

VERTICILLIUM WILT OF RASPBERRIES

by

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CHAPTER I

INTRODUCTION

In the past ten years acreage of bramble fruits throughout Michigan, especially in the southwestern section of the state, has steadily increased. A corresponding increase in bramble plant losses, in part due to a vascular wilt, has also been noted. Isolations indicated that this vascular wilt disease was frequently associated with the fungus Verticillium albo-atrum Rke. and Bert. (43).

The writer in his investigations has found two factors mainly responsible for these losses. The first factor is the high degree of susceptibility of the commercial raspberry varieties now being grown in Michigan. The other factor is that on all of the farms visited in this investigation there was a history of crop rotations using potatoes, tomatoes, or eggplants at some time prior to the planting of raspberries. These crops are well noted for increasing the Verticillium content of lesser contaminated soils.

It appeared desirable to examine existing literature on Verticillium wilt and conduct as many experiments as possible that would lead to verification or refutation of existing theories about the wilting mechanism, infection, and classification of the causal organism. From this extensive study it was hoped that an accurate description of the symptoms of Verticillium wilt of red and black raspberries could be interpreted for growers, county agents, and state inspectors. Finally, additional information on possible measures for the control of this disease was desired.

CHAPTER II

REVIEW OF LITERATURE

The earliest recorded plant species associated with a wilt disease caused by Verticillium is the potato (Solanum tuberosum). In 1879, two German investigators, Reinke and Berthold (43), isolated a fungus from a potato plant affected with a disease called "Krauselkrankheit". On the basis of Hoffman's (26) findings, they named the causal organism Verticillium albo-atrum.

It was not until 1904 that a case of hadromycosis "(form of disease in which the parasite is confined almost exclusively to the xylem hadrome)" was reported in ginseng (Panax quinquefolium) by Van Hook (55). However, Van Hook reported the causal organism as Acrostalagmus albus which is actually synonymous with the older genus Verticillium (38). In the same year Verticillium was first recorded in North America. Lawrence (31), in the Puget Sound area of western Washington, described this fungus as the causal organism of "black raspberry (Rubus occidentalis) bluestem". However, he gave the name Acrostalagmus caulophagus to the causal organism. This is the earliest record of Verticillium attacking any species of the genus Rubus. Lawrence briefly described the symptoms of the disease on the Cumberland variety and directed most of his attention to the study of the causal organism.

In 1918, Carpenter (9) referred the bluestem organism to the genus Verticillium with the implication that it is identical with, or at least closely related to, Verticillium albo-atrum. In 1923, Hockey (24) isolated the organism from red raspberry in the Niagara district of Ontario, Canada.

In his report he supported Carpenter's views, saying, "The organism differs very little from and probably is identical with Verticillium albo-atrum Reinke and Berthold." In the same year the disease was reported in California (8 and 27), although it had been observed at an earlier date.

In 1924, Verticillium was reported on red raspberry (Rubus idaeus) in England by Harris (23). He called the disease "blue stripe" for it was a better term than "bluestem" (31) due to the fact that most commercial varieties of the red raspberry have a general bluish coloration normally on the new canes near the end of the growing season. This maturing coloration is also present on the new canes of black raspberries. The term "blue stripe" has not been adopted by plant pathologists in North America. They reject the term because black raspberries can be infected with a virus that causes a disease, "eastern bluestem" (58), that shows occasional symptoms of pencil-like blue streaks on the new canes. The disease caused by Verticillium has therefore been called "western bluestem" to differentiate between the two.

The term, "bluestem", is sometimes confusing to plant pathologists because they are not always sure which disease is referred to. Because the organism Verticillium causes deep blue stripe or band symptoms on the canes and because one of the striking characteristics of this disease is a yellowing and wilting of the leaves, the general term "blue stripe wilt" disease seems justified. An incorporation of all the terms or part is offered. These terms leave much to be desired since raspberry plants may also wilt from many causes, either physiological, fungus, or of insect origin. The

presence of discoloration on the canes is not an infallible proof of the absence of the disease, throughout the remainder of this thesis referred to as "wilt" for brevity.

In 1926, Berkely and Jackson (3) reported a new species of the organism, which they called Verticillium ovatum, parasitic on five North American red raspberry varieties (Rubus strigosus) growing in New York and the Niagara-Ontario district of Canada.

In 1931, Rudolph (46) published a monograph on Verticillium albo-atrum. His studies on Rubus included methods of inoculation and the host range of Verticillium isolated from raspberry.

In 1936, Zeller (65) presented data of eight years research on Verticillium wilt of cane fruits including symptoms, varietal resistance, field spread, and crop rotation studies.

Recent investigations on the Verticillium wilt problem in Rubus have been carried on by Wilhelm (60, 61, and 62). His studies included the vertical distribution of Verticillium in soils, varietal resistance studies on both raspberries and blackberries, and types of crops in relation to the infestation of land with the wilt Verticillium.

CHAPTER III

THE CAUSAL ORGANISM

The genus Verticillium was created by Nees von Esenbeck (38) in 1816. In the year 1838, Corda (11) described the genus Acrostalagmus to accommodate an organism Acrostalagmus cinnabarinus. It was described as an organism that differed from Verticillium by forming its conidia in heads at the tips of the conidiophores. Hoffman (26), in 1854, advanced evidence that there was no actual difference existing in the manner in which the conidia are united in heads.

Hoffman noted that the conidia are held together by a slime which absorbs water in a moist atmosphere, forming a globule of water at the tip of each sterigma. Within this globule of water the conidia appear to float about. If the humidity of the environment is in excess of the maximum for the moisture drops to retain there globular form they will soon collapse, leaving in most cases one conidium on the sterigma tip, as described for the genus Verticillium. This finding indicates that there is no basis for a distinction between the genera Verticillium and Acrostalagmus.

Verticillium albo-atrum Rke. and Bert. has been classified by Engler and Prantl (16) in the section Eu-Verticillium of the genus Verticillium of the Mucedinaceae - Hyalosporae - Verticillieae. The conidiophores are verticillately branched; conidia are formed at the tips of all branches and fall off readily. The distinction between the three sections of the genus Eu-Verticillium Sacc., Oncocladium Wallr., and Gliocephalum Sacc., has not been sharply drawn. In the latter section the conidia are held

together by a hygroscopic slime, while in the former two sections they are not. Therefore, in following Engler and Prantl's classification, if the characters are to be determined in their natural environment then Verticillium albo-atrum would be placed in the section Gliocephalum Sacc..

If, however, they are examined in water mounts, rarely more than one conidium would be found on each sterigma tip, and the fungus would therefore be erroneously placed with the section Eu-Verticillium.

Carpenter (9), Klebahn (29), and Van der Meer (56) support the view that Saccardo in 1886 (47) had set aside the section of the genus Verticillium - Gliocephalum for forms where the conidia are held together in heads.

In this investigation on the morphology of the causal organism both types of conidia formations on the sterigma tips were noted. Also some of the original isolates made from raspberry began to form resting structures. Possibly similar structures were noted by Reinke and Berthold in their original study of Verticillium. They noted that a blackening of certain cultures was due to blackened, septate, somewhat swollen hyphae which they named "Dauermycelium". From these observations it seemed advisable to concur in the name of Verticillium albo-atrum Rke. and Bert..

In 1913, Klebahn (29) isolated a Verticillium from diseased dahlias and found it to be different from the species of Verticillium isolated by Reinke and Berthold. The isolate of Klebahn's produced microsclerotia while that of Reinke and Berthold formed resting mycelium. It was named by the finder Verticillium dahliae Klebahn. Since the classification of

this possibly new species there has been a controversial taxonomic treatment of the two vascular Verticillia.

Carpenter (9), Rudolph (46) in their publications have stated that the original description by Reinke and Berthold was meant to include both types of resting structures. This is substantiated by the fact that Reinke and Berthold illustrated microsclerotium-like forms in their original plates. Rudolph (46) and Nelson (39) consider the presence or absence of microsclerotia an unreliable character and relate that the two forms should be classified as Verticillium albo-atrum with the addition of a varietal specificity if indicated. The evidence of existing strains of Verticillium differing in their pathogenicity has been reported by Nelson on peppermint and by Snyder et al. (51) on different varieties of vegetable crops.

Van der Meer (56), Berkeley, Madden, and Willison (4), and Ludbrook (33) support the views of Klebahn and classify the microsclerotial producing forms as Verticillium dahliae Kleb..

Berkeley et al. (4) made a cultural and taxonomic study of the many strains of Verticillia and from these studies divided them into two groups: (1) Verticillium albo-atrum group which produces the resting mycelium form, but loses the power of producing this resting stage under continued culturing on artificial media; (2) Verticillium dahliae group which produces the microsclerotial type of resting structure even under conditions of continued culturing on artificial media. However, the writer's observations concur with Nelson's (39) that either one or both types may be present in the same culture.

Such fungi as Verticillia are composed of complex mixtures of established biotypes and repeated isolations from this complex will result simply in separating from the mass either single biotypes or new combinations of them. Presley (41) reported saltants from monosporic cultures of Verticillium albo-atrum. By repeated transfers from a monoonidial culture the writer has been able to isolate similarly four main biotypes of the Verticillium fungus, which are:

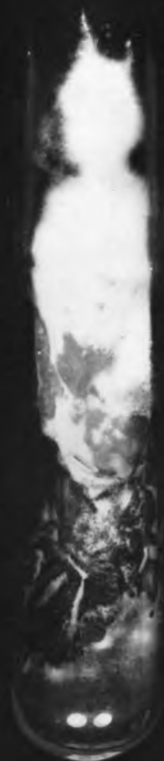
1. Microsclerotial dominant, slight mycelium
2. Fluffy aerial mycelium
3. Appressed mycelium
4. Microsclerotial, abundant aerial mycelium

Further transferring from biotype-4 soon sectors this isolate into any one of the other three biotypes. The biotypes-1, 2, and 3 have remained constant in transferring over a ten month period. Throughout this study these three constant biotypes will be referred to as S1, S2, and S3. (Figure 1).

Prolonged cultivation in the laboratory induces in many species of the fungi imperfecti an abrupt change in type of growth. This has been reported by many investigators on the genus Verticillium (21, 40, 41, and 46). Hansen and Smith (21) suggested that the basic unit of the individual is the nucleus and not the cell. Therefore, a multinucleate conidia as has been noted in Verticillium (21) is not an individual but a colony, and it will not give rise to a genetically pure culture unless all of its nuclei are genetically identical. From this it may be hypothesized that the

FIGURE 1

Biotypes S1, S2, and S3 of Verticillium albo-atrum



S-1

S-2

S-3

variable forms of Verticillium may owe their instability not to mutation but to nuclear heterogeneity (heterocaryosis).

It is widely known that the classification of Verticillia cannot be established on the basis of gross cultural characteristics on standard media. Since gross morphological characteristics cannot be used as a means of separation among Verticillium isolates from different species of plants, another means of classification must be found. The host-pathogen relationship can be used to distinguish between species as is found in the classification of the vascular Fusaria group (51). This would be of more value in the case of identification of this pathogenic fungus since no pronounced cultural or physiological differences have been reported by other investigators or noted in this study. The Verticillium pathogen isolated from raspberry in this study has a limited host range confined to the genus Rubus and a few species of the Solanaceous family.

Another factor that could be used to further classify this fungus pathogen is the host - temperature relationship. Bennett (2) and Zeller (65) have reported that generally the most severe outbreaks of the wilt disease on raspberries occur in cool wet seasons and are correlated with the minimum temperatures and the number of days of below freezing temperatures occurring in any particular winter. This is at variance with the results obtained in this study for both laboratory and field data indicate that the most severe symptoms of Verticillium wilt on raspberry, induced by inoculations with the writer's Verticillium isolate, occurred during periods of high mean daily temperatures. Therefore, it is possible that

the isolate used in this study differs in temperature requirements from those used by Bennett and Zeller and thus temperature differences may possibly be used in the classification in the Verticillia.

Since the morphological observations in this study concur with those of Reinke and Berthold and the isolates were not specific to certain plant species as reported in Verticillium dahliae Kleb., it seems advisable to name the causal organism in this study Verticillium albo-atrum Pke. and Bert. (43).

CHAPTER IV

SYMPTOMS OF THE DISEASE

- A. In Black Raspberries: The following symptoms are described from the Cumberland variety but the symptoms on the other varieties of black raspberry were generally similar.

1. General Symptoms

The earliest signs of this disease on the black raspberry variety Cumberland are noticed on the current year's growth in late June or early July. The first symptoms seen are a dull green cast of the lower leaves when compared with the bright normal green color found on leaves of healthy shoots.

The entire stool may be affected, but more often the disease is confined to a few of the young shoots. Then too, this disease works rather slowly and usually from one to three years are required to kill a plant. The disease progresses upward contrary to the observations of Czarnecki (13) and Lawrence (31) who reported that defoliation took place from the top of the plant downward. The symptoms of the disease are yellowing of the first leaves and stunting of growth caused by the shortening of the internodes, and finally the entire cane becomes wilted and the affected shoots usually develop a dark blue color, entirely or limited to stripes.

In the advanced stages of wilt the vascular tissue is discolored and has a water-soaked appearance. There is a definite line of demarcation between the vascular tissue and pith, the former is brownish in color and the latter usually a white normal color. In severe cases the pith may be slightly brownish in color.

2. Leaf Symptoms

The amount of leaves which will show symptoms will depend entirely upon the amount of root infection. If the entire root system is infected the whole plant shows visible wilt symptoms, (Figure 2). However, if only part of the root system is infected only that side of the plant above the crown in line with the infected roots will show visible signs of infection in the leaves. Generally, the lower leaves are the first to show signs of the disease. The uppersurface of the leaves develops an "off-green" or yellowish bronze cast which may be manifested by yellow and green, or brown and green stripes. This striped effect is separated by the prominent lateral veins of the leaflets and is generally confined to one-half of the leaflet. A whole leaf consists of a terminal leaflet and one to two pairs of lateral leaflets.

The pattern on the black raspberry leaf extends from the midrib of the leaflet to its margin in a diagonal fashion following the lateral veins. The striping may be confined to one-half of the leaflet; but it may and does affect other leaflets of the compound leaf. Coinciding with the striped pattern symptom, the leaflets occasionally tend to curl upward (noted in both black and red raspberry varieties) thus exposing the silvery underside of the leaflet, (Figure 3).

A compound leaf may have one-half of the terminal leaflet and the lateral leaflet on the corresponding side affected with this striped pattern while the other half appears normal. There are many

FIGURE 2

Wilt symptoms in Cumberland raspberry resulting from
severe root infection following inoculation with
Verticillium albo-atrum, biotype Sl.



FIGURE 3

Upward curling of leaf margins in Indian Summer red
raspberry following inoculation with Verticillium
albo-atrum, biotype S2.



possible combinations of the striped pattern effect. This observation corroborates the hypothesis of others that the effects of Verticillium disease are concerned with the vascular system.

Following the beginning "off-green" color there is generally a marginal chlorosis, the leaflets turning from a yellow-green to a chocolate brown. This type of tissue discoloration may be erratically distributed throughout a single leaflet or envelope the entire compound leaf. The progressive pattern of necrotic areas is generally similar in all cases, beginning with a marginal and tip chlorosis of the leaflets soon followed by complete necrosis of the affected leaflet. It was also noted that when marginal necrosis occurred the entire leaf had a tendency to curl downward and resist pressing into a flat normal plane of position. Nelson (39) in his studies of Verticillium on mint reported similar curling of the leaves.

Leaflets additionally infected with one of the leaf spot fungi (Septoria rubi) or (Gleosporium veneta) tend to wither and die with more rapidity than noninfected leaflets. The lesions tend to act as focal points for the spread of the necrosis.

The petioles may or may not show the blue discoloration characteristic of an affected cane. However, a pinching of the base of the petioles prior to actual leaf symptoms has been observed frequently.

3. Primo Cane (current year's growth) Symptoms

A discoloration of the primo cane will occur generally at the same time or earlier than the leaf symptoms. This cane symptom may

begin at the groundline if only one or more laterals of the secondary roots are infected. The symptom may begin several inches above the groundline if the infection is confined to the small secondary roots which are produced directly from the main crown of the plant. If this latter type of infection takes place the entire crown may be destroyed in one growing season. If the causal organism does not attack the secondary lateral roots the crown is not totally destroyed and will produce small shoots from the adventitious buds. These small shoots will generally become infected before the growing season is over and they may defoliate from the base up or die back from the tip.

The intensely blue to purple discoloration extends in varying heights at variable rates in the black raspberry primo canes. These rates which vary from zero to nine inches per day, according to Lawrence (31), depend upon the environmental factors of temperature and moisture. A high mean temperature and low soil moisture content generally are conducive to higher daily spread of cane discoloration. The rate of climb is much slower in the fruiting wood than in the primo canes and this may be correlated with the lower transpiration rate in the former. The discoloration generally forms a longitudinal stripe that touches every node along that side of the cane contiguous with infected roots. Occasionally the blue stripe symptom advances into the petioles in varying degrees. The discolored stripe gradually narrows and terminates to a point.

The bluish discoloration noted on the primo canes is not due to a pigment produced by the fungus or the host but is the result of the brownish-blue discoloration of the diseased bark as seen through the waxy bloom covering the cane. When the bloom is rubbed off, the purple color disappears (31, 46, and writer's observations).

The boundary between the normal and discolored portions, while rather irregular, is quite distinct. Discoloration in the cane may extend all around the cane. When two inches of a cane becomes encircled, the entire cane above the encirclement will wilt and die.

In the fall it is necessary to distinguish between the blue stripe symptom and the maturing color of the cane. As previously noted, blue discoloration appears on the cane near the groundline and is continuous in its pattern upwards, whereas change of color due to maturity often takes place irregularly in patches. This also holds true for red raspberry varieties.

Generally, the infected growth of the current season often appears healthy if the season has been moderate. However, the following spring one finds most of these canes dead at the tips and for varying lengths down the cane and to the base. New growth is usually noted at the base of these dead canes and the shoots appear healthy, though dwarfed or stunted.

4. Fruiting Cane Symptoms

The effects of the disease are finally climaxed in the fruiting canes. In the spring, the diseased canes are conspicuous. Some canes

are dead, others have shrivelled and poorly developed buds. On some canes the dead buds lie in a longitudinal line. On sectioning such a cane, a strip of dead wood was found beneath the dead line of buds while the opposite side of the cane was green. The buds on the green side develop normally at first but later there is a stunting in both lateral shoot growth and leaves.

The wilting symptoms vary in their time of appearance on the fruiting cane. In some experimental plots the symptoms did not appear until the fruit was almost mature. On these canes the leaves become prematurely discolored and die followed by a drying up of the fruit and death of the cane. In other cases as soon as the first leaves had unfolded they became yellow and withered up with no production of flower buds.

Wilhelm (60) observed that brambles are most seriously affected in the first and second fruiting years. If the fruiting canes are not dead before maturity they will usually yield small tasteless fruit. In some cases, the berries, when partially developed in size, become perfectly dry except for the placentae which contains considerable moisture but is woody in texture.

- B. In Red Raspberries: The following symptoms are described from the Latham variety but the symptoms on the other varieties of red raspberry were generally similar and in some cases were more severe (St. Regis and Indian Summer varieties).

1. General Symptoms

The red raspberry varieties have many symptoms of Verticillium wilt that are identical with those symptoms found on the black raspberry varieties. The initial symptom is generally noticed on the current year's growth in the later part of July or early August. The lower leaves begin to change from a rich green to a light green color with a yellowish margin. Within a period of fourteen days the leaves begin to fall off. A distinguishing characteristic is that the terminal tuft of leaves are invariably the last to be affected. These terminal leaves often survive the rest of the leaves for a considerable period of time.

The primo cane discoloration found quite prominent in black raspberries is often absent on red raspberry varieties, or if present it is hard to distinguish from the normal bronze to reddish coloration. The fruiting wood generally has a deep brown to blue normal mature color that would mask any striping effect resulting from a Verticillium infection. In the red raspberry varieties a moderate infection of Verticillium may be present for years without depriving the plantation of its commercial value. This is not the case in the black raspberry varieties.

2. Leaf Symptoms

The striped pattern effect as noted on black raspberry leaves is more noticeable on red raspberry leaves because of the more prominent interveinal areas. This pattern has been well described previously.

It may be necessary occasionally to distinguish the defoliation of the Verticillium wilt disease from Spur Blight caused by Didymella applanata. In both cases the dead leaflets fall leaving the petioles attached. The petiole may be persistent for three more weeks.

In the case of the Spur Blight disease a chocolate brown to darkened blue discoloration develops around the base of the petioles on the new canes. Pycnidia of the Spur Blight fungus can be observed with the aid of a hand lens in these discolored areas. In the case of Verticillium the discoloration generally appears in stripes and no fructifications of the fungus can be observed.

The symptom of the entire compound leaf curling downward as in the raspberry virus leaf curl (2) was noted occasionally on black raspberries. However, with all the red raspberry varieties tested there was a pronounced downward curling of the terminal leaflet prior to actual color change in the leaflets. This downward terminal leaflet curling was also observed in the laboratory on tip cuttings of red raspberries placed in a filtrate of the toxin produced by the Verticillium fungus.

3. Root Symptoms

In the red raspberry varieties as in the black raspberry the infected vessels of the xylem are usually discolored. In severe cases most of the woody tissue in the roots have a pronounced brownish-red to black discoloration. The black raspberries do not reproduce by rhizomes, consequently a diseased plant is more likely to die faster

than those plants of the red raspberry varieties. In these observations the rhizomes frequently were free of the causal organism. However, as the disease spreads in the roots these disease-free rhizomes eventually were attacked. It was also observed in confirmation of reports by Harris (23) and Rudolph (46) that red raspberry varieties infected with Verticillium do not sucker as plentifully as those free from this organism.

Three red raspberry plants of the Indian Summer variety showing visual wilt symptoms were tagged in comparison with three plants that appeared normal in growth. Counts were made in the late fall of that growing season on the number of suckers produced by the parent plants. The entire plants were then carefully dug up keeping the root-sucker systems intact. Agar plantings were then made from the rhizomes of all six parent plants at the points of sucker attachment and also from the individual sucker plants. The results from this experiment are recorded in Table I.

TABLE I

Isolations from normal appearing and diseased plants and the reduction of suckers resulting from Verticillium infection

	Infected Parent Plants	Normal Parent Plant
Plants Used	3	3
Total Suckers Present	23	35
<u>Verticillia</u> isolates from parent plants	3	0
SUCKER PLANTS		
<u>Verticillia</u> isolates	14	4
Other organisms	9	31

From the foregoing table it is concluded: (1) Suckers from infected parent plants may be entirely free of the causal organism; (2) When red raspberries are infected with Verticillium the amount of suckering is reduced.

It will be noted from Table I that sucker plants from apparently healthy parents had become infected. This possibly resulted from natural infections from organisms in the soil.

This table suggests a point of interest in regards to field spread of the wilt disease organism. Sucker plants from infected parents can disseminate the fungus over the extent of their radius. The infected sucker plants growing in the aisles may be torn out of the ground by cultivating tools and dragged for some distance which may further spread the causal organism.

CHAPTER V

METHODS OF ISOLATION

A characteristic of the Verticillium fungus is that it does not develop fast on artificial media. As a result the body of the culture medium may become densely overrun with bacteria and rapidly growing contaminant fungi (Fuaria, Alternaria, Gleosporium, et cetera) that inhibit the growth of the Verticillium isolate. The following methods of isolation were tested to determine which method would prove the most efficient to obtain pure cultures of Verticillium from diseased plant tissues.

A. Preparation of Plant Tissue Specimen

Since the surface of the diseased tissue is often contaminated with secondary fungi it is desirable to kill or remove these organisms without injuring the pathogen in the inner tissues. A series of three different chemicals possessing disinfective qualities were tested for their efficiency on surface disinfection of raspberry tissue. The chemicals tested were 0.1 per cent mercuric chloride, 3 per cent sodium hypochlorite, and 1 per cent Chlorazene. (All were tested in aqueous solutions).

Tested in combination with these chemicals were two procedures for the handling of the diseased plant tissues.

1. Small sections of the plant tissue were soaked in the specific chemical for one minute. Then, using a flame-sterilized scalpel, sections of the cane were cut and transferred to poured agar plates.
2. In addition to the foregoing procedure the plant tissue was run through four series of sterile distilled water rinses - this aids

in removing the residual disinfectant and possible surface contaminants not previously killed by the chemical.

Procedure Number 2 gave the least contamination and more Verticillium isolates. Sodium hypochlorite or Chlorazene were suitable disinfectants as they were foot acting and left little toxic residue.

Many of the plant tissues used in this isolation work were thin leaflets and fibrous roots that could be penetrated by the disinfectant with the probability of killing the pathogen. Therefore, it was necessary to prepare the tissue without using a chemical disinfectant. Rinsing the plant tissues under running tap water for thirty to sixty minutes and then running the material through a series of four sterile distilled water rinses gave little contamination and proved satisfactory for isolation of the fungus.

An 80 per cent increase in pure cultures of the causal organism were noted when the tissue plantings were cut from the region on the cane bordering the diseased and normal tissue. This area is apparently free from the secondary invaders. Over 75 per cent of all isolations made from cane tissue already discolored failed to yield Verticillium and most of the isolates were fungi of the Fusaria group. The Verticillium fungus could be obtained two to three inches beyond the discolored portion. No isolations of Verticillium were obtained from plant portions higher than one foot above the crown line unless so previously inoculated.

B. Selection of the Agar Medium for Plating

Since the Verticillium fungus develops slowly a medium must be used that eliminates bacteria. Such a medium must be lacking in proteins

and have a pH around 5.0 to prevent excessive bacterial growth. The rapidly growing contaminant fungi thrive on media that are rich in carbohydrates; therefore, reduced carbohydrate formulation is desirable.

Five different types of media tested were: (1) 2 per cent malt extract, (2) corn meal, (3) lima bean, (4) potato-dextrose, and (5) prune infusion, in 2 per cent agar. In addition, a natural media consisting of Cumberland black raspberry foliage chopped into small pieces about three-eighths of an inch square and mixed in 2 per cent agar. The raspberry foliage was first sterilized in propylene oxide vapors (52).

The best type of media for isolation of the Verticillium fungus was prune agar. Prune agar is slightly acid in nature and very low in proteins, two characteristics that limit bacterial growth. The raspberry leaf media was fairly satisfactory for Verticillium isolation but proved of most value for microsclerotial production from S1 biotype. The microsclerotia were used in mass inoculations for varietal resistance studies.

C. Single-Conidial Isolation Techniques

There are many methods for single-conidial isolations. Two methods used in these Verticillium studies were as follows:

1. A modified Hansen and Smith (21) procedure: Cultures of the biotypes were maintained on 2 per cent malt extract agar slants. 10 ml. of sterile distilled water was added to a test tube culture and the tube was vigorously shaken. This water which then contained many

conidia was transferred to a clean sterile test tube. The approximate concentration of a conidial suspension was determined by direct microscopic counts of conidia carried in a 4 mm. transfer loop. Counts were made using the Howard counting cell. This conidial concentration was diluted by a series of loop transfers to sterile distilled 10 ml. water blanks. When a loop suspension contained approximately 50 to 75 conidia, as determined by count, it was transferred to a test tube containing 10 ml. of melted Coon's (42) medium made with 0.5 per cent agar. This extremely low percentage of agar made it possible to cover the bottom of three 90 mm. Petri plates with the 10 ml. of inoculated medium giving approximately 15 to 25 conidia per plate.

The thinness of the agar in this technique greatly minimized the possibility of conidia being situated directly above one another and was especially advantageous for the extremely small Verticillia conidia. Most of the conidia germinated within a twenty hour period. At the end of this period the Petri plate was placed on the stage of a compound microscope, the cover removed, and the germinating conidia picked up singly on the tip of a flattened inoculating needle and transferred to 2 per cent malt extract agar slants.

This technique was used in these studies as it gave a greater possibility of a single cell isolate.

2. The dilution blank technique: Following the conidial dilution

procedure as previously described, direct loop transfers are made to previously poured and solidified agar plates. Small rings previously drawn on the bottom outside of the plate with a wax pencil aid in placing the loop transfers on the medium and also facilitates the location of germinating conidia for isolation.

This technique has one disadvantage for single-conidial Verticillium isolations. The extremely small conidia of Verticillium may lie directly above one another without detection and there is a possibility that the resulting transfer would not be a single-conidial isolate.

The single-conidial transfer needle was made in the laboratory. An inoculating needle was heated red-hot and then hammered flat. The needle was then given a very sharp point by grinding on a whetstone. A point of such fineness must be sterilized by chemical means (1 per cent Chlorazene) as it is immediately destroyed in a flame.

CHAPTER VI

METHODS OF INOCULATION

The Verticillium fungus is soil borne (61) and can penetrate the roots directly (39). However, a variation of three plant inoculation methods were studied. The methods used are outlined below. For each series of inoculations comparable check inoculations were made using sterile distilled water. The fungus was later reisolated in pure culture from each plant to prove the actual pathogenicity of the fungus according to the principles of Koch's postulates (30).

Method Number 1

The entire root system was simply dipped into a microsclerotial or conidial suspension and the plant potted immediately.

Method Number 2

The plant was potted and inoculated after it had become established. For inoculation a six inch iron rod was pushed into the soil manually near the base of the growing raspberry plant, making three or four such holes around the plant. The pathogenic suspension was then poured into each hole and the top of the hole covered with soil.

Method Number 3

Using a flame-sterilized scalpel, a T-shaped incision was made into a cane to a depth of about one-eighth of an inch. Into this incision was placed a cotton wad previously soaked in a conidial or microsclerotial suspension of the causal organism. The treated incision was then covered with a proprietary self-sticking latex tape called Sealtex. This tape not only sticks to itself but also seals against air and water loss from

the covered area.

The relative incubation period using these three methods with three biotypes of the Verticillium fungus are presented in Table II. The shorter the incubation period the more efficient the method.

Table II indicates that the cane wound method has the shortest incubation period of the biotypes used for inoculation. The S1 biotype exhibited the highest degree of pathogenicity as indicated by the shortest incubation period. Of further interest, this biotype showed the highest toxicity in laboratory studies.

TABLE II

Relative incubation period following the use of three different biotypes and three different methods of inoculation.

Method of Inoculation	BIOTYPES USED					
	S1		S2		S3	
	Plants Used NUMBER	Incubation Period DAYS	Plants Used NUMBER	Incubation Period DAYS	Plants Used NUMBER	Incubation Period DAYS
Root Dip	10	34	4	42	4	32
Soil Punch	10	29	4	37	4	29
Cane Wound	10	24	4	33	4	26

CHAPTER VII

MODE OF INFECTION

Reinke and Berthold (43) demonstrated the ability of the Verticillium mycelium to penetrate healthy root tissue. Their technique was to simply place mycelial bits in direct contact with potato roots grown in humid chambers. However, under similar conditions they were not able to achieve infection with conidial suspensions.

Recent work of several investigators (6, 39, and 56) has independently confirmed the view upheld by Reinke and Berthold. Also, Nelson (39) has shown by histological studies that the Verticillium conidia can penetrate roots directly within a period of six hours. Other investigators (3, 9, 13, and 28) have advanced this theory on assumption only as they offer no investigational data to support their contentions.

A. Root Infection Studies

Experiment Number 1

Nelson (39) showed that Verticillium conidia were attracted to mint roots, presumably by chemical or electrical phenomena.

Following Nelson's technique, but using Cumberland black raspberry instead of mint, healthy, fibrous roots were placed in a Verticillium conidial suspension. The roots were then subjected to a series of sterile distilled water rinses and vigorously shaken. After this procedure, slide mounts were made and examined. Many conidia were still found to be appressed to the surface of the roots.

Experiment Number 2

An experiment was designed to determine whether isolates of

Verticillium could penetrate directly into the roots. Two Cumberland black raspberry plants were grown in sand culture to develop a more fibrous root system and to enable an easier removal of the roots from the sand with a minimum of injury.

Two inch portions of these roots were placed in pairs on thirty-two aseptically clean glass slides in Petri dish moist chambers. A series of eight slides (sixteen inoculations) per biotype was used and bits of mycelium from the three biotypes were placed upon the apparently uninjured root sections. The remaining eight slides were used as checks, the roots being subjected to small bits of sterilized cotton instead of inoculum.

Within a thirty-six hour period, brown lesions developed next to and under the inoculum in the S2 and S3 inoculations. After a sixty hour period similar lesions were noted with the S1 biotype which consisted mainly of microsclerotia. As microsclerotia are considered to be a resting stage of the fungus (23, 43, and 61), it seems reasonable and is corroborated by the data that this thick-walled structure will take a longer period of time to germinate and infect.

Isolations made from the foregoing diseased root portions readily yielded the Verticillium organism. There is little doubt that the organism penetrated for the isolations were made from surface sterilized roots, using the chlorine compounds and water rinses. Further evidence in support of this data is that no Verticillium isolates could be obtained from dilution plates made of the rinse waters. Following the final rinse,

portions of the stelar tissue were teased from the roots by aseptic techniques and plated.

The results of this experiment are recorded in Table III.

TABLE III

BIOTYPES	Roots Inoculated NUMBER	Roots Containing <u>Verticillium</u> NUMBER
S1	16	10
S2	16	14
S3	16	13
Check	16	0

The above table indicates that all three biotypes are capable of penetrating root tissue. The lower amount infected in the S1 biotype possibly results from the longer period required for the germination of microsclerotia.

Experiment Number 3

Two inch sections of healthy fibrous black raspberry roots, obtained by the same procedure given in Experiment Number 1, were subjected to the following test. Using a flame-sterilized forceps, a section of the root was picked up by its basal cut-end, the terminal unwounded end then being dipped into a conidial suspension of Verticillium to a one-half inch depth. The inoculated root was then placed on an aseptically clean glass slide in a Petri dish moist chamber. Other roots were similarly treated using eight roots per biotype. A check consisted of eight roots

that had been dipped into sterile distilled water.

After a two day period necrotic specks were noted on the treated portions of all roots, while the check roots appeared normal. On the fourth day isolations were made and the Verticillium was obtained in pure culture. This experimental evidence substantiates the results obtained in the foregoing experiment. No table is presented for all roots dipped into a conidial suspension yielded Verticillium upon isolation.

Experiment Number 4

In 1922, Bewley (6) grew tomato seedlings in a nutrient agar and when the roots were well developed he added a conidial infusion of Verticillium. The roots were readily attacked and the plants began to wilt in nine days. Van der Meer (56) conducted a similar experiment using cucumber seedlings and potato tubers. He concluded with the statement, "the roots were attacked either directly through the epidermal cells or through the root hairs."

Using tomato seedlings for the test plants a modified technique from that given above was adopted. Aseptically clean Bonny Best tomato seeds were germinated under aseptic conditions in sterile Petri dish moist chambers. After thirty-two of the young seedlings had developed their primary organs they were transferred singly to large sterile test tubes that contained a 15 ml. conidial suspension of the Verticillium fungus. Eight seedlings per biotype were used. The remaining eight seedlings used as checks were placed in sterile test tubes containing

15 ml. of sterile distilled water.

The roots were apparently attacked by the organism, evident by the appearance of many small brown spots scattered over the roots submerged in the conidial suspensions. The wilt symptoms, as noted by Bewley (6) in nine days did not appear until the thirteenth day in this study.

Isolations from the affected root systems of the seedlings yielded the Verticillium organism upon plating. Following is the data obtained from this experiment on tomato seedlings.

TABLE IV

Isolations of Verticillium following inoculation
of unwounded tomato seedling roots

BIOTYPE	Seedlings Used NUMBER	Yielding <u>Verticillium</u> in Culture NUMBER
S1	8	4
S2	8	6
S3	8	3
Check	8	0

This experiment shows that the raspberry isolate of Verticillium is not only capable of root penetration but is also pathogenic to tomato (Solanum lycopersici).

Experiment Number 5

A few investigators (13, 23, and 46) have reported actual field observations on the presence of microsclerotia on the epidermis of

infected cane tissue. Isolation studies were not included in their investigational data to justify this field observation. Then too, their observations may have been confused with the fruiting bodies of Coniothyrium sp. and Didymella applanata which are often found on diseased canes of raspberry.

Recent investigations have shown that no fructifications of the Verticillium fungus have been observed on diseased canes in the field or greenhouse (61, 62, and 65). This observation, in agreement with the writer's, then eliminates to a great extent the possibility of wind blown infective conidia. However, there is a possibility that microsclerotia may be disseminated by water or dust erosion of the soil. Microsclerotia are highly resistant to desiccation for they can withstand a constant temperature of 120 F for several months (61) and still be viable. Their average measurement is from twenty-five to seventy-five microns in diameter so they are not only small in size but capable of being wind blown. Their occurrence in surface cultivated soils substantiates the wind and water dissemination theory (61).

A series of three black and three red raspberries were grown in the greenhouse. After they had become established, the unwounded canes of the above plants were atomized with pathogenic suspensions of the three biotypes. A similar series of check plants were atomized with sterile distilled water. Vaseline barriers were used on all atomized canes to prevent the suspensions from flowing down the canes and into the soil where infection has been proven to take place.

All treated plants were placed in a moist chamber for twenty-four hours, as conidial germination studies indicated a 65 per cent germination within twenty hours.

After a forty day period no visual wilt symptoms were noted on any of the treated plants. Isolations were made from the treated areas on the canes with negative results.

Previous experimental trials described in Chapter VI show that infection will take place on the aerial portions of the plant if the organism is placed in a wound on the cane. This type of infection, if present, would generally be found near the crown line within range of the microsclerotia known to be present in surface cultivated soils.

CHAPTER VIII

MECHANISM OF WILTING

A. Nutrient Theory

In 1892, Atkinson (1) formulated the first important hypothesis on the nature of vascular wilts. He stated that infected plants wilted because the fungus utilized the available nutrients present in the tracheal fluid and thereby starved the host. Klebahn (29) took a similar view and even went as far as stating that the causal organism inhabited the vessels as a saprophyte.

B. Thrombosis Theory

1. Mycelial Thrombosis

This theory is based on a mechanical plugging of the xylem elements by mycelial wefts or strands, especially in the sieve plates and pits present in these elements. The first investigator to advance the idea that plugging of the vessels brought about a wilting symptom was Klebahn in 1913 (29). "Mycelial development in the vessels," he stated, "eventually plugged them to such an extent that translocation of water and food materials to the upper parts of the plant was rendered impossible."

Other investigators who were found to agree with the mycelial thrombosis theory are: Reinke and Berthold (43), Van Hook (55), Berkeley and Jackson (3), Pethybridge (40), and Maclean et al (34).

Arguments against this mycelial theory have been noted by Van der Meer (56), Bewley (6), Brandes (7), and others. Their investigational data pointed out that plants suffering from severe "wilt" may

have very few hyphae in their vessels. However, those plants showing only a mild form of "wilt" may have a pronounced congestion of their xylem elements.

2. Gum and Tylose Thrombosis Theory

This theory is substantiated by many investigators (15, 40, 49, 56, and others) who state that other types of occlusions, such as the formation of gums or tyloses in the xylem, are responsible for the blockage of the flow of water and food material upwards in plants so affected.

Are tylose formations a primary cause of wilting or are they secondary factors? Tyloses are of common occurrence in many of the angiosperms. The development of tyloses either normally or as a result of wounding may be due to one or combinations of the following factors: (1) difference in pressure in the cells on each side of a pit membrane; (2) reduction of pressure; (3) cessation of conduction in the vessel thus permitting the membrane to expand into the cell. To disprove this theory one needs to only observe a diseased plant in the field or greenhouse. On the affected plant the lower leaves may be dead or dying but the topmost leaves are generally turgid and show no visual "wilt" symptoms. These former symptoms are associated with a lack of moisture and one would expect the terminal leaves to be similarly affected if an actual blockage did occur in the vascular system.

Further investigational data providing strong evidence against the thrombosis theory (mycelial, gum, or tylose) was reported in 1922

by Dowson (15). By experimentation, Dowson, inactivated the conducting channels of lilac and privet for long distances without a subsequent symptom of wilting.

The writer repeated Dowson's experiment on the inactivation of conducting channels using raspberry. Wedge-shaped pieces were cut from the canes of raspberry on alternate sides. This operation was done under water so as not to break the capillary flow of water. The cut surfaces were rubbed with vaseline and the canes were then removed from the water and the wounds were covered with Sealtex tape. After a period of thirty-two days (average Verticillium incubation period) not the slightest trace of wilting occurred on the treated canes although their conducting tissues were inactivated over a long distance by mechanical plugging.

Another argument against the thrombosis theory is that the disease "wilt" is more severe during the cooler portion of the growing season (33). Bennett (2) and the writer have noted in their observations and experiments that Verticillium wilt of raspberries occurs most during the hottest period of the summer with an apparent recovery during the cooler days in autumn. Bewley (6) stated that greenhouse tomato plants wilted at high temperatures but they recovered at still higher temperatures. Using the thrombosis theory as the criterion, how would one explain why a tomato plant would require less water to retain turgidity at a higher rather than at a lower temperature?

C. Gas Formation Theory

Another theory that had been advanced by investigators is that a gas, such as carbon dioxide, is formed by the fungus and results in the formation of a gas pocket in the tracheae. Such a formation would thereby break the transpiration stream and a flaccid, wilted condition would result. In 1926, Tochizaki (54) discovered that a large amount of carbon dioxide was produced by a vascular wilt fungus, Fusarium lini.

In view of this theory a series of tests were then initiated to see if the biotypes of Verticillium isolated from raspberry would produce a gas, (Figure 4). The Durham type of fermentation tubes were used. This apparatus consists of pyrex test tubes in which are placed in an inverted position small glass vials (two inches long and one-quarter inch in diameter). If a gas is formed by saccarolytic enzyme action it will displace the nutrient broth in the vial. The amount of gas formed may be roughly estimated but the kind of gas cannot be determined by the use of the Durham fermentation tubes.

This type of apparatus possesses some advantages over the Smith fermentation tube, if only the presence of gas production is to be noted, as the tubes are easily cleaned, sterilized, and requires no special apparatus other than a test tube rack.

To overcome the possibility of the vial resting on the bottom of the test tube so firmly that it would exclude the fungus, the open end of the vial was cut off at an angle. The inoculated tubes were periodically shaken.

The media used for these test consisted of nutrient broth (3.0 gm. beef extract, 5.0 gm. peptone, 1,000 ml. distilled water) to which was added at a 1 per cent concentration the fermentable sugar.

The results of these gas production studies as recorded in Table V show that no gas was formed by any of the three biotypes of the causal organism. However, data was obtained on the production of microsclerotia and the amount of mycelial growth. This is presented in Table V.

From these studies it was concluded that no gas was formed by Verticillium and that the gas production theory is untenable for the wilting resulting from Verticillium infection.

TABLE V

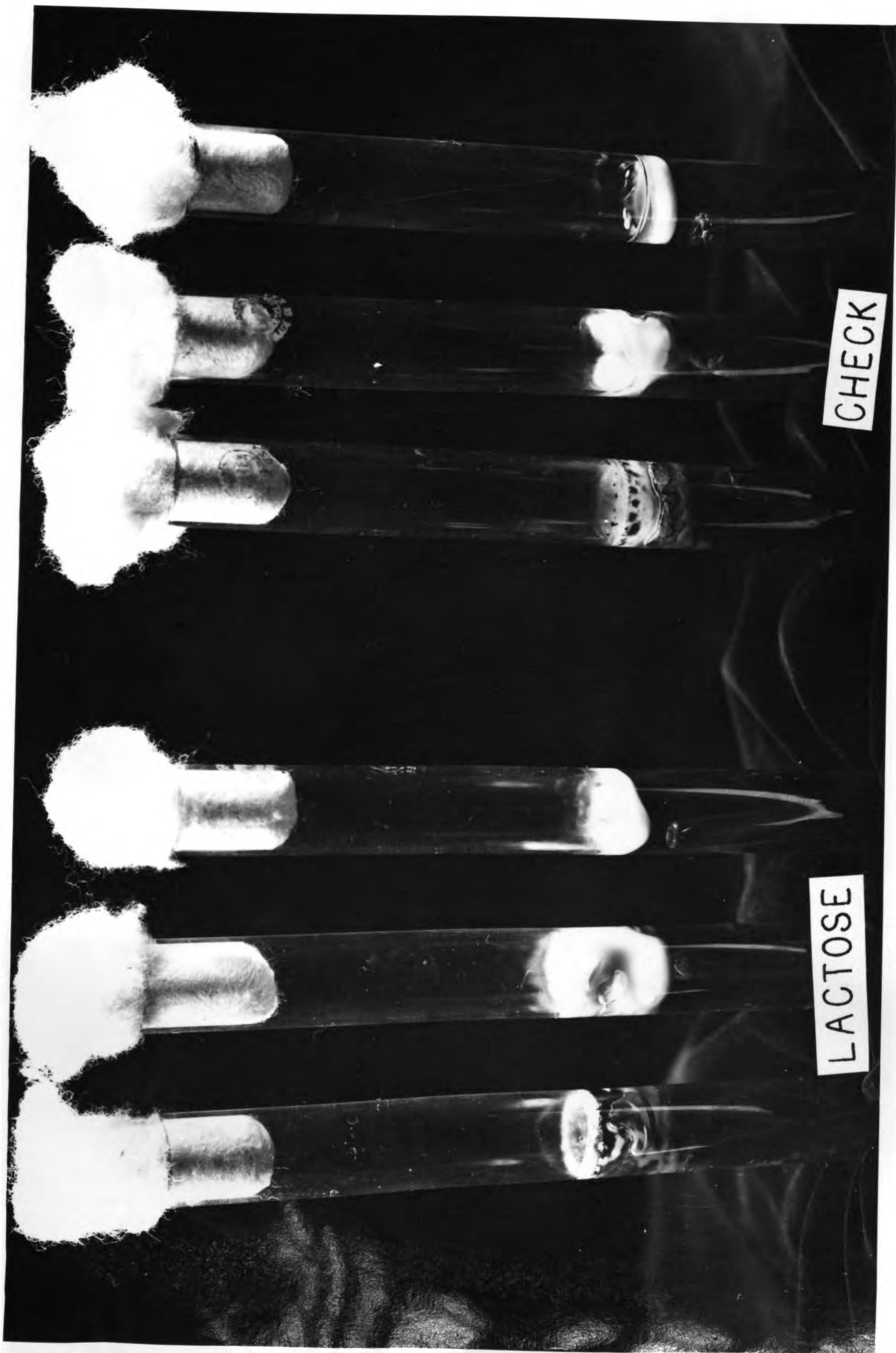
Gas production and fungus growth in media containing various standard sugars using
Biotypes S1, S2, and S3.

Fermentable Sugars	S1			S2			S3		
	Microsclerotia *RATING	Gas Production *RATING	Mycelium Growth *RATING	Microsclerotia *RATING	Gas Production *RATING	Mycelium Growth *RATING	Microsclerotia *RATING	Gas Production *RATING	Mycelium Growth *RATING
Dextrose	2	0	2	2	0	2	0	0	0
Galactose	1	0	3	3	0	2	0	0	0
Lactose	4	0	4	4	0	4	0	0	0
Levulose	5	0	5	5	0	5	0	0	0
Maltose	3	0	3	3	0	3	0	0	0
Raffinose	2	0	2	2	0	3	0	0	0
Saccharose	1	0	0	0	0	2	0	0	0
Soluable Starch	0	0	0	0	0	1	0	0	0
Sucrose	1	0	0	0	0	2	0	0	0
Xylose	0	0	3	3	0	1	0	0	0
Check (no sugar)	2	0	1	1	0	1	0	0	0

*Rating scale from 0 to 5 with the lower number indicating the least growth

FIGURE 4

Sample of gas production experiment also showing variations in growth of the different biotypes. Left to right in each series, biotypes S1, S2, and S3 respectively.



CHECK

LACTOSE

D. Toxin Theory

The toxin theory is rather generally accepted at the present time (6, 14, 19, 31, and 57). It is based on the findings that toxins are produced by vascular fungi when grown on synthetic nutrient media and that these toxins are able to wilt excised shoots.

Gottlieb (19), in recent fusarium wilt studies reported the presence of toxins in the xylem elements of wilted plants and their total absence in the fluids obtained from noninoculated turgid plants.

Wellman (57) reported differences in the toxicity of culture filtrates of mild and virulent strains of Fusarium lycopersici, increased toxin production being associated with greater virulence. This finding has also strengthened the toxin theory.

The following experiments attempted in part to repeat the work of other investigators and to test the validity of the toxin theory using only the isolates of Verticillium obtained from raspberry.

Experiment Number 1

Dowson (14) reported that a toxin of Verticillium acts on the leaf tissues in Michaelmas Daisies. He observed that prior to actual wilting the leaves took on an intense yellow coloration due to chloroplast degeneration.

In this experiment it was attempted to compare Dowson's (14) observations on daisy with the Verticillium disease of raspberry. The results were similar. Hand sectioning of affected leaves showed that the actual chloroplasts were completely absent from the mesophyll and palisade tissues

and only a yellow disorganized mass of plastic material was present in the affected cells.

Leaves from both healthy black and red raspberries were carefully scraped with a sharp scalpel. The scrapings were placed in a water mount and many intact leaf cells with brilliant green chloroplasts were present. A portion of a filtrate from the liquid media in which the Verticillium fungus had been growing was pipetted and dropped onto the water mount containing the leaf tissue.

Constant checking under the microscope indicated that the chloroplasts were affected first. They collected at the cell walls, lost their green color, and soon disintegrated to a yellow mass of unorganized material. The cell walls did not appear to be ruptured or otherwise affected.

Experiment Number 2

Wellman (57) and Wolf (63) reported from their studies with Fusarium isolates that a growth period, on synthetic media, of at least one month is required before the filtrate attains its maximum toxicity.

A similar study was conducted with the virulent strains of the three Verticillium biotypes. All isolates of biotypes S1 and S3 were found to be pathogenic to raspberry. This virulence was determined by actual plant inoculation, wilt symptoms, and reisolation from the infected plant. Finding a virulent strain of biotype S2 proved to be difficult for many isolates of this biotype failed to infect raspberry.

One strain was finally isolated that proved pathogenic. It was interesting to note that this isolate had a high aerial mycelial growth in contrast to the relatively small aerial mycelial growth of the other S2 isolates. Cultures with slight or low aerial mycelial growth proved non-pathogenic to raspberry.

The virulent strains of the three biotypes were cultured in 100 ml. of Coon's synthetic media (42) contained in 250 ml. Erlenmeyer flasks for varying periods, namely, 5, 10, 15, 20, 30, 45, and 90 days. At the end of a specified time the mycelial accumulation was removed by filtration using four layers of cheese cloth and one layer of filter paper as the complete filter pad. Loop transfers from the filtrate, examined under the microscope, were completely free of conidia and mycelium.

Ten-inch shoots of similar age and cane diameter, excised from Cumberland black raspberry, were placed into the filtrates. The following table indicates the time required for maximum toxin production using the three biotypes of Verticillium.

TABLE VI

The time required for maximum toxin content is indicated by the rating scale - the higher the rating number the greater the toxin activity present in the filtrate.

Toxin Production (*) at Progressive Intervals

DAYS	5	10	15	20	30	45	90
Biotypes							
S1	0	0	1	2	4	3.5	2
S2	0	0	0	1	2	2.3	1
S3	0	1	1	2	3	3.5	2

*Rating Scale for 48 hours' observation:

- 0 - no wilting in 48 hours
- 1 - slight wilt in 36 hours
- 2 - slight wilt in 12 hours
- 3 - complete wilt in 4 hours
- 4 - complete wilt in 2 hours

The above table shows that approximately 30 days in culture are required for a significantly demonstrated toxin content in the filtrate with some increase up to 45 days and a decrease after 90 days.

Experiment Number 3

Nelson (39) reported that excised shoots of mint when placed in toxic filtrates wilted faster in the light than when exposed to darkness. Also, filtrates prepared from cultures grown in darkness were impotent to cause wilt in contrast to filtrates from cultures grown in exposed light which cause severe wilt.

The foregoing report prompted an investigation to determine the influence of light on the wilting phenomenon using the raspberry Verticillium isolates.

A series of cultures from the three biotypes, S1, S2, and S3 grown in Coon's synthetic solution, were exposed to continuous light. Another series was cultured in amber flasks in complete darkness. The mycelial accumulation was removed at the end of a thirty day period (57 and 63) and saved separate from the filtrate.

All filtrates were diluted to twice their original bulk to offset the effects of exosmosis which may interfere with the results when the filtrates are used at their original concentrations. Checks, consisting of sterile synthetic nutrient solution with excised shoots placed in them, were included in all of these experiments.

Ten-inch excised raspberry shoots were placed into the filtrates that were cultured under continuous light conditions. These flasks were then placed in bright sunlight and within a four hour period all shoots had wilted, indicating that a toxin was present in all of the three Verticillium biotypes, (Figure 5).



FIGURE 5

Progressive wilting of raspberry shoots following
ten, twenty, and thirty minute exposures to the
toxin of Verticillium albo-atrum, biotype Sl.
Control shoot on left.



10 MIN.
S 1



20 MIN.
S 1



30 MIN.
S 1



The filtrates cultured in darkness in amber flasks were tested under similar conditions and no wilting was observed.

Different combinations of light and dark exposures were then tried using the two series of filtrates.

a. Light Exposed Culture Filtrates

Filtrates were placed in a dark room with excised raspberry shoots immersed in them. After a thirty-six hour period not a wilt symptom was evident on the shoots. The flasks containing the filtrates were covered with black paper, exposed to sunlight, and all shoots demonstrated positive wilt symptoms. This shows that shoots subjected to the toxin will not wilt unless under the influence of light.

In addition to these tests on the light exposed filtrates a similar series of filtrates were tested as follows. Excised raspberry shoots completely swabbed with vaseline, except for the basal cut-end were used. This procedure prevented transpiration of the shoot by mechanical blocking. No wilting whatsoever was produced and indicates that under reduced transpiration the cells do not lose their turgor.

b. Filtrates from Cultures Grown in Darkness

The filtrates produced in absence of light were placed in a dark room with excised shoots immersed in them. No wilting was observed during forty-eight hours of observation.

The filtrates from darkness produced cultures were trans-

ferred to clear glass flasks. These were then exposed to continuous light for seventy-two hours. After a thirty hour period, wilt symptoms were present in the S1 filtrate but none in those from the other two biotypes. Further light exposure did not increase the wilt symptoms resulting from this biotype filtrate.

Thus, the non-toxic filtrate from S1 biotype became toxic after exposure to light, showing that the influence of light acts directly on some chemical product in the filtrate and synthesizes the toxin. Therefore, the formation of toxins is not necessarily the result of growing the fungus in light, but results from the exposure of the filtrate to light. As far as the writer is aware, this observation has not been mentioned by other workers.

Why the filtrate from the S1 biotype was unique in this phenomenon is not known but the S1 filtrate was always more toxic than the filtrates from the other biotypes in these studies. However, it has been noted by both Bewley (6) and Nelson (39) that sclerotia-forming biotypes are more toxic and in accord with these observations. The S1 biotype is a sclerotia former.

Experiment Number 4

Wolf (63) reported that the mycelial mat that accumulated on the synthetic solution induced wilting. The technique used by Wolf was to simply grind the mycelial mat in quartz sand and extract it in distilled water.

This technique was then repeated with the light exposed and dark series of mycelial mats accumulations. The results were identical as as noted for the filtrates; namely, the light exposed mycelium produced wilting and the extract from non-light exposed mycelium did not.

Experiment Number 5

Bewley (6) using a toxin, produced by a Verticillium isolated from tomato, greatly reduced its toxicity by heating at 100 C for five minutes. He concluded that the toxin was thermolabile.

Nelson (39) and Wolf (63) noted that heating of the toxins produced by the vascular fungi increased their toxicity, contrary to Bewley's findings.

Using the technique of Wolf, the filtrates of both the light exposed and the dark series were evaporated to dryness on the steam table. The individual residues were redissolved in distilled water to bring them to a volume equal to that of the original filtrate.

The heat treated residues were then tested with excised raspberry shoots. All light exposed heat treated residues still retained their toxic activity and the S1 biotype residue was even more potent after this heat treatment. All residues of the dark series were non-toxic to raspberry.

The results indicate that the light exposed biotypes of Verticillium are thermostable and that heating tends to increase the toxin activity of the microsclerotia form of biotype S1.

Experiment Number 6

It was questioned whether these toxins were permanent in their action and could the shoots acquire resistance or immunity to the toxin. Excised shoots were removed from the toxic filtrates at various periods of time after wilting had begun, and placed in distilled water. If no recovery of the shoot took place it indicated a possible permanent disorganization of the tissues.

The data obtained from this experiment showed that if fifteen to twenty-five minutes elapsed after the first visual symptom of wilt was evident, the excised shoot would not recover its turgidity when placed in distilled water. Excised shoots removed immediately, or up to approximately fifteen minutes after wilt symptoms occurred, did not acquire resistance or immunity to the toxin. This was demonstrated by placing the shoots back into the toxin and having wilt occur.

Experiment Number 7

Hodgson et al (25) reported that there was a direct relationship between molecular weights and wilt-inducing activity of the synthetic polymer glycols. The largest in molecular weight being the most toxic as demonstrated by wilt and a greater accumulation of the synthetic compound in the region of wilt.

Nelson (39) stated that the mint Verticillium toxin is comparable to large dye molecules. He noted that only one-half of a mint shoot was affected (coloration) by a Fast Green dye, the other half appearing normal in color. This demonstrated that the dye could not move laterally

and must be composed of large molecules.

Following Nelson's procedure (39) but using raspberry shoots instead of mint, an excised shoot was split vertically at the base for three inches and the cut-end of one-half of the stem placed in distilled water, the other end placed in a suspension of Fast Green dye. The phenomena exhibited in mint did not occur in raspberry for the entire shoot was colored. The reason for this difference is not known at the present time.

Similarly, the two ends of a split shoot were each immersed in a toxin filtrate and distilled water. The entire shoot wilted, showing the same lateral penetration exhibited with the dye.

If the toxin consists of large molecules there is a possibility that these molecules could be removed by ultra-filtration. It was decided to test toxins passed through a Seitz bacteriological filter. Prior to this ultra-filtration the toxins were tested and found to cause a complete wilt of shoots within a two hour period. The symptoms of this type of complete wilt is with the lowermost leaves and progresses upward as in field wilt symptoms. The symptoms include a drooping and wilting of the leaves, curvature of the stem tip, interveinal necrosis, and in the case of red raspberries, a terminal leaf downward curling.

Following ultra-filtration the solutions caused no typical wilt symptoms in raspberry shoots following twenty hours of exposure as the shoots were still upright and the leaves turgid. However, the main

vascular elements in the stems and leaves were colored an unnatural brown to black. This coloration persisted for thirty-six hours when the observations were ended.

The Seitz filter pad was placed in a flask and macerated in distilled water and tested with an excised shoot. Wilt symptoms occurred in twelve hours although not quite as severe as found in the original filtrate.

This test indicates that the toxin may be complexes of several compounds of different molecular size - the smaller sized compound causing only discoloration but no wilting and the other compound either consisting of larger molecules or perhaps electrically or mechanically absorbed by the filter pad and causing general wilt symptoms. No definite conclusion could be made regarding the relative size of the toxin molecules.

CHAPTER IX

CULTURAL STUDIES

A determination of the different environmental factors influencing the causal organism may provide useful information on the development of the wilt disease in the field and on possible control measures.

A. Effect of Temperature

In reviewing the data of many investigators (2, 6, 10, 13, and 59), it was noted that no uniformity existed in the results of their temperature studies. These discrepancies may possibly result from the use of different strains or biotypes of the organism. Therefore, it was desirable to know whether differences occurred in the temperature constants of the three biotypes isolated from raspberry. In this study the series of seven electrically controlled incubators used were set at 10, 14, 16, 22, 24, 26, and 28 degrees centigrade.

The inoculum for the S1 and S3 biotypes was prepared as follows: The two biotypes were cultured on malt extract agar until the colonies were approximately three inches in diameter. Using a flame-sterilized brass cork bore, 6 mm. disks of the given biotypes were cut from the agar. A uniform disk of inoculum was then obtained for the culture plates. S1 and S3 biotypes have a characteristic appressed mycelial or sclerotial type of growth and the use of actual bits of the media provide the only assurance for getting uniform amounts of inoculum.

The S2 (fluffy mycelial) inoculum was prepared by a different method. The fungus was cultured on malt extract agar and when the

colonies were approximately three inches in diameter, 6 mm. filter paper disks were placed onto the colonies. These disks were previously autoclaved in a 10 per cent glucose solution. A few days after the fungus had grown into the disks they were transplanted onto the culture plates as uniform amounts of inoculum.

Each test had four replications for each biotype and temperature. Three complete tests were run. The results of all the tests are averaged and the data is presented in Table VII.

TABLE VII

Influence of temperature on the growth of the Verticillium Biotypes S1, S2, and S3

BIOTYPES	Increase in diameter in mm. Temperature in degrees C.						
	<u>10</u>	<u>14</u>	<u>18</u>	<u>22</u>	<u>24</u>	<u>26</u>	<u>28</u>
S1	Test 1	6.5	12.0	16.5	17.0	20.0	17.0
	Test 2	7.0	13.5	17.5	17.5	21.0	15.0
	Test 3	<u>7.5</u>	<u>14.0</u>	<u>17.0</u>	<u>17.5</u>	<u>21.0</u>	<u>17.0</u>
	Average	7.0	13.16	17.0	17.33	20.66	16.33
S2	Test 1	6.5	15.5	22.5	23.0	25.5	23.5
	Test 2	7.5	18.0	24.0	24.5	25.0	24.0
	Test 3	<u>7.5</u>	<u>17.5</u>	<u>23.5</u>	<u>24.0</u>	<u>25.0</u>	<u>24.5</u>
	Average	7.16	17.0	23.33	23.83	25.16	24.0
S3	Test 1	7.5	14.0	18.5	24.0	27.5	30.0
	Test 2	7.5	16.5	19.0	24.5	28.5	30.0
	Test 3	<u>7.5</u>	<u>16.0</u>	<u>19.0</u>	<u>24.0</u>	<u>27.0</u>	<u>29.0</u>
	Average	7.5	15.5	18.83	24.16	27.66	29.66
							33.0
							33.5
							33.0
							<u>32.5</u>

Table VII indicates the optimum ranges for the three biotypes, S1 (22 C to 24 C), S2 (24 C to 26 C), and S3 (26 C to 28 C). However, the S3 biotype may have a higher temperature range than indicated, but higher ranges were not included.

The optimum for the S1 biotype agreed with the findings of Bewley (6), who classified his isolate as Verticillium albo-atrum and stated that it formed microsclerotia. Ch audhuri (10) and Czarnecki (13) using isolates of Verticillium albo-atrum reported almost identical optimum ranges which averaged 21.5 to 25 degrees C. Their data overlap the S1 and S2 biotype optimum ranges reported here. Bennett (2) reported an optimum of 27 C to 32 degrees C which is similar to the findings in this study using the S3 biotype. The S3 is more pathogenic to raspberry than the other two biotypes and may explain the cases of severe wilt during high mean daily temperatures noted in Michigan in these studies and by Bennett.

In addition to the rate of growth the following observations on the effect of temperature on the morphological characteristics were noted. The microsclerotial formation in the S1 biotype was predominant at a temperature range of 18 to 22 degrees C and the mycelial growth in this biotype increased with the higher temperatures. This concurs with Wilhelm's findings (59) on cultures producing microsclerotia. The other two biotypes appeared consistent in their morphological characteristics throughout the temperature range of this study.

B. Effect of Cultural Media

To determine if certain biotypes, namely S1, S2, and S3, are stable it is only necessary to plate them out on a series of various synthetic and natural infusion medias. If the biotypes retain their characteristics under these treatments, their stability is indicated.

The following are the gross general characteristics of the three biotypes, noted throughout the series of cultural media tests.

S1 - Swollen, closely septate darkened mycelium which at first contains many oil guttules but these tend to disappear as the individual cells enlarge. The cells become almost spherical in shape, thick-walled and tend to heap into separate compact black masses called sclerotia. The mycelium is greyish in color.

S2 - The distinguishing characteristic of this biotype is the snowy white fluffy mycelial growth.

S3 - The mycelium is appressed to the media and forms anastomosing ribbons with scattered aerial synnemata through the colony. The colony is waxy in appearance and becomes wrinkled or buckled and gives off a yeast-like odor. The wrinkling radiates from the center or source of inoculum to the outer edge of the colony.

The cultural media consisted of malt extract, corn meal, lima bean, oat, potato-dextrose, prune, Cumberland black raspberry, and Latham red raspberry leaf infusion. All media were solidified in 2 per cent agar. The *Verticillium* biotypes were incubated at their approximate optimum temperatures, namely, S1 - 22 C, S2 - 24 C, and S3 - 26 C.

Each cultural media had four replications for each biotype.

The *Verticillium* biotypes were capable of growing on all the media tested and did not lose their individual identity over this range of eight cultural medias. However, differences in the amount of growth were observed as shown in Figure 6. These differences are recorded in Table VIII.

TABLE VIII

Growth of the *Verticillium* Biotypes on Different Cultural Media

<u>CULTURAL MEDIA</u>	<u>POOR</u>	<u>MEDIUM</u>	<u>EXCELLENT</u>
Corn Meal		S1, S2	S3
Lima Bean	S1	S2	S3
Malt Extract			S1, S2, S3
Oat	S1, S2	S3	
Potato-Dextrose		S3	S1, S2
Prune		S1, S2, S3	
Cumberland Inf.		S1, S2	S3
Latham Inf.	S1	S2	S3

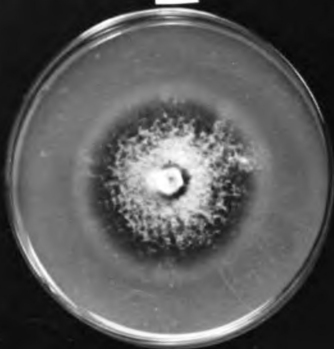
The data presented in the above table are of value for the selection of media desirable for growing large quantities of a given biotype. Such quantities are useful for mass inoculation studies of plants and Petri-dish seeding.

Zimm (66) reported that oat agar gave the best production of microsclerotia which is contrary to the findings of this study. This difference probably results from the use of different biotypes.

FIGURE 6

Variations in growth of the three biotypes S1,
S2, and S3 as influenced by different culture media.

S-1



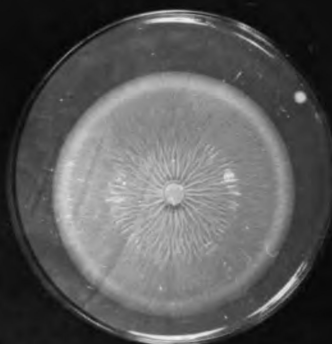
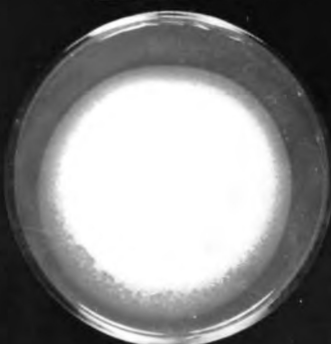
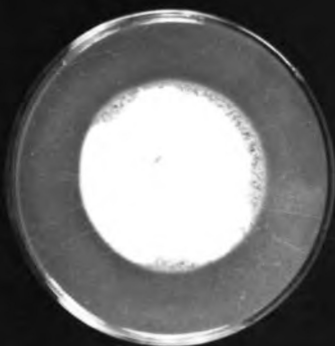
S-2



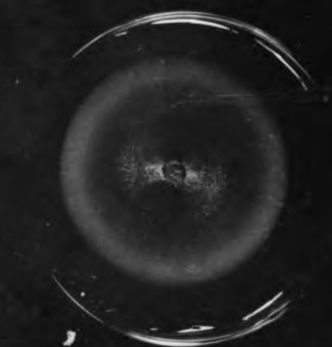
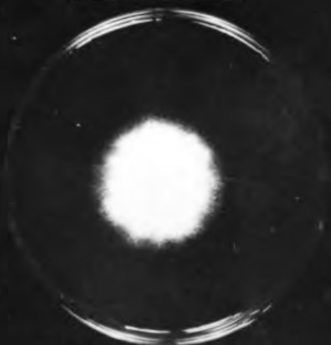
S-3



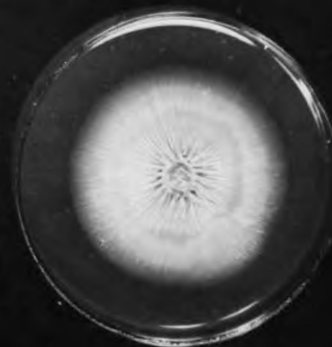
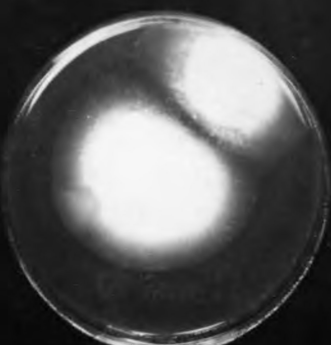
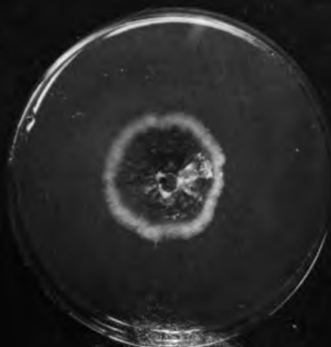
CORN MEAL



LIMA BEAN



LATHAM INFUSION



MALT EXTRACT

C. Effect of Acidity

Haenseler (20) reported that keeping the soil pH as low as possible reduced development of the *Verticillium* wilt organism in the soil. He treated diseased soil plots for four years, one series with sulfur and the other with lime. The results indicated that artificial acidification will reduce severity of wilt, but can not be offered as a practical means of control on soils. The limed plots greatly increased the wilt infection over the four year test period. In the laboratory cultures, Haenseler noted little growth at or below pH 4.0, and best growth from pH 6.0 to pH 8.0

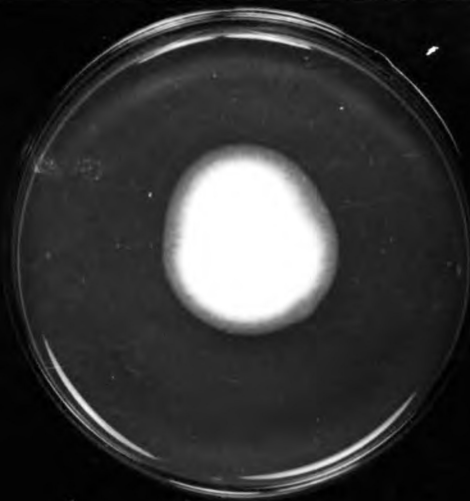
An acidity study on the *Verticillium* organism was made in the laboratory. The technique given by Rawlins (42) for solid media acidity study was followed. It consists simply of adjusting the malt extract media before and after sterilization using a 1 normal solution of hydrochloric acid for low pH and a 1 normal solution of sodium hydroxide for a high pH. The pH value was constantly checked with the aid of the Beckman pH meter. The pH range used was 4.0, 5.5, 6.5, 7.0, 8.0, and 9.0.

Only the *Verticillium* biotype S2 was used because of its easily recognized growth, uniform inoculum, and in trial experiments it was noted that this biotype had the greatest effect on alteration of pH in the media.

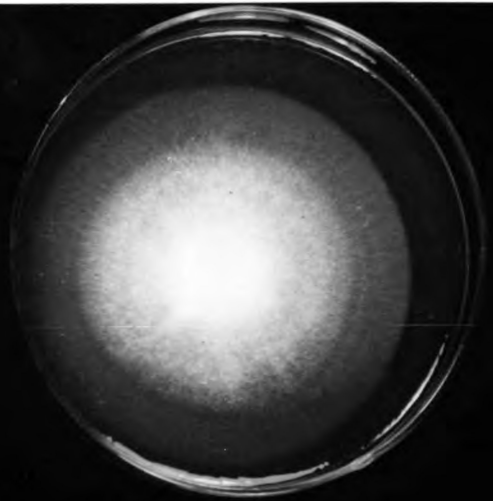
The results obtained are illustrated in Figure 7 and concur partially with those found by Haenseler. There was no appreciable growth

FIGURE 7

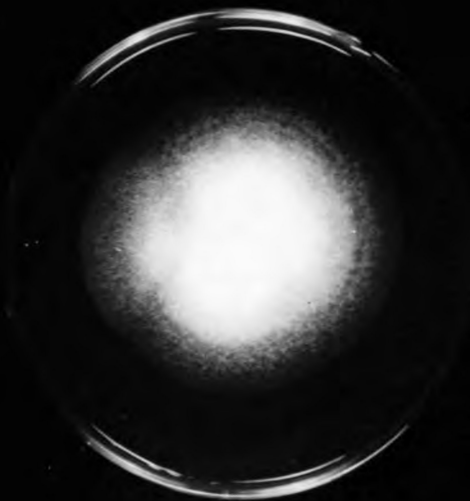
Growth of Verticillium albo-atrum, biotype S2 as
affected by the acidity of the media.



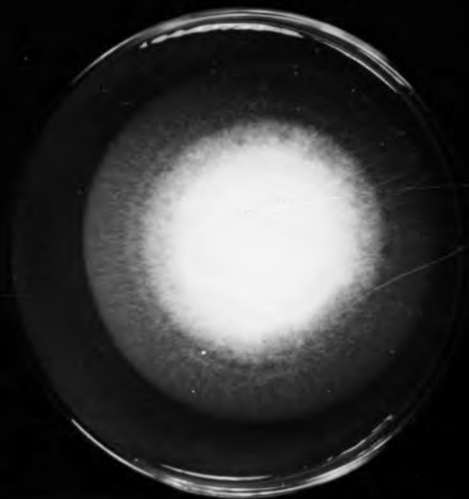
pH 4.0



pH 5.5



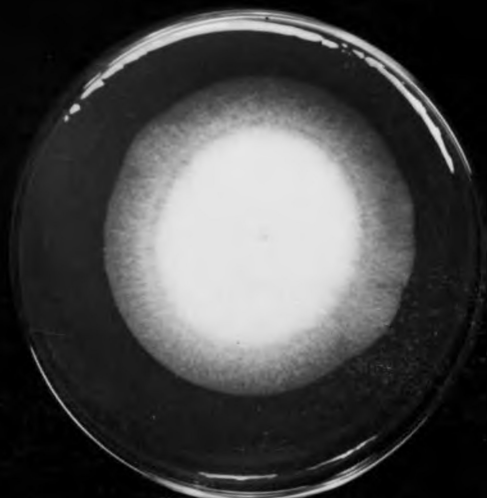
pH 6.5



pH 7.5



pH 8.0



pH 9.0

at a pH 4.0 and the optimum was at a pH 8.0. However, two optimums were noted, mainly at pH 5.5 and at pH 8.0. The reason for this phenomenon is unknown but the lower may be an adaptation to the pH of cell sap which in most plant species is about 5.5.

Another laboratory study was made on the alteration of the H-ion concentration by the *Verticillium* biotypes in the presence of different fermentable sugars. This study was a continuation of the gas production experiment described previously on Page 40. The results of this study are recorded in Table IX.

TABLE IX

The change in H-ion concentration resulting from the growth of *Verticillium* biotypes on nutrient broth with the addition of various sugars.

SUGAR ADDED	S1 pH	S2 pH	S3 pH	Control pH
Dextrose	7.52	8.10	7.65	6.55
Galactose	8.19	8.22	8.35	6.55
Lactose	8.18	8.15	8.20	6.55
Maltose	8.10	7.51	7.91	6.55
Saccharose	7.76	7.96	7.53	6.55
Sucrose	7.61	7.71	7.70	6.55
None	8.31	8.33	8.48	6.55

From the above table it can be concluded that all three biotypes have the ability of decreasing the H-ion concentration of the broth. In these studies it was noted that the fungus had an optimum growth at a pH of 8.0 and the fungus has the ability to change the media to

suit its optimum requirements. The S2 biotype seems to have the most effect on altering the pH of the media.

D. Enzyme Activity

Crabil and Reed (12) developed a method to show the presence and action of products of cellular activity upon substances incorporated in thin layers of agar in Petri plates. They used a standard stock solution consisting of magnesium sulfate 0.5 gm., potassium acid phosphate 1.0 gm., potassium chloride 0.5 gm., ferrous sulfate 0.01 gm., agar 20.0 gm., and 1,000 ml. of distilled water. This stock solution contained no carbohydrate nutrients and consequently supported little fungal growth. By adding substances requiring specific enzymes for hydrolysis, the various enzymes produced could be determined.

Bewley (6) by using methods corresponding to those of Crabil and Reed showed that Verticillium albo-atrum, isolated from tomato, was capable of producing the following enzymes. They are amidase, amylase, emulsin, erepsin, inulase, lipase, and protease.

The report of a similar study follows. However, tests were included only for amylase, inulase, and protease. The three biotypes S 1, S 2, and S 3 used in this study are shown in figure 8.

1. Amylase Test

To 500 ml. of stock solution, 10 gm. of corn starch, dissolved in a little cold water, was added. This gives a clear white substratum in which the starch is suspended. This media was autoclaved and poured directly into sterilized Petri plates. The plates were seeded

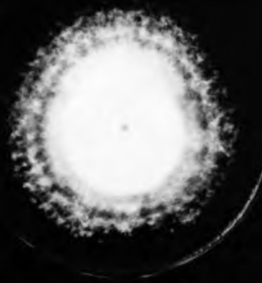
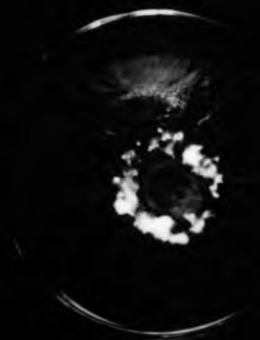
FIGURE 8

Variations in growth of biotypes S1, S2, and S3 on different media suggesting enzyme production suitable for different carbohydrates.

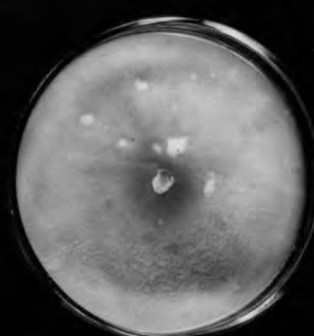
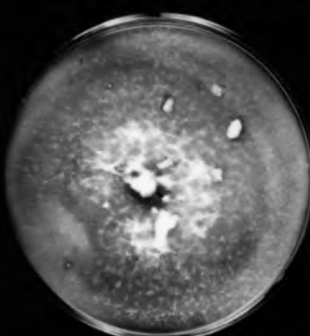
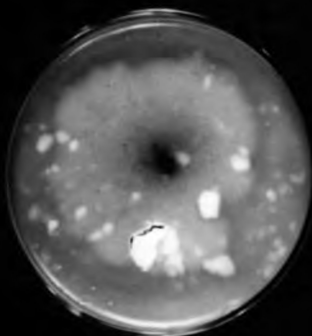
S-1

S-2

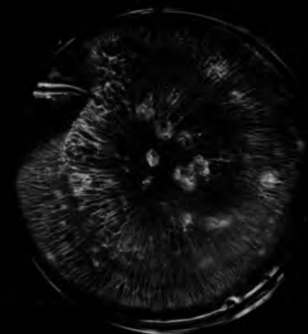
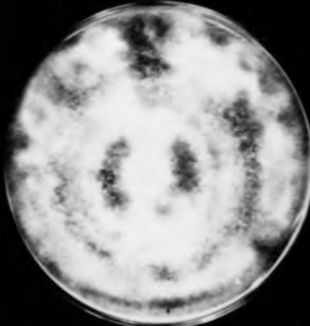
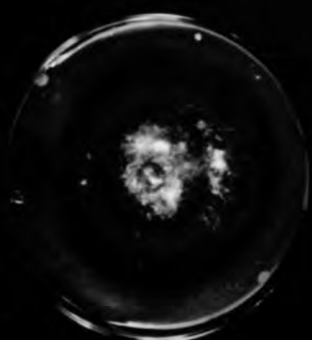
S-3



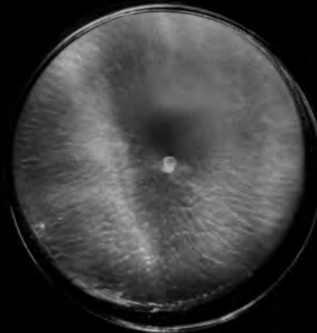
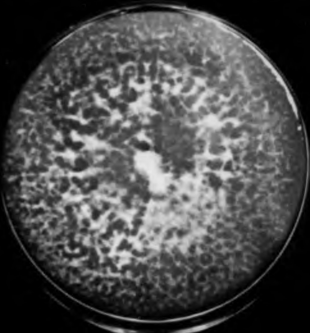
MALT EXTRACT



CORN STARCH



GROUND WHEAT SEED



INULIN

individually with each of the three biotypes of *Verticillium*. The plates were then incubated at the optimum temperature for the biotype contained.

If a readily diffusible extracellular amylase is produced, the starch is hydrolyzed and the space surrounding the fungus colony is clear, giving a halo-like effect.

The *Verticillium* biotypes S1, S2, and S3 were capable of growing on this media. The S2 biotype produced the clearest and largest halo thus indicating a greater production of amylase.

2. Protease Test

The media for this test consisted of 1.0 gm. of ground wheat seed added to a liter of stock solution making a .1 per cent wheat grain media containing protein. Following a similar laboratory technique as used above, this study was carried on. If the fungus grows on this media and dissolves the wheat particle it is said to produce protease. All three biotypes tested grew well on this media as illustrated in Figure 8.

However, wheat grains contain carbohydrates and other foods in addition to proteins. Therefore, fungus growth does not identify protease unless protein hydrolysis is ascertained. Visual examination indicated that the wheat particles were almost entirely utilized strongly indicating protease.

3. Inulase Test

To a liter of stock solution, one-half gram of pure inulin was

added. Inulin is soluble in hot water and consequently dissolves during sterilization. The three biotypes were seeded and tested as previously described.

The inulin incorporated in the media is hydrolyzed to reducing sugars, and as such may be utilized by the fungus. The evidence of the ability of the organism to dissolve inulin is discerned only by the amount of growth occurring.

The S2 and S3 biotypes grew well on this media. However, the S1 biotype produced only a few mycelial strands, indicating a weak production of inulase.

CHAPTER X

CONTROL MEASURES

After a complete review of the literature on the *Verticillium* wilt problem in raspberries, it appears that the control consists mainly in prevention of infection in the future planting rather than effecting a cure in an established plantation.

The fungus is believed to thrive in the soil as a saprophyte on the remains of plant parts. This fact was substantiated by the following test. Small pieces of leaves and canes of seven varieties of black and red raspberries were sterilized in propylene oxide vapors (22), then mixed with 2 per cent sterilized agar and plated. The three biotypes S1, S2, and S3 were seeded, with four replications, to each variety of the raspberry incorporated in the media.

At the end of twenty-five days the relative amount of fungal development was used as a criterion of the suitability of each substrate as a growth medium. No significant differences were noted either between biotypes or substrate of medium. This indicates that the causal organism can live saprophytically on the debris of these common varieties of raspberry.

McKay (35) and Zeller (65) reported the crop rotation studies of *Verticillium albo-atrum*. Their trials indicated that a three or four year rotation with two to three non-susceptible intervening crops, mostly grains, was effective in greatly eliminating the fungus from the soil.

Miles and Persons (37) reported that the heavier sedimentary and alluvial soils were more favorable to the disease than the sandy soils.

Bewley (5 and 6), Leyendecker (32), and Sherbakoff (40) support the same view that the heavier soils are favorable to the spread and propagation of the fungus. A possible reason for the greater prevalence of Verticillium in heavier soils is they are better supplied with plant nutrients and humus to nourish saprophytic fungi, and are neutral to alkaline in nature which is a requirement for best Verticillium growth.

Apart from utilization of genetic resistance most of the recent investigations on Verticillium control have been directed at the soil. Keyworth (28) has recommended removal of the soil from an infected area to a depth of three feet. Wilhelm (61) found that on the whole the zero to twelve inch depths of the soil contained three to four times more infestation than the lower depths. Information on the depth of fungus penetration is essential before efficient use can be made of soil fumigants.

Richardson (44) tested many fungicides against Verticillium albo-atrum and the best results were obtained with mercuric chloride as a soil drench. This did retard the disease development but did not completely prevent it and was not commercially feasible. Garrett (18) and Keyworth (28) working with Hops, reported the use of 2 per cent formalin as a erradicant for small outbreaks of the disease in a plantation. Young (64) noted that soil fumigation with chloropicrin or carbon bisulfide delayed the development of the disease and reduced the incidence of wilted plants. Smith (50) greatly reduced cotton wilt using ethylene dibromide. However, he also reduced the nematode population with this chemical which may be a contributing factor to the incidence of the wilt disease.

Another means of control may possibly be that of host nutrition. Gallegly (17) using sand-drip culture technique reported that an increase of nitrogen, potash, phosphorous also increased the disease and in unbalanced solutions only the low nitrogen decreased the incidence of disease. Nelson (39) noted that increasing nitrogen improved the growth of mint with no increase in wilt.

Varietal resistance studies were used to determine whether any of the common varieties were possibly immune to attack by the *Verticillium* organism. The varieties tested were Latham, Indian Summer, Chief, St. Regis, and Cuthbert red raspberries and the Cumberland and Morrison black raspberries. All these varieties were susceptible to the isolates of *Verticillium albo-atrum*. However, the incubation period for the symptoms was the longest with the Latham red raspberry variety.

Raspberries are a marginal economic crop because yields are not commensurate with prices received and the costs incurred with growing the crop.

Low yields are frequently traced to disease and insects, and certainly the *Verticillium* if present contributes additionally to low yields - in fact, it can be the contributing factor to practically a complete crop loss. As costs are already high, soil disinfection is impractical unless it can be shown that yields are materially increased or if by there use on a small area the disease can be eliminated or restricted. Further exploration of economically feasible soil treatments are needed.

It has been definitely shown in these studies and elsewhere that the

strains of Verticillium attacking Solanaceous crops are also pathogenic to raspberry. Therefore, the one precaution that is definitely indicated is to avoid preceding raspberries with Solanaceous crops. This precaution is not well understood by growers and appears to be the principal source of infection in Berrien County.

The symptoms of the disease are obscure and confused with soil deficiencies. To what extent the disease is spread by nursery stock is not clear. However, Bennett (2) indicated that nursery stock was responsible in many cases for he traced infections to new plantings not preceded by tomatoes or potatoes. This needs additional study but is certainly worth every precaution by growers to avoid propagation from possibly infected material.

Biological control by antagonistic cover crops have not been indicated for Verticillium on raspberry, but rotations using grasses have cleared the soil of the organism to allow further profitable potato production. This method is also indicated for raspberry as the same organism is involved.

CHAPTER XI

SUMMARY

This paper reports the results of investigations on the fungus Verticillium albo-atrum Eke. and Bert. and its pathogenic relationship to black and red raspberries.

Three constant biotypes of the Verticillium fungus were isolated. They are S 1 (microsclerotia dominant), S 2 (fluffy mycelium), and S 3 (appressed mycelium). All three biotypes were pathogenic to raspberry with the S 1 and S 3 biotypes being the more potent.

The symptoms of the disease on black and red raspberries are fully described. The general symptoms of yellowing and drooping of the leaves progressing from the base upwards is characteristic of both species investigated.

Root studies on diseased red raspberry showed that some of the sucker plants may be entirely free of infection and the amount of suckering is reduced as a result of the disease.

Methods of surface sterilization preliminary to isolation of the fungus are compared. Chlorazene or sodium hypochlorite appeared satisfactory and a series of water rinses were used with success in root studies. Single-conidial isolation was accomplished from dilution plates of thinly spread agar.

Plant inoculations were accomplished in the roots with and without wounding but in the canes only through wounds.

Experiments on the mechanism of wilting indicated that the toxin theory is the most plausible.

Light was necessary for toxin production and it is believed that this study is the first to show that light activates the toxin in the substratum rather than within the tissues of the fungus.

Ultra-filtration of toxins indicated that they may be composed of complexes of several compounds of different molecular size as the filtrate was less toxic than the original solution. From this it was postulated that there was a partial absorption of toxin particles by the filter pad.

The effect of temperature, cultural media, acidity, and possible enzyme activity were studied using the three biotypes isolated. Optimum temperatures ranged from 22 to 28 degrees centigrade, with the higher temperatures accorded to the S 3 biotype. Malt extract and potato-dextrose agar afforded the most growth when compared with corn meal, lima bean, oat, prune, Cumberland leaf infusion, and Latham leaf infusion.

Two optimum acidity requirements were indicated at pH 5.5 and 8.0. The biotypes had the property of changing an acid media to a more alkaline state, which more nearly suited their optimum requirements.

It was indicated that all three of the enzymes, amylase, inulase, and protease were produced. Although the S 1 biotype appeared to produce less inulase.

Crop rotations of raspberry following Solanaceous plants were conducive to the disease and should be avoided. No other practical control measures were indicated as far as studied.

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