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Title: COMPARISON OF PECTIC ENZYMES IN RESISTANT AND

SUSCEPTIBLE MINTS INFECTED BY VERTICILLIUM

DAHLIAE

Abstract approved:

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Verticillium dahliae Kleb. incites a vascular wilt disease of

mints. Susceptible Mentha piperita L., resistant M. crispa L.,

and intermediate hybrid 148 were used to study 1) the site of differ-

ential resistance and 2) the role of polygalacturonase (PG) in the

disease syndrome.

Many more V. dahliae propagules were recovered from surface

sterilized, finely fragmented, susceptible mint roots than from resis-

tant roots. Therefore, the vascular system of susceptible mint was

more extensively invaded than resistant mint. Increase of Verticilli-

um in the stems was dependent on the extent of root invasion; sever-

ity of foliar symptoms, in turn, was related to pathogen prolifera-

tion in stems. These results indicate that the roots are the first

important site of differential resistance.

Inoculation through excised shoots showed that mint stems differ in their resistance to <u>Verticillium</u>. Although <u>Verticillium</u> increased similarly in stems of shoot inoculated resistant and susceptible mints, severe symptoms developed only in susceptible <u>M</u>. <u>piperita</u>; resistant <u>M</u>. <u>crispa</u> developed only slight symptoms.

PG activity was detected only by the reducing group assay in extracts from diseased mint prepared without a phenolic adsorbent, polyvinylpyrrolidone (PVP). In extracts prepared with PVP, PG was detected by both the viscosity reduction and reducing group assays. Enzyme extracts prepared late in disease when phenol oxidase activity was high showed considerable decrease in viscosity reducing power but not a decrease in reducing groups released from substrate. These results indicate that <u>Verticillium</u> produced an exo-PG in mint that was less affected by phenol oxidation products than the endo-PG.

Increase of <u>Verticillium</u> and PG production was similar in shoot inoculated resistant and susceptible mints. Foliar symptoms developed only in susceptible <u>M</u>. <u>piperita</u> and intermediate resistant hybrid <u>148</u>. No foliar symptoms occurred in resistant <u>M</u>. <u>crispa</u>. Resistance of mint to <u>Verticillium</u> apparently is not due to inactivation of fungal PG.

PG production in mints during disease development showed no relationship between symptom severity and PG activity in infected

mint stems. A nonpathogen of mint produced PG activity in mint similar to that produced by Verticillium but no wilt symptoms developed. Hypotheses regarding the contribution of PG to wilt symptoms depend on the formation of PGs in diseased plants that hydrolyze pectic substances to large fragments. Enzyme extracts prepared from plants as symptoms increased, liberated a high number of reducing groups from substrate while the viscosity reducing power decreased. This indicated that large pectic fragments are not formed in plants. It was proposed that the role of PG in the Verticillium wilt of mint disease is to provide an additional carbon source for the pathogen.

Comparison of Pectic Enzymes in
Resistant and Susceptible Mints
Infected By Verticillium dahliae

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

COMPARISON OF PECTIC ENZYMES IN RESISTANT AND SUSCEPTIBLE MINTS INFECTED BY VERTICILLIUM DAHLIAE

INTRODUCTION

Verticillium wilt, caused by Verticillium dahliae Kleb. (=V. albo-atrum var menthae Nelson), was the primary factor in the migration of commercial peppermint, Mentha piperita L., production from the midwest into Washington and Oregon. The disease was first reported on the West Coast by Boyle in 1945 (14). Since then wilt has become progressively severe and now constitutes the most serious threat to continued production of high quality peppermint oil in the Pacific Northwest.

V. dahliae persists in the soil almost indefinitely after it becomes established in a field. It infects mint plants by penetrating the roots, ramifies within the cortex and later invades the xylem systemically to the apex of the stem. Infection is generally followed by wilting and death of part or all the shoot system. After the plant dies, the fungus develops saprophytically, forms microsclerotia, and is returned to the soil in crop debris.

Control of wilt by crop rotation is ineffective because of the long survival of microsclerotia in soil and the nonpathogenic invasion of nonhost plants. Chemical soil treatment, although effective, has not been used because it is cheaper to plant new land than to

treat soil chemically. Resistant varieties would be the most effective method to control Verticillium wilt of mint. However, resistant varieties now available do not produce desirable oils. Therefore, further studies into the nature of the disease and of resistance to Verticillium wilt of mint are necessary to provide a sound scientific basis for disease control.

The work presented in this thesis was done to extend our knowledge of the Verticillium wilt syndrome and had the following objectives:

1. Quantitatively determine the proliferation of Verticillium in stems and roots of susceptible, intermediate resistant and highly resistant mints and relate this to symptom severity.

Previous work (55) has shown that Verticillium appears to penetrate the roots of resistant species nearly as readily as susceptible species and can be isolated throughout resistant plants. Determining quantitative differences in the build-up of Verticillium in the stems and roots of the different strains of mint would provide information on the site of resistance and the importance of pathogen build-up in contributing to disease severity.

2. Determine if the advance in wilt symptoms is related to the amount of polygalacturonase (PG) produced by the pathogen in the host and in turn if PG is related to the amount of fungus within the plant.

Assays of diseased plants for hydrolytic enzymes usually contrast conditions in healthy and diseased plants after symptoms are well developed. Tissue maceration occurs late in wilt diseases and is not a diagnostic symptom even though it may be easier to isolate pectic enzymes from this late stage of disease (104, p. 307). If wilt symptoms result from the activity of fungal PG, then the advance in symptoms should be related to the amount of fungus in the plant and in turn to PG activity in infected stems.

3. Compare PG activity in infected susceptible and resistant mints in relation to the amount of Verticillium present.

Susceptibility to Verticillium wilt has been correlated with formation of PG on tomato stem tissue by Deese and Stahmann (24, 25, 27). Inhibition of the formation of this enzyme on resistant tissue was associated with resistance. However, one big weakness in their experimental design was that the amount of fungus on the various tissues was not taken into consideration. I wished to measure the amount of fungus in relation to the amount of enzyme.

4. Determine the phenol oxidase activity of resistant and susceptible mints, before and after infection, and note the effect of phenol oxidase extracts and oxidized and unoxidized phenols on PG activity.

In several instances, a high phenol oxidase activity was correlated with disease resistance (17, 24, 68). It has been proposed

that the oxidation products of phenols are toxic directly to the fungus
or to enzymes necessary for pathogenicity (53, p. 22). Oxidation
products of phenols have been shown to inhibit pectic enzymes in
some diseases (17, 18, 72). Information on the effects of phenol
oxidase extracts and various phenols on PG activity might provide
further insight into the defense mechanism of resistant plants.

REVIEW OF LITERATURE

Penetration and Infection

Penetration refers to the initial invasion of the host by an organism and does not imply a successful infection (102, p. 6). In the vascular wilts a successful infection necessarily includes invasion of the xylem.

Few critical studies have been carried out on the penetration and infection of hosts by pathogens. However, host resistance to many potential pathogens may be determined at this stage and a better understanding of it could provide a new insight into disease control (35, p. 256). According to Nelson (70, p. 65-69), Verticillium enters roots of peppermint by direct penetration of surface cells along the young root or through wounds made by emerging lateral roots, tillage implements, or insects.

Talboys (95) found the splitting apart of hop cells by V. albo-atrum to be a mechanical rather than an enzymatic process. In cotton infected by V. albo-atrum, Garber and Houston (36) noted that direct cell penetration was sometimes accompanied by appresorium-like swellings followed by peg-like hyphae projections where the fungus passed through the wall. They also observed that where cells of the epidermis or cortex were invaded by a mass of hyphae, cell

breakdown occurred, but if invasion was limited to only a few

hyphae, the cells remained intact.

After entry, the infection hyphae grow through intervening

tissues to the xylem and penetrate the xylem. In order to accom-

plish this growth, food must be obtained from the tissues invaded.

The principal components of the cell wall are cellulose, mucilage,

pectic substances, lignin, hemicelluloses, suberin and cutin (88, p. 145,

33, p. 33-63). An invading organism such as Verticillium must there-

fore have enzyme systems to break down the components present in

the wall, or the ability t o obtain food by diffusion if infection is to

be successful (88, p. 145). Once Verticillium penetrates the xylem,

its subsequent growth is limited mainly to the vascular elements

until the later stages of the disease when it ramifies throughout the

host (70, p. 67).

The Disease Syndrome

In most wilt diseases, the first symptoms are epinasty of the

petioles followed by progressive wilting from older to younger

leaves. The entire plant eventually collapses and dies (29, p. 330).

Nelson (70, p. 40-62) discusses symptom expression in pepper-

mint in detail. One of the earliest observable symptoms is a short-

ening of internodal spaces near the tip of the shoot giving the plant

a stunted appearance. Terminal leaves are small and may become

chlorotic or bronzed.

The asymmetrical development of young leaves is the most typical diagnostic symptom of Verticillium wilt of peppermint. The leaves on a peppermint shoot are arranged in alternating bifoliate whorls with each member of a pair attached to the opposite side of the stem. Unilateral development of the young leaves, which curl and twist toward or away from each other, destroys the symmetry of the young shoot. This type of symptom is apparently due to unilateral infection of vascular bundles on one side of the leaf, allowing only one side of the leaf to develop normally.

Whether or not the plants wilt in the later stages of disease depends on how severely the plant was initially infected (93, 95). When wilting doesn't occur, the leaves are stunted and curled downward; they become necrotic and eventually fall from the plant.

It is generally agreed that in the vascular wilt diseases an infected plant suffers from a derangement in water balance and that the pathogen triggers this and other abnormalities by the release of metabolic products. However, there is much controversy as to the means by which this is brought about. Three hypotheses have been presented to account for wilting in Verticillium and Fusarium wilt diseases: 1) root damage, 2) foliar damage by toxins and 3) impaired water transport in conductive elements. The first hypothesis can apparently be discarded as there is rather conclusive evidence

that root damage does not play a significant part in wilting. Kramer
(52, p. 481-492) found that absorption of water by a dead root system
was faster than a living one when the root systems cut from tops were
connected to a vacuum pump and the rate of water absorption meas-
ured in terms of the water collected above the cut end of the stem.
A comparable system is involved in an intact plant. The roots of
Verticillium infected plants sometimes show no external symptoms
and die only when the plant dies (70, p. 58).

Evidence for the second hypothesis has been quite unsatisfac-
tory (29, p. 329-350). Some workers claim to have isolated toxins
from culture filtrates of Verticillium which cause wilting (40, 70,
p. 94, 85, 94) but these claims must be treated with caution because
the wilting of a shoot is by no means a specific response (52).
Talboys (94) showed that the action of toxic substances in culture
filtrates in inducing symptoms in hops was not related to the patho-
genicity of the strain of the fungus or to the wilt tolerance of the
hop variety used.

Impaired Water Transport in Conductive Elements

There is considerable evidence that dysfunction of conductive
elements causes a reduction of water movement through vessels of
diseased stems causing a water shortage in the leaves. This may
be brought about by various physical and chemical causes, such as

9

mycelium and spores, tylosis, gums and gels, and gas embolism. The gas embolism theory is based entirely on CO_2 production of the pathogen in culture (29, p. 332). Many vessels have been found to be gas-locked in healthy plants (76, p. 257-321). Thus, the amount of gas liberated by the pathogen would be small relative to that freed by living host cells (29, p. 332). Tylosis may also be discarded as contributing to wilting in Verticillium wilt of pepper-mint since tylosis seldom occurs in this disease.

Mycelium. The role of mycelium in obstructing water flow in vessels is undecided. The distribution of organisms in vascular tracheae is generally found to be vertical rather than lateral and, therefore, the chances of the vessels getting blocked by the lateral spread of the pathogen are negligible and vessels that are not infected initially remain comparatively free of mycelium (92, p. 313-348). Generally, workers have found many more vessels free of mycelium than are invaded in diseased plants and mycelium alone could not account for vascular occlusion (29, p. 332; 70, p. 70).

Using scale models, Waggoner and Dimond (29) showed that the transpiration stream has laminar and not turbulent flow. There-fore, they concluded that obstacles in vessels do not interfere by increasing turbulence but instead cause a small resistance to flow by the frictional drag created. The resistance to flow created if all the vessels contained mycelium would contribute to water shortage

in a diseased plant but could not be the sole cause.

Pectic Substances. From the time of de Bary (5), it has been assumed that secretion of extracellular enzymes and toxins play an important role in pathogenicity of fungi on higher plants. De Bary demonstrated the presence of an active principle in extracts of tissues parasitized by Sclerotinia libertiana which produced disease. Since boiling of these extracts destroyed their activity, he concluded that the effect on the cell was due to an enzyme. The nature of the killing substance was less clear and de Bary left that question open.

Bewley (11) found in culture filtrates of Verticillium albo-atrum a substance that could be precipitated by addition of an equal volume of 95% ethyl alcohol and was capable of inducing a wilt reaction in tomato stems. The activity after resuspending in water could be inactivated by heating at 100° C for five minutes. He showed the presence of several hydrolytic enzymes in the 30-day-old culture but did not find pectic enzymes.

Porter and Green (75) originally reported that the toxic fraction in Verticillium albo-atrum was a complex polysaccharide but later Green (40) reported that a proteinaceous material was responsible for wilting of tomato cuttings and a polysaccharide responsible for vascular discoloration.

The results of Kamal and Wood (50) with young cotton plants and V. dahliae were similar to those of Scheffer et al. (87) with

V. albo-atrum and tomato plants. Vascular browning was associated

with a thermolabile substance, presumably a protein and possibly a

pectic enzyme. Wilting seemed to be caused by a thermostable

substance. When Scheffer et al. examined Verticillium for hydro-

lytic enzymes similar to those produced by Fusarium, they discov-

ered that Fusarium produced much pectinmethylesterase (PME) and

polygalacturonase (PG), whereas Verticillium produced PG but very

little PME. PME demethylates pectinates, and PG hydrolyzes the

α -1,4 glycosidic linkages of the pectic substances.

Wood (105) reported the secretion of pectic and cellulolytic

enzymes by V. albo-atrum on a variety of synthetic media. He

showed that culture filtrates with high PG activity produced some

of the symptoms found in naturally diseased plants. Filtrates from

cultures on dead tomato stems, however, had relatively low PG and

cellulolytic activities.

Information from studies of culture filtrates of organisms

grown on synthetic liquid media suggest, then, that extracellular

pectolytic enzymes might function in Verticillium and Fusarium

diseases. However, Waggoner and Dimond (101) note that the

data were obtained by inducing wilt symptoms from unpurified

culture filtrates and thus fail to satisfy Koch's postulates, i.e.,

identification in the host, purification, and production of symptoms

by the pure material. Therefore, the mere demonstration that

culture filtrates contain components that lead to wilting of cuttings is not sufficient to show that these components are involved in the syndrome of a wilt disease.

The postulation of an in vivo role of pectic enzymes in vascular wilt diseases requires evidence of their presence in diseased plants. Thus, Waggoner and Dimond (101) identified PME and possibly PG in Fusarium diseased tomato. Further, they discriminated between PME produced by the fungus and that present in healthy tomato plants, and detected PME of fungal origin in diseased plants. Matta and Dimond (67) also found increased PME in Fusarium infected tomato plants but detected no PG.

Wood (105), studying Verticillium wilt of tomato, demonstrated that when shoots wilt, the activity of PME increases. PME was concluded to be of host origin as Verticillium produces very little PME in culture. Wood failed to detect PG in infected stems.

Deese and Stahmann (27) obtained evidence for a PG that produced monogalacturonic acid in tomato stem tissue inoculated with V. albo-atrum. From sap of potato plants inoculated with V. albo-atrum, they (28) isolated a PG that produced monogalacturonic acid and large pectic fragments. Blackhurst and Wood (13) obtained a preparation with weak PG activity from susceptible and resistant tomato varieties inoculated with V. albo-atrum, but did not characterize these enzymes. In contrast to Blackhurst and Wood, Deese

and Stahmann (24), failed to detect PG on resistant tomato tissue.

Young (106) and McIntyre (62) present the first conclusive evidence of PG in Fusarium and Verticillium infected tomato plants. Both of these workers found PGs that carried hydrolysis to mono-galacturonic acid.

Two hypotheses have been presented regarding the role of pectolytic enzymes in the wilt syndrome. Gothoskar et al. (38) suggested that a depolymerase PG is produced in the vessels of infected plants. The enzyme diffuses out of the vessels and hydrolyzes the pectic substances in the middle lamella and primary cell walls of xylem parenchyma. Partially hydrolyzed pectic fragments diffuse into the vessels, increase the viscosity of the vascular sap, and eventually form gels which slow the transport of water. Impaired upward flow of water in vessels then causes the diseased plants to wilt.

Beckman et al. (8) suggested that formation of gels in vessels of the roots could block the opening in the vessel end-walls and thus prevent upward movement of the pathogen. The application of this hypothesis depends on spread of the pathogen through its host as microconidia. Before the pathogen can advance from one vessel element to the next, the microconidia must germinate and penetrate the pectic gel at the vessel end-wall. This could be done mechanically or by dissolving the plug through the action of pectic enzymes.

Acceptance of these hypotheses depends on proof that PGs

produced by the pathogen hydrolyze pectic substances to large

fragments that could form gels. From studies to date, it appears

that both Fusarium and Verticillium produce PGs in intact plants

that carry hydrolysis to monogalacturonic acid.

Vascular Discoloration

Vascular browning consists of the deposition of dark pigments

in the cell walls of vessels and in those of the surrounding xylem

parenchyma. The relationship between phenolics and browning

in Verticillium and Fusarium infected plants has been well estab-

lished (22, 23, 29, 41). According to existing data, phenolic mater-

ials occur in xylem primarily as glycosides (22, 41, 100). As host

cells become disorganized through the action of the pathogen, glyco-

sides may be hydrolyzed, phenols liberated and these move to living

cells where they are oxidized to various colored products, including

melanins and tannins (23, 41).

Although several workers have found a high positive correla-

tion between the extent of vascular discoloration and foliar symp-

toms of wilt diseases (29, 67, 103), the role, if any, that these

phenol oxidation products play in the wilt syndrome is not firmly

established. Vascular discoloration products could possibly take

part in the occlusion of conducting vessels (23, 29, 100). Corden

and Chambers (20) suggest a more indirect role for phenol oxidation

products. They propose that these substances prevent lateral move-
ment of water and thus a single occlusion would render a vessel non-
functional for axial translocation. Gubanov (41) hypothesizes that
newly formed aglycones and quinones have a toxic action on the in-
fected plant and eventually cause wilting and death. The slowly dying
cells lose their capacity for osmotic regulation, and as a result
materials pass out of them and into the vessels. These materials
are then used by the fungus.

Resistance

Resistance mechanisms can be classified as passive, in which
the defense factor is already present prior to infection, or active,
in which defense factors develop in the host as a response to infec-
tion (1, 2).

The active type of resistance seems to be a more general resis-
tance mechanism. The importance of metabolic activities in disease
resistance was indicated by the lowering of resistance to both obligate
and faculative parasites by narcotics and inhibitors of oxidative meta-
bolism (98).

Active resistance does not depend on an accumulation of fungi-
toxins in the healthy plant but on the ability of the plant to produce
toxins or antitoxins in response to infection (1, 2). Some parasites
have developed means of by-passing these host reactions by

inactivation of toxins or fail to induce their formation. Successful

invasion then involves a disruption of normal host mechanisms which

regulate oxidative pathways and prevent undue expenditures of re-

serves while still maintaining the cell's life processes and growth.

The food reserves and intermediate products then become available

to the parasite (42). Thus, the aggressiveness of the parasite, the

presence of inhibitors and toxic substances produced by either the

host or the parasite and the availability of required nutrients at the

infection site determine successful invasion (42).

Mint species are available which are resistant to Verticillium

wilt but none produces mint oil of acceptable commercial quality.

Although the mechanism of wilt resistance has been studied very

little in mint, resistance has been studied in hop, tomato and

potato. In a soil-borne fungus, such as Verticillium, resistance

may take the form of exclusion in the rhizosphere or at the root

surface of the host plant, between the root surface and the vascular

elements and within the vascular system.

Exudates and the Rhizosphere as Factors in Resistance

Rhizosphere population of pathogenic fungi in host and non-host

plants have received much attention recently because increase of

pathogens in the rhizosphere could lead to greater inoculum potential

for infection, and in non-host plants could provide an important

mechanism of survival for the pathogen in the absence of host plants (91, p. 340-341). The reasons for preferential stimulation of micro-organisms in the rhizosphere of some plants and its relation to re-sistance is still a matter of conjecture. Although hypotheses have been presented concerning the occurrence and selective action of toxic substances in exudates against pathogenic fungi, no adequately confirmed evidence is available to indicate that toxic components of exudates are a major factor contributing to disease resistance.

Lacy and Horner (56) observed that susceptible mint species supported similar populations of V. dahliae in the rhizosphere, indicating that susceptible mint species do not selectively stimulate the pathogen near the root surface. Also it was shown that Verticil-lium penetrates the root surface of resistant mint plants nearly as readily as the root surface of susceptible mints. Other workers report similar observations (3, 36, 93). Thus, resistance to Verticillium wilt of peppermint does not appear to be located in the rhizosphere or at the root surface.

Inhibition Between the Root Surface and the
Vascular Tissue as a Factor in Resistance

Garber and Houston (36) traced the progress of Verticillium in resistant and susceptible cotton roots and found that growth of the fungus was identical until the time of initial xylem colonization.

Significant differences in fungal growth were observed as the disease

progressed from the initial xylem colonization. Other workers have

reported similar results (3, 93).

With mint, Lacy and Horner (56) showed that when roots of

susceptible and resistant mint were finely fragmented to release the

contents of the vascular system, many more Verticillium propagules

were recovered from susceptible mint roots than from the resistant

roots. The vascular systems of susceptible mint roots were invaded

to a much greater extent than those of resistant mint.

Movement Within the Vascular System

There have been several studies on the movement of wilt fungi

in resistant and susceptible varieties, and varieties have been com-

bined by grafting to obtain information on the site of resistance.

Keyworth (51), Harris (43, p. 37-43) and Talboys and Wilson

(96) using intervarietal grafts, have shown that resistance in hops

to Verticillium apparently depends upon the characteristics of the

root system. Resistant vines grafted on susceptible rootstocks

showed severe wilt symptoms, and direct stem inoculations caused

severe symptoms in both resistant and susceptible varieties.

Keyworth (51) concluded that the progress of a wilt disease was

like a chain reaction in which the effects of root invasion governed

the extent of stem invasion which in turn governed the severity of

leaf symptoms. Stem invasion is an essential link in the chain and leaf symptoms cannot be caused solely by the action of the fungus in the roots.

In cotton, Garber and Houston (36) noted a pronounced difference in xylem colonization between a resistant and susceptible variety. The susceptible variety was more extensively and intensively colonized, vessels often were packed with free-floating conidia. Leaf vessels of susceptible cotton also were invariably more heavily colonized than those of resistant cotton.

Blackhurst and Wood (12) found no evidence with a resistant and susceptible tomato variety that resistance was localized in the roots. In plants inoculated through the shoots or roots, the pathogen spread rapidly up the stem of both resistant and susceptible varieties despite the fact that only slight disease symptoms appeared in resistant plants. In the susceptible variety, this rapid spread was always closely associated with vascular browning, but in the resistant variety there was much less browning. Microscopic examination showed that the fungus moved almost immediately into the petiole of the susceptible variety accompanied by vascular browning but in the resistant variety the lower petioles were invaded to a lesser degree and the upper petioles remained free of the pathogen. Dimond and Edgington (30) observed that a 50% reduction in the effective radius of the vascular bundles of the petioles reduced water transport 500

times more effectively than a similar reduction in the stem.

From the results of experiments with inoculated resistant
and susceptible cross-grafted tomato plants, Blackhurst and Wood
(12) also showed that shoots as well as roots were resistant; this
agrees with the work of Snyder et al., (89) and Scheffer (83, 84)
with cross-grafts of tomato plants resistant and susceptible to
Fusarium oxysporum f. sp. lycoperscii.

Berry and Thomas (10) and Horner (47) present evidence that
the site of differential resistance to Verticillium in mint is located
in the stem as well as in the roots. Using symptoms as a criterion
for degree of invasion, Berry and Thomas found that unrooted and
rooted plants responded similarly to inoculation by V. albo-atrum.
Horner reported that non-virulent strains of Verticillium progress
up mint stems less than virulent ones, both in root-inoculated or
stem-inoculated plants.

Inhibitory Substances as Factors in Resistance

To prove that an inhibitory substance is associated with resis-
tance to a particular disease, it must be isolated from resistant
hosts at the site where infection occurs and then be reintroduced
into susceptible hosts where it must confer resistance (2). It may
act by inhibiting spore germination, host penetration, or production
of toxins and enzymes by the fungus. Existence of fungitoxic plant

substances does not necessarily denote resistance. For example,

23 percent of the plant extracts obtained from 1,915 species of

angiosperms were found to be fungitoxic to Ventura inaequalis,

the causal agent of apple scab (99). All extracts prepared from

apple were inactive whether from resistant or susceptible trees.

The material may inhibit both virulent and avirulent strains, it may

not be present in sufficient quantity at the infection site, or it may

be more plentiful in susceptible than resistant hosts (42).

Inactivation of Fungal Toxins. Pringle and Scheffer (78, p.

133) define a host-specific toxin as a metabolic product of a patho-

genic microorganism which is toxic only to the host of that pathogen.

To date host-specific toxins have been found for Helminthosporium

victoriae, Alternaria kikuchiana and Periconia circinata.

A toxin is produced only in wilt-susceptible tomato varieties

by V. albo-atrum even though the fungus invades resistant plants

and spreads up the stem without inducing disease symptoms(12).

Since cell-free culture filtrates produce disease symptoms in both

resistant and susceptible varieties, resistant plants may somehow

reduce toxin formation by the fungus.

Phenols and Related Compounds. Almost every infection or

mechanical damage induces phenol accumulation including anthocy-

anins, leucoanthocyanins, flavonoids, aromatic amino acids, glycos-

ides, coumarin derivatives, alkaloids and terpenoids (65). Because

of the general occurrence of phenols at infection and wounding sites,

and because many phenols and their oxidation products (quinones)

exhibit antibiotic properties, it is evident why researchers have

often implicated these compounds with disease resistance mechan-

isms in plants (42). Kuc' (53) believes that the basic role of phenolics

in disease resistance of plants is to stop growth of the pathogen since

limited growth of the pathogen is one observation common to all

resistance mechanisms.

Resistance to Verticillium wilt of potato is correlated with

phenolic metabolism (58, 64, 73). McLean et al. (64), using histo-

chemical tests for phenols, found that potato varieties resistant to

Verticillium wilt contained more chlorogenic acid than susceptible

varieties. Chlorogenic acid concentrations of 2500-10,000 ppm were

reported in the vascular systems of potato plant roots. Since germi-

nation of V. albo-atrum spores was significantly reduced at 50 ppm,

they believed that chlorogenic acid in the vascular system of plants

could inhibit the pathogen after its entry. Lee and LeTourneau (58)

and Patil et al. (73), however, showed that V. albo-atrum could grow

in 1,000 ppm of chlorogenic acid in still culture, and in fact, could

utilize chlorogenic acid as a sole source of carbon. Patil et al.

found 60-day-old plants of resistant as well as susceptible varieties

invaded systemically by V. albo-atrum, which would not be possible

with 2500-10,000 ppm chlorogenic acid in the vascular system if

chlorogenic acid per se was toxic. Also, Patil et al. reported very little difference in chlorogenic acid concentration with a highly resistant and highly susceptible potato variety. It was postulated that because quinones are highly toxic, even at low concentration, the quinone form of chlorogenic acid was responsible for resistance of young potato plants. The quinones may be produced when the pathogen wounds the cortical root tissue during entry.

Many phenols and related compounds have been tested for fungitoxicity (59). Against Verticillium albo-atrum, pyrogallol (a trihydroxyphenol) was the most toxic phenol. Catechol was the most toxic dihydroxyphenol. Quinones were more toxic than phenols and their toxicity was increased by chlorination. The most toxic material tested, 2,3-dichloro-1,4-naphthoquinone, was inhibitory at less than 1 ppm.

The importance of phenol oxidase in the phenolic defense mechanism has already been emphasized. When this enzyme is inhibited, resistance is decreased (71). Oxidation of phenols to quinones should improve resistance, but further polymerization may or may not favor resistance (42). Resistance in cotton to V. dahliae and Fusarium vasinfectum (41) and in tomato to F. oxysporum (68) was related to greater amounts of toxic phenols and their oxidation products, and to greater phenol oxidase activity in resistant than in susceptible plants.

Enzyme Inhibition. Inhibition of pectic enzymes by compounds of phenolic origin has been reported extensively (18, 19, 49, 72). Oxidized phenols (quinones) have been found to be particularly inhibitory.

There is some evidence to support the concept of resistance due to the presence or appearance of compounds not necessarily directly fungitoxic but that have the ability to inactivate the extracellular enzymes of a fungus attempting to invade the plant. Studies on the nature of resistance of apple to Sclerotinia fructigena (17) revealed that resistance may result from inhibition of pectic enzymes by oxidized phenols. The relationship between the rate of browning of injured tissues and their resistance to infection indicated that the phenol-phenol oxidase system might be involved as a defense mechanism. Phenol-containing extracts from resistant tissue did not exert an inhibitory effect on spore germination. Not even oxidation of the extracts led to formation of toxic products. However, the extracellular pectolytic enzymes of Sclerotinia fructigena, necessary for its pathogenicity, were markedly inhibited by phenolic compounds and particularly by their oxidation products. Resistance in this case depended on the interaction of the host's phenol-phenol oxidase systems with the pectic enzymes of the pathogen and this resulted in the inhibition of tissue-macerating activity.

Deese and Stahmann (24) correlated susceptibility to

Verticillium wilt with the formation of PG. On stem tissue from susceptible tomato plants PG was formed whereas on resistant tomato tissue, PG was inhibited. The inhibition or suppression of the enzyme on resistant tissue appeared to be associated with a high oxidizing power found in stems of resistant varieties. They hypothesized that resistance to Verticillium was due to quinone-like substances that inhibit the pectic enzymes of the invading pathogen. Patil and Dimond (72) found Verticillium PG to be inhibited by quinones in vitro.

Thus, there is circumstantial evidence that inactivation of pectolytic enzymes contributes to the resistance mechanism of plants.

GENERAL METHODS AND MATERIALS

Preparation of Culture Filtrate Enzyme and Inoculum

Isolate 95 of Verticillium dahliae, collected from Mentha piperita, in 1964 by Dr. C. E. Horner, Corvallis, Oregon was used in this study. Stock cultures of the isolate were incubated at 25° C on potato-dextrose agar (PDA) in test tubes and the tubes were stored at 4° C after sufficient growth of the fungus.

Culture Filtrate

Verticillium was grown in Erlenmyer flasks in a modified Richard's medium containing the following salts: 5 g NH_4NO_3, 2.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $ZnSO_4$, and 0.06 g $FeCl_3$ per liter. The carbon source was one percent (wt/v) Sunkist Growers pectin N. F. (Product No. 3442). Ten grams of pectin were added to 500 ml of mineral solution containing all the salts except KH_2PO_4 and mixed for one minute in a Waring Blendor. One hundred ml of KH_2PO_4 were placed in 250 ml Erlenmyer flasks and 100 ml of the mineral solution containing the pectin were placed in 500 ml Erlenmyer flasks. The flasks were autoclaved, cooled and sufficient 1.0 N NaOH was added to the KH_2PO_4 solution to give a final pH of 5.0 when the KH_2PO_4 solution was added aseptically to the pectin mixture.

The medium was inoculated and the cultures were grown for six days on a shaker. At the end of this period, the medium was combined aseptically into one flask, and aliquots removed and plated in PDA to check contamination. The remainder of the culture filtrate was squeezed through cheesecloth and centrifuged for 20 minutes at 6500 rpm. The supernatant was filtered through Whatman No. I paper on a Büchner funnel and then passed successively through 1.2 μ, .8 μ, and .45 μ pre-sterilized Millipore filters.

Toluene (1 ml/l) was added to the culture filtrate to prevent contamination. The filtrate was placed in dialysis tubing and dialyzed against running tap water for 16 hours and distilled water for six hours. The filtrate was checked for contamination by plating in PDA and then frozen until used.

Inoculum

To obtain inoculum, Verticillium was grown in Erlenmyer flasks in 200 ml of Czapek-Dox medium with 5% (wt/v) yeast extract for seven days. Bud cells were removed from the medium by centrifuging at 6000 rpm for 20 minutes. The supernatant was discarded, the bud cells washed on a Büchner funnel using Whatman No. 3 filter paper, and the cells resuspended in distilled water. If plants were to be inoculated by root or stem dipping, the bud cell suspension was adjusted to 5×10^{6} cells/ml. If infested soil was to be

used as inoculum, the cell suspension was mixed with soil, the

mixture covered, and after seven days incubation passed through a

screen. This inoculum was mixed with natural soil with the aid of a

concrete mixer at the rate of 50,000 to 100,000 viable propagules

per gram of soil.

Assay Procedures

Fungus Assays

Vascular proliferation of Verticillium in stems and roots was

estimated by fragmenting surface sterilized tissue in a Servall Omni-

mixer. Macerated tissue was diluted into ethanol-streptomycin

sodium polypectate agar (ESPA) for detection of Verticillium (107).

Colonies were counted two weeks later for estimation of fungus.

Determination of Polygalacturonase
Activity by Viscosity Reduction

One percent (wt/v) sodium polypectate was added to 0.1 M

acetate buffer (pH 5.0), and mixed for one minute in a Waring

Blendor. Five ml of pectate were then placed in an 18 X 120 mm

tube and 5 ml of enzyme added. The reaction mixture was agitated

on a Vortex Mixer for about 20 seconds and then 8 ml of the mixture

were pipetted into an Ostwald-Fenskie No. 300 viscosimeter sus-

pended in a waterbath at 30° C ± 0.1°. The first viscosity reading

was taken one minute after addition of the enzyme to the substrate.
Additional readings were made periodically, depending on enzyme
activity. The time (seconds) necessary to reduce the viscosity of
the substrate by 20 or 50 percent was designated as n-R_{20} and n-R_{50},
respectively. Enzyme activity was expressed as the reciprocal of
this value multiplied by 10^6.

Determination of Polygalacturonase by Reducing Group Method

Liberation of reducing groups from pectate was determined by
Nelson's modification of the Somogyi method (69, 90). Substrate was
prepared by mixing one percent (wt/v) sodium polypectate with 0. 1 M
acetate buffer (pH 5. 0) in a Waring Blendor for one minute. Fresh
Copper Reagent was prepared by combining four parts of Somogyi
Reagent I with one part of Somogyi Reagent II. At zero time 5 ml
of enzyme were combined with 5 ml of substrate and agitated for
approximately 20 seconds by a Vortex Mixer. Then a two ml sample
was removed and added to two ml of the Copper Reagent in a Folin-Wu
tube. The reducing group result obtained with this sample and a
boiled enzyme were the controls for the experiment. The reaction
mixture was incubated at 30° C. Two ml samples were withdrawn
periodically from the enzyme reaction mixture to follow the course
of substrate hydrolysis. Tubes containing the Copper Reagent and

enzyme reaction mixture were capped with aluminum foil and placed

in a boiling water bath for 20 minutes. The tubes were then removed

from the boiling bath, foil caps removed and tubes allowed to cool

ten minutes. Two ml of arsenomolybdate reagent (69) were added to

each tube and agitated until fizzing stopped. The mixture was diluted

to exactly 25 ml with distilled water, mixed on a Vortex Mixer and

filtered through Whatman No. 4 paper into 8 inch tubes. Optical

densities (O.D.) were then determined at 520 mμ. Samples were

diluted within the O.D. range 0.100 to 0.600 so that more accurate

readings could be made. A standard curve was prepared by plotting

O.D. against concentration of monogalacturonic acid. The activity

of the PG was expressed as micrograms (μg) of reducing groups

(as monogalacturonic acid) released in one hour from 100 milligrams

(mg) of substrate by the enzyme from five grams fresh weight of

plant tissue.

Determination of Polygalacturonase
by the Cup-plate Method

A cup-plate assay was used for rapid determination of PG

activity (32). One gram of ammonium oxalate, two grams of sodium

polypectate and three grams of agar were mixed in a Waring Blendor

in 200 ml of 0.1 M acetate buffer (pH 5.0). The mixture was placed

in a 500 ml flask, covered with cheesecloth and aluminum foil, and

autoclaved for five minutes at 15 psi. Hot media was poured into a prepared plate consisting of a 10 X 10 inch piece of glass with one inch pieces around the outside edges. When the medium had solidified, a No. 3 cork-bore was used to cut cups.

One-tenth ml of enzyme was pipetted into each cup using a long tipped measuring pipette. After filling the cups, the plate was topped with a single glass plate. A paper towel, saturated with water, was layed on top of the plates and the plate placed in a sealed double plastic bag. Plates were incubated at 37° C for 14 hours.

Following incubation, HCl (1:2 dilution of concentrated HCl) was poured over the plate. After approximately three minutes, the plates were rinsed with cold water. The diameter of the cleared zone was measured with calipers and recorded. Diameters were converted to enzyme activity by running a dilution of the enzyme and plotting zone diameter against log of the enzyme concentration.

Polygalacturonase Assay by Paper Chromatography

The presence of breakdown products of pectic substrates was determined by the chromatographic method of Young (106). Fifty and 100 μl aliquots of enzyme-substrate mixtures were withdrawn at predetermined intervals, and spotted on Whatman No. 4 paper. The chromatograms were developed using an ascending solvent system containing 60 mg of sodium formate and 50 mg of brom

phenol blue dissolved in a mixture of 15 ml of 88 percent formic acid and 85 ml of 77 percent ethanol. Chromatograms were removed from the solvent after the solvent front had migrated approximately 30 cm from the origin. The wet, yellow chromatographs were hung in a fume hood to dry. The developed chromatographs showed bright yellow spots of pectic breakdown products on a blue background.

Determination of Pectinmethylesterase Activity

The method of Glasziou (37) was used to measure activity of PME in plant extracts. Substrate was prepared by mixing 0.5 percent pectin N.F. with distilled water in a Waring Blendor for one minute. The mixture was filtered through Whatman No. 1 paper on a Büchner funnel. One hundred ml of substrate and 3 ml of 1.0 M $CaCl_2$ were placed in a 250 ml beaker containing a stirring bar. The electrodes of a Beckman pH meter were inserted and the mixture adjusted to pH 7.5 with 1 N NaOH. Ten ml of extract were added to the mixture and the pH of the enzyme substrate mixture was readjusted to 7.5 and a timer started. The pH was adjusted continually to pH 7.2-7.5 during the course of the assay by adding 0.005 N NaOH from a burette. This assay was carried on for four hours, and the total ml of NaOH were recorded. A control consisted of 10 ml of boiled extract substituted for the active enzyme sample. The titration difference in ml of alkali required by active

extract and that of the heated control multiplied by the concentration

of alkali equaled the milliequivalents (meq) of acid released by

enzymatic action. PME activity was expressed as microequivalents

(meq × 1000) of carboxyl groups released in one hour from 500 mg of

substrate by the enzyme from one gram fresh weight of plant tissue.

Determination of Phenol Oxidase Activity

Tissue was cut into 3-5 mm sections and then homogenized

for one minute in 0.1 M (pH 7.0) potassium phosphate buffer con-

taining 0.001 M ethylenediaminetetraacetate in a Servall Omni-

mixer. The chamber of the omni-mixer was immersed in an ice

bath. Three ml of buffer were used per gram fresh weight of tis-

sue. The homogenate was strained through a flour sack, the filtrate

centrifuged in a refrigerated Servall for five minutes at 3800 g and

the resulting supernatant again centrifuged at 17,000 g for 20 min-

utes. The supernatant constituted the crude enzyme extract.

A colorimetric procedure was used to determine phenol oxidase

activity (67). One-tenth ml of extract was added to 10 ml of a freshly

prepared 0.5 percent catechol solution. Activity was measured at

400 mμ in terms of the rate of formation of brown pigment in the

interval between 30 and 90 seconds after the addition of the extract.

Preparation of Plant Material

Three mint genotypes differing in resistance to Verticillium were used: Mentha piperita (susceptible), M. crispa (resistant) and hybrid 148 (intermediate). Hybrid 148 was obtained from a cross of M. piperita × M. crispa (70). The three mint clones were main- tained in soil beds in the greenhouse. Cuttings from vigorously growing shoots were rooted in sand. Two to three-week-old cuttings were used in all tests where roots were inoculated.

At harvest time, plants were cut at the ground-line, side branches removed and stems weighed. Roots were washed 24 hours with running tap water, blotted dry and placed on paper towels until enough excess imbibed water evaporated to return them approxi- mately to their original state. The roots were then weighed.

RESULTS

Phenol Oxidase Assays

Phenol Oxidase Activity in Healthy Stems

Because high phenol oxidase activity in resistant plant stems
is correlated with PG inhibition (24), phenol oxidase activity was
determined in the stems of the three mint varieties. Rooted cuttings
of M. piperita, hybrid 148 and M. crispa were potted in soil and
grown in the greenhouse. After four weeks, the stems were col-
lected, extracted and assayed for phenol oxidase activity.

There was about three times as much phenol oxidase activity in
stems of M. crispa as in M. piperita and about twice as much in
M. crispa as in hybrid 148 (Figure 1). The three mint types were
suitable, then, to test the hypothesis that wilt resistance may be
due to high phenol oxidase activity of resistant stems forming prod-
ucts which inactivate the PG of the invading pathogen.

Phenol Oxidase Activity in Infected M. piperita

Matta and Dimond (67) found that phenol oxidase activity
increased rapidly with progress of disease in Fusarium infected
tomato, and they found a high positive correlation of phenol oxidase
activity with severity of symptoms and the amount of fungal mycelium.
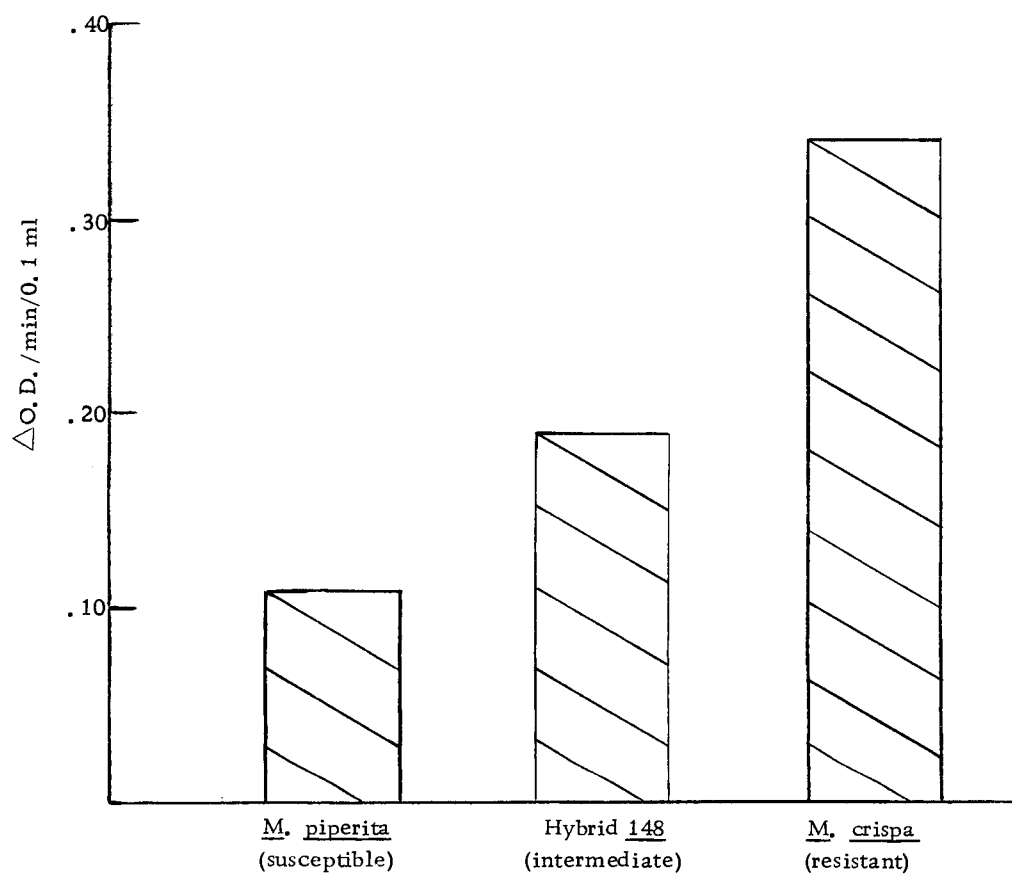
Figure 1. Phenol oxidase activity (absorbence change $[\triangle O. D.]$ /min per
0. 1 ml of extract at 400 mμ) measured in extracts of healthy
mint stems.

During extraction of PG from diseased tissue, the enzyme would be
exposed to these phenol oxidase reaction products. Thus, changes
in phenol oxidase activity with progress of disease in Verticillium
infected M. piperita were studied. The relationship of phenol oxidase
activity and quantity of fungus to symptom development was deter-
mined in this experiment.

Rooted cuttings of M. piperita were potted in infested soil in
the greenhouse. Control plants were potted in noninfested soil at
the same time. After inoculation, triplicate plants were periodically
harvested as severity of symptoms progressed. Four harvests of
stems and roots were made. The severity of symptoms was assessed
when plants were harvested using the following scale: 0 = no symp-
toms, 1 = mild symptoms (asymmetry of leaves on the shoot apex),
2 = moderate symptoms (down-curled, dying leaves), 3 = severe
symptoms (severe stunting of shoot, many dead leaves). The amount
of fungus in stems was estimated from a 12 mm sample taken from
the basal part of diseased stems and from three 10 mm sections of
roots. Crude phenol oxidase extracts were prepared from the remains
of the roots and from 15 cm samples from the bases of stems.

Phenol oxidase activity in stems of uninoculated plants remained
uniform throughout the experiment (Figure 2). Phenol oxidase activ-
ity and fungus proliferation increased in a similar fashion with prog-
ress of disease in inoculated plants; both increased rapidly with
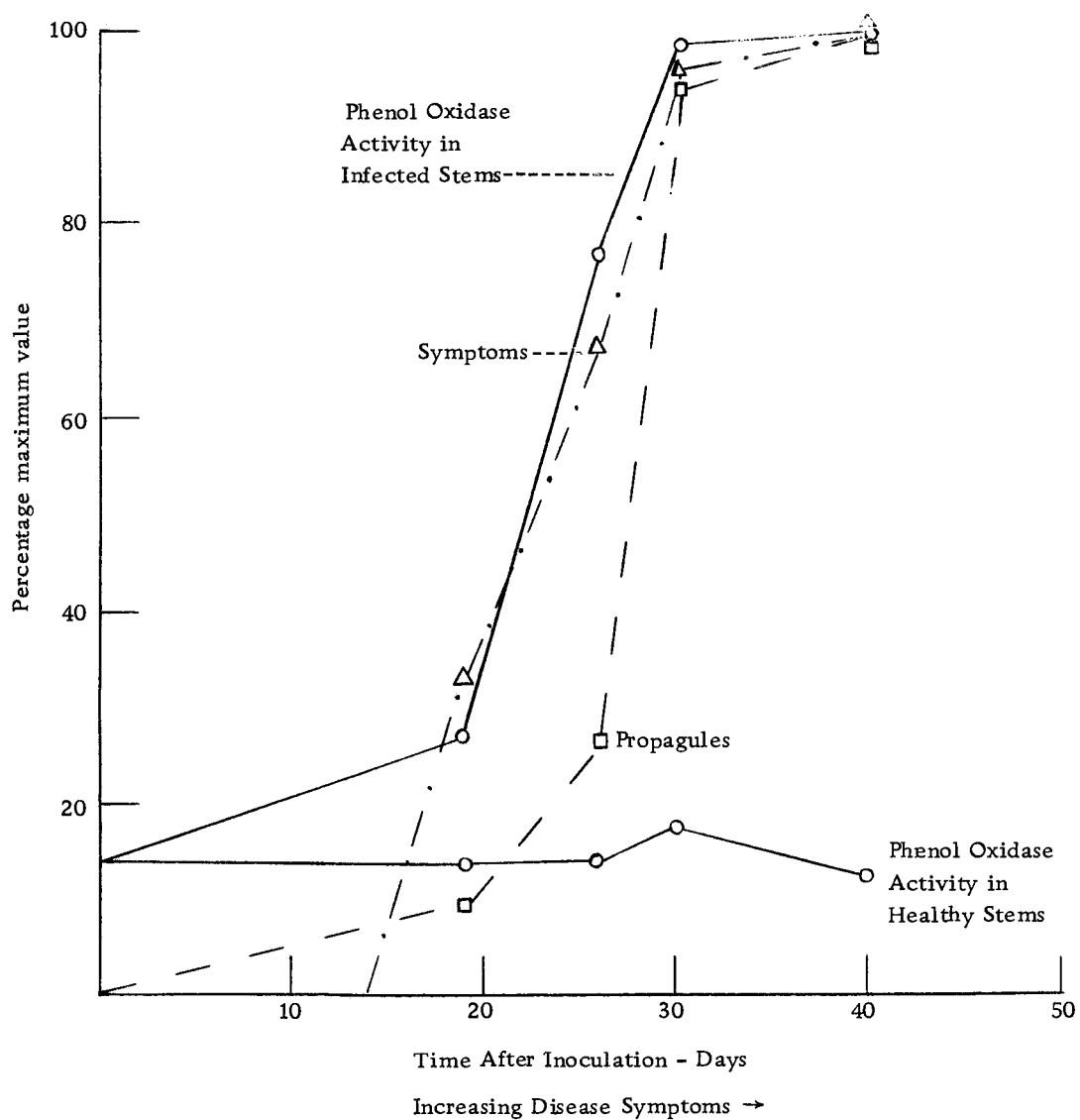
Figure 2. Development of symptoms, phenol oxidase activity and propagule numbers associated with disease in stems of M. piperita. Data are expressed as a percentage of the maximum values attained. Phenol oxidase activity in healthy stems is expressed as a percentage of the maximum value attained in infected stems. Maximum values: phenol oxidase, change in absorbence $[\triangle O. D.]$ /min from 50 mg of substrate by the enzyme from 0.10 gr fresh wt of plant tissue, 1.98; propagules/gr fresh wt of plant tissue, 3,516,000; symptoms, 3.

symptom development and leveled off when symptom expression

reached a maximum. This is not to imply that there is a cause and

effect relation between appearance of symptoms and increased

phenol oxidase activity, but merely that both are measures of the

effect of fungal invasion of the stem.

Fungal invasion of the roots of M. piperita had little influence

on phenol oxidase activity (Figure 3). Patil et al. (73) also found

that infection of potato roots by Verticillium had little effect on

phenol oxidase activity.

Interaction Between Crude Phenol Oxidase
Extracts and Polygalacturonase Activity

Plant extracts may inhibit PG (16), particularly when the

extracts have high phenol oxidase activity (18, 19, 54). Because of

the high phenol oxidase activity in stem extracts from M. crispa and

infected M. piperita, the effect of crude phenol oxidase extracts on

culture filtrate PG was determined.

PG from culture filtrates of Verticillium was incubated 14

hours with crude phenol oxidase extracts from stems of diseased

M. piperita, healthy M. piperita, healthy hybrid 148 and healthy

M. crispa. PG activity was assayed by viscosity reduction and cup-

plate methods. No differences were observed in PG activity of these

mixtures and that of an equivalent mixture of the enzyme with distilled
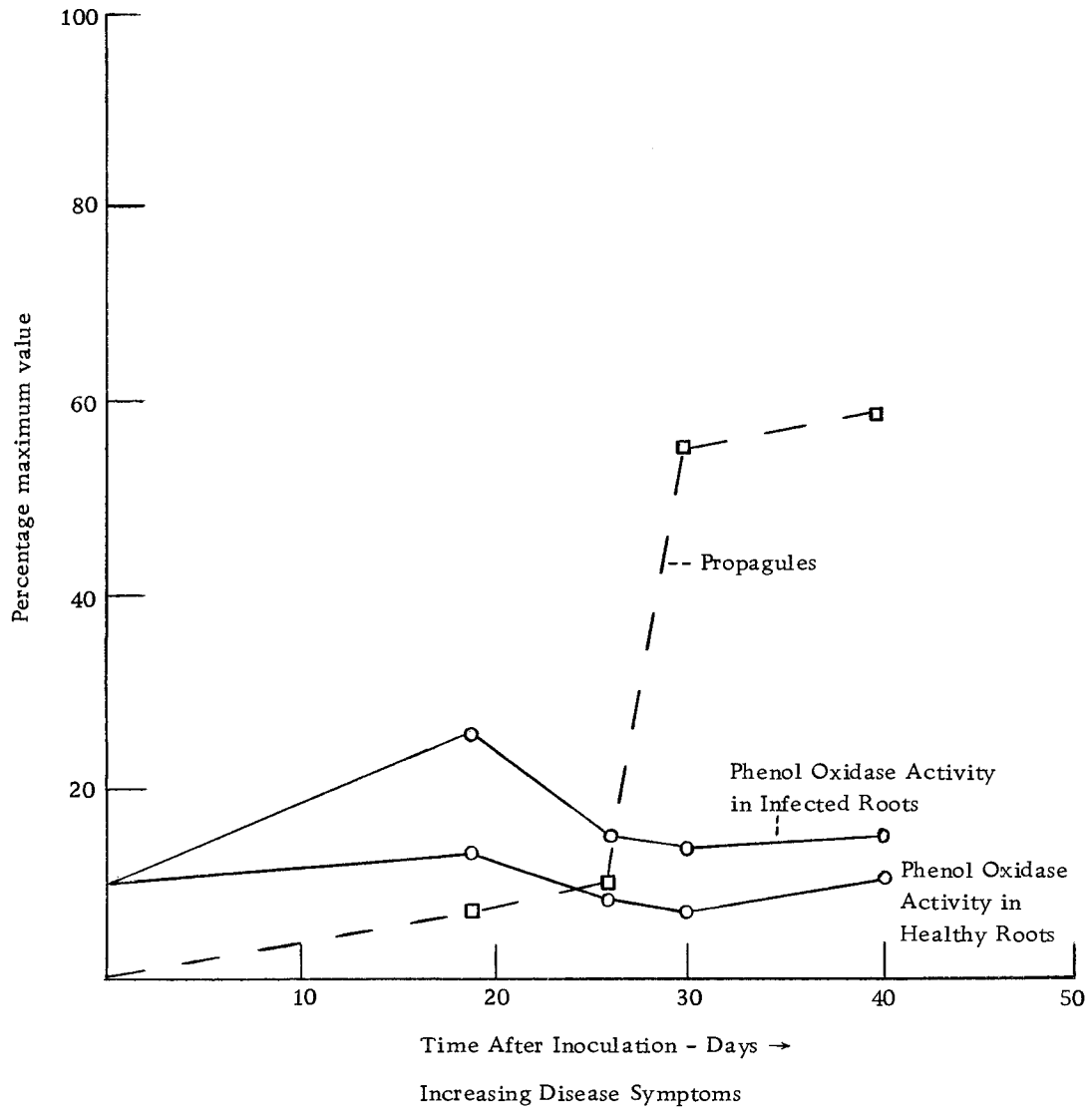
Figure 3. Development of phenol oxidase activity and propagule numbers associated with disease in roots of M. piperita. Data are expressed as a percentage of maximum values attained in stems (see Figure 2).

water. Blackhurst and Wood (12) and Matta and Dimond (67) also
found that extracts from tomato did not inactivate PG in vitro. I
assumed that inhibitors to PG were not present in mint extracts.

Polygalacturonase and Fungus Assays of Field Grown Mints

This phase of the study had two main objectives: (1) determine
if advance in wilt symptoms is related to PG activity and quantity of
Verticillium and (2) determine if PG formation is inhibited in resis-
tant mint plants.

Deese and Stahmanns' (24) technique of culturing the fungus on
propylene oxide sterilized host tissue was attempted but the method
proved of no value. They recommended 3-4 hours under propylene
oxide to sterilized tomato tissue, but mint tissue could not be steri-
lized in this time. Longer sterilization periods resulted in death
of the mint tissue which defeated the purpose of the experiment.
In all of the remaining experiments, extracts from intact plants
were used for enzyme analysis.

Preliminary Experiments

Several preliminary experiments were done to find a method
for extraction of PG from infected plants. Of all the extraction
methods used, only the reducing groups assay detected PG activity.
This presented a dilemma because Verticillium produces PGs in

culture that attack the pectic chain randomly and thus reduce the viscosity of the substrate rapidly. Chromatograms of reaction mixtures were of little value in characterizing the enzyme from plant extracts because of considerable streaking and buffer interference. There did appear to be a very light yellow spot on chromatograms that migrated the same distance as the monogalacturonic acid standard. Streaking on chromatograms made it uncertain if other breakdown products were present on chromatograms. Verticillium has been reported to form an exo-PG (terminal hydrolysis) on tomato tissue by Deese and Stahmann (24), and studies with other fungi report differences between PG from diseased plants and culture filtrates (6, 106). It was possible, then, that a PG may have been extracted from infected mint that differed from the enzyme from the culture filtrate.

Extraction of Polygalacturonase and Fungus Estimation

The following procedure was used to extract PG and estimate fungus quantity from Verticillium infected mint plants:

1. Stems and roots were cut into 3-5 mm sections and homogenized for five minutes in 0.01 M (pH 7.6) potassium phosphate buffer in a Servall Omni-mixer. The chamber of the mixer was immersed in an ice bath. Three ml of buffer were used per gram fresh weight of tissue.

2.   Aliquots were withdrawn for estimation of fungus and plated with ESPA media.

3.   NaCl was added to the extraction mixture to obtain a concentration of ten percent and the mixture homogenized for two more minutes in an ice bath.

4.   The homogenized tissue was filtered through a flour sack, toluene added (1 ml/l), and the filtrate centrifuged at 6500 rpm for 20 minutes.

5.   The supernatant was collected and sufficient ascorbic acid was added to obtain a final concentration of 0.01 M.

6.   The supernatant was dialyzed for 16 hours against running tap water, followed by dialysis against distilled water for six hours.

7.   The dialysis tubing containing the crude enzyme preparation was then suspended in front of a fan at 22° C and concentrated to one ml per gram fresh weight of plant material.

8.   The concentrated enzyme preparation was passed successively through 1.2 μ, .8 μ and .45 μ pre-sterilized Millipore filters. Toluene was added at the rate of 1 ml/l.

Field Experiment

A field plot infested with Verticillium was planted with rooted cuttings of the three mint types.   Control plants were planted in a noninfested plot at the same time.   The field plot was infested so

that an inoculum gradient was created insuring differences in disease severity. Four harvests of stems and three harvests of roots were made from the infested plot over the summer and 150-200 grams of tissue collected at each harvest for each of the following disease classes: no symptoms, moderate symptoms (asymmetry of leaf formation and downcurled, dying leaves), and severe symptoms (severe stunting of shoot, many dead leaves). M. piperita was harvested in all three disease classes. Hybrid 148 was harvested in two disease classes, no symptoms and moderate symptoms. M. crispa never developed adequate leaf symptoms so was harvested in only one disease class. Two harvests of stems and one harvest of roots were made from control plants.

Although the primary objective was to determine PG activity in infected tissue, pectinmethylesterase (PME), the enzyme that demethylates pectin, was also assayed in enzyme extracts since increase in activity of this enzyme has been noted in wilt diseased plants (67). In the past PME has been associated with the cause of wilt symptoms (29, 38, 57), and some workers have even attributed vascular browning to it (103). PME is produced by Verticillium in small amounts early in culture but is absent later so any change in activity of this enzyme is probably of host origin (63, 105).

The build-up of Verticillium in the stems with disease progres- sion was considerably higher in M. piperita than in hybrid 148 and

M. crispa (Table 1). Severity of disease was dependent on the extent of fungus proliferation in the stem. There were extremely low propagule numbers recovered from M. crispa stems; much less than from symptomless M. piperita. Although lower propagule numbers were obtained in roots than in stems, the pattern of proliferation was similar to stems (Table 2). This data suggests, then, that the effects of root invasion govern the extent of stem invasion which in turn governs the severity of leaf symptoms.

As in preliminary experiments, the only PG assay that provided evidence of PG activity in plants was the reducing group method. PG activity was found only in the roots and stems of M. piperita with moderate and severe infection (Tables 3 and 4). However, it was not possible to test the hypothesis that resistance is due to inactivation of PG in resistant stems because of the very low propagule numbers recovered from M. crispa. PG activity was variable in M. piperita from harvest to harvest and there were no trends with increasing disease severity. There was a decrease in PG activity in the last two harvests of M. piperita with severe symptoms. Extracts from these plants were extremely dark, indicating high phenol oxidase activity. Results of this experiment indicate that Verticillium produces a PG in infected mint which can cause a release of reducing groups from sodium polypectate but not a viscosity reduction and so the enzyme would be classified as an exo-PG. The enzyme also is

Table 1. Vascular proliferation of V. dahliae in stems of resistant, intermediate, and susceptible mints.

M. piperita Stems (Susceptible)

Days After Planting	No Symptoms		Moderate Symptoms		Severe Symptoms	
	Propagules[1]	% Max[2]	Propagules	% Max	Propagules	% Max
50	38,280	1.25	465,800	15.1	850,000	27.7
64	42,900	1.39	753,600	24.5	2,276,000	74.0
78	120,548	3.94	2,121,520	69.2	2,827,600	92.0
92	96,532	3.15	2,273,200	72.6	3,068,800	100.0

Hybrid 148 Stems (Intermediate)

Days After Planting	No Symptoms		Moderate Symptoms	
	Propagules	% Max	Propagules	% Max
50	488	0.0156	26,000	0.85
64	422	0.0140	86,000	2.8
78	776	0.0253	490,000	16.0
92	9,388	0.3060	1,167,600	38.0

M. crispa Stems (Resistant)

Days After Planting	No Symptoms	
	Propagules	% Max
50	55	0.0018
64	732	0.0240
78	1,550	0.0505
92	212	0.0069

[1] Propagules/gr fresh wt of plant tissue.
[2] Percentage of the maximum value attained.

Table 2. Vascular proliferation of V. dahliae in roots of resistant, intermediate, and susceptible mints.

M. piperita Roots (Susceptible)

Days After Planting	No Symptoms Propagules[1]	% Max[2]	Moderate Symptoms Propagules	% Max	Severe Symptoms Propagules	% Max
50	6,056	2.2	156,200	5.55	353,800	12.6
64	12,914	0.46	281,200	10.00	762,840	27.1
78	-	-	-	-	-	-
92	180,880	6.30	1,624,400	58.00	2,816,000	100.0

Hybrid 148 Roots (Intermediate)

Days After Planting	No Symptoms Propagules	% Max	Moderate Symptoms Propagules	% Max
50	0	0.00	28,000	0.99
64	1,220	0.43	123,200	4.30
78	-	-	-	-
92	12,572	0.45	386,500	13.70

M. crispa Roots (Resistant)

Days After Planting	No Symptoms Propagules	% Max
50	110	0.0039
64	186	0.0066
78	492	0.0175
92	124	0.0044

[1] Propagules/gr fresh wt of plant tissue.
[2] Percentage of the maximum value attained.

Table 3. PG activity in crude extracts of M. piperita stems.[1]

Days After Planting	Reducing Groups from Disease Classes			
	Healthy	Symptomless	Moderate Symptoms	Severe Symptoms
50	0	0	6.5	11.3
64	-	0	10.0	16.0
78	0	0	9.0	10.0
92	-	0	15.0	9.6

[1] μg of reducing groups (as monogalacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

Table 4. PG activity in crude extracts of M. piperita roots.[1]

Days After Planting	Reducing Groups from Disease Classes			
	Healthy	Symptomless	Moderate Symptoms	Severe Symptoms
50	-	0	0	8.6
64	-	0	0	6.8
78	-	-	-	-
92	0	0	4.7	4.3

[1] μg of reducing groups (as monogalacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

produced in such small amounts that it is detectable only after a large build-up of the pathogen.

Although Matta and Dimond (67) found PME remained constant with increase in maturity of healthy tomato, PME activity in roots and stems of healthy mint plants varied with age (Tables 5 and 6). PME activity increased in all healthy controls and in symptomless mints as the plants matured, and reached particularly high values in M. crispa. PME activity was initially highest in stems of M. piperita with severe symptoms, but as disease progressed, PME fell to levels below that present in healthy stems. Matta and Dimond (67) found that PME in Fusarium infected plants reached a maximum in stems at 16 days but decreased rapidly again. They attributed the decline in PME activity to hyphal invasion of living cells exposing the mycelium to a medium rich in glucose and protein. The amount of PME formed by Fusarium on a glucose casein hydrolysate medium is one percent of that on a pectin medium (101). Since PME appears to be primarily of host origin in Verticillium wilt diseases and less PME activity was detected in severely infected M. piperita stems than healthy, PME may be inactivated with progression of disease severity.

Table 5. PME[1] activity in stems of resistant, intermediate and susceptible mints.

PME in <u>M.</u> <u>piperita</u> Stems (Susceptible)

Days After Planting	Healthy	Symptomless	Moderate Symptoms	Severe Symptoms
50	1. 70	1. 72	1. 86	3. 25
64	-	3. 64	2. 62	2. 90
78	2. 10	2. 92	4. 56	1. 14
92	-	2. 70	1. 10	1. 25

PME in Hybrid <u>148</u> Stems (Intermediate)

Days After Planting	Healthy	Symptomless	Moderate Symptoms
50	2. 20	2. 25	2. 36
64	-	2. 16	4. 50
78	2. 86	2. 73	6. 12
92	-	3. 14	5. 80

PME in <u>M.</u> <u>crispa</u> Stems (Resistant)

Days After Planting	Healthy	Symptomless
50	2. 45	2. 40
64	-	2. 96
78	5. 04	4. 18
92	-	4. 70

[1]Microequivalents of carboxyl groups released in one hr from 500 mg of substrate by the enzyme from one gr fresh wt of plant tissue.

Table 6. PME[1] activity in roots of resistant, intermediate, and
susceptible mints.

PME in M. piperita Roots (Susceptible)

Days After Planting	Healthy	Symptomless	Moderate Symptoms	Severe Symptoms
50	-	1. 72	0. 736	0. 890
64	-	1. 83	1. 000	0. 990
78	-	-	-	-
92	2. 12	2. 10	0. 970	0. 870

PME in Hybrid 148 Roots (Intermediate)

Days After Planting	Healthy	Symptomless	Moderate Symptoms
50	-	2. 07	1. 14
64	-	2. 60	2. 06
78	-	-	-
92	3. 03	2. 75	2. 44

PME in M. crispa Roots (Resistant)

Days After Planting	Healthy	Symptomless
50	-	1. 90
64	-	2. 02
78	-	3. 32
92	4. 45	4. 00

[1]Microequivalents of carboxyl groups released in one hr
from 500 mg of substrate by the enzyme from one gr fresh
wt of plant tissue.

Inhibitor Studies

Effect of Polyvinylpyrrolidone on
Polygalacturonase Extraction

Because of the dark browning of extracts prepared from dis-
eased tissue and the low PG activity, detectable only by the reducing
groups method, the possibility of enzyme inhibition was investigated
again. To determine if PG may have been inhibited by compounds of
phenolic origin during extraction, culture filtrate PG was homogen-
ized with mint tissue under two condition : 1) without a phenolic
adsorbent and 2) with a phenolic adsorbent, polyvinylpyrrolidone
(PVP)(10% wt/v) and 0.01 M ascorbic acid (60). Enzyme homogen-
ized without tissue was used as a control. PG extracts were pre-
pared as previously described. Residual fiber was incubated with
0.01 M potassium phosphate buffer (pH 7.6) at the rate of three ml
per gram original fresh weight to determine the amount of enzyme
adhering to the fiber. Sufficient NaCl was added to the fiber to obtain
a final concentration of ten percent NaCl. Enzyme activity was as-
sayed by the cup-plate method.

Table 7 shows that PG recovery from mint tissue homogenized
without PVP decreased with heightened phenol oxidase activity of
the tissue (see Figures 1 and 2). Addition of PVP enhanced recovery
of PG and recovery was similar from all tissues. Extracts prepared

with PVP also eliminated browning. Thus, inhibition may be of

phenolic origin. If this is so, then PG may not have been inhibited

by addition of extracts because phenols are very reactive in aqueous

solution and probably polymerized to inactive products before ex-

tracts were added to PG. Addition of PVP, by allowing enzymes to

be exposed to more similar conditions from extraction to extraction,

would permit more accurate comparison between enzyme activity in

extracts early and later in disease development.

Table 7. Recovery of PG from mint tissue homogenized with and
without PVP. [1]

Extraction Mixture	PG Activity
PG alone	820
PG + PVP	820
PG + diseased M. piperita	98
PG + diseased M. piperita + PVP	500
PG + healthy M. piperita	280
PG + healthy M. piperita + PVP	500
PG + healthy hybrid 148	236
PG + healthy hybrid 148 + PVP	500
PG + healthy M. crispa	165
PG + healthy M. crispa + PVP	500
Fiber	10

[1]Enzyme activity determined by the cup-plate assay.

Verticillium infected M. piperita plants with moderate symptoms were extracted without and with PVP (10% wt/v) to determine if PVP would enhance recovery of fungal PG from diseased plants. PG activity was detected in extracts from diseased M. piperita stems prepared with PVP by both the reducing group and viscosity reduction assays (Table 8). As in earlier experiments without a phenolic adsorbent, PG activity was detected in diseased plants only by the reducing group assay.

Table 8. Effect of PVP on extraction of PG from stems of
Verticillium infected M. piperita.

Source of Enzyme	PG activity		Propagules[3]
	Reducing Group Assay[1]	Viscosity Reduction Assay[2]	
M. piperita + PVP	12.15	290	470,000
M. piperita alone	5.50	0	463,000

[1]PG activity expressed as μg of reducing groups (as mono-galacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

[2]PG activity expressed as viscosity reduction $[\frac{1}{n-R_{20}}] \times 10^6$

[3]Number of Verticillium propagules/gr fresh wt of plant tissue.

Effect of Phenolic and Oxidized Phenolic
Compounds on Polygalacturonase Activity

The effect of several commonly occurring phenols, with and without crude phenol oxidase stem extract, on Verticillium PG from

culture filtrate was determined. Catechol, pyrogallol, gallic acid, chlorogenic acid, ferulic acid, quinic acid, protocatechuic acid, and tannin were the phenols tested. Crude phenol oxidase extracts were prepared as before. Phenols with and without phenol oxidase extracts were added to culture filtrate enzyme and activity assayed by the cup-plate method. Final phenol concentrations of 333 ppm, 1666 ppm, and 3333 ppm were used.

Without addition of plant extract, only tannin inhibited PG, but when phenol oxidase extracts were added, catechol, quinic acid and tannin were inhibitory (Table 9). M. crispa extract caused the greatest inhibition upon addition to the above three phenols. These results emphasized the importance of PG inhibition by oxidized phenols.

Polygalacturonase Activity from Culture Filtrates of Verticillium using M. piperita, hybrid 148 and M. crispa Extracts as Carbon Sources

Because previous results suggest that as phenol oxidase activity increases in plant tissue, PG inhibition increases, the effect of extracts from the three mint varieties on production of PG by Verticillium was determined.

Sliced stems (5% wt/v) of M. piperita, hybrid 148 and M. crispa were suspended in modified Richard's medium without a carbon source and homogenized in an ice bath for five minutes. The solutions were

56

Table 9. Effect of phenolics plus crude phenol oxidase extracts on
          PG activity.

Concentration[1]	Phenolic	Phenol Oxidase Extract	Relative Activity[2]
333	Catechol	M. crispa	77. 0
1666	Catechol	M. crispa	8. 0
3333	Catechol	M. crispa	2. 7
333	Catechol	Hybrid 148	100. 0
1666	Catechol	Hybrid 148	60. 0
3333	Catechol	Hybrid 148	16. 1
333	Catechol	M. piperita	60. 0
1666	Catechol	M. piperita	20. 3
3333	Catechol	M. piperita	8. 0
3333	Quinic Acid	M. crispa	32. 0
3333	Tannin	None	3. 2
3333	Tannin	M. crispa	1. 0
3333	Tannin	Hybrid 148	2. 0
3333	Tannin	M. piperita	1. 8

[1]Final concentration in parts/million of phenolic compound.

[2]Expressed as a percentage of enzyme activity with water.
Enzyme activity determined by the cup-plate assay.

sterilized by successive filtration through 1.2 mμ, .8 mμ and .45

mμ Millipore filters and the pH adjusted to 5.0. Fifty ml aliquots

were placed in flasks and inoculated with <u>Verticillium</u>. The organism

was grown for ten days on a shaker. At the end of the incubation per-

iod an aliquot was withdrawn from flasks, plated with ESPA media

and colonies counted two weeks later to estimate fungus growth.

The culture filtrate was centrifuged at 7500 rpm for 20 minutes, the

supernatant filtered through a Millipore filter (0.45 mμ) and then

dialyzed for 16 hours in tap water and six hours in distilled water.

Enzyme activity was assayed by the cup-plate method.

Eighty-three percent more PG was formed with <u>M. piperita</u>

as a carbon source than <u>M. crispa</u> and 50 percent more than with

hybrid <u>148</u> as a carbon source (Table 10). There was considerable

browning of <u>M. crispa</u> nutrient solutions during the incubation period

and only slight browning of <u>M. piperita</u> and hybrid <u>148</u> nutrient solu-

tions. In a previous experiment (Figure 1) <u>M. crispa</u> extracts con-

tained more phenol oxidase activity than hybrid <u>148</u> and hybrid <u>148</u>,

in turn, had more phenol oxidase activity than <u>M. piperita</u>. Thus,

there appears to be an inverse relationship between the amount of

PG activity in the culture filtrate and the phenol oxidase activity of

added mint extract.

Table 10. PG activity in liquid culture, using mint extracts as
carbon sources.

Extract	Phenol[1] Oxidase Activity	PG Activity	Growth
M. piperita	32[2]	100	96
Hybrid 148	56	50	100
M. crispa	100	17	94

[1] Assays for phenol oxidase activity made on a separate group
of plants (see Figure 1).

[2] Percentage of maximum value attained. Maximum values:
phenol oxidase, change in absorbence/min per 0.1 ml, 0.34;
PG, 300; growth, propagules/gr fresh wt of plant tissue,
5.8×10^7.

Enzyme Activity and Proliferation of Verticillium

in Stem Inoculated Mints

Fungal Proliferation

In experiments with root inoculated plants, very small numbers
of propagules were recovered from resistant mint stems. To test
the hypothesis that resistance may be due to inhibition of PG, it was
necessary to obtain fungus growth in resistant stems. Berry and
Thomas (10) have reported that M. crispa developed only light symp-
toms when stem inoculated. Estimations of the quantity of Verticil-
lium in stems were not made. The objective of this experiment was

to determine fungus growth in the stems of the three varieties of

mint after stem inoculation.

Unrooted cuttings of M. crispa, hybrid 148 and M. piperita

were dipped in a suspension of Verticillium spores for four hours,

the stems were washed and then placed in Hoagland's nutrient solu-

tion. The nutrient solution consisted of 0.96 gm Ca $(NO_3)_2 \cdot 4H_2O$,

0.61 gm KNO_3, 0.49 $MgSO_4 \cdot 7H_2O$, 0.12 gm $NH_4H_2PO_4$, 0.005 gm

ferric tartrate, 1 ml of A-Z solution of minor elements, and 1000 ml

of distilled water. Stems were assayed periodically for quantity of

fungus.

Growth of Verticillium in the three varieties of mint was simi-

lar (Figure 4). There was a very rapid increase in propagule num-

bers from 7 to 12 days and then a plateau followed by a drop in num-

bers. Foliar symptoms in hybrid 148 and M. piperita developed

rapidly in the period of most rapid growth of the pathogen. M. crispa

developed only a slight yellowing of lower leaves. However, growth

of Verticillium in the stems of M. crispa caused slight stunting com-

pared to healthy controls.

PG Activity in Stems of M. piperita,

Hybrid 148 and M. crispa

Similar increase of Verticillium in stem inoculated M. piperita

and M. crispa with only slight foliar symptoms in M. crispa suggested
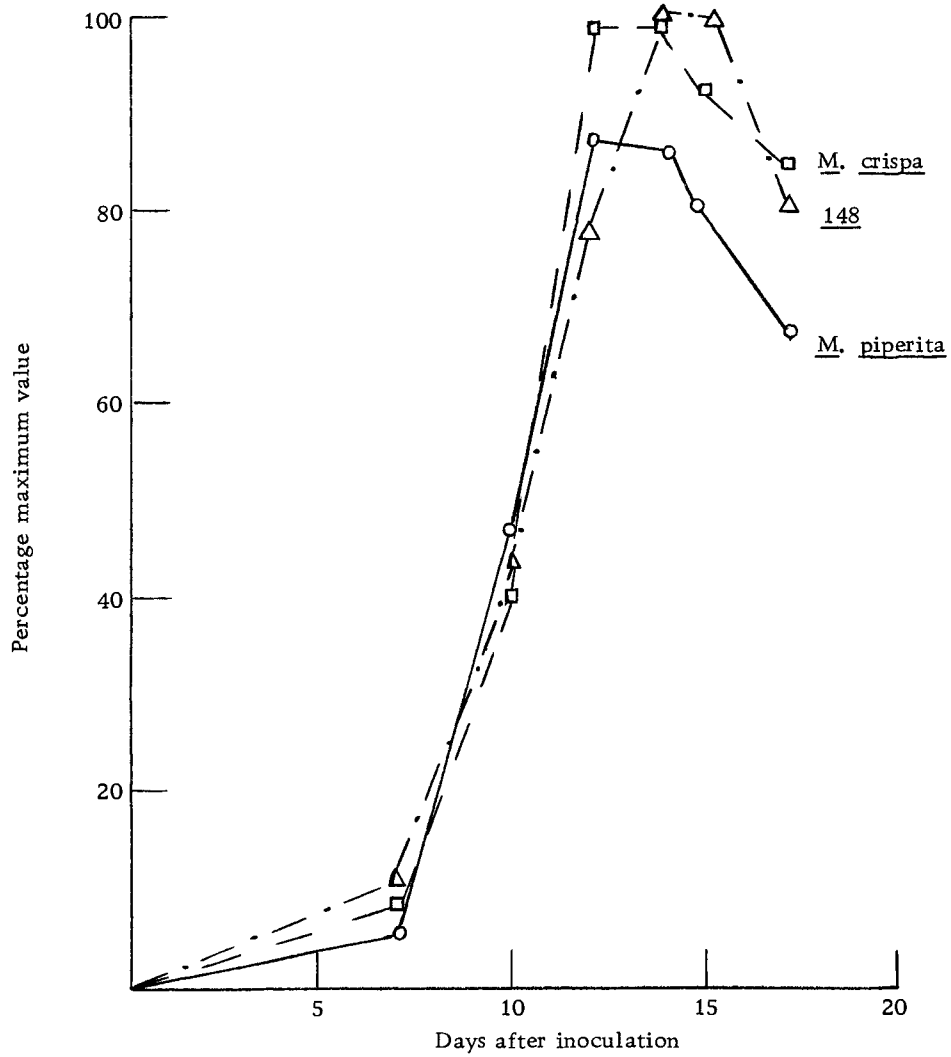
Figure 4. Verticillium proliferation in the stems of inoculated mints.
Data expressed as a percentage of the maximum value
attained, 2,9000,000 propagules/gram fresh wt of plant tissue.

that resistance may be due to inhibition of PG. Therefore, the

excised shoots of each variety were dipped for four hours in a

Verticillium cell suspension and then placed in Hoagland's nutrient

solution. PG extracts were prepared from plants 12 days after

inoculation. Preparation of the PG extracts from plant tissues was

the same as before with PVP and ascorbic acid.

Similar propagule numbers were obtained from infected stems

of the three mint varieties (Table 11). PG activity, determined by

reducing group and viscosity reduction assays, was also similar

among infected plants. Differences between susceptible and resis-

tant plant symptoms cannot be ascribed, therefore, to differences

in concentration of PG produced by Verticillium in infected plants.

Although there was no viscosity reduction of sodium polypectate by

healthy extracts, there was an increase of reducing groups released.

Table 11. PG activity from stem inoculated resistant and susceptible mints.

	M. piperita	Diseased Hybrid 148	M. crispa	Healthy
Symptoms	Severe	Moderate	None	None
Propagules[1]	1,800,000	2,000,000	1,890,000	0
Reducing Groups[2]	13.0	12.2	13.2	6.0, 5.8, 6.1[4]
Viscosity Reduction[3]	104	102	104	0

[1]Number of Verticillium propagules/gr fresh wt of plant tissue.

[2]PG activity expressed as μg of reducing groups (as monogalacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

[3]PG activity expressed as viscosity reduction $\left[\frac{1}{n\text{-}R_{50}} \right] \times 10^5$.

[4]M. piperita, hybrid 148, and M. crispa, respectively.

Phenol Oxidase Activity in M. piperita,

Hybrid 148 and M. crispa

It was found in an earlier experiment that appearance of foliar symptoms in diseased M. piperita is related to increased phenol oxidase activity of stems. If there is a cause and effect relation between appearance of symptoms and heightened phenol oxidase activity in stems, then phenol oxidase activity in infected M. crispa stems should not increase with pathogen growth. The three mint varieties were stem inoculated and phenol oxidase activity and propagule counts determined with advance of disease.

Phenol oxidase activity increased in diseased M. piperita stems as before (Figure 5). M. crispa and hybrid 148 initially had higher phenol oxidase activity than M. piperita, but as disease progressed, phenol oxidase activity became similar in extracts from all three varieties. Fungus growth was also similar in the three types of mint. However, leaf symptoms developed only in hybrid 148 and M. piperita; M. crispa remained symptomless. Thus, increased phenol oxidase activity appears to be a measure of mycelium invasion of the stem and does not contribute to foliar symptom development. Increase phenol oxidase activity is a response of the host and not a product of the pathogen itself.
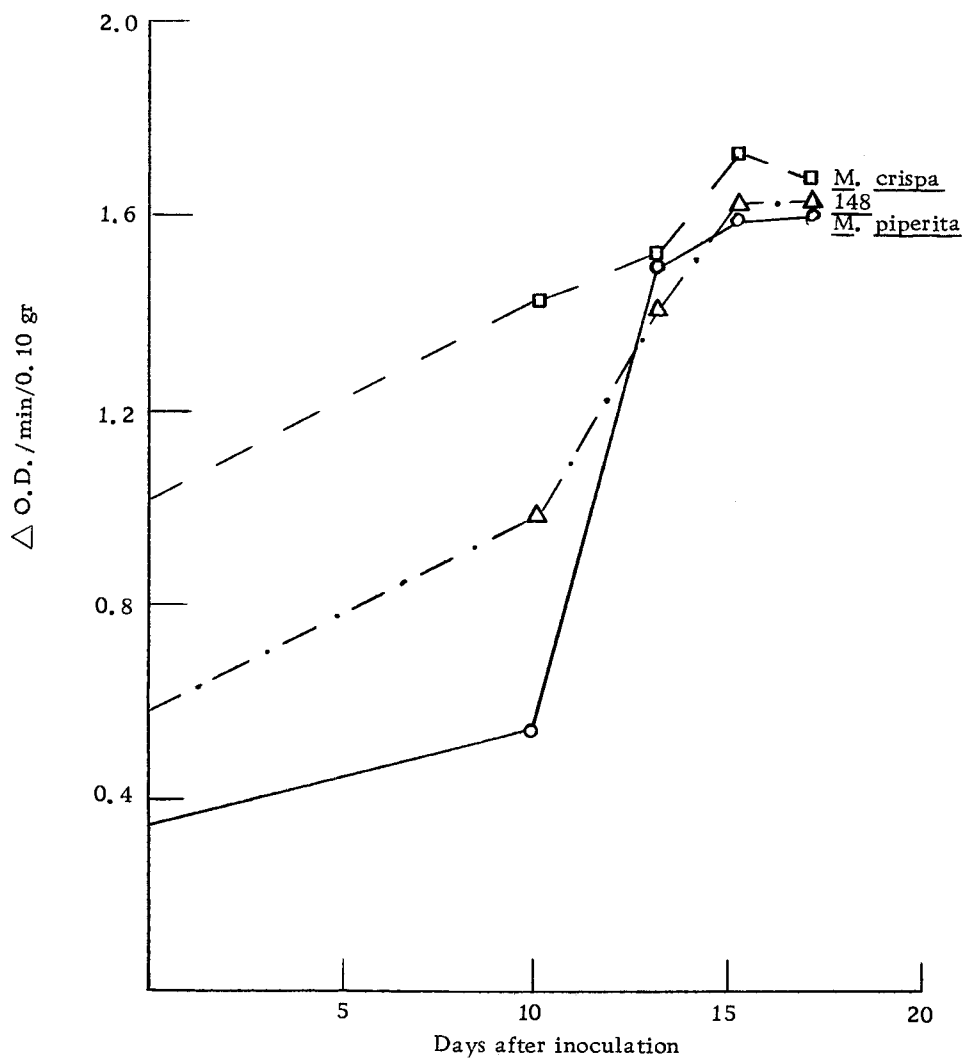
Figure 5. Phenol oxidase activity (absorbence change $\left[\triangle\text{O. D.}\right]$ /min from 50 mg of substrate by the enzyme from 0. 10 gr fresh wt of plant tissue) measured in extracts of Verticillium infected mints.

Greenhouse Studies

Polygalacturonase Activity in Roots and Stems
of Verticillium Inoculated Mint Plants

The objective of this experiment was to determine PG activity

in the roots and stems of the three varieties of mint during disease

development.

Plants were root-dipped for five minutes in a Verticillium bud-

cell suspension. The roots were severed before dipping to insure

infection since bud-cells of Verticillium are readily taken into the

plant through freshly cut roots. Plants were then potted in Verticil-

lium infested soil. Uninoculated control plants were potted in non-

infested soil at the same time.

Originally four harvests were planned, but symptoms developed

so rapidly in hybrid 148 and M. piperita that only two harvests were

made. Plants were collected three and five weeks after inoculation.

After three weeks, hybrid 148 and M. piperita had developed moder-

ate leaf symptoms and M. crispa no leaf symptoms. However, M.

crispa plants were moderately stunted compared to the healthy con-

trols. After five weeks, symptoms in hybrid 148 and M. piperita prog-

ressed to very severe and M. crispa showed only stunting. Roots from

all inoculated plants at both harvests were severely stunted with decaying

tissues. Enzyme extracts were prepared as previously described

with PVP and fungus assays were also made.

After three weeks, PG activity in stems, determined by the

viscosity reduction assay, was twice as high in inoculated hybrid

148 and M. piperita as in inoculated M. crispa (Table 12). However,

the population of Verticillium was considerably higher in hybrid 148

and M. piperita stems than in M. crispa stems. At five weeks,

viscosity reducing power of extracts from hybrid 148 and M. piperita

fell below that of M. crispa. Propagule counts also declined in

hybrid 148 and M. piperita. The enzyme extracts of M. piperita

and hybrid 148, tested by the reducing group assay, had slightly

more PG activity than M. crispa at three weeks but fell below

M. crispa at five weeks. Healthy stem extracts produced no vis-

cosity reduction of sodium polypectate but the reducing group assay

showed an increase in reducing groups. Similar PG activity in

stems of M. piperita, hybrid 148 and in infected but symptomless

M. crispa is additional evidence that PG does not contribute to wilt

symptoms.

Very low PG activity was obtained in roots of the three mint

varieties compared to stems (Table 13). PG activity was similar

in the roots of all infected varieties. As in healthy stems, PG

activity in roots could not be detected by the viscosity reduction

assay but some activity was indicated by the reducing group assay.

Table 12.   PG activity in stems of Verticillium inoculated and uninoculated mints.

Source of Enzyme	PG activity Reducing Group Assay[1]	Viscosity Reduction Assay[2]	Propagules[3]
Inoculated			
M. piperita (3 weeks)	19.7	476	2,100,000
M. piperita (5 weeks)	17.5	127	446,000
Hybrid 148 (3 weeks)	15.4	476	1,670,000
Hybrid 148 (5 weeks)	12.9	143	360,000
M. crispa (3 weeks)	15.5	218	950
M. crispa (5 weeks)	20.6	185	15,900
Uninoculated			
M. piperita (3 weeks)	7.8	0	0
M. piperita (5 weeks)	9.6	0	0
Hybrid 148 (3 weeks)	8.1	0	0
Hybrid 148 (5 weeks)	9.8	0	0
M. crispa (3 weeks)	8.3	0	0
M. crispa (5 weeks)	8.0	0	0

[1] PG activity expressed as μg of reducing groups (as mono-galacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

[2] PG activity expressed as viscosity reduction $[\frac{1}{n-R_{20}}] \times 10^6$.

[3] Number of Verticillium propagules/gr fresh wt of plant tissue.

Table 13. PG activity in roots of Verticillium inoculated and
uninoculated mints.

Source of Enzyme	PG Activity		
	Reducing Group Assay[1]	Viscosity Reduction Assay[2]	Propagules[3]
Inoculated			
M. piperita (3 weeks)	8.9	133	150,000
M. piperita (5 weeks)	13.7	166	366,000
Hybrid 148 (3 weeks)	10.8	117	66,000
Hybrid 148 (5 weeks)	10.0	133	104,100
M. crispa (3 weeks	8.4	55	30,000
M. crispa (5 weeks)	10.0	116	5,280
Uninoculated			
M. piperita (3 weeks)	5.2	0	0
M. piperita (5 weeks)	5.0	0	0
Hybrid 148 (3 weeks)	5.2	0	0
Hybrid 148 (5 weeks)	5.3	0	0
M. crispa (3 weeks)	4.8	0	0
M. crispa (5 weeks)	5.0	0	0

[1] PG activity expressed as μg of reducing groups (as mono-galacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

[2] PG activity expressed as viscosity reduction $[\frac{1}{n-R_{20}}] \times 10^6$.

[3] Number of Verticillium propagules/gr fresh wt of plant tissue.

The above experiment failed to produce a gradation of foliar

symptoms in M. piperita and, therefore, the relation of the amount

of fungus and activity of PG to symptom severity was not determined.

In an attempt to obtain a more gradual development of disease,

M. piperita plants were planted in Verticillium infested soil without

root dipping. This method of inoculation permitted collection of

M. piperita in several disease classes. Six harvests of inoculated

plants were made. Enzyme extracts were prepared with PVP and

the fungus populations were estimated.

PG activity in diseased M. piperita stems, tested by viscosity

reduction, reached a maximum when foliar symptoms were mild

and the number of propagules relatively low (Table 14). Thereafter,

activity decreased as symptoms increased. Extracts of M. piperita,

tested by the reducing group assay, had similar PG activity until

symptoms became severe, then PG increased slightly. The lack of

relationship between the viscosity reduction and reducing group

assays as symptom severity increased suggested more than one

type of enzyme action in diseased stems. An explanation would be

that as disease progresses and the fungus invades the xylem parenchy-

ma, oxidation compounds inhibit the PG responsible for viscosity re-

duction or modify this enzyme to a type hydrolyzing the pectic chain

in a terminal fashion.

In contrast to stems, PG activity in roots determined by the

Table 14. Relation of the amount of fungus in the stem and activity of PG to symptom severity in <u>Verticillium</u> inoculated <u>M</u>. piperita.

Days After Planting	Symptoms	PG Activity		Propagules[3]
		Reducing Group Assay[1]	Viscosity Reduction Assay[2]	
	Inoculated			
10	None	10.7	80	5,000
14	Light	9.7	63	48,300
26	Mild	12.5	370	108,000
32	Moderate	11.2	280	1,450,000
37	Severe	16.7	78	1,430,000
42	Very Severe	15.0	52	343,000
	Uninoculated			
14	None	8.0	0	0
42	None	8.3	0	0

[1] PG activity expressed as μg of reducing groups (as mono-galacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

[2] PG activity expressed as viscosity reduction $[\frac{1}{n\text{-}R_{20}}] \times 10^6$.

[3] Number of <u>Verticillium</u> propagules/gr fresh wt of plant tissue.

viscosity reduction assay did not decrease as symptoms became severe (Table 15). Extracts from healthy roots assayed by the reducing group method produced no increase in reducing groups. This is contrary to the preceding experiment but the small increase in reducing groups in the earlier experiment may not be significant.

The data show that PG production by Verticillium in stems of infected M. piperita plants varies considerably with disease progression and that there is not a relationship between PG production in stems and symptom severity. The results also indicate that more than one type of PG may be produced in diseased stems.

Polygalacturonase Activity in Roots and Stems of Fusarium Inoculated Mint Plants

In preliminary experiments, considerable PG activity was detected by the reducing group method in Verticillium inoculated M. crispa. Instead of isolation of Verticillium from plants, a Fusarium was isolated and it appeared to be systemic. To determine if PG was formed in mint by a nonpathogen, an experiment was done in which PG activity was assayed in the stems of three mint varieties inoculated with the previously isolated Fusarium.

Plants were inoculated by root dipping for five minutes in a spore suspension prepared as described in the materials and methods section. Roots were severed before dipping. Plants were then

Table 15. Relation of the amount of fungus in the root and activity of PG to symptom severity in <u>Verticillium</u> inoculated <u>M. piperita</u>.

Days After Planting	Symptoms[1]	PG Activity		Propagules[4]
		Reducing Group Assay[2]	Viscosity Reduction Assay[3]	
	Inoculated			
10	None	6.0	45	390
14	Light	7.7	66	6,640
26	Mild	11.2	90	8,250
32	Moderate	12.0	78	102,000
37	Severe	12.5	99	143,000
42	Very Severe	15.9	238	430,000
	Uninoculated			
10	None	0	0	0
42	None	0	0	0

[1]Foliar symptoms.

[2]PG activity expressed as μg of reducing groups (as mono-galacturonic acid) released in one hr from 100 mg of substrate by the enzyme from five gr fresh wt of plant tissue.

[3]PG activity expressed as viscosity reduction $[\frac{1}{n\text{-}R_{20}}] \times 10^6$.

[4]Number of <u>Verticillium</u> propagules/gr fresh wt of plant tissue.

potted.   Five weeks after inoculation stems and roots were collected.

Inoculated plants were indistinguishable from uninoculated plants.

Plants were extracted with the addition of PVP and assays of fungus

populations were also made.

Much lower fungus counts were obtained from Fusarium

inoculated mints than from Verticillium inoculated mints (Tables

16 and 17).   However, slightly more PG activity was detected in

the roots and stems of the Fusarium inoculated varieties than in

Verticillium inoculated plants grown for the same period of time.

A non-pathogen, then, can produce more PG in the roots and stems

of mint than Verticillium, a pathogen.

Table 16. PG activity in stems of uninoculated and Fusarium
inoculated mint.

Source of Enzyme	PG Activity		
	Reducing Group Assay[1]	Viscosity Reduction Assay[2]	Propagules[3]
Inoculated			
M. piperita	18.8	276	400
Hybrid 148	11.3	100	100
M. crispa	15.9	240	1,720
Uninoculated			
M. piperita	9.6	0	0
Hybrid 148	9.8	0	0
M. crispa	8.0	0	0

[1] PG activity expressed as μg of reducing groups (as mono-galacturonic acid) released in one hr from 100 mg of sub-strate by the enzyme from five gr fresh wt of plant tissue.

[2] PG activity expressed as viscosity reduction $[\frac{1}{n-R_{20}}] \times 10^6$.

[3] Number of Verticillium propagules/gr fresh wt of plant tissue.

Table 17. PG activity in roots of uninoculated and <u>Fusarium</u>
inoculated mint.

| Source of Enzyme | PG Activity | | |
	Reducing Group Assay[1]	Viscosity Reduction Assay[2]	Propagules[3]
Inoculated			
M. piperita	12.2	214	3,200
Hybrid 148	20.5	590	9,200
M. crispa	13.0	214	2,400
Uninoculated			
M. piperita	5.0	0	0
Hybrid 148	5.2	0	0
M. crispa	5.0	0	0

[1]PG activity expressed as μg of reducing groups (as mono-
galacturonic acid) released in one hr from 100 mg of sub-
strate by the enzyme from five gr fresh wt of plant tissue.

[2]PG activity expressed as viscosity reduction $[\frac{1}{n-R_{20}}] \times 10^6$.

[3]Number of <u>Verticillium</u> propagules/gr fresh wt of plant tissue.

DISCUSSION

Lacy and Horner (56) found that V. dahliae penetrated roots

of resistant M. crispa nearly as readily as susceptible M. piperita.

Vascular invasion, however, was much greater in susceptible than

in resistant mints. My work confirmed that vascular invasion was

much greater in susceptible plants. Proliferation of Verticillium

in the vascular system of susceptible M. piperita roots was greater

than in intermediate resistant hybrid 148, and proliferation of

Verticillium in hybrid 148, in turn, was greater than in highly re-

sistant M. crispa. Increase of Verticillium in the stems was de-

pendent on the extent of root invasion; severity of foliar symptoms,

in turn, was related to pathogen proliferation in stems. These re-

sults indicate that the roots are the first important site of differen-

tial resistance.

Berry and Thomas (10), using symptoms as a criterion for

fungal invasion, reported that unrooted and rooted mint cuttings

responded similarly to inoculation with Verticillium. In tomato

plants inoculated either through the shoots or roots, Blackhurst

and Wood (12) found similar spread of Verticillium up the stems of

both resistant and susceptible plants. The above workers concluded

that resistance was common to roots and stems. In contrast Keyworth

(51) presented evidence that the site of differential resistance of hops to

Verticillium is located in the roots. My results agree with Berry

and Thomas (10) and Blackhurst and Wood (12) that stems differ in

their resistance to Verticillium. Inoculation through the shoots or

roots resulted in severe symptom development in susceptible M.

piperita and only slight symptom development in resistant M. crispa.

Verticillium increased similarly in stems of shoot inoculated resis-

tant and susceptible mints; growth was also similar in synthetic

media amended with stem extracts from resistant and susceptible

mints. These results indicated that adequate nutrients are available

and that fungitoxic compounds are absent in resistant stems. Where-

as Blackhurst and Wood (12) found similar fungal invasion of resis-

tant and susceptible tomato roots, I found less Verticillium in resis-

tant mint roots. This is in agreement with Keyworth (51), who ob-

served that fungal growth in roots of resistant hops was suppressed.

My results indicate that the defense mechanism operating in resis-

tant mint roots may be different than in stems.

Although active stem invasion is a prerequisite to symptom

development in Verticillium wilt of mint, other factors appear to

be important. The parasite may not produce toxins (including en-

zymes) in sufficiently high concentrations in resistant stems to

induce disease symptoms. The high phenol oxidase activity in re-

sistant stems could lead to the formation of products that inactivate

a toxin necessary for maximum symptom expression.

Extracellular pectic enzymes produced by <u>Verticillium</u> and <u>Fusarium</u> may be important in the wilt syndrome (24, 38, 50, 67). Resistance to attack by a vascular pathogen may also depend in part on suppression of fungal pectic enzymes by phenolic oxidation products formed in resistant tissue (24, 25, 26, 27).

PG activity was detected in extracts from diseased <u>M</u>. <u>piperita</u> prepared without a phenolic adsorbent only after a large build-up of the pathogen. PG from diseased tissue could be detected by the reducing group assay but not by viscosity reduction. This suggests that the fungal PG formed in mint hydrolyzes the sodium polypectate chain terminally and is thus an exo-PG. By contrast, the enzyme from culture filtrates of <u>Verticillium</u> hydrolyzes the substrate in a random manner and is thus an endo-PG.

Pectic enzymes can be inhibited by oxidized phenols (18, 19, 72). Phenol oxidase activity was initially high in resistant mint stems and also increased in resistant and susceptible mints following infection. Extracts from stems of susceptible and resistant <u>Mentha</u> varieties failed to inhibit the PG from culture filtrates of <u>Verticillium</u>. Other workers report similar results (12, 67). However, when PG was homogenized with mint tissue only a small proportion of enzyme was recovered by treatments which remove enzyme adsorbed by fiber. The amount of PG inhibition increased with increase in the phenol oxidase activity of the mint tissue, suggesting

that inhibition may be due to phenolic compounds. Of several phenols

tested, only tannin inhibited PG in the absence of phenol oxidase from

mint. Others have reported inhibition of pectinase by tannin (19, 44).

When phenol oxidase extracts were added, catechol, quinic acid and

tannin were inhibitory. Patil and Dimond (72) found that PG from

Verticillium was inhibited by the oxidized form of chlorogenic and

caffeic acid when the phenolase and the phenol were added to the

PG simultaneously. When the phenol and the phenolase were incu-

bated prior to addition to PG they found that compounds were formed

that had no inhibitory effect on PG, probably due to polymerization

of the phenols to inactive compounds before they were added to the

enzyme. Thus, failure to obtain inhibition of PG when mint extracts

were added to PG may be explained by polymerization of inhibitory

compounds to inactive substances. When PG was homogenized with

tissue, it was exposed to inhibitory substances before they polymer-

ized.

Mint tissue homogenized with PG and a phenolic adsorbent,

PVP, yielded extracts that did not become brown colored and con-

tained greater amounts of PG. Recovery of PG was similar from

mint tissues with different phenol oxidase activities. In the intact

plant, phenols and enzymes are separated but extraction leads to

mixing of materials which may result in inhibition or modification

of the enzyme. This may explain why Deese and Stahmann (24)

failed to obtain PG activity from resistant tomatoes containing high oxidative power. During extraction of tomato tissue, PG was exposed to more inhibitory compounds in resistant tomato tissue than in susceptible tissues. PG may have been formed by the pathogen in resistant tomato plants but was lost during preparation of the enzyme extract. Extracts prepared with PVP from infected M. piperita showed PG activity by both the viscosity reduction and reducing group assays.

PG production by Verticillium was similar in shoot inoculated resistant and susceptible mints extracted with the addition of PVP. Increase of Verticillium in the stems of the three varieties was similar but foliar symptoms developed only in susceptible M. piperita and intermediate resistant 148. No foliar symptoms occurred in resistant M. crispa. Blackhurst and Wood (13) also found similar PG production by Verticillium in resistant and susceptible tomatoes. Therefore, Deese and Stahmann's (24) hypothesis that resistance to Verticillium is due to inactivation of fungal PG does not appear to be valid.

A study of fungal PG production in mints during disease development shows no relationship between symptom severity and PG activity in infected mint stems. Changes in enzyme activity as disease progresses emphasizes the importance of studying PG production in infected plants at several stages of disease. Further,

enzyme extracts prepared late in disease showed considerable de-

crease in viscosity reducing power and an increase in reducing

groups released from sodium polypectate. This suggests more

than one type of PG action in <u>Verticillium</u> infected mint stems.

When the pathogen invades xylem parenchyma, oxidation com-

pounds may be formed that inhibit the PG attacking the pectic chain

randomly (endo-PG) or modify the enzyme to a type attacking the

pectic chain terminally (exo-PG). The exo-PG is less affected by

these oxidation compounds. This may explain why PG activity was

detected in extracts from diseased mint plants prepared without PVP

only by the reducing group method. Cole and Wood (17), working

with <u>Botrytis</u> <u>cineria</u> soft rot of apple, found no viscosity reduction

by enzyme extracts but galacturonic acid was released from the

substrate. They proposed that the exo-PG was unaffected by phenol

oxidation products that inactivated the endo-PG. This supports my

view that with advance of disease, oxidation compounds are formed

in the plant that are more inhibitory to endo-PG than exo-PG. In

roots where phenol oxidase activity remained uniform with disease

development, PG activity, measured by viscosity reduction, did not

decrease.

A nonpathogen of mint produced PG in mint equivalent to that

produced by <u>Verticillium</u> but no wilt symptoms developed. Since

PG can be formed in plants by nonpathogenic organisms, isolations

for microorganisms should be made on material used for enzyme extractions. Sal'kova and Guseva (80), working with Verticillium wilt of cotton, found much higher PG activity in resistant cotton than in a susceptible variety. Although the high PG production in resistant plants was attributed to Verticillium, it is uncertain what was producing PG because isolations were not made. Their work illustrates, however, that PG can be formed in plants without relation to wilt symptom development.

Extracts from healthy plants produced a small release of reducing groups from the sodium polypectate substrate. Possible causes of this increase in reducing groups are: 1) noninfected plants may contain an exo-PG. 2) Nonpathogenic organisms may have produced PG. It was found that a nonpathogen could produce PG in mint without a deleterious effect. 3) Since the sodium polypectate substrate contains impurities, enzymes in addition to PG may have been extracted that caused a release of reducing groups from the substrate.

The hypotheses presented by Gothosker et al. (38) and Beckman et al. (8) regarding the role of pectolytic enzymes in the wilt syndrome depend on the production by the pathogen of pectic enzymes that hydrolyze pectic substances to large fragments. These fragments can form gels which obstruct the vessels to the passage of water or microconidia. Deese and Stahmann (28) have

reported a PG of this type in potato infected with Verticillium,

but others (24, 63, 87, 105, 106) have found that Verticillium pro-

duces a PG that carries hydrolysis to monogalacturonic acid.

McIntyre (62) observed that tomato cuttings treated with partially

purified PG from Verticillium failed to wilt. Histochemical exam-

ination of PG treated stems revealed no pectin-like plugs. The

ability of enzyme extracts prepared from mints as symptoms in-

creased to liberate a high number of reducing groups from substrate

with a decrease in viscosity reducing power also supports the con-

tention that large pectic fragments are not formed in infected plants.

Therefore, the hypotheses advanced by Gothoskar et al. (38) and

Beckman et al. (8) are not applicable to Verticillium wilt and another

explanation may be considered for the role of these enzymes.

In Verticillium diseases, symptoms are relatively slow in

development. This means that the vascular pathogens must invade

and colonize the host to a certain extent before it can produce wilting.

To accomplish this colonization nutrients are required. Nutrients

may be released from the cell walls of the vascular tissue or from

reserve food in the xylem parenchyma by action of hydrolytic en-

zymes of the pathogen. In addition to PG, other hydrolytic enzymes

such as amylase, cellulase, protease and ribonuclease have been

detected in culture media of fungi (81). The ability to parasitize a

plant depends on available nutrients in which these hydrolytic enzymes

may play an important role. From my results and the work of others (63, 87, 105, 106), I propose that PG does not contribute directly to wilt symptoms in <u>Verticillium</u> infected mint plants but functions in providing an additional nutrient source for the pathogen.

The data suggest that the pathogen confined to the vascular elements of mint secretes pectic enzymes and possibly other hydrolytic enzymes which attack the cell wall to release simple carbohydrates. These substances are used by the fungus to produce more invading hyphae which block the xylem and petiolar vessels (97). The fungus also secretes toxic metabolites which contribute to wilting (12, 40, 94). In resistant plants these metabolites appear to be inactivated (12). The invasion of living xylem cells by mycelium presumably involves the action of pectic enzymes on pectic substances exposed at pits. Phenols and phenolases are then brought together to form colored oxidation products. The exo-PG of <u>Verticillium</u> is less affected by these products than the endo-PG. The colored products are responsible for the vascular browning of wilt diseases and may also restrict the activity of the pathogen in xylem parenchyma.

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