

AN ABSTRACT OF THE THESIS OF

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Title AN IMPROVED SELECTIVE MEDIUM FOR THE ISOLATION
OF VERTICILLIUM DAHLIAE KLEB.

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Sodium polypectate (SPP) was added to an ethanol-streptomycin agar (ESA) medium to determine if it would provide a selective substrate for the growth of Verticillium dahliae and for isolation of the fungus from soil and plant tissues in the presence of saprophytic fungi. Sodium polypectate improved greatly the formation and pigmentation of microsclerotia in four different isolates of V. dahliae. Adding SPP to ESA medium also improved the recovery rate of the fungus from soil and from infected plants. At high inoculum densities, SPP did not increase colony numbers, and ESA without SPP apparently allowed the recovery of all viable fungus propagules. However, adding SPP to the medium, even at levels as low as 0.25 g/l, increased the size, microsclerotial formation, and pigmentation of the colonies in every case. All the colonies on ESA-SPP medium were very distinct and they could be observed and counted easily and rapidly with the unaided eye. The time required for formation

and pigmentation of microsclerotia was shortened by five to seven days when SPP was added to the medium. The results suggest that V. dahliae utilizes SPP primarily to form pigmented resting structures rather than vegetative mycelium.

In soil dilutions, addition of SPP up to 1.0 g/l of ESA increased the recovery of V. dahliae. At higher concentrations of SPP, vigorous growth of saprophytic soil fungi inhibited the growth and masked the presence of V. dahliae.

Microsclerotial production by V. dahliae was obtained in shake cultures by adding SPP to a 0.1 percent water agar or a 0.1 percent Czapek Dox solution, both containing 0.76 percent ethanol and 101 ppm streptomycin. The intensity of production was always related to the level of SPP in the media. Water agar containing SPP yielded many more microsclerotia than Czapek Dox containing SPP, at all tested levels.

For best microsclerotial production the combined effect of SPP and ethanol was necessary in all experiments.

Improvement of ESA medium by addition of appropriate amounts of SPP will allow more refined studies in the biology of V. dahliae and the knowledge gained from such experiments should enhance our understanding of this important plant pathogen and eventually aid in its control.

AN IMPROVED SELECTIVE MEDIUM FOR THE
ISOLATION OF VERTICILLIUM DAHLIAE KLEB.

by

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AN IMPROVED SELECTIVE MEDIUM FOR THE
ISOLATION OF VERTICILLIUM DAHLIAE KLEB.

INTRODUCTION

The imperfect fungus Verticillium dahliae is one of the most economically important pathogens of many crop plants. It causes vascular wilt and death of susceptible hosts, and is capable of long survival in soil and plant debris by forming resting structures, called microsclerotia, in the dead host plant.

A review of literature on the disease cycle of V. dahliae in susceptible plants (13; 20; 21; 32; 43; 46; 55) reveals that the fungus enters the host by direct penetration of the root and progresses through the cortex with later systemic invasion of the xylem. When the infected plant dies, V. dahliae grows out transversely from the vessels and tracheids of the xylem, passing through the pits toward the cortex and pith parenchyma. After death of tissues, the fungus forms typical black microsclerotia, primarily in parenchyma tissue.

The isolation and recovery of V. dahliae from soil, roots, and infected plants or plant debris has been a difficult task in the past. This is because such materials are always contaminated with large numbers of saprophytic bacteria and fungi which grow rapidly on ordinary nutrient media and thus reduce or inhibit the

growth of V. dahliae. A medium suitable for successful isolation of this pathogen from soil, infected plants and plant debris has long been needed. The development of such a medium would allow many biological studies of V. dahliae, such as survival in soil and dead plants, to be undertaken. Also, with a favorable medium developed, the response of the pathogen to chemical and physical agents can be studied. In plant pathology the development of effective control and curative measures against a disease is dependent upon an understanding of the biological and physiological capabilities of the causal organism and its reaction to varying environments.

Nadakavukaren and Horner (31) used an ethanol-streptomycin agar (ESA) medium to recover V. dahliae from soil. This medium was used by Martinson (27) to recover V. dahliae from infected plant debris in soil, and by Lacy (23) to study rhizosphere populations. One disadvantage of ESA medium is that under certain conditions, and especially in the presence of certain soil fungi, V. dahliae colonies are diffuse and produce few microsclerotia.

Verticillium dahliae is believed to use cellulose and pectic substances for its growth during the process of infection, stem maceration, and later saprophytic existence in the dead hosts. This concept is supported by the fact that in parenchyma cells of a dead host plant cellulose and pectic substances are the major carbon sources available for growth of the fungus. With this idea in mind,

some preliminary experiments were carried out to determine the possibility of using cellulose and pectic materials as selective substrates for growing V. dahliae in culture. Cellulose in ESA media did not cause any improvement in the growth or pigmentation of the fungus. On the other hand, striking results were observed by using sodium polypectate. The colonies on the media containing sodium polypectate were more numerous and produced more microsclerotia with a higher degree of pigmentation in comparison with those on ESA without sodium polypectate.

The objective of the experiments presented in this thesis was to develop an improved culture medium selective for V. dahliae by using sodium polypectate.

REVIEW OF LITERATURE

In 1913 Klebahn (22) described a vascular wilt disease in dahlia and named the causal fungus Verticillium dahliae Kleb. One of the characteristics of this fungus was formation of small, black pseudosclerotia (microsclerotia) that did not appear in the type culture of V. albo-atrum, described earlier by Reinke and Berthold (37), as the cause of vascular wilt in potato. Rudolph (39) and Wilhelm (47) were two of several who questioned the validity of V. dahliae as a separate species. The controversy is based upon the two types of resting structures formed: 1) resting mycelia (Dauermycelien), formed by V. albo-atrum, which consist of oblong or rounded cells that originate only by transverse division and form thickened, dark cell walls, and 2) microsclerotia, formed by V. dahliae, which consist of almost spherical cells formed by cell division in all planes resulting in a nearly spherical, multi-cellular resting body with thickened and darkened cell walls (14, p. 138-140; 21, p. 347).

Rudolph (39, p. 252-254), in 1931, expressed his view about the validity of V. dahliae and maintained that a difference in resting structure should not entitle a fungus to specific rank. Wilhelm (49), from his experiments on the effect of temperature on microsclerotial formation, concluded that the resting structures, especially

microsclerotia, are not reliable characters upon which to separate species. In his experiments, microsclerotia formed readily in culture at lower temperatures, but above 25^oC only sparse microsclerotial formation occurred.

Isaac (14, p. 137-157; 16; 17, p. 180-195) and Keyworth (21, p. 346-457) have accepted V. dahliae as a valid species. To support this view, Isaac has demonstrated that the maximum growth temperatures as well as pH optima for growth of the two forms of Verticillium are different. He also has presented evidence showing a difference in the preference of carbon sources for optimum growth by the two fungus forms.

Ludbrook (26, p. 122-123) showed that the upper temperature limit for growth of V. albo-atrum in culture was 28^o to 30^oC. At this temperature range, V. dahliae made fair growth and the upper limit was much higher. Thus he demonstrated that V. dahliae and V. albo-atrum could be distinguished by temperature response as well as resting stage morphology.

Martinson (27, p. 10) stated that "since the microsclerotial and resting mycelium types are constant, have differing responses to environment, and vary in pathogenicity, they should be differentiated taxonomically." It appears that V. dahliae is the most internationally accepted name for the microsclerotial type of the fungus (27).

Verticillium dahliae infects plants by penetration of the roots, ramification within the cortex, and subsequent systemic invasion of the xylem (13; 32, p. 65-69; 46; 55, p. 363). Direct penetration of root tips occurs, (13; 32, p. 66), but the fungus also enters through wounds, especially those made by the emergence of adventitious roots, insects, and tillage operation (32, p. 66). However, wounds are not necessary for penetration (13).

After the fungus enters the root system, it causes a systemic infection that progresses upward to the stem apex (32, p. 69). While the fungus is confined to the xylem, characteristic symptoms appear on the foliage (32, p. 39-56). The symptoms of disease depend to some extent upon the host plant and conditions of growth, but infection is generally followed by wilting, yellowing of the foliage, and death of a part or all of the shoot system (7). In peppermint (Mentha piperita L.), symptoms of the disease are dwarfing, shoot and leaf asymmetry, twisting and curling of the shoot and leaf, blanching, wilting and defoliation, stem and rhizome cankers, root rot, and finally death (32, p. 39-58). The most typical diagnostic symptom of Verticillium wilt of peppermint is the asymmetrical development of young leaves (23, p. 12). Wilting may occur in the later stages of the disease. Under normal moisture conditions wilting seems to depend on the severity of the initial infection of the plants. Lacy (23, p. 12-13) feels that the more

extensive the infection of the root system, the more suddenly the onset of symptoms and more likely the occurrence of wilting.

Plant pathologists are not in agreement on the cause of wilting induced by Verticillium. Concepts suggested by different workers to explain wilting include: 1) toxins produced by the pathogen which cause wilting (11; 32, p. 94-101; 45, p. 415-537), 2) production of growth hormones by the fungus in the vascular system which play a role in wilting by inducing tylosis in the vessels (35), 3) prevention of water transport by hyphae of the fungus growing in the vessels (2; 48, p. 58), and 4) obstruction of vessels by plugs of pectic or other materials (32, p. 69; 40; 48, p. 68).

Production of pectic enzymes by Verticillium has been shown in vitro (1; 6; 20, p. 325-327; 29, p. 26-76) and in vivo (5; 7; 28). If these enzymes are produced by the pathogen in the host xylem vessels, pectic and other substances of relatively high molecular weight might be released into the vascular sap (8, p. 346). Wood (56, p. 120-139) found that very dilute solutions of undegraded pectic substances, even at concentrations as low as 30 ppm, caused wilting of cut shoots of tomato. Blackhurst (1, p. 79-88) showed that solutions of sodium polypectate induced slightly less water loss than solutions of pectin and were more active in causing wilt of tomato shoots.

Kamal and Wood (20, p. 322-340) observed that the production

of pectic enzymes by V. dahliae was more active when media contained pectic substances. McIntyre (29, p. 26-76) discovered that V. albo-atrum produced twenty times as much polygalacturonase with sodium polypectate as the carbon source than when grown on pectin. Assays of polygalacturonase activity suggested that V. albo-atrum produced at least two endo-polygalactruonases in culture. One endo-polygalacturonase preferentially hydrolyzed sodium polypectate to large fragments, the other preferentially hydrolyzed small fragments from the substrate. In Kamal and Wood's studies (20) there was a close relationship between the protopectinase activity of culture filtrates and toxicity of these filtrates to parenchyma cells. These results were confirmed by Blackhurst (1) who found that filtrates of the fungus on pectic substrates which were known to have a very high polygalacturonase activity, caused rapid wilting of cut shoots of tomato, maceration of stem tissue, and vascular browning. Also a solution of commercial pectic enzyme preparation, pectinol 100D known to have a high polygalacturonase and pectin methyl esterase activity, induced wilting and vascular discoloration in both resistant and susceptible varieties of tomato.

Kamal and Wood (20) reported that the addition of pectin into the basal medium increased the growth of V. dahliae by about 25 percent. Wood (56) later demonstrated that the fungus grew

poorly in the xylem sap, presumably because of the shortage of carbon, since when glucose was added growth increased greatly. The fungus was able also to use pectin and polypectate under these conditions, and growth on polypectate was almost as good as on glucose. Wood (56, p. 136) concluded "it seems that pectic enzymes would be produced in xylem vessels provided that relatively small quantities of appropriate substrates were available, from what is known about the structure and composition of xylem elements this is very likely to be the case." McIntyre (28) in 1964 found that the crude enzyme preparation of V. albo-atrum from infected tomato plants yielded pectic fragments as well as monogalacturonic acid. This indicated the presence of an endo-polygalacturonase that hydrolyzed small fragments from the substrate.

From these studies it appears that Verticillium produces polygalacturonase abundantly when it is supplied with pectic substances. Blackhurst (1) feels this enzyme is the most active component of culture filtrates in inducing the disease symptoms. Deese and Stahmann (7, p. 53-70) studied the pectic enzyme formation of V. albo-atrum grown on surface-sterilized living stem tissues of resistant and susceptible tomato plants. They found that the fungus formed a large amount of polygalacturonase on susceptible tissue but little or none was formed on resistant tissues. They indicated that culture filtrates from resistant tissues contained a high

oxidizing power which was absent from tissues of the susceptible variety. In resistant varieties the inhibition or suppression of polygalacturonase formation appeared to be associated with this high oxidizing power. Deese and Stahmann (7, p. 66) from their studies proposed the following hypothesis in relation to the cause of wilting in infected plants:

The fungus confined to the vascular system of susceptible tomato plants secretes pectic enzymes and possibly other hydrolytic enzymes which attack the cell wall to release simple carbohydrates in the transpiration stream which may not cause wilting. These substances may be used by the fungus to grow and produce more invading hyphae which block the xylem and petiolar vessels. The small amount of PME produced by the young growing pathogen and that already present in the susceptible plant demethylates pectin to yield low-methoxy pectin and pectic acid; this process enhances the initial polygalacturonase activity. If this process continues, the combined action of hydrolytic enzymes, like polygalacturonase, and the concomitant synthesis of polysaccharides by the invading fungus would reduce transport of nutrients in vascular system. The decrease in nutrient and water transport may produce dwarfing and wilting of the leaves which become yellow and epinastic.

It is likely that dysfunction of the stem involves the degradation of pectic substances of the middle lamella and cell wall. Since wilting can be induced experimentally by the uptake of compounds of high molecular weight which prevent the upward water flow (1, 20), therefore Blackhurst (1) believes that it is reasonable to assume that the action of pectic enzymes able to hydrolyze compounds of high molecular weight might lead to the formation of insoluble

pectinates or pectates which, if persisting, will impede the flow of the vascular sap and consequently cause water shortage and wilting.

In late stages of the disease the fungus invades surrounding tissues, especially the pith (32, p. 56).

In studying wilt of lucerne caused by different species of Verticillium, Isaac (18, p. 552-558) observed that in the initial stages of disease the fungus is confined to the vessels and tracheids of the xylem. When the shoots were nearly dead, the pathogen grew out transversely from the vessels and tracheids, passing through the pits toward the cortex. After invasion of the cortex, superficial conidiophores developed. When the stem died, hyphae in the wood rays and cortex became black and carbonized and formed either the typical black resting mycelium of V. albo-atrum or the black microsclerotia of V. dahliae.

Microsclerotia form by septation and budding of certain hyphal cells which enlarge and become thick and highly pigmented (54). When infected plants die, V. dahliae readily permeates the surrounding tissues (18, p. 552-558; 21, p. 348; 32, p. 69; 43). After death, microsclerotia form throughout the infected plant tissues (18; 50; 53). Their formation is favored by a moist cool environment, and they function as inoculum for inciting disease at a later time (50; 53; 54).

Verticillium dahliae is not capable of saprophytic growth through natural soil (15; 51; 52). Martinson (27; p. 92-98), however, showed that the fungus is capable of saprophytically colonizing a low percentage of pieces of crop residues introduced into the soil. Isaac (15, p. 635) demonstrated that a progressive reduction in the amount of viable material of V. dahliae occurs when it remains in the soil for several months. The fungus cannot survive for long periods of time as mycelium and conidia (13; 41), but microsclerotia are capable of passive survival for many years (13; 50; 53).

Wilhelm (53) found that microsclerotia of Verticillium could survive for 13 years in culture and 12-14 years in soil in the absence of known host plants.

Verticillium dahliae can maintain a high inoculum potential by infecting many different plants. Russian workers Soloveva and Polyarka (44) found that V. dahliae attacked 27 different plants, but cereals were immune.

Martinson and Horner (28) have shown that many plants, including members of the family Gramineae were susceptible to infection by V. dahliae without showing any disease symptoms. They indicated that the fungus could form microsclerotia, pathogenic to peppermint, in the roots of these plants.

Horner (12) has demonstrated that mint could serve as a potential reservoir for isolates of Verticillium other than the mint

pathogen without showing symptoms.

Verticillium dahliae is normally transmitted to new areas on propagative material or within infected plant debris (21, p. 350; 30, p. 464; 32, p. 162; 43).

Isaac (13, p. 630-638) demonstrated that spread of V. albo-atrum and V. dahliae throughout soil and subsequent infection of the new host plants was related to the ability of these pathogens to kill and cause the decay of roots of the infected hosts with consequent liberation of the fungus into the soil. He concluded that since these pathogens appear to be incapable of spreading as soil saprophytes, the rapid spread of the disease from an infected plant must be due, not to the growth of fungal mycelium in the soil toward new hosts, but to the growth of the roots of these potential hosts into the infected soil around the initially diseased plants.

Information about the sporulation of V. dahliae is very limited. McKay, in 1926, reported sporulation of V. dahliae in soil. He buried infected potato stems in soil under field conditions and observed the fungus sporulating freely on the infected plant materials. According to McKay the sporulation continued for at least six months.

Keyworth (21, p. 346-357) working on wilt of hop caused by V. albo-atrum, observed verticillate conidiophores and conidia on dead diseased leaves. Spores were formed plentifully on diseased vines and leaves lying on the ground after harvest. Keyworth (21)

was able to induce the disease in hops by adding infected plant debris to the soil.

Isaac (18, p. 550-558) and Sewell (43, p. 312-321) also studied the sporulation of V. albo-atrum. Their studies revealed that any fragmentary plant material containing the fungus, when kept under moist conditions, becomes covered by conidiophores, bearing enormous numbers of spores. Sewell (43) found that the sporulation of the fungus on roots of infected tomato plants occurred shortly after the onset of root degeneration in soil. The duration and density of sporulation was related to the quantity and rapidity of degeneration of the tissues.

One of the important factors affecting the development of a fungus in soil is the principle of fungistasis. In 1953, Dobbs and Hinson (9) showed that germination of fungal spores is inhibited in natural soil, apparently by a biological factor or factors. The fungistatic factor(s) can be removed from soil by dry heat, prolonged drying, autoclaving, adding a dilute glucose solution, wetting with acetone, and adding charcoal at the rate of ten percent (w/w).

Lockwood (25, p. 327-331) showed that germination of V. albo-atrum conidia was inhibited when in contact with natural soil. Also mycelia of the fungus when covered with soil for 14 days, was lysed partially or completely. Sterilized soil had no effect. When the sterilized soil was inoculated with mycolytic isolates of

Streptomyces sp. and incubated for 14 days, it produced lytic and inhibitory effects on test fungi similar to those produced by natural unsterilized soil. Lockwood (25) felt that fungistasis in natural soils is related to diffusible fungitoxic substances, suggesting a causal role for antagonistic isolates of Streptomyces sp. in natural soil fungitoxicity.

The phenomenon of fungistasis has been noted to occur in all soils tested, except some deep subsoils (42).

Information on germinability of Verticillium microsclerotia is meager and conflicting. Thomas (47) was unable to germinate them without prior cold treatment; and even then germination percentages were low.

Jackson (19, p. 96-97) and Rovira (38, p. 53-63) demonstrated that seedling roots and root exudates stimulated germination of fungal spores in soil. Schreiber and Green (42, p. 260-264) found that the fungistatic principle of all natural soils prevented germination of both conidia and microsclerotia of Verticillium and this fungistatic effect was overcome to varying degrees by plant root exudates. Root exudate from tomato (a host) overcame fungistasis to a greater degree than that of the non-host (wheat). Fractionation of tomato root exudate suggested that amino acids or other nitrogen-containing compounds were responsible for overcoming fungistasis.

Lacy (23) recently showed that the rhizosphere of soils from all plants tested had significantly higher populations of V. dahliae than did non-rhizosphere soils. The host plants supported large populations of V. dahliae in the rhizosphere, but plants such as wheat, corn, and beans (non-hosts) tended to support fewer numbers of Verticillium propagules in the rhizosphere. Lacy (23) felt that the larger fungal population in the rhizosphere was related to the stimulation of reproduction of propagules by roots.

MATERIALS AND METHODS

Media Preparation. Nadakavukaren and Horner (31) discovered that ethanol added to streptomycin water agar induced Verticillium dahliae to form abundant black microsclerotia in culture. Ethanol was added to obtain a concentration of 0.5-1.0 percent just prior to pouring the plates. This method has been employed successfully in quantitative determinations of V. dahliae, especially in soil. Streptomycin eliminates or reduces the development of bacteria and ethanol allows growth of V. dahliae whereas most rapidly growing fungi do not develop.

In all experiments presented in this thesis a modification of Nadakavukaren and Horner's ethanol-streptomycin agar was used. The pectic substance employed in these studies was sodium polypectate manufactured by Sunkist Growers. Throughout the thesis ethanol-streptomycin agar will be referred to as ESA and sodium polypectate as SPP. The SPP is directly soluble in water forming a very viscous solution. It contains pectate with a higher molecular weight than that formed by pectic acid and sodium carbonate. It is susceptible to precipitation with excessive amounts of salts, alcohol, acid and alkaline earth or heavy metals.

The desired amounts of SPP were added to one percent Difco water agar in 500 ml flasks. Flasks containing the media were then

autoclaved for 20 minutes at 15 psi and cooled to 40°C in a temperature-controlled water bath. Sixteen ml of 47.5 percent ethanol containing 6300 ppm streptomycin sulfate were added per liter of medium just prior to pouring the plates. This resulted in a final concentration of 0.76 percent ethanol and 101 ppm streptomycin. Adding ethanol to media containing SPP caused formation of a white, gel-like precipitate especially at higher concentrations of SPP. Vigorous shaking of the flasks was required to break the gel into a homogenous suspension. In one experiment ethanol was excluded from the media and streptomycin was added in the form of a water solution at the same concentration indicated above.

In preliminary tests, an attempt to cause V. dahliae to produce microsclerotia at low nutrient levels in shake culture was unsuccessful. An experiment was performed that included SPP in the media. In this test, two kinds of media were used. One contained one g/l Czapek Dox and the other contained one g/l Difco agar. The water agar at this concentration does not solidify after autoclaving. The levels of SPP used in both media were 0.0 (control), 0.25, 0.5, 1.0, and 2.0 g/l. The prepared media were autoclaved for 20 minutes at 15 psi in cotton-plugged flasks. After cooling, a solution of ethanol and streptomycin (16 ml of 47.5 percent ethanol containing 6300 ppm streptomycin sulfate) was added to all flasks. The flasks were then inoculated with V. dahliae and attached to a shaker.

Inoculum. Four different isolates of V. dahliae were used as inoculum in the experiments:

1. Number 86, originally isolated from infected hops (Humulus lupulus L.) grown in England.
2. Number 95, isolated from peppermint (Mentha piperita L. 'Mitcham') then inoculated and reisolated from bean (Phaseolus vulgaris L.).
3. Number 121, originally isolated from infected peppermint showing severe wilt.
4. Number 129, originally isolated from severely infected cotton (Gossypium hirsutum L.) plants in California.

The isolates were maintained on potato dextrose or Czapek Dox agar media in screw cap tubes, and when used in the experiments, a shake culture of the given isolate was prepared. The medium in the shake cultures contained 35 grams Czapek Dox broth and three grams yeast extract per liter. This medium, after being autoclaved for 20 minutes in 500 ml cotton plugged flasks, was cooled and inoculated. The inoculation was done by transferring a small piece of the fungus from culture tubes into the prepared media with a sterile needle. The flasks were then attached to a wrist-action shaker.

In some of the experiments the inoculum was a dilution of spores produced in shake cultures. The number of spores per ml

of the media was determined with a hemocytometer after shake cultures had grown five to seven days at room temperature. Spore concentrations was adjusted to the desired level by successive dilutions in distilled sterile water. The spore suspension obtained in the above manner was used to inoculate plates by placing one ml in each plate. The inoculated plates were then covered with appropriate media in each experiment and incubated. Aseptic techniques were used in all steps of the experiments to prevent contamination.

Stem Assay. Cuttings of healthy peppermint, grown in the greenhouse, were inoculated with isolate 121 by dipping their roots in a spore suspension of the fungus for one minute. This isolate causes severe disease in peppermint. The cuttings were planted in pots after inoculation and were kept in the greenhouse under favorable conditions for growth. All the inoculated plants showed severe wilt symptoms and stunting after six to eight weeks. When the disease was at maximum intensity and the plants were nearly dead, the foliage was removed just above the soil level and several stem segments each two to three cm long were obtained at random. These segments were chopped and fragmented in 100 ml of water with a high speed Omni-Mixer. The prepared material was further diluted 100 or 1000-fold with sterile distilled water and used to inoculate plates by placing one ml of the final dilution in each plate. The inoculated plates were covered with ESA containing various

concentrations of SPP and incubated in the dark at room temperature.

Soil Assay. Chehalis sandy loam field soil was air dried in the laboratory and was artificially infested with V. dahliae isolate 95 by mixing 350 ml of shake culture of the fungus with 2,000 grams of soil. The infested soil was stored at room temperature until completely dry then screened through a 14-mesh screen. This constituted the stock inoculum. The dilutions were made by mixing one part of stock with 9, 49, and 99 parts of noninfested field soil in a laboratory tumbler for 20 minutes. One gram of each prepared soil mixture was suspended in 100 ml of a 0.5 percent carboxymethyl cellulose solution. Further dilutions of the suspension were made with distilled water. The final dilutions were used for inoculation of the plates.

In another experiment the plates were inoculated by direct dilutions of a shake culture of isolate 95 and prior to pouring the plates 0.8 gram of a natural field soil was added per liter of media.

Incubation, Observation, and Data Analysis. In all experiments, the plates were incubated at room temperature. Since in the preliminary experiments the plates incubated in the dark had significantly higher numbers of colonies than those in light, all plates in later experiments were incubated in the dark by placing them in sealed drawers. All the shake cultures were maintained at room temperature and no effort was made to control light conditions.

In each experiment, the cultures were observed frequently and the data on the number of Verticillium colonies were taken after the colonies had sufficiently darkened.

Statistical analysis of data obtained from the experiments was done by the analysis of variance method and the least significant difference (LSD) values obtained were based on the comparison of the means.

RESULTS

Effect of Sodium Polypectate on the
Number of *Verticillium dahliae* Colonies

In this experiment, plates were inoculated with a fixed concentration of spores obtained by the dilution of a shake culture of *V. dahliae* isolate No. 95. The plates were covered with ESA containing different amounts of SPP. All cultures were then separated into two groups with the same number of plates for each treatment. One group was incubated in the dark, the other in normal light conditions in the laboratory. The data was taken after about two weeks when the colonies in all cultures had darkened sufficiently. Data on the effect of SPP on recovery of *Verticillium* conidia is shown in Table 1.

The media containing one and 2.5 g/l SPP supported significantly higher numbers of colonies in comparison to ESA control. The data revealed also that, in general, the cultures incubated in the dark had higher numbers of colonies than those incubated in the light. In this respect, the differences between the number of colonies in the higher rates of SPP (one and 2.5 g/l) were statistically significant. However, no interaction between light conditions of incubation and SPP was found.

The most outstanding effect of SPP was on color and appearance

of the colonies as shown in Figure 1. Increasing the level of SPP in the medium caused a considerable increase in the intensity of pigmentation and in the abundance of microsclerotia. The colonies were very distinct and were larger in comparison to those on ESA control. The difference was noticeable even with the lowest rate of polypectate (0.1 g/l). Another interesting observation was the incubation time necessary for the colonies to turn dark. This was much shorter with SPP in the medium (five to eight days depending on the amount of SPP) in comparison to ESA control (10-15 days).

Table 1. The effect of SPP concentration on the number of colonies of V. dahliae.

SPP G/liter	Number of colonies (a)	
	Dark incubation	Light incubation
0.0 (ESA control)	148	145
0.1	173	145
0.5	164	157
1.0	237	203
2.5	321	291

(a) Average of three plates.

LSD for the effect of SPP 5% = 20
1% = 27

LSD for the effect of dark and light
5% = 13
1% = 17

LSD for the effect of different concentration of SPP
5% = 28
1% = 38

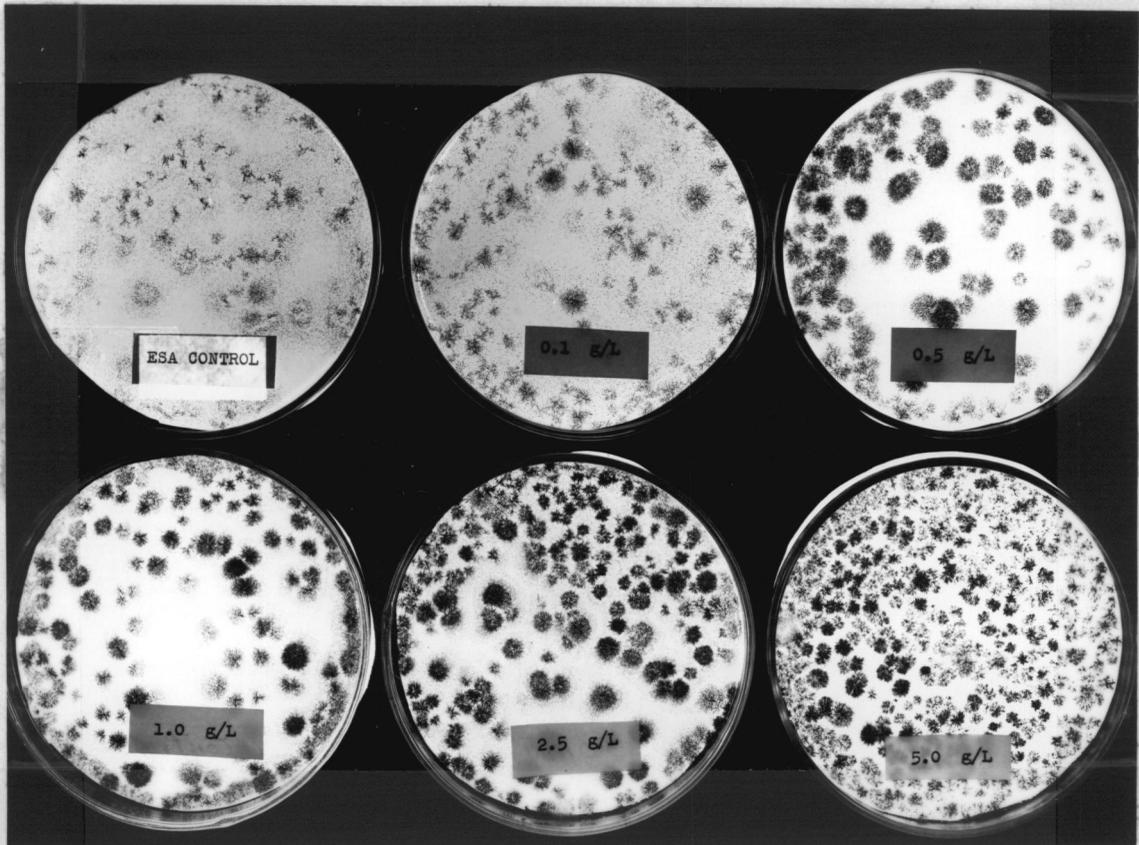


Figure 1. The effect of different concentrations of sodium polypectate on microsclerotial development by *V. dahliae* in ethanol-streptomycin agar medium.

Effect of Sodium Polypectate on Different
Isolates of *Verticillium dahliae* in Culture

SPP as indicated by the previous experiment, had a considerable effect on the development of *Verticillium* colonies and also on the quantity and pigmentation of microsclerotia formed in culture. In order to determine whether this was the case with different isolates of the fungus, an experiment was performed involving three isolates of *V. dahliae*. The isolates used were 86 from England, 121 from Oregon, and 129 from California.

Shake cultures of the three isolates were diluted with water, then used to inoculate plates. All plates were incubated under identical conditions. Data on the effect of SPP on the number of colonies is given in Table 2.

Table 2. The effect of SPP on the number of colonies of different isolates of *V. dahliae*.

SPP G/liter	Number of colonies (a)		
	Number 86	Number 121	Number 129
0.00	11	21	9
0.25	13	22	9
0.50	11	19	7
1.00	10	22	7

(a) Average of five plates.

LSD for number 86	5% = 5
	1% = 7
LSD for number 121	5% = 5
	1% = 6
LSD for number 129	5% = 4
	1% = 5

In this experiment the spore concentration of the three isolates used in inoculation was not the same, therefore no comparison in number of colonies can be made among the three isolates. There was no significant difference, however, between the number of colonies of a specific isolate that developed on media containing different amounts of SPP, but differences in the size of the colonies, degree of pigmentation and abundance of microsclerotia in all isolates were impressive. Depending on the amount of SPP used, the colonies were more distinct and had darker and more abundant microsclerotia in comparison to ESA controls (Figure 2). There were differences in the length of incubation period required for the colonies to fully darken, depending on the isolate and the level of SPP in the media. The ESA control cultures of isolate No. 129 were slowest to form microsclerotia and required 16 days for the colonies to darken. In general, adding SPP to the media hastened the darkening of the colonies and microsclerotial formation. In all three isolates, the cultures with the highest amount of SPP required the shortest time for microsclerotial formation (five to seven days).

All cultures of the three isolates were examined under the microscope for formation of conidiophores. No conidiophores were observed in the ESA control cultures, while the cultures with 0.5 gram and 1.0 g/l SPP showed some conidiophore formation. Providing the colonies with a droplet of a nutrient solution containing

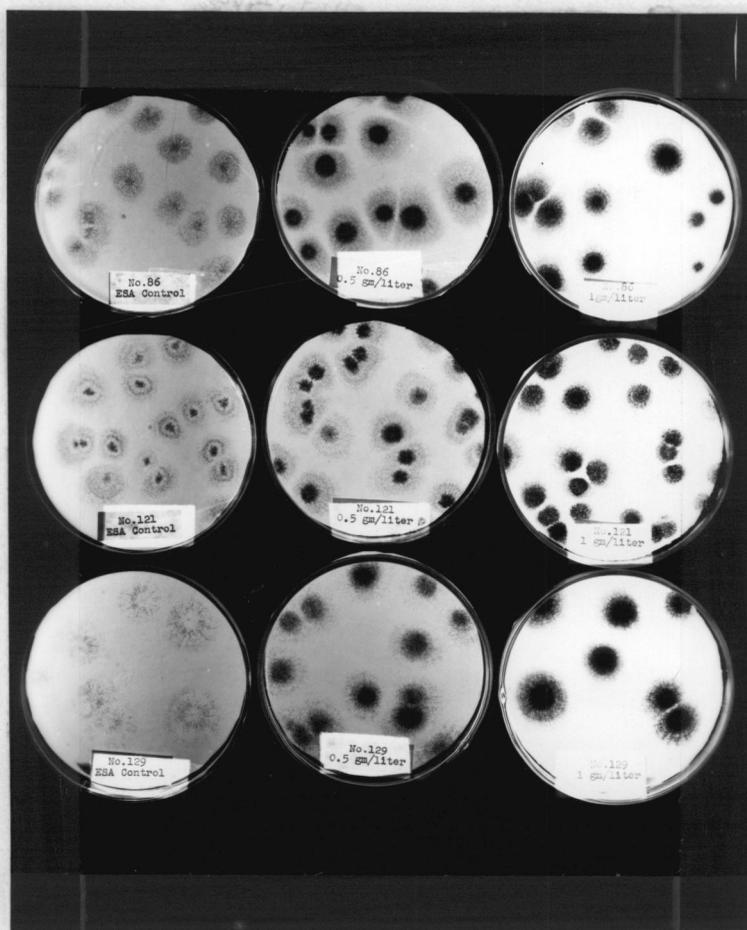


Figure 2. The effect of different concentrations of sodium polypectate on microsclerotial development by three isolates of *V. dahliae* growing in ethanol-streptomycin agar medium.

35 grams Czapek Dox broth and three grams yeast extract per liter, caused abundant conidiophore formation by all three isolates, including ESA controls. However, the intensity of conidiophore production was closely related to the amount of SPP in the medium, being maximum at the level of one g/l. The same results were obtained when the above procedure was used with V. dahliae colonies grown on SPP media in the presence of soil saprophytes.

Effect of Sodium Polypectate on the
Development of *Verticillium dahliae*
in ESA Medium Without Ethanol

Isolate 95 was used in an experiment to determine if SPP could be used to replace ethanol in the culture medium. One series of plates contained ESA plus varying amounts of SPP; another series contained only streptomycin in water agar with the same rates of SPP as the first. Equal amounts of shake culture inoculum were added to each plate and all plates were incubated under identical conditions. Data on the number of colonies is shown in Table 3.

In general there was no significant difference between the number of colonies developed on media with or without ethanol at any of the SPP levels tested. Also, no interaction was found between SPP and ethanol in relation to the number of colonies. The outstanding effect of ethanol was on the quantity and pigmentation of microsclerotia. More abundant and much darker microsclerotia

were formed in the cultures with ethanol and SPP than in those without ethanol or without SPP (ESA control). This effect can be seen clearly in Figure 3. The size of colonies, however, was not affected by ethanol at comparable levels of SPP.

Table 3. The effect of SPP and ethanol on the number of colonies of V. dahliae.

SPP G/liter	Number of colonies (a)	
	With ethanol	Without ethanol
0.25	28	35
0.50	31	25
1.00	29	28

(a) Average of three plates.

LSD for the effect of ethanol	5% = 6
	1% = 9
LSD for the effect of SPP	5% = 8
	1% = 11
LSD for the effect of different levels of SPP	5% = 11
	1% = 16

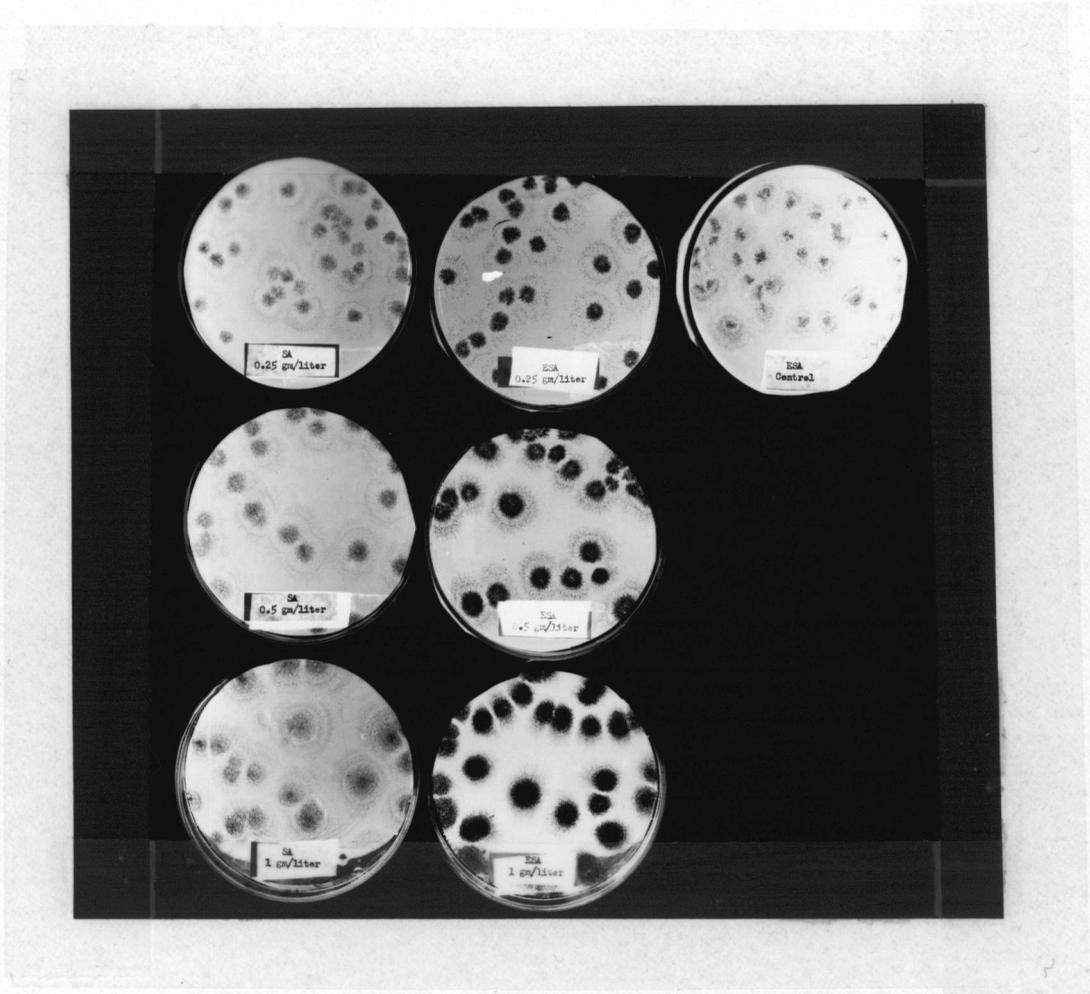


Figure 3. The effect of different concentrations of sodium polypectate on the development of *V. dahliae* with (ESA) and without (SA) ethanol in the medium.

Effect of Sodium Polypectate on Recovery
of *Verticillium dahliae* from
Infected Plants

Infected peppermint stems were finely chopped with a high speed blender in 100 ml water. The prepared suspension was used to inoculate the plates after dilution with distilled water. ESA medium containing various concentrations of SPP was poured and mixed with the stem inoculum. Plates were incubated at room temperature in the dark. Data on the number of colonies is presented in Table 4.

Table 4. The effect of SPP on recovery of *V. dahliae* from chopped infected peppermint stems.

SPP G/liter	Number of colonies (a)
0.0 (ESA control)	374
0.1	395
0.5	427*
1.0	430*

(a) Average of three plates.

LSD at 5% = 27

1% = 39

* Indicates a significant increase in the number of colonies at 1% probability compared to ESA control.

The number and appearance of colonies were affected by SPP in the medium. Cultures with 0.5 and 1.0 g/l SPP had significantly more colonies in comparison to ESA. The improvement in color, size, and sharpness of colonies was outstanding (Figure 4).

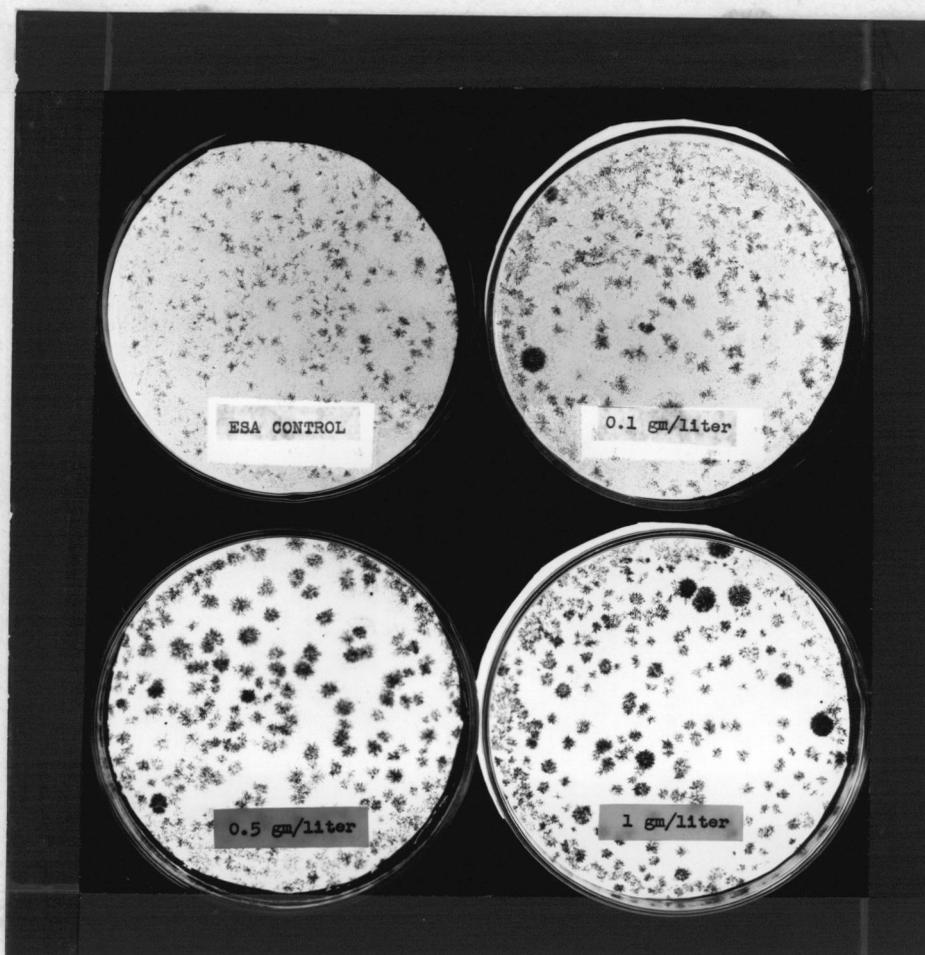


Figure 4. The effect of different concentrations of sodium polyepectate on recovery of V. dahliae from chopped infected peppermint stems in ethanol-streptomycin agar medium.

Effect of Sodium Polypectate on Recovery
of *Verticillium dahliae* from Soil
by Dilution Plates

To test the effect of SPP on the recovery of *V. dahliae* from soil in the presence of saprophytic soil fungi, the following experiment was performed. Plates were inoculated with equal amounts of spore suspension obtained by dilution of a shake culture of isolate No. 95. Prior to pouring the plates, 0.2 g natural soil was added to each flask containing 250 ml of ESA with desired levels of SPP. Data on the number of colonies of *V. dahliae* obtained is shown in Table 5.

Table 5. The effect of SPP on recovery of *V. dahliae* in the presence of soil saprophytic fungi.

SPP G/liter	Number of colonies (a)
0.0 (ESA control)	258
0.1	349
0.5	330
1.0	340
2.5	155*
5.0	176*

(a) Average of six plates.

LSD at 5% = 112

1% = 151

* Indicates a significantly lower number of colonies at 1% probability in comparison to lower levels of SPP.

The numbers of Verticillium colonies in plates containing 0.1, 0.5, and 1.0 g/l SPP were not significantly higher than those in the ESA control. Increasing the level of SPP in the media caused considerable increase in the growth of saprophytic fungi, mostly Penicillium sp. In the ESA control the growth of soil saprophytes was hardly detectable, while in the cultures with 2.5 and 5.0 grams per liter SPP their growth was very vigorous and extensive causing a significant reduction in the number of V. dahliae colonies observed. However, at all levels of SPP tested, the colonies of V. dahliae were sharp and distinct in contrast to soil saprophytic fungi and the pigmentation of microsclerotia was increased by increasing the amount of SPP in the medium.

In another experiment natural field soil was artificially infested with V. dahliae isolate No. 95 by mixing a shake culture of the fungus with the soil. The infested soil was mixed with 9, 49, and 99 parts of non-infested field soil and soil dilution plates were prepared using ESA alone and ESA plus various concentrations of SPP. Data on the recovery of V. dahliae propagules is presented in Table 6.

No significant difference was found between the number of colonies that developed on media containing SPP and the ESA controls. However, there was a considerable improvement in the size and degree of pigmentation of colonies growing on ESA plus

SPP (Figure 5). The soil saprophytic fungi (mostly Penicillium) also grew more vigorously with increased amounts of SPP.

Table 6. The effect of SPP on recovery of V. dahliae by soil dilutions.

Test number	Dilution treatments		SPP G/liter	Number of colonies (a)
	Dilutions with soil	Further dilutions with water		
One	1:10	1:1000	0.00	159
			0.25	156
			0.50	173
			1.00	153
Two	1:50	1:500	0.00	63
			0.25	74
			0.50	57
			1.00	62
Three	1:100	1:100	0.00	49
			0.25	58
			0.50	44
			1.00	46

(a) Average of three plates.

$$\text{LSD}_1 \text{ at } 5\% = 20$$

$$1\% = 29$$

$$\text{LSD}_2 \text{ at } 5\% = 24$$

$$1\% = 35$$

$$\text{LSD}_3 \text{ at } 5\% = 15$$

$$1\% = 21$$



Figure 5. The effect of concentration of sodium polypectate on the recovery of V. dahliae by soil dilution. Growth and micro-sclerotial development by V. dahliae is superior with 0.5 and 1.0 g/l of sodium polypectate, but growth of saprophytic soil fungi becomes stronger at 1.0 g/l, and at 2.5 and 5.0 g/l (not shown) is excessive.

Effect of Sodium Polypectate on Formation
of Microsclerotia by Verticillium dahliae
in Shake Culture

The effect of SPP on production of microsclerotia by V. dahliae in shake culture was studied using two kinds of media. One medium contained 0.1 percent Difco agar and the other contained 0.1 percent Czapek Dox broth. After a period of two weeks microsclerotia were produced in flasks containing water agar plus SPP with the intensity closely related to the level of SPP in the media. In order to break the clusters of microsclerotia into more or less individual structures, a 30-second fragmentation period in a high-speed Omni-Mixer was required. Data on the amount of microsclerotia produced in three weeks with different levels of SPP is presented in Table 7. The values given in this table were estimated by counting microsclerotia present in 0.1 ml of the diluted media on a filter paper. Figure 6 shows the comparison between the amounts of microsclerotia produced after three weeks. The materials in this figure were obtained by filtering 15 ml of the test media through glass filter papers. Conidia production of V. dahliae was observed also in the cultures. Quantity of conidia was related to the level of SPP in the media. More conidia were formed in cultures containing high levels of SPP.

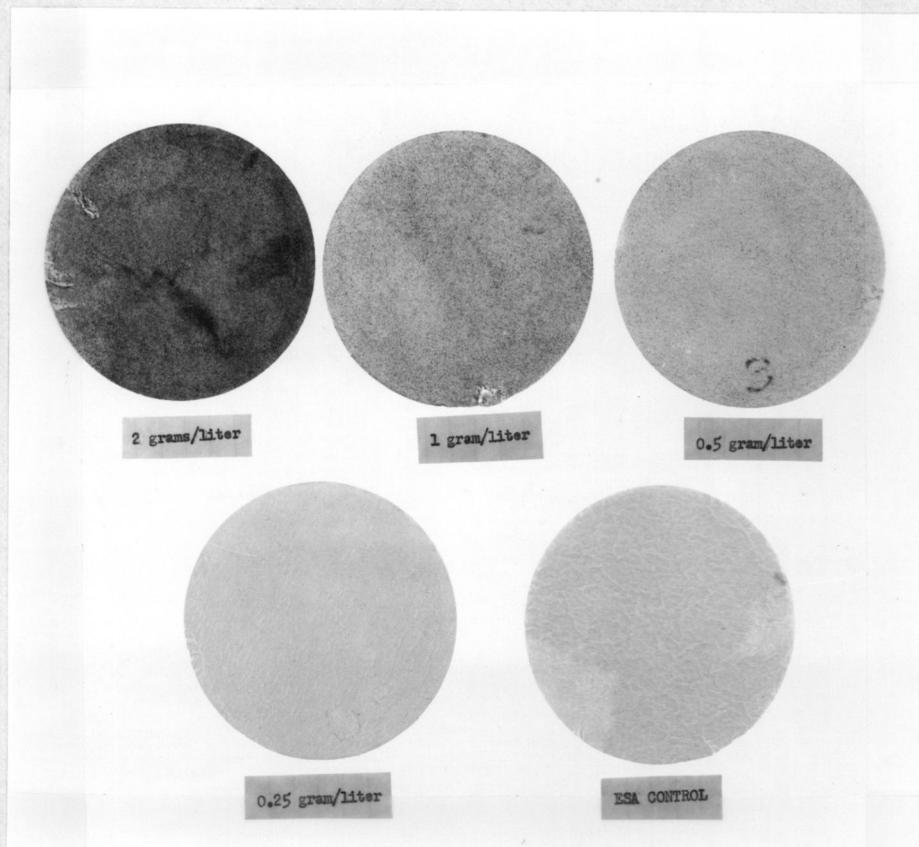


Figure 6. The effect of concentration of sodium polypectate on microsclerotial production by V. dahliae in shake cultures with the medium containing 0.1 percent agar, 0.76 percent ethanol, and 101 ppm streptomycin. The figure shows a comparison between the quantities of microsclerotia recovered by filtration of 15 ml of media.

Table 7. The estimated number of microsclerotia produced in ESA shake culture with different levels of SPP.

SPP G/liter	Number of microsclerotia per ml
0.0 (ESA control)	None
0.25	2,000
0.50	6,000
1.00	26,000
2.00	45,000

Microsclerotial production in Czapek-Dox was very slow and small in quantity in comparison to ESA cultures. The estimated number of microsclerotia produced in three weeks is shown in Table 8.

Table 8. The estimated number of microsclerotia produced in Czapek Dox shake culture with different levels of SPP.

SPP G/liter	Number of microsclerotia per ml
0.0 (control)	None
0.25	150
0.50	800
1.00	6,000
1.00 (without ethanol)	None
2.00	16,000

The quantity of microsclerotia was closely related to the amount of SPP in the cultures. When ethanol was excluded from the media containing one g/l SPP, no microsclerotia were produced. Growth of the fungus in this case was in the form of white mycelial pellets. Figure 7 shows a comparison of the amounts of microsclerotia produced after three weeks in 100 ml of Czapek Dox media with different levels of SPP. The few microsclerotia present in the control culture shown in Figure 8 are part of the original inoculum.

In ESA cultures many microsclerotia were formed individually. In contrast, microsclerotia in Czapek Dox were formed on the surface of pellets of varying sizes. Production of conidia was observed in all cultures with the quantity related to the amount of SPP in the media. Abundant conidia were formed at higher levels of SPP.

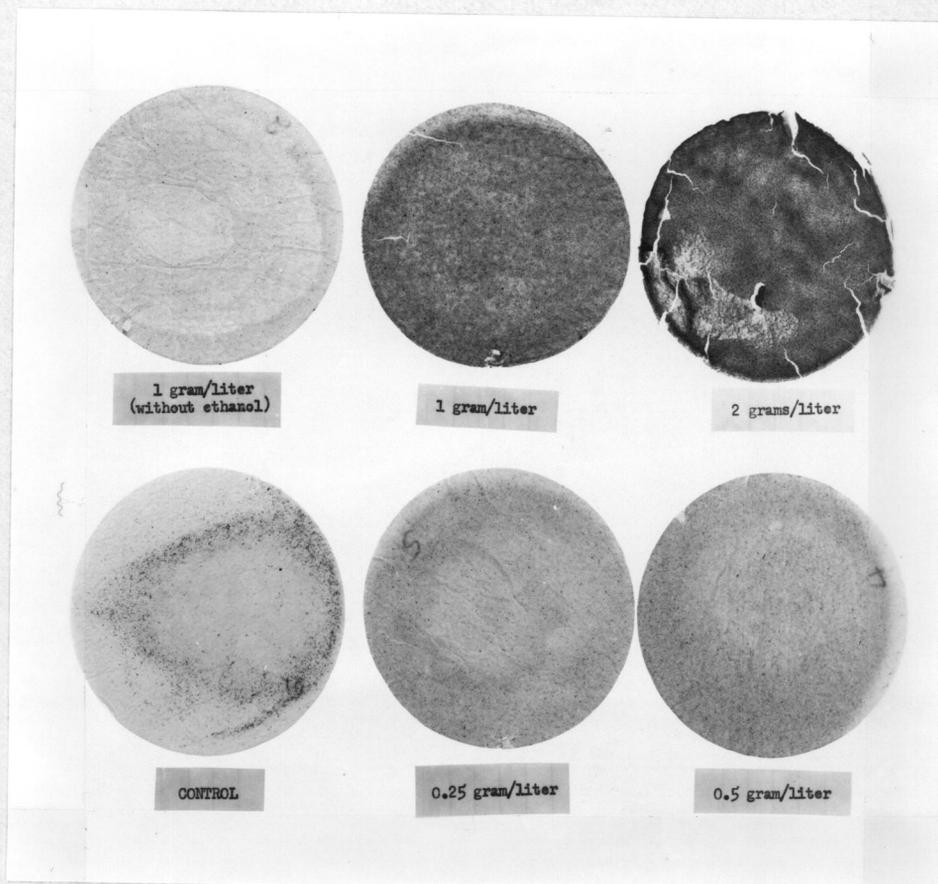


Figure 7. The effect of concentrations of sodium polypectate on microsclerotial formation of *V. dahliae* in 100 ml Czapek Dox broth in shake culture. Filter disc in upper left from medium without ethanol; filter at lower left from medium with ethanol but without sodium polypectate. Other filters show effect of various concentrations of sodium polypectate in Czapek Dox with ethanol.

DISCUSSION

Sodium polypectate added to ethanol- streptomycin agar medium improved greatly the formation and pigmentation of microsclerotia of V. dahliae. Adding SPP to ESA medium also improved the recovery rate of the fungus from cultures, soil, and infected plants. The results suggest that V. dahliae utilizes SPP primarily to form pigmented resting structures rather than vegetative mycelium.

In nature, V. dahliae forms microsclerotia primarily in parenchyma tissue of the host by saprophytic growth after death of the infected plant. Pectic substances and cellulose would be available as substrates in such tissue. The failure of cellulose to stimulate microsclerotia formation in culture suggests that pectic substances are the primary substrate of the fungus when it saprophytically invades dead parenchyma tissues in nature.

Several workers have shown that Verticillium albo-atrum and V. dahliae produce pectic enzymes in vitro and in vivo (1; 6; 7; 20; 29; 56). In culture the production of pectic enzymes increases greatly when pectic substances are added to the media. Kamal and Wood (20) discovered that the addition of pectin to the basal medium increased the growth of V. dahliae by about 25 percent. It appears that V. dahliae, by producing pectic enzymes, is able to hydrolyze

the pectic substances to fragments usable as a carbon source in the metabolism of the fungus. McIntyre (29) found that V. alboatrum produced at least two endo-polygalacturonases in culture. One endo-polygalacturonase hydrolyzed small fragments from the substrate. Wood (56) found that V. dahliae grew poorly in xylem sap of tomatoes because of the carbon shortage, but when SPP was added to the xylem sap growth improved greatly.

At high inoculum densities ESA containing 1.0 and 2.5 g/l SPP supported a significantly higher number of V. dahliae colonies than ESA alone (Tables 1 and 4). Repeated experiments showed the same results. This phenomenon can be related to the abundance of food supply, especially carbon source, provided by SPP in the media. In ESA medium alone spore germination, and consequently the number of colonies, were lower presumably because of a shortage of carbon and possibly other metabolites. It may also be true that in ESA the low number of colonies was caused by the principle of "self-inhibition" of the spores (5) at high inoculum density and that SPP overcame this factor. Since any tested amount of SPP at any inoculum density improved the growth and pigmentation of V. dahliae in comparison to ESA, it seems that the increase in the number of colonies can best be explained by the concept of increased food supply which was provided by SPP.

At lower inoculum densities, SPP did not cause any significant

increase in the number of colonies, and ESA without SPP apparently allowed the recovery of all viable propagules of the fungus (Tables 2, 3, and 6). However, adding SPP to the medium, even at levels as low as 0.25 g/l, increased the size, microsclerotial formation, and pigmentation of the colonies in every case. This was true with four different isolates of V. dahliae tested. All the colonies on ESA-SPP medium were very distinct. They could be observed and counted readily with the unaided eye. Another important effect of addition of SPP was the length of time necessary for darkening of the colonies. In general, depending on the level of SPP and the given isolate, the colonies turned dark five to seven days earlier than those on ESA alone. This would be of importance in experiments where a rapid quantitative study of V. dahliae is desired.

Colonies of V. dahliae were observed to form typical verticilliate conidiophores in culture when they were provided with a droplet of Czapek Dox plus yeast extract. The intensity of conidiophore formation was closely related to the level of SPP in the media. More abundant conidiophores were formed by increasing the level of SPP. The conidiophore formation of the colonies could be of great advantage in successful identification of V. dahliae in isolation procedures.

Ethanol was necessary for microsclerotial formation by V. dahliae in cultures with SPP, and it could not be replaced by

SPP. Although SPP improved the growth of colonies in cultures without ethanol, the quantity of microsclerotia was much lower in comparison to the cultures with ethanol and SPP (Figure 3). These results show that ethanol and SPP in a water agar media increase the growth of V. dahliae and also improve the production and pigmentation of microsclerotia.

In assays of infected peppermint stems SPP improved the recovery, pigmentation, and development of V. dahliae. The number of colonies that developed on ESA containing one g/l SPP were significantly higher than those on ESA control cultures.

In soil assays a different pattern was observed. Adding SPP to the media up to one g/l improved the recovery of V. dahliae and also stimulated growth of soil saprophytes. When the rate of SPP was increased to 2.5 or 5.0 g/l, the growth of soil saprophytic fungi, mostly Penicillium sp. , was so vigorous and rapid that it masked the growth of Verticillium colonies and caused a significant reduction in the observable colonies of V. dahliae (Table 6). From these results it seems that up to a certain concentration SPP could be used to improve the recovery and growth of V. dahliae in soil dilutions. At higher levels of SPP (2.5 and 5.0 grams), the amount of substrate allows for extensive growth of soil saprophytes which inhibit growth and mask the presence of V. dahliae. However, at any level of SPP tested, the developed colonies of V. dahliae were

more distinct in comparison to ESA controls and the pigmentation and size of the colonies were increased by increasing the level of SPP.

Microsclerotia production by V. dahliae was obtained in shake cultures by adding SPP to a 0.1 percent water agar or a 0.1 percent Czapek Dox solution. The intensity of production was always related to the level of SPP in the media. Water agar plus SPP yielded many more microsclerotia than Czapek Dox plus SPP at all tested levels (Tables 7 and 8). For good microsclerotia production the combined effect of SPP and ethanol appeared to be necessary. In shake cultures containing 0.76 percent ethanol, streptomycin, and 0.1 percent agar, microsclerotia did not form (ESA control, Figure 6). Also microsclerotia were not formed when ethanol was excluded from 0.1 percent Czapek Dox containing one g/l SPP, (Figure 7, without ethanol). This supports the results of the experiments discussed previously where excluding ethanol from the media reduced microsclerotial formation in solid agar cultures (Figure 3).

The promotive effect of SPP on the growth and development of V. dahliae can not be related to any specific fragment of this substance at present time. The structure and composition of pectic substances are not fully known at present. Therefore the only explanation for the effect of SPP would be that it provides a

selective carbon source and possibly other essential substances for the metabolism of the fungus.

The results obtained in this study have direct application in research on the biology of V. dahliae. The development of ESA medium by Nadakavukaren and Horner (31) permitted quantitative studies, not previously feasible, on the ecology of V. dahliae. These included survival in soil (31), saprophytic activity in soil (28), rhizosphere effects (24), and root infection (34). Improvement of ESA medium by addition of appropriate amounts of SPP will allow more refined studies in the same and in other facets of the biology of V. dahliae. The knowledge gained from such experiments should enhance our understanding of this important plant pathogen and eventually aid in its control.

SUMMARY

Sodium polypectate (SPP) was added to an ethanol-streptomycin agar (ESA) medium to determine if it would provide a selective substrate for the growth of Verticillium dahliae and for isolation of the fungus from soil and plant tissues in the presence of saprophytic fungi. The following results were obtained:

1. SPP added to ESA improved the recovery rate of V. dahliae from cultures, soil, and infected plants.
2. Increasing the level of SPP in the medium improved the size of V. dahliae colonies and also improved the formation and pigmentation of microsclerotia in four different isolates of the fungus.
3. Adding SPP to the medium hastened the darkening of the colonies by five to seven days depending on the isolate tested.
4. The intensity of conidiophore formation by V. dahliae in ESA was closely related to the amount of SPP in the medium.
5. In soil assays, adding up to one g/l of SPP to the medium improved the recovery of V. dahliae. When the rate of SPP was increased to 2.5 or 5.0 g/l, growth of soil saprophytic fungi was so vigorous and extensive that it masked the presence of Verticillium colonies and caused a significant reduction in the number of observable colonies.

6. Microsclerotial production by V. dahliae was obtained in shake cultures by adding SPP to a 0.1 percent water agar or a 0.1 percent Czapek Dox solution, both containing 0.76 percent ethanol and 101 ppm streptomycin sulfate. The intensity of production was always related to the level of SPP in the media. Ethanol-streptomycin agar plus SPP was superior to Czapek Dox plus SPP in microsclerotia production.

7. For good microsclerotial production the combined effect of SPP and ethanol appeared to be necessary in all experiments.

BIBLIOGRAPHY

1. Blackhurst, F. M. Induction of Verticillium wilt disease symptoms in detached shoots of resistant and susceptible tomato plants. *Annals of Applied Biology* 52:79-88. 1963.
2. Blackhurst, F. M. and R. K. S. Wood. Resistance of tomato plants to Verticillium albo-atrum. *Transactions of the British Mycological Society* 46:385-392. 1963.
3. _____ . Verticillium wilt of tomatoes--further experiments on the role of pectic and cellulolytic enzymes. *Annals of Applied Biology* 52:89-96. 1963.
4. Boyle, L. W. Verticillium wilt of mint in Oregon. *Plant Disease Reporter* 28:1095. 1944.
5. Cochrane, V. W. *Physiology of fungi*. New York, Wiley, 1958. 524 p.
6. Deese, D. C. and M. A. Stahmann. Role of pectic enzymes in susceptibility and resistance to Fusarium and Verticillium wilt of plants. (Abstract) *Phytopathology* 50:633. 1960.
7. _____ . Formation of pectic enzymes by Verticillium albo-atrum. *Phytopathologische Zeitschrift* 46:53-70. 1962.
8. Dimond, A. E. Pathogenesis in the wilt diseases. *Annual Review of Plant Physiology* 6:329-350. 1955.
9. Dobbs, C. G. and W. H. Hinson. A widespread fungistasis in soils. *Nature* 172:197-199. 1953.
10. Garrett, S. D. *Biology of root infecting fungi*. Cambridge, Cambridge University Press, 1956. 239 p.
11. Green, R. J., Jr. A preliminary investigation of toxins produced in vitro by Verticillium albo-atrum. *Phytopathology* 44:433-437. 1954.
12. Horner, C. E. Pathogenicity of Verticillium isolates to peppermint. *Phytopathology* 44:239-242. 1954.

13. Isaac, I. Verticillium wilt of sainfoin. *Annals of Applied Biology* 33:28-34. 1946.
14. _____. A comparative study of pathogenic isolates of Verticillium. *Transactions of the British Mycological Society* 32:137-157. 1949.
15. _____. The spread of diseases caused by species of Verticillium. *Annals of Applied Biology* 40:630-638. 1953.
16. _____. Studies in the interactions between species of Verticillium. *Annals of Applied Biology* 40:623-629. 1953.
17. _____. A further comparative study of pathogenic isolates of Verticillium: V. nubilum Pethybr. and V. tricorpus sp. nov. *Transactions of the British Mycological Society* 36:180-195. 1953.
18. _____. Wilt of lucerne caused by species of Verticillium. *Annals of Applied Biology* 45:550-558. 1957.
19. Jackson, R. M. Fungistasis as a factor in the rhizosphere phenomenon. *Nature* 180:96-97. 1957.
20. Kamal, M. and R. K. S. Wood. Pectic enzymes secreted by Verticillium dahliae and their role in the development of the wilt disease of cotton. *Annals of Applied Biology* 44:322-340. 1956.
21. Keyworth, W. G. Verticillium wilt of the hop (Humulus lupulus) *Annals of Applied Biology* 29:346-357. 1942.
22. Klebahn, H. Beitrage zur Kenntnis der Fungi Imperfecti. 1. Eine Verticillium Krankheit auf Dahliem. *Mycologisches Zentralblatt* 3:49-66. 1913.
23. Lacy, M. L. Influence of host and non-host plants upon population of Verticillium dahliae in soil. Ph. D. thesis. Corvallis, Oregon State University, 1965. 95 numb. leaves.
24. Lacy, M. L. and C. E. Horner. Reproduction of Verticillium in the rhizosphere and subsequent root infection of resistant and susceptible mint species. (Abstract) *Phytopathology* 52: 739. 1962.

25. Lockwood, J. L. Streptomyces spp. as a cause of natural fungitoxicity in soils. *Phytopathology* 49:327-331. 1959.
26. Ludbrook, M. V. Pathogenicity and environmental studies on Verticillium hadromycosis. *Phytopathology* 23:117-154. 1933.
27. Martinson, C. A. Active survival of Verticillium dahliae in soil. Ph. D. thesis. Corvallis, Oregon State University, 1964. 143 numb. leaves.
28. Martinson, C. A. and C. E. Horner. Importance of non-host in maintaining the inoculum potential of Verticillium. (Abstract) *Phytopathology* 52:742. 1962.
29. McIntyre, G. A. Production of pectic enzymes by Verticillium albo-atrum. Ph. D. thesis. Corvallis, Oregon State University, 1964. 86 numb. leaves.
30. McKay, M. B. Further studies of potato wilt caused by Verticillium albo-atrum. *Journal of Agricultural Research* 32:437-470. 1926.
31. Nadakavukaren, M. J. and C. E. Horner. An alcohol agar medium selective for determining Verticillium microsclerotia in soil. *Phytopathology* 49:527-528. 1959.
32. Nelson, R. Verticillium wilt of peppermint. *Plant Disease Reporter, Supplement* 50:474. 1926.
33. _____ . Verticillium wilt of peppermint. East Lansing, 1950. 259 p. (Michigan. Agricultural Experiment Station. Technical Bulletin no. 221)
34. Patil, S. S. , R. L. Powelson and R. A. Young. Relation of chlorogenic acid and free phenols in potato roots to infection by Verticillium albo-atrum. *Phytopathology* 54:531-535. 1964.
35. Pegg, G. F. and I. W. Selman. An analysis of the growth response of young tomato plants to infection by Verticillium albo-atrum. II. The production of growth substances. *Annals of Applied Biology* 47:222-231. 1959.

36. Porter, C. L. and R. J. Green. Production of exotoxin in the genus Verticillium. (Abstract) *Phytopathology* 42:472. 1952.
37. Reinke, J. and G. Berthold. Die Zersetzung der Kartoffel durch Pilze. Universitat Gottingen, Untersuchungen Botanisches Laboratorium 1:1-100. 1879.
38. Rovira, A. D. Plant-root excretions in relation to the rhizosphere effect. *Plant and Soil* 11:53-63. 1959.
39. Rudolph, B. A. Verticillium hydormycosis. *Hilgardia* 5:197-361. 1931.
40. Scheffer, R. P. et al. Physiological aspects of Verticillium wilt. *Phytopathology* 46:83-87. 1956.
41. Schreiber, L. R. and R. J. Green, Jr. Comparative survival of mycelium, conidia, and microsclerotia of Verticillium albo-atrum in mineral soil. *Phytopathology* 52:288-289. 1962.
42. _____. Effects of root exudates on germination of conidia and microsclerotia of Verticillium albo-atrum inhibited by the soil fungistatic principle. *Phytopathology* 53:260-264. 1963.
43. Sewell, G. W. F. Direct observation of Verticillium albo-atrum in soil. *Transactions of the British Mycological Society* 42:312-321. 1959.
44. Soloveva, A. I. and L. V. Polyarka. Wilt of cotton. Tashkent, U. S. S. R., Tashkent Agricultural Publishing Department, Uzbekistan Soviet Republic, 1940. 63 p. (Abstracted in *Review of Applied Mycology* 27:19. 1948)
45. Talboys, P. W. The possible significance of toxic metabolites of Verticillium albo-atrum in the development of hop wilt symptoms. *Transactions of the British Mycological Society* 40:415-427. 1957.
46. _____. Some mechanisms contributing to Verticillium resistance in the hop root. *Transactions of the British Mycological Society* 41:227-241. 1958.

47. Thomas, J. Factors relating to the development and germination of certain fungus sclerotia. Ph. D. thesis. Lafayette, Ind. Purdue University, 1952. 96 numb. leaves.
48. Threlfall, R. J. Physiological studies on the Verticillium wilt disease of tomato. *Annals of Applied Biology* 47:57-77. 1959.
49. Wilhelm, S. The effect of temperature on the taxonomic characters of Verticillium albo-atrum R. & B. (Abstract) *Phytopathology* 38:919. 1948.
50. _____. Vertical distribution of Verticillium albo-atrum in soils. *Phytopathology* 40:368-376. 1950.
51. _____. Is Verticillium albo-atrum a soil invader or soil inhabitant? (Abstract) *Phytopathology* 41:944-945. 1951.
52. _____. Some factors affecting substrate colonization and growth of the Verticillium wilt fungus in soil. (Abstract) *Phytopathology* 45:696. 1955.
53. _____. Longevity of the Verticillium wilt fungus in the laboratory and field. *Phytopathology* 45:180-181. 1955.
54. _____. A sand culture technique for the isolation of fungi associated with roots. *Phytopathology* 46:293-295. 1956.
55. _____. Parasitism and pathogenesis of root-disease fungi. In: *Plant pathology, problems and progress 1908-1958*, ed. by C. S. Holton. Madison, University of Wisconsin Press, 1959. p. 356-366.
56. Wood, R. K. S. Verticillium wilt of tomatoes - the role of pectic and cellulolytic enzymes. *Annals of Applied Biology* 49:120-139. 1961.