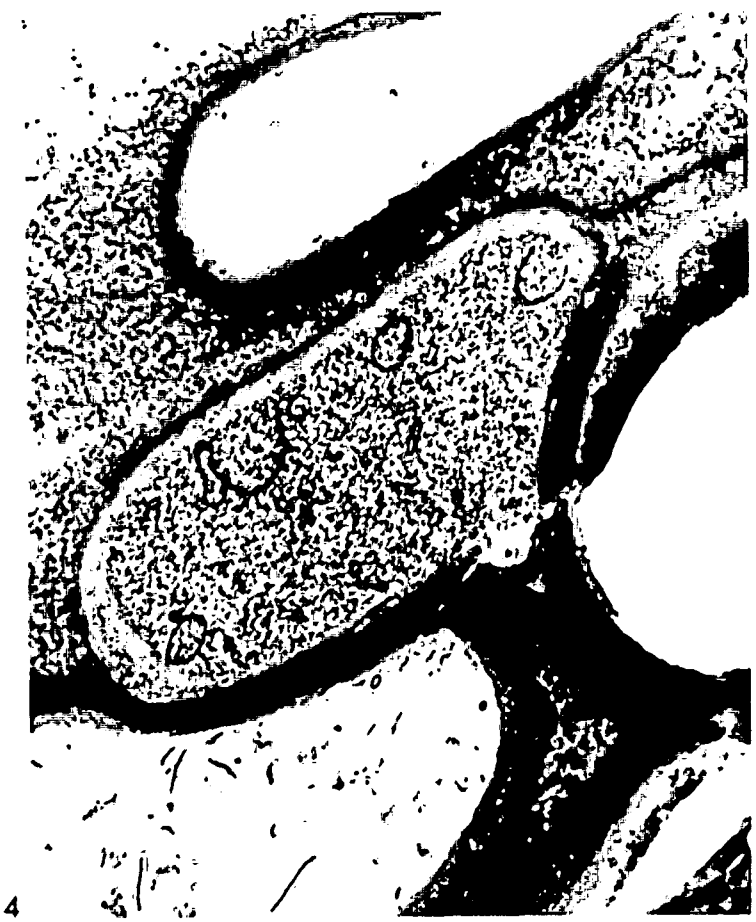
Plate 33 - Unusual structural detail of MS cells observed in Plate 31. A second cell (C2) seems to have been formed within a primary cell (C1). Bacteria (B) can be seen between and within the cells.

Plate 34 - Unidentified structural detail of a MS cell recovered from low pH soil after incubation for 30 days.



1 μm

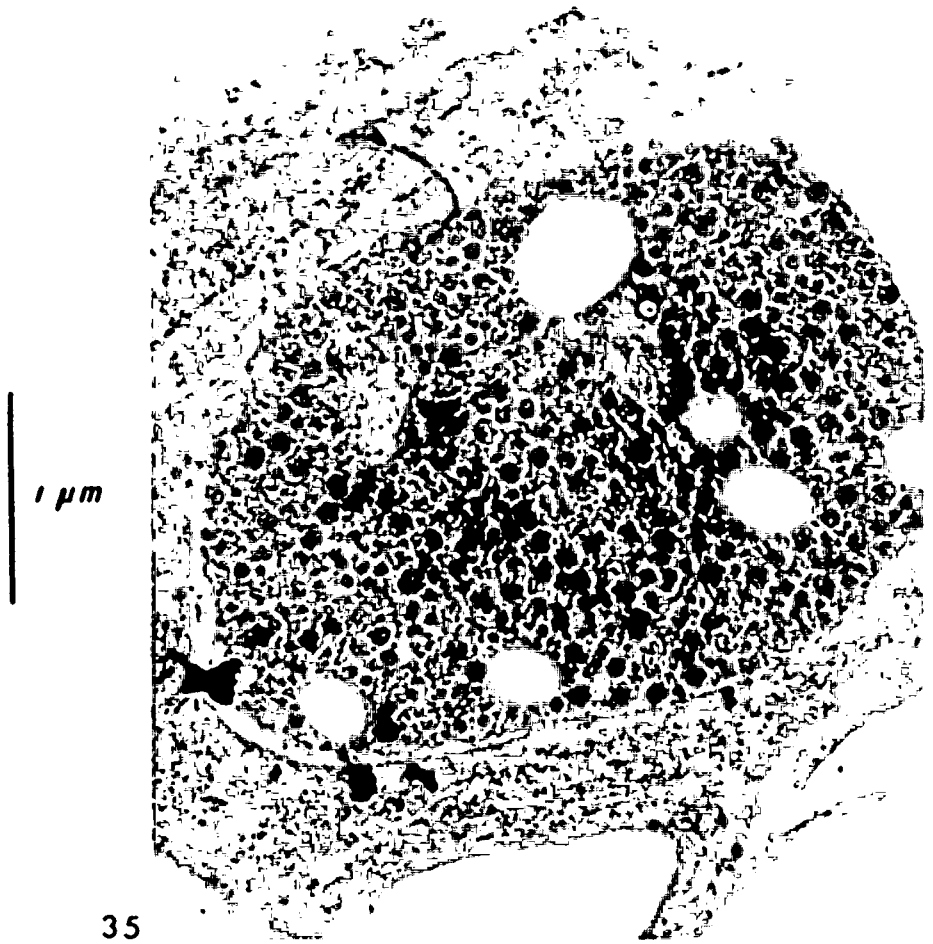
33



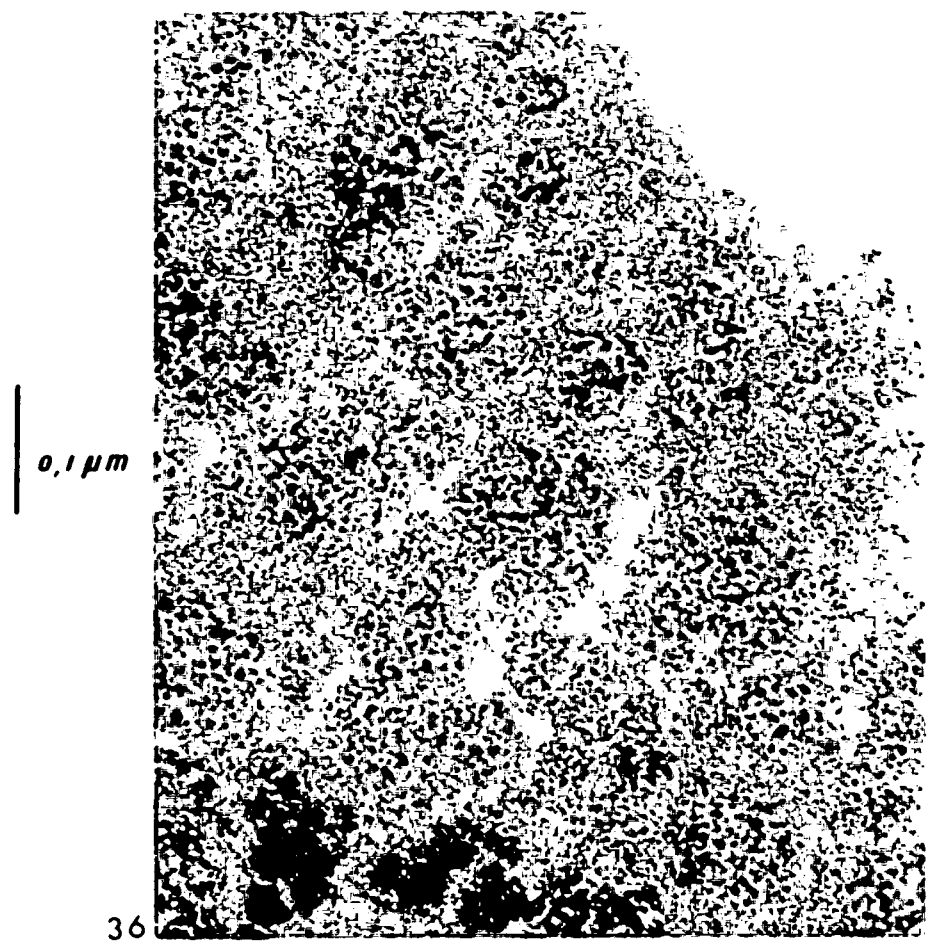
1 μm

34

Plates 35 & 36 - Unidentified (virus ?) particles found within a MS cell. (High pH soil, incubated for 36 h)



35



36

8.3 DISCUSSION

8.3.1 Germination and viability of MS incubated on "Nuclepore" membranes in soil

The behaviour of V. dahliae with respect to the germination of its MS in soil, is not fully understood (p. 29). For instance, Schreiber & Green (1963) found that 11,5% of the MS which were subjected to soil fungistasis, had germinated after 24 h, while Emmatty & Green (1969) reported 17% germination after 18 h. On the other hand, Farley et al. (1971) observed 50% germination after one day and Jordan et al. (1972) 58 - 65% after 72 h.

In the present study the germination percentages of MS in high and low pH soils after 36 h, were 34 and 18% respectively (Fig. 28). These results indicate that factors such as soil type and pH may be involved and responsible for the conflicting results reported by the above mentioned workers. Germination percentages were 62 after 7, 59 after 15, and 12% after 30 days in alkaline soil. The MS between the membranes were relatively free from colonizing fungi in alkaline soil (Plate 1) and the hyphae observed, probably were germ tubes from the MS. If this interpretation is correct, the present results are in corroboration with previous findings that MS sporulate in soil over an extended period of time (Menzies & Griebel, 1967) or in seasonal cycles (Carlstrom, quoted by Powelson, 1970).

In low pH soil germination percentages were initially lower than in high pH soil, but after 30 days the apparent germination was higher. However, colonization of MS by fungi became evident after the seventh day of incubation and it can not be

stated with certainty that the hyphae observed in low pH soil, were germ tubes of V. dahliae.

Emmatty & Green (1969) reported that V. dahliae germinated in soil in two different ways: After 18 h in soil, thin-walled germ tubes as well as chains of chlamydospore-like cells were observed. The latter were transformed to thick-walled pigmented cells which they considered to be secondary MS. They also reported that the thin-walled hyphae were quickly lysed. They did not find any evidence of conidial formation. On the other hand, Farley et al. (1971) recorded that conidia were formed by germinated MS in soil. However, they could find no evidence that secondary MS were produced.

In the present study conidiophores with conidia, attached to MS, were occasionally found. Free spores resembling conidia of V. dahliae were abundant, but no secondary MS were observed.

Air-drying of the soil for at least 48 h is recommended to kill hyphae and conidia of V. dahliae (Farley et al. 1971; Benson & Ashworth, 1976). However, population increases were recorded after the soil had been incubated and air-dried and this was ascribed to conidia being formed by germinating MS (Menzies & Griebel, 1967; Farley et al. 1971) but not by hyphae (Farley et al. 1971). In the present study it was found that hyphae, originating from MS, still appeared viable after air-drying the soil for one month. Extensive lysis of germ tubes was found to occur and bacteria were found in large numbers on these lysed hyphae in alkaline soil. These bacteria

probably contributed to the larger numbers of antagonists which were recorded after the incorporation of MS in soil in previous experiments. From the present studies it would appear that MS which were incorporated into the soil, germinate at a fairly high rate until the cells of the MS most susceptible to germination stimuli, have exhausted themselves. Studies on survival (Fig. 16, high pH soil) support this conclusion. It was found that a sharp decline in viability of MS occurred during the first four weeks in the soil which was then followed by a much slower decline over the next 28 weeks. It is possible that the less dormant cells, probably the viable thin-walled ones, from individual MS, germinate first and in the absence of a susceptible host, succumb to lysis by the soil microflora. The most dormant cells remain ungerminated in the MS. This conclusion is supported by the fact that when MS were placed on agar medium, germ tubes originated from the thinner-walled, less-pigmented cells, whereas the more highly pigmented cells remained inactive (Schreiber & Green, 1963; Isaac & McGarvie, 1966). Further support for this deduction is found in the fact that MS often require shock stimulation to initiate germination, indicating that dormancy might be involved. For instance, Thomas (Schreiber & Green, 1963) reported that MS would not germinate without prior cold treatment while Isaac & McGarvie (1966) found that a pre-soaking in water was necessary to stimulate germination. MS collected by processes involving the use of water, germinate readily without further treatment (Congly & Hall, 1967). In the present study it was found that MS recovered by the use of water and NaOCl (to remove soil fungistasis) (Butterfield, 1975) germinated better

after 6 weeks of cold storage (Fig. 27).

The conflicting reports concerning viability and germination of MS (p. 29) can probably be accounted for if the morphology of a MS is taken into account. In a MS of 63 µm diameter, approximately 88 individual cells were counted (Plate 2). It is thus conceivable that the number of cells within a MS is extremely high and would increase with the size of the MS. Cells vary in size and cell-wall thickness, and each cell, if viable, has an opportunity to germinate. The theoretical opportunities to germinate would thus be equally high. However, in sucrose amended soil in which 90% of the MS germinated, only 4 - 6 germ tubes were produced per MS. When MS were stimulated to germinate on 9 successive occasions, 1 - 3 germ tubes were still produced per MS during the 9th germination period (Farley et al. 1971). This is indicative of the resistance to germination posessed by individual cells. Cells on the periphery of the MS are usually thinner-walled and less pigmented than those towards the centre and in young MS these cells are presumably viable (Gordee & Porter, 1961; Nadakavukaren, 1962; Schnathorst, 1965) and can germinate (Schreiber & Green, 1963; Isaac & McGarvie, 1966). It is possible that these cells are responsible for the high percentage of germination of MS after they are placed into the soil. When these cells are exhausted, only the thick-walled, highly pigmented cells with more resistance to germination, remain.

Brown & Wyllie (1970) have shown that the mucilaginous matrix which cements MS cells together, is the result of melanin accumulation. As the matrix deteriorates (Plate 31),

individual cells from the periphery of the MS, are freed in the soil. These cells are then more exposed to germination stimuli such as nutrients. It is even possible that the release of individual cells may relieve them from the effects of an auto-inhibitor (Isaac & McGarvie, 1966). Some cells may germinate early in the life of the MS, while others may remain dormant for extended periods. Changing environmental conditions may also stimulate otherwise dormant cells to germinate. Dormancy of individual cells apparently confers on V. dahliae the ability to survive for long periods, under widely differing conditions. Studies on the survival of V. dahliae also support this theory. When MS of various sizes were added to the soil (Fig. 16, high pH soil), a fast attrition rate at the start of the experiment was followed by a much slower attrition rate after one month of incubation, whereas, when relatively large MS (45 - 75 µm) were selected (Fig. 29, pH 8), the attrition rate was slow from the start of the experiment. The resistance of individual MS to attrition is evident even in low pH soil which is extremely detrimental to MS (Fig. 29).

8.3.2 Structure and lysis of microsclerotia in soil

Electron photomicrographs of MS of V. dahliae from pure culture and from soil, incubated at different pH levels, revealed interesting structural characteristics. Biodegradation by microorganisms was also evident.

Nuclei and other cell organelles were frequently observed in young MS cells (Plate 4). Older cells of MS from both pure

culture and soil appeared to have undergone considerable structural changes. Nuclei were rarely observed in these cells. However, other inclusions such as mitochondria, lipid drops, the endoplasmic reticulum, and vacuoles were sometimes still present (Plates 5 - 7). In most of the cells which still contained cytoplasm after one year in pure culture, the cytoplasm became extremely electron-dense and organelles became indistinguishable (Plate 8). Large vacuoles became evident and the cytoplasm was pressed against the cell wall. The cells appeared to be non-viable.

It also appeared that cell-wall thickness varied considerably (Plates 4, 6, 7). Sometimes the walls apparently consisted of a single layer (Plate 4), or three layers (Plate 6), or in some cases secondary thickening had occurred (Plate 7). Griffiths (1970) could find no evidence of an appreciable increase of cell-wall thickness. He ascribed the variation in cell-wall thickness described by Nadakavukaren (1963) to melanin deposition between the cell walls which gave the appearance of cell walls varying in thickness. The present results are thus in corroboration with those of Nadakavukaren (1963).

There is some controversy about the origin of the melanin granules embedded in and surrounding MS cell walls. Griffiths (1970) suggested that melanized particles were extruded from living cells and deposited within the matrix between the cell walls. Wheeler, Tolmsoff & Meola (1976) could find no evidence for cytoplasmic synthesis of melanin and suggested that melanin is formed extracellularly in association with the

fibrillar network. In the present study older MS which were subjected to biodegradation in the soil seem to have formed smaller secondary MS cells within primary MS cells (Plate 33). These secondary MS cells were as heavily pigmented as the primary cells, indicating that the origin of the melanin particles, in the present case at least, is probably cytoplasmic.

The formation of secondary cells within primary cells when these were subjected to biodegradation in the soil, seems to be a further mechanism by which the pathogen could extend its longevity in the soil.

The internal structural detail of some MS cells was sometimes unusual. Cells recovered from low pH soil appeared granular and disorganized internally (Plate 34), possibly because of the effect of pH on the cell. Cells recovered from high pH soil after 36 h of incubation, also appeared granular internally, but some organization which appeared to resemble virus particles, was observed (Plates 35, 36). Such structures were not found in photomicrographs published by other investigators.

Bacteria as well as fungi were found to colonize MS which had been buried in both high and low pH soils (Plates 10 - 33). Bacteria were found within the cell lumina, embedded within the cell walls, attached to cell walls, and in the matrix between the cells. The cell wall areas surrounding bacteria embedded within these walls, were less electron dense than the intact walls and seemed to have been eroded by bacterial action (Plates 14, 15). A slight swelling or thickening of cell walls was seen where bacteria were attached to the walls (Plates 16, 17), indicating that bacterial enzymes might be involved.

Bacterial penetration of cell walls were often observed (Plates 11 - 14). Similar perforations were also found in conidia of various fungi (Old & Wong, 1976), and in chlamydospores of Thielaviopsis basicola (Clough & Patrick, 1972). The diameter of the perforations found in the present study compares well with the size of the bacteria penetrating the cells. Larger cavities which might have been caused by soil amoebae (Old, 1977a,b) were not seen in the present study.

The bacterial and fungal colonization of R. solani sclerotia as shown in an electron microscopic study (Naiki & Ui, 1975) was similar to the colonization of the MS of V. dahliae. However, perforations in the cell walls of R. solani sclerotia were not observed (Naiki & Ui, 1975). The present work seems to support the suggestion of Naiki & Ui (1975) that the bateria produced enzymes which initiated the degradation of sclerotial cell walls (Plates 14 - 17, 21). In view of the evidence pre= sented in this study and that presented by Naiki & Ui (1975), the statement that melanins in the cell walls have the ability to inhibit the activity of cell-wall-lysing enzymes (Kuo & Alexander, 1967; Bull, 1970) does not seem to be valid. This view is further supported by the deterioration of pigmented material found in MS incubated in soil for extended periods (Plate 31).

It was impossible to identify the bacteria from the electron= photomicrographs. They varied in shape and size, but bacteria with a wall and capsule (Plate 18) similar to those described by Naiki & Ui (1975) and others, apparently similar to the heli= cally lobed bacteria found by Old & Wong (1972) were observed

(Plate 19).

Fungal structures, mycelia and spores, were found within and in close association with MS, especially when they were incu= bated in low pH soil (Plates 20, 22, 23). However, neither fungi nor bacteria were found within or in the process of pene= trating a MS cell which had the appearance of a typically viable cell. It is thus not clear whether they are capable of penetra= ting viable cells. It is possible that the cells were killed by some other mechanism of antibiosis and invaded afterwards by bacteria and fungi. Aluko & Hering (1970) have shown that Gliocladium virens deposited lethal concentrations of antibiotics on sclerotia of R. solani and that direct parasitism was less important than antibiosis. It was evident from the present study, however, that micro-organisms are involved in the degradation of melanized MS of V. dahliae in soil.

CHAPTER 9

SUMMARIZED DISCUSSION AND CONCLUSIONS

The survival of V. dahliae in soil is a controversial subject. Apparently the pathogen relies mainly on its MS for survival. These structures consist of numerous thin- and thick-walled cells, the walls of which are melanin impregnated. MS appear to be adapted to prolonged survival under adverse conditions.

In the field it was found that individual MS survived in the soil for more than two years. Regression analysis of these survival data indicated that the MS are capable of surviving up to 43 months in the soil in the absence of host plants. Data on temperature and the moisture content of the soil indicated that these factors could not be related to the attrition of the pathogen. However, extensive colonization of the MS by bacteria, fungi, and actinomycetes could have had an effect on the attrition of the pathogen.

Propagules of the pathogen were released into rhizosphere soil after the plants had been killed by the pathogen or by frost. During this period fungi of many genera, antagonistic to V. dahliae, and consistently associated with the roots of diseased plants, did not respond by increasing appreciably.

Bacteria and actinomycetes antagonistic to V. dahliae could be stimulated to increase in high pH soil by the addition of MS and fertilizers containing phosphate. In low pH soil, which favoured fungal antagonists, these tendencies were less obvious. The largest numbers of bacterial and actinomycetous antagonists

occurred in high pH soil in which V. dahliae survived best.

In low pH soil where attrition was the fastest, fungal antagonists prevailed. However, suppression of antagonists, e.g. Trichoderma spp. had no effect on the number of MS which could be recovered from the soil. This suggested that antagonists were not the primary factor responsible for the rapid attrition of V. dahliae in acid soil. This conclusion was also supported by circumstantial evidence.

Electron microscopic studies of MS buried in high and low pH soil for various periods indicated active invasion by fungi and bacteria. They were found in the matrix between individual cells, attached to cell walls, within apparently lysed cell walls, and within the lumina of the cells. Eroded areas within the walls in the immediate vicinity of bacteria were often observed, indicating that enzymes may be involved in the deterioration of cell walls. It is concluded that antagonists are capable of actively destroying MS in soil, but this probably is a much slower process than that which was observed in acidified soil.

Various techniques were used to establish the fact that attrition was much faster in acidified than in alkaline soil. The use of several techniques demonstrated that the effect of low pH on the attrition of the pathogen was real and not a reflection of the inadequacy of a single technique. It was also evident that the effect of low pH was fungitoxic and not fungistatic to the pathogen.

Experiments to test the validity of the claim that the Al-ion is toxic to V. dahliae at very low concentrations indicated that the attrition of V. dahliae was as fast in low pH soil devoid of aluminium salts as in aluminium-amended soil.

Soil acidification may be considered as a control measure. However, practical and economic considerations will prohibit its implementation. Apart from the cost factor, most plants do not tolerate such a low soil pH. Liming of the soil would be necessary, with the result that favourable conditions are again created for renewed increase in pathogen numbers.

In a green-house study it was found that varying the moisture content of the soil and incorporating N and P, were ineffective as measures to reduce Verticillium populations. However, in flooded and air-dried soils, significant decreases occurred. The addition of urea at 0,25% or higher to the soil, reduced Verticillium populations appreciably. Various organic soil amendments gave diverse results. Maize residues, followed by soyabean pods caused the fastest attrition of MS in the soil.

The addition of urea to soil at high rates would not be practical as a control measure. In situations where cotton can be produced in rotation with paddy rice, flooding may be of practical use. Air-drying of the soil will depend on weather conditions, but it may be possible to devise agronomic practices to speed up the drying-out process and thereby reduce pathogen populations. This finding should, however, be studied under field conditions to verify the results obtained in the laboratory.

The reduction of MS in the soil after amendment with plant residues holds promise as a control measure. Various crops could effectively be used in rotation with cotton and when the residues are incorporated into the soil, a significant attri= tion rate could be expected. However, the large quantity (1%) of residues required to effectively reduce the population of V. dahliae suggests that attrition would not be as fast under field conditions as it was under laboratory conditions. A long term rotation might thus be necessary.

Electron microscopic studies on the fine structure of the MS indicate that they are composed of numerous thin- and thick-walled cells. The cell walls vary in thickness and are impregnated with melanin which also occurs in the matrix between the individual cells. These properties confer resis= tance to attrition to the MS. In the soil they apparently germinate over extended periods and give rise to limited hyphal growth which exhausts the reserves of the less resistant cells. However, some of the more resistant cells may remain dormant and retain the viability of the MS. This may explain the survival of some propagules even under the adverse conditions to which they were subjected in the present study. It also explains why V. dahliae is such a difficult pathogen to eradicate under normal agronomic conditions.

CHAPTER 10

SUMMARY

1. Various aspects concerning the survival of V. dahliae in soil, in the field, green-house, and laboratory, were studied.

2. With biological control in mind, attention was paid to factors affecting antagonists of V. dahliae. Possible activation of antagonists during the period of absence of host plants was considered.

3. The literature concerning the etiology of the disease, disease control, the effect of antagonists on plant pathogens in soil, factors affecting survival, structure and germination of MS, and the problem of determining survival, was reviewed.

4. MS incubated on glass fibre disks in soil survived for more than two years. A regression line fitted to the data indicated that V. dahliae would survive in soil for 43 months. Field moisture and temperature conditions could not be related to the attrition rate of the MS.

5. In the field V. dahliae could be isolated from the rhizosphere soil from moribund cotton roots after the plants started to die from the disease or in particular after the plants had been killed by frost in April.

6. Fungi recovered from rhizosphere and root-zone soil of diseased cotton plants contained large numbers of antagonists. However, an appreciable increase in antagonistic types after Verticillium propagules had been released from the diseased plants, was not observed.

7. Numbers of bacterial and actinomycetous antagonists were

reduced by acidifying the soil, while fungal antagonists were increased.

8. Aeration and temperature changes, within the ranges tested, did not have an appreciable effect on numbers of antagonists.

9. In high pH soil with bacterial and actinomycetous antagonists prevailing, antagonists normally increased after the incorporation of Verticillium propagules, but in low pH soil which favoured the development of fungal antagonists, this tendency was less obvious.

10. The effect of fertilizers on numbers of antagonists in high and low pH soil, varied considerably. Increases in antagonist numbers after amendment with fertilizers, were more evident in high pH soil than in low pH soil. The indications were that P, added alone or in combination with other fertilizers, was mainly responsible for the significant increases.

11. Survival of V. dahliae in high and low pH soils over a period of 32 weeks, was determined by direct plating on ESA. In high pH soil attrition rate was very fast during the first four weeks. Thereafter the attrition rate slowed down and after 32 weeks c. 600 MS/g soil still survived.

12. In acidified soil the attrition rate was much faster than in alkaline soil and no MS could be recovered by plating on ESA after four weeks of incubation.

13. During the same period, numbers of antagonists increased initially in high pH soil, and then declined. In low pH soil antagonists decreased initially, followed by an increase

after 16 weeks and a further decline up to 32 weeks.

14. Neither gypsum (26 000 kg/ha) nor sulphur (5 500 kg/ha) had an appreciable effect on either the survival of V. dahliae or the numbers of antagonists in the soil after an incubation period of two months.

15. The possible implication that antagonists might be involved in the attrition of V. dahliae in soil was discussed and the conclusion was reached that bacterial and actinomycetous antagonist numbers, prevailing in high pH soil, could not be related to the attrition of V. dahliae. In low pH soil where fungal antagonists prevailed, increased activity and numbers of antagonists could possibly be related to the attrition of the pathogen. However, circumstantial evidence indicated that antagonists were not the most important cause of the attrition of V. dahliae in acidified soil.

16. The flotation technique was used in conjunction with plating on VIA to determine if acidification of the soil would have an effect on the survival of V. dahliae embedded in ground cotton stalks and to test the effectiveness of the isolation technique.

17. After 3 months c. 85% more of the MS which were embedded in cotton residues, survived in high pH soil than in low pH soil and c. 77% of the MS could be recovered by the flotation technique.

18. The survival of V. dahliae, as determined by various techniques, was detrimentally affected by acid soils, whether it was naturally acid soil or artificially created by the

addition of aluminium sulphate, sulphur or sulphuric acid.

19. An experiment with PCNB incorporated into acidified soil showed that PCNB-sensitive fungi such as Trichoderma spp. were not responsible for the decline of V. dahliae in low pH soil.

20. It was established that low soil pH itself, and not the Al ion, which might have had a toxic effect on the pathogen, was responsible for the decline in viability of MS in acidified soil.

21. V. dahliae did not tolerate concentrations of urea at 0,25% (m/m) or higher.

22. Varying the moisture content of the soil did not appreciably reduce the population of V. dahliae in soil. However, when soil was kept at constant moisture levels for 3 months, it was found that the attrition rate of MS was the in air-dried and flooded soils.

23. Fertilization with N and P caused a significant decrease in Verticillium populations in air-dried soil but not in soils in which the moisture was varied or kept constant at 20%.

24. Organic soil amendments (1% m/m) had varied effects on the survival of V. dahliae in soil. Maize, followed by soyabean-pod residues were the most effective in reducing the population of V. dahliae.

25. During the investigation it became clear that the techniques as well as the media used for the recovery of V. dahliae from soil, affected the apparent numbers of the pathogen which could be recovered.

26. Storage at 4°C for 6 weeks before numbers of viable propagules were determined, caused an increase in the numbers recovered, indicating that dormancy may be involved.

27. Germination of MS in high and low pH soils with MS enclosed in "Nuclepore" membrane packets, was studied. In both soils germination of MS increased from 1,5 to 7 days, but thereafter decreased. In high pH soil 34, 62, 59, and 12% MS were germinated after 1,5, 7, 15, and 30 days, respectively. In low pH soil initial germination was lower than in high pH soil, but it became evident from plating of MS on isolation media that the MS became colonized by volunteer fungi after 7 days of incubation. Thus it can not be stated with certainty that the "germ tubes" observed on MS from low pH soil after this period, were those of V. dahliae.

28. Electron photomicrographs are presented which show the internal structure as well as lysis of MS by bacteria and fungi. Young MS cells have a nucleus and various organelles. In older cells nuclei were rarely seen, but other organelles were still evident. After one year in pure culture, vacuoles were large and the cytoplasm was compressed to the cell wall. Organelles were indistinguishable.

29. Unknown particles were sometimes observed within cells of the MS. Some of these structures appeared to resemble virus particles.

30. What appeared to be secondary MS cells which had formed within primary cells, were observed, and the possibility that this was a mechanism of survival, was considered.

31. Cell-wall thickness varied from cell to cell and individual cells also varied in shape and size.

32. In both high and low pH soils MS became colonized by bacteria and fungi which were found attached to cell walls, within apparently lysed walls, and within the cell lumina of apparently dead MS cells. Indications that enzymes might be involved in the deterioration of cell walls in soil, have been found.

33. Melanin granules situated between individual MS cells appeared to deteriorate after the MS had been buried in the soil for three months.

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ABSTRACT

STUDIES ON THE SURVIVAL OF VERTICILLIUM DAHLIAE IN SOIL

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Various aspects concerning the survival of Verticillium dahliae Kleb. in soil, in the field, green-house, and laboratory were studied. With biological control in mind, attention was paid to factors affecting antagonists of V. dahliae and to colonization of microsclerotia (MS) in soil. Possible activation of antagonists during the period of absence of host plants was considered.

Regression analysis of survival data indicated that individual MS are capable of surviving up to 43 months in soil in the absence of host plants. Soil moisture and temperature could not be related to the attrition of the pathogen. However, microbial colonization of the MS could have had an effect.

Pathogen propagules were released into rhizosphere soil after the plants had been killed. Antagonistic fungi did not appreciably increase in the rhizosphere soil after the release of pathogen propagules.

Bacteria and actinomycetes antagonistic to V. dahliae could be stimulated to increase in high pH soil by the addition of MS and fertilizers containing phosphate. In low pH soil, which favoured fungal antagonists, these tendencies were less obvious. The largest numbers of bacterial and actinomycetous antagonists occurred in high pH soil in which V. dahliae survived best.

The attrition rate of MS was fastest in low pH (c. pH 4,5) soil. However, it was established that fungal antagonists were not mainly responsible for the attrition.

Active microbial invasion of MS in soil was established by electron microscopic studies. Apparently lysed cell walls and eroded areas in the immediate vicinity of bacteria indicated that enzymes may be involved in the deterioration of the cell walls. It is concluded that antagonists are capable of actively destroying MS in soil, but this probably is a much slower process than that which was observed in acidified soil.

Various techniques were used to establish the fact that attrition was much faster in acidified than in alkaline soil. The use of several techniques demonstrated that the effect of low pH on the attrition of the pathogen was real and not a reflection of the inadequacy of a single technique. It was also evident that the effect of low pH was fungitoxic and not fungistatic to the pathogen.

Experiments to test the validity of the claim that the Al-ion is toxic to V. dahliae at very low concentrations indicated that the attrition of V. dahliae was as fast in low pH soil devoid of aluminium salts as in aluminium-amended soil.

Soil acidification may be considered as a control measure. However, practical and economic considerations will prohibit its implementation. Apart from the cost factor, most plants do not tolerate such a low soil pH. Liming of the soil would be necessary, with the result that favourable conditions are again created for renewed increase in pathogen numbers.

In a green-house study it was found that varying the moisture content of the soil and incorporating N and P, were ineffective as measures to reduce Verticillium populations. However, in flooded and air-dried soils, significant decreases occurred. The addition of urea at 0,25% or higher to the soil, reduced Verticillium populations appreciably. Various organic soil amendments gave diverse results. Maize residues, followed by soyabean pods caused the fastest attrition of MS in the soil.

The addition of urea to soil at high rates would not be practical as a control measure. In situations where cotton can be produced in rotation with paddy rice, flooding may be of practical use. Air-drying of the soil will depend on weather conditions, but it may be possible to devise agronomic practices to speed up the drying-out process and thereby reduce pathogen populations. This finding should, however, be studied under field conditions to verify the results obtained in the laboratory.

The reduction of MS in the soil after amendment with plant residues holds promise as a control measure. Various crops could effectively be used in rotation with cotton and when the residues are incorporated into the soil, a significant attrition rate could be expected. However, the large quantity (1%) of residues required to effectively reduce the population of V. dahliae suggests that attrition would not be as fast under field conditions as it was under laboratory conditions. A long term rotation might thus be necessary.

Electron microscopic studies on the fine structure of the MS indicated that they are composed of numerous thin- and thick-walled cells. The cell walls varied in thickness and were impregnated with melanin which also occurred in the matrix between the individual cells. These properties confer resistance to attrition to the MS. In the soil they apparently germinate over extended periods and give rise to limited hyphal growth which exhausts the reserves of the less resistant cells. However, some of the more resistant cells may remain dormant and retain the viability of the MS. This may explain the survival of some propagules even under the adverse conditions to which they were subjected in the present study. It also explains why V. dahliae is such a difficult pathogen to eradicate under normal agronomic conditions.
