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EFFECT OF CEPHALOSPORIUM ON SEVERAL BIOCHEMICAL ASPECTS OF FUSARIUM WILT IN RESISTANT AND SUSCEPTIBLE TOMATO PLANTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University

1964

Approved by

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INTRODUCTION

In agricultural areas where complete soil sterilization is either impractical or impossible, the pathogens causing the wilt diseases are perhaps the most difficult to cope with. The use of resistant varieties is a common practice, but it is not the ultimate solution for at least two reasons. First of all, plant breeders have not been able to select resistant plants of all phenotypes. As a result an aster variety with desirable flower color or a high yielding tomato variety may lack resistance to one of the wilt disease pathogens. Secondly, when genetic resistance is achieved, it is often specific to one race or biotype of the pathogen. Since the wilt disease pathogens are variable and already exist in many physiological races, genetic resistance is often only a temporary condition.

Perhaps a more lasting solution to the wilt disease problem would be a practical biological or chemical control. In order to achieve this goal, plant pathologists must learn as much as possible about the complex relationship that exists between the wilt-pathogens and their host plants, and discover some way to interrupt this relationship before it results in symptom expression. In preliminary studies at The Ohio State University, it appeared that inoculation of susceptible tomato plants, Lycopersicon esculentum Mill., with a Cephalosporium species, before inoculating
with *Fusarium oxysporum f. lycopersici* (Sacc.) Snyd. and Hans., might become a practical control for Fusarium wilt. However, further studies have failed to advance this treatment to a practical level. It still remains an interesting phenomenon and, perhaps, what is more important, a means of studying the mechanism of Fusarium wilt. A major portion of the earlier work was concerned with the effect of environmental changes on this *Cephalosporium*-Fusarium relationship both *in vitro* and in the tomato plant. Although environmental studies are important in pioneer work, there is a limit to the amount of information which can be learned from them.

In order to understand more fully any host-pathogen relationship one must study the physiology of the organisms involved and try to discern alterations in normal metabolism which may result in symptom expression by the host. A study of the *Cephalosporium*-Fusarium-tomato plant relationship is slightly more complicated, for the *Cephalosporium* may alter the tomato plant making it more tolerant to the *Fusarium* or it may alter the pathogenicity of the *Fusarium*. In either situation, the net result would be a decrease in Fusarium wilt symptoms in the host.

Although it is not generally believed that pectic enzymes are the primary cause of Fusarium wilt, their importance in the Fusarium wilt syndrome cannot be disregarded.

The objective of this research was to determine whether *Cephalosporium* affects pectic enzyme production *in vivo*, and
from this information attempt to explain more fully the Cephalosporium-Fusarium-tomato plant relationship.
LITERATURE REVIEW

The physiology of the wilt diseases has been studied for the past fifty years, but still there is no general agreement on the mechanism of wilting. Since there are a number of comprehensive reviews concerning the wilt diseases (2, 6, 13, 16, 22, 24, 36, 43), the historical aspects of the subject will not be discussed here. Much research in the last decade on wilt diseases has dealt with the Fusarium wilt of the tomato plant, Lycopersicon esculentum Mill., caused by Fusarium oxysporum f. lycopersici (Sacc.) Smyd. and Hans. Presently there seem to be two principal explanations for the mechanism of wilting. First, there are those of the Gäumann school who believe toxins are of primary importance. They have isolated from culture filtrates of Fusarium, a peptide, lycomarasmin, an enzymic protein, vasinfuscarin, and fusaric acid, and maintain that at least one of these compounds initiates the Fusarium wilt syndrome. In this explanation pectic enzymes and vascular plugging are of secondary importance. On the other hand, Gothoskar et al. (19) found that only culture filtrates and other preparations with pectic enzyme activity produced disease symptoms in tomato cuttings. Vascular occlusion by mycelium, tyloses, or gums and gels is of primary importance in this explanation and toxins, if they are present, may cause secondary symptoms. The pectic enzymes of primary concern are pectin methylesterase (PME)
and polyglacturonase (PG). This latter enzyme exists in several forms which make the literature on the subject confusing. Deuel and Statz (10) have designated three types of polygalacturonase. Type I and II are liquefying polygalacturonases which split the 1-4 glycosidic linkages of the pectin molecule at random. Type I acts primarily on low ester pectin molecules, while Type II is more active in the presence of highly esterified pectin. Types I and II are also known as pectin depolymerases (DP) and endopolygalacturonases. Type III is a saccharifying polygalacturonase which hydrolyzes the pectin molecules only at the ends. This results in free galacturonic acid in the medium. Type III is also known as exopolygalacturonase. The possible role of these enzymes in plant disease has been reviewed by Wood (43, 44) and Husain and Kelman (22). *Fusarium oxysporum f. lycopersici* produces both PME and Type I endopolygalacturonase in culture when pectic materials are added to the culture medium. The presence of these enzymes in diseased tissues has also been demonstrated. Deese and Stahmann (9) and Matta and Dimond (26) reported higher quantities of PME in diseased tomato stems than in healthy tissues. It is believed that this increase is due to fungal PME because of enzyme activity at pH 5.5 and in the absence of NaCl. PME from higher plants is most active at pH 7 and must be activated by NaCl. Deese and Stahmann (9) have also reported high PG activity in stem juice expressed from diseased plants. Other workers (26, 45) have failed to find any PG activity in diseased tissues. They believe that this may be due either to the method of analysis or
the presence of an inhibitor in the stem tissue which inactivates the enzyme soon after it is produced. The quantities of PG present in culture filtrates suggest that this enzyme is also produced in the diseased plant. However, one must proceed with caution when comparing in vitro and in vivo work. This point is emphasized by the work of Hancock et al. (21). They examined the pectolytic enzyme production of three Botrytis species on potato dextrose broth, and in excised and intact onion leaves. They found that all three species produced PME under all conditions, but one of the three species produced polygalacturonase only in excised and intact leaves. Two of the three species produced both endo- and exopolygalacturonase in both excised and intact leaves, while the third species produced only exopolygalacturonase in excised leaves.

Faquin and Coulombe (32) studied pectic enzyme synthesis in vitro in relation to the virulence of F. oxysporum f. lycopersici with both virulent and avirulent strains. They found the PME activity was greater in culture filtrates from virulent strains but there was no correlation of virulence with PG activity. McDonnell (27, 28) and Mann (25) studied mutants of F. oxysporum f. lycopersici which were unable to produce pectic enzymes in vitro but could still cause mild disease symptoms in susceptible hosts. This, however, may only be another indication of the incompatibility of in vitro and in vivo experiments. Edgington et al. (15) found that calcium deficient plants which were more susceptible to Fusarium wilt than plants which were not calcium deficient,
contained more water-soluble pectin than normal plants. The pectic substances in calcium-deficient tissues were also more easily released by pectic enzymes than were pectic materials of normal tissues, but treating the calcium deficient tissues with $10^{-6}$ M naphthaleneacetic acid reversed this. Yoda (46) also observed changes in pectin enzyme activity while using auxins to promote and inhibit water absorption. This suggests that pectic enzymes may affect water relations in ways other than producing materials which block the xylem vessels. Grossman (17, 18) has added support to the enzyme theory of Fusarium wilt with his study of pectinase inhibitors. He found that rufianic acid (1,4-deoxyanthroquinone-sulfonic acid) was effective in controlling Fusarium wilt symptom development in both tomato cuttings and intact plants when applied prior to inoculation. He believed this chemical inhibited pectolytic enzymes of the fungus, because the decrease in wilting symptoms was much greater than the decrease in vascular discoloration and spread of fungus in the plant. There was also little inhibition of *F. oxysporum* growing in a synthetic medium.

There are numerous reports (3, 4, 29, 31, 33, 38, 39) of several aspects of the *in vivo* inhibition of Fusarium wilt symptoms by a species of *Cephalosporium*. Chisler (4) and Phillips (33) have suggested that the mechanism of this inhibition might be the inactivation of toxins produced by the *Fusarium*, however, there was no direct evidence of this occurring *in vivo*.

Vascular discoloration is a very characteristic symptom of Fusarium wilt of tomato. It is thought that this discoloration is
due to brownish black melanoid pigments formed by the oxidation of polyphenols to quinones, which polymerize forming melanins. Davis et al. (5) have shown that many phenolic compounds will cause vascular discoloration of cuttings but do not cause wilting. They suggested that, in the diseased plant, the phenols are freed from such natural conjugated phenols as glycosides, tannins, or lignins by β-glucosidase which is present in the sap of diseased plants but not in the sap of healthy plants. Possibly, the carbohydrate fraction of the conjugated phenol has a function in the wilting mechanism or it may become a carbon source for the fungus in the plant. Matta and Dimond (26) and Sanwal (37) have demonstrated that the increase in activity of polyphenol oxidase in the diseased tomato plants parallels the development of visible leaf symptoms. This increase in activity precedes the increase in PME activity by about six days. Dimond (14) discusses two interesting interactions involving polyphenol oxidase and other substances associated with the wilt syndrome. Bossi (1) demonstrated that polyphenoloxidase is competitively inhibited by fusaric acid in vitro, and Deverall (12), and Deverall and Wood (11) have shown that phenolases are inhibitory to polygalacturonase. In chocolate spot of bean caused by Botrytis sp., the polygalacturonase releases substances which activate the phenolase in the bean leaves. This phenolase in turn inhibits further polygalacturonase activity. They believed this limited the size of the spots on the bean leaf. This inhibition of polygalacturonase by phenolase has been suggested as a mechanism of resistance in other tissues also.
MATERIALS AND METHODS

Source of fungi

The fungus isolates used in this research were from stock cultures maintained in the plant pathology area of the Botany and Plant Pathology Department at The Ohio State University. Fusarium isolate C was obtained in 1962 during reisolation from WR-7 tomato plants inoculated with Race I which is not pathogenic to WR-7. The Cephalosporium isolates 13 and 16 were biotypes from the Cephalosporium originally isolated by Smith (38). Cephalosporium isolate 88 was from a carnation plant and isolated at the Ohio Agricultural Experiment Station in 1963. Stock cultures of these isolates were maintained under oil on PDA slants in test tubes stored at room temperature. Each month transfers were obtained from these stock cultures and maintained on PDA in petri dishes. Transfers from the petri dish cultures were used in the research.

Inoculum preparation

The Cephalosporium was cultured for five days at 70°F on 100 ml of dilute potato dextrose broth (12 g dehydrated mashed potatoes, 5 g dextrose, 1,000 ml deionized water) in 250 ml Erlenmeyer flasks placed on a reciprocating shaker operating at 110 oscillations per minute. The five-day old cultures were filtered through two layers of cheese cloth and then centrifuged at 3,000 X g for 5 minutes. The supernatant was discarded and
the residue, which was primarily conidia, was resuspended in deionized water to the original volume. This suspension which contained approximately $5 \times 10^8$ conidia per ml was used as inoculum. The Fusarium inoculum was prepared in a similar manner except that the cultures were on the shakers for only three days at $80^\circ F$. After centrifugation, the residue, again primarily conidia, was resuspended in deionized water equal to ten times the original volume. This suspension, which contained approximately $5 \times 10^6$ conidial per ml, was the Fusarium inoculum.

**Plants**

Seeds of the Bonny Best and WR-7 tomato plants were planted in the greenhouse in a steam-sterilized soil mix, (2 parts sand, 4 parts soil, and 1 part peat). Two weeks later the seedlings were transplanted into steam-sterilized vermiculite in metal flats and placed in a controlled environment room, with 16-hour light periods (1,200-1,500 f.c.) at $75^\circ F$ and an 8-hour dark period at $65^\circ F$. The vermiculite was watered regularly with full strength Meyer solution (30). When the plants were 4-6 weeks old they were removed from the vermiculite and the roots washed with tap water. The plants were then placed in containers of water until time of inoculation.

**Inoculation procedures**

The inoculation procedures varied with the experiment but basically the procedure was that of exposing the roots of the tomato plants to a suspension of *Cephalosporium* conidia in water.
In earlier work (3) it was observed that the Cephalosporium treatment was most effective when the roots of the plants were placed in the conidial suspension and then the flask containing both plants and conidial suspension was agitated. This treatment became too severe when larger plants were used; thus other methods of exposing the plants to Cephalosporium were tested. In the majority of the experiments, the plants were left in containers of Cephalosporium for 48 hours. In order to ascertain whether the increased effect of Cephalosporium when plants were agitated was due to increased aeration in the suspension, two other methods of inoculation were tried. One of these was to place the plants in a device that regularly dipped the plant roots in the Cephalosporium conidial suspension. The other procedure was to bubble air through the suspension during the time the plants were in it. As a check in all experiments the roots of a similar group of plants were suspended in water.

In all experiments, the method of inoculating the plants with Fusarium was the same. After the plants had been treated with Cephalosporium for 48 hours they were removed from either the Cephalosporium suspension or water and placed for five minutes in the suspension of Fusarium conidia which was described earlier. The plants were removed and planted in a soil mix in metal flats which had been prepared and steam-sterilized prior to the time of inoculation.

1Two parts greenhouse soil mix, 1 part vermiculite, and 1 part perlite.
The roots of another similar group of plants were placed in water prior to planting and served as controls. The flats were placed in either the greenhouse or a controlled environment room depending upon the experiment.

**Harvest**

At intervals ranging from 5 to 25 days after planting, 12 to 16 plants to be used for future enzyme studies were harvested by cutting at the soil line. After these plants were measured and checked for vascular discoloration, the leaves were removed and the stems wrapped in aluminum foil and frozen at -10°C until analyzed for enzyme activity.

**Culturable mycelium**

In a few experiments the amount of culturable mycelium present in the stems was calculated by using a modification of the method of Saaltink as described by Matta and Dimond (29). Before the plant stems were frozen, a section just above the cotyledonary node was removed and surface sterilized by placing it in a 20 per cent clorox solution for 5 minutes. Aseptic techniques were used to cut a 5 mm sample from the middle of each section and four of these 5 mm samples were macerated with 50 ml of sterile water in a Waring blender. Five ml of this suspension were added to 50 ml, 100 ml, or 300 ml of sterile water depending upon the dilution desired, and then a 1 ml sample of this suspension was mixed with PDA in a petri dish. After 48 hours incubation at room temperature the number of *Fusarium* colonies growing
in each petri dish was counted and, after correcting for the appropriate dilution, the result was an indication of the amount of mycelium in the stem.

**Enzyme studies**

The stem juice analyzed for enzyme activity was extracted in the following manner. The frozen stems were cut into small sections and weighed. The stem segments were then placed in a volume of 0.15 M NaCl solution (pH 8) equal to the weight of the stem. After 30 minutes the solution and stem segments were placed in a hydraulic press and subjected to a pressure of 15,000 psi for 2 minutes. The expressed sap was then centrifuged at 15,000 X g for 15 minutes. The supernatant was carefully decanted and tested for PME, PG, and polyphenol oxidase activity.

**Pectin methylesterase activity.**—The activity of PME was determined by measuring the increase in acidity due to deesterification of pectin by the enzyme. The method used was similar to that of Deese and Stahmann (9). Three-tenths ml of stem juice was incubated at 30°C with 5 ml of 0.5 M citrate-phosphate buffer at pH 5.5, and 15 ml of 1.5 per cent pectin solution previously adjusted to pH 5.5. After 3 hours the mixture was titrated to pH 6 with 0.025 N NaOH. The pectin methylesterase activity was calculated as microequivalents of methoxyl groups liberated in 3 hours per 1 ml of stem juice.

**Polygalacturonase activity.**—The polygalacturonase activity was determined in two ways. The first was the iodometric method
of Willstätter and Schudel as modified by Jansen and MacDonnell (24) which measures the increase in reducing groups produced by the hydrolysis of pectin. The other method was to measure the reduction in viscosity of a pectin solution to which the stem juice had been added. In this assay, 5 ml of the stem juice were added to 5 ml of 0.15 M citrate-phosphate buffer, pH 4.5, and 10 ml of 1.5 per cent pectin solution adjusted to pH 4.5. This mixture was incubated at 30°C and viscosity measurements were made at regular intervals with an Ostwald viscometer.

**Polyphenol oxidase activity.**—A modification of the method of Matta and Dimond (26) was used to measure the polyphenol oxidase of the stem juice. Five-tenths ml of the stem juice was added to 10 ml of 0.5 per cent catechol solution and a Klett-Summerson Colorimeter with filter No. 42 was used to measure the increase in optical density during the time interval between 30 and 90 sec after adding the stem juice to the solution.
RESULTS

It was the plan of this research to study the physiology of the Cephalosporium-Fusarium-tomato plant relationship in a standardized environment. To achieve this, one isolate of Cephalosporium and one isolate of Fusarium were chosen to be used throughout the research. Preliminary experiments summarized in Table 1 indicated isolate 13 of Cephalosporium was the most effective in the preventing of the characteristic vascular discoloration of Fusarium wilt. This isolate decreased vascular discoloration by 32 per cent while isolates 16 and 88 caused decreases of 21 and 24 per cent respectively.

In one experiment, tomato cuttings, instead of intact plants, were placed in the Cephalosporium conidial suspension. Since this treatment did little to alter the effectiveness of the Cephalosporium, it was not used in further experiments.

Earlier work (4, 33) demonstrated the desirability of using whole cultures of Cephalosporium rather than conidia re-suspended in water. Due to the difficulty of obtaining the proper checks for the whole cultures, conidial suspensions were used. The effects of whole cultures, cultural filtrates, and conidial suspensions on vascular discoloration were compared (Table 2). The 5-day-old whole cultures and cultural filtrates had little
effect on vascular discoloration, but the conidial suspensions decreased vascular discoloration by 20 per cent.

**TABLE 1.** Effect of three isolates of *Cephalosporium* in preventing Fusarium wilt of tomato, Bonny Best variety, as measured by extent of vascular discoloration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Per Cent Vascular Discoloration*</th>
<th>Per Cent Decrease in Vascular Discoloration Due to Fusarium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalosporium</em> and <em>Fusarium</em> C 13</td>
<td>53 ± 1.3</td>
<td>32</td>
</tr>
<tr>
<td><em>Cephalosporium</em> and <em>Fusarium</em> C 16</td>
<td>62 ± 2.0</td>
<td>21</td>
</tr>
<tr>
<td><em>Cephalosporium</em> and <em>Fusarium</em> C 88</td>
<td>59 ± 0.6</td>
<td>24</td>
</tr>
<tr>
<td><em>Cephalosporium</em> only</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Water only</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Water and <em>Fusarium</em> C</td>
<td>78 ± 1.8</td>
<td>0</td>
</tr>
</tbody>
</table>

*Average of four replications with four plants in each replicate.*

In order to understand better why agitation during *Cephalosporium* inoculation makes the treatment more effective, two experiments were conducted. In the first, the tomato plants were dipped in a *Cephalosporium* conidial suspension at the rate of 20 times per minute for 12 hours. After this treatment the conidial suspension was diluted with water, and the plants remained in the solution until inoculated with *Fusarium* 36 hours later. Plants dipped in *Cephalosporium* had 10 per cent less vascular discoloration than those plants remaining motionless in the *Cephalosporium* (Table 3). To determine whether this increase in ef-
fectiveness was due only to aeration of the root systems, the
effect of bubbling air through the conidial suspension in which
the plants were suspended was examined as a second experiment
(Table 4). Aeration had the greatest effect on PME activity, for
aerated plants inoculated with only *Fusarium* had 55 per cent less
PME than unaerated plants, while there was little change in the
amount of culturable mycelium in the stem or in the percentage of
vascular discoloration. In plants inoculated with *Fusarium* plus
*Cephalosporium* there was over twice as much culturable mycelium
in the stems of the aerated plants but only a 40 per cent increase
in PME, as compared with unaerated plants.

### TABLE 2.—Effect of various *Cephalosporium* culture fractions in
preventing *Fusarium* wilt of tomato, Bonny Best variety, as
measured by extent of vascular discoloration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Per Cent Vascular Discoloration*</th>
<th>Per Cent Decrease in Vascular Discoloration Caused by <em>Fusarium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalosporium</em> cultural filtrate (autoclaved) and <em>Fusarium</em> C</td>
<td>88 ± 3.2</td>
<td>4</td>
</tr>
<tr>
<td><em>Cephalosporium</em> cultural filtrate (autoclaved)</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td><em>Cephalosporium</em> whole culture and <em>Fusarium</em> C</td>
<td>88 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td><em>Cephalosporium</em> whole culture</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td><em>Cephalosporium</em> conidia suspended in water and <em>Fusarium</em> C</td>
<td>74 ± 5</td>
<td>20</td>
</tr>
<tr>
<td><em>Cephalosporium</em> conidia suspended in water</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td><em>Fusarium</em> C only</td>
<td>92 ± 0.8</td>
<td>0</td>
</tr>
<tr>
<td>Water only</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

*Average of three replications of four plants in each replicate.*
TABLE 3.—Effect of continuously dipping tomato plants during Cephalosporium inoculation in preventing Fusarium wilt of tomato, Bonny Best variety, as measured by extent of vascular discoloration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Per Cent Vascular Discoloration*</th>
<th>Per Cent Decrease in Vascular Discoloration Caused by Fusarium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium only</td>
<td>92 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>Cephalosporium (dipping)</td>
<td>64 ± 3.3</td>
<td>30</td>
</tr>
<tr>
<td>and Fusarium C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalosporium (still)</td>
<td>74 ± 5</td>
<td>20</td>
</tr>
<tr>
<td>and Fusarium C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cephalosporium only (dipping)</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Water only</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

* Average of four replications of four plants in each replicate.

In the unaerated plants the PME activity was almost twice as high in the plants inoculated with only Fusarium as that found in those inoculated with Cephalosporium prior to the Fusarium inoculation. There was also about 50 per cent more culturable mycelium in plants inoculated with Fusarium alone, and 30 per cent more vascular discoloration. However, when the roots were aerated there was 50 per cent more culturable mycelium in the plants inoculated with both Fusarium and Cephalosporium than in plants inoculated with only Fusarium. There was also over 30 per cent more PME activity in the Fusarium and Cephalosporium inoculated plants but 7 per cent less vascular discoloration than in the aerated plants inoculated with Fusarium alone.

Since the tomato variety WR-7 is highly resistant to Fusarium wilt, a study was made to compare the metabolic effects
TABLE 4.—Effect of aeration during Cephalosporium inoculation on several aspects of Fusarium wilt of tomato, Bonny Best variety

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vascular Discoloration</th>
<th>Culturable Mycelium</th>
<th>PME Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>NA</td>
<td>A</td>
</tr>
<tr>
<td>Fusarium C only</td>
<td>82</td>
<td>84</td>
<td>1900</td>
</tr>
<tr>
<td>Cephalosporium and Fusarium C</td>
<td>76</td>
<td>58</td>
<td>2540</td>
</tr>
<tr>
<td>Cephalosporium only</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water only</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A = Aerated  NA = Not Aerated

1Average of three replications of four plants in each replicate (measured as percentage of total plant height).
2Average of two replications of three samples in each replicate (measured as number of colonies per 20 mm stem + 50).
3Average of three determinations (measures as μeq. methoxyl groups per ml stem juice).

It has already been demonstrated (7, 8, 9, 26) that there is a significant increase in pectic enzyme and polyphenoloxidase activity in plants with Fusarium wilt symptoms. The pectic enzyme and polyphenoloxidase activities in Bonny Best and WR-7 tomato varieties with and without Cephalosporium inoculation prior to Fusarium inoculation are compared in Table 5. There was little difference in the enzyme levels in the WR-7 plants but in the Bonny Best plants, the PME and polyphenoloxidase activities were 370 and 250 per cent higher in diseased plants than in healthy plants, respectively. In diseased plants inoculated with Cephalosporium these levels were
TABLE 5. Effect of Cephalosporium on several aspects of Fusarium wilt in resistant (WR-7) and susceptible (Bonny Best) tomato varieties, twenty-five days after inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vascular^1 Discoloration</th>
<th>Culturable^2 Mycelium</th>
<th>PME^3 Activity (Per Cent of Maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
</tr>
<tr>
<td>Fusarium only</td>
<td>6  91</td>
<td>21 1320</td>
<td>35 103 21 100</td>
</tr>
<tr>
<td>Fusarium and Cephalosporium</td>
<td>- 72</td>
<td>23 780</td>
<td>40 78 23 71</td>
</tr>
<tr>
<td>Cephalosporium only</td>
<td>- - -</td>
<td>- - -</td>
<td>37 30 17 38</td>
</tr>
<tr>
<td>Water only</td>
<td>- - -</td>
<td>- - -</td>
<td>32 28 21 40</td>
</tr>
</tbody>
</table>

^1 Average of three replications of four plants in each replicate (measured as percentage of total plant height).

^2 Average of two replications of three samples in each replicate (measured as number of colonies per 20 mm stem + 50).

^3 Average of three determinations (measured as µeq. methoxyl groups per ml stem juice).

about 25 per cent lower. There was also less culturable mycelium in the plants inoculated with Cephalosporium plus Fusarium than in the plant inoculated with only Fusarium. To obtain a better picture of what takes place in the plants as Fusarium wilt is developing, the enzyme changes in both WR-7 and Bonny Best tomato varieties were measured at intervals during disease development. These results, along with the amount of culturable Fusarium mycelium in the plant and per cent vascular discoloration, are presented in Table 6 and Figures 1-3.
TABLE 6. Effect of *Cephalosporium* on amount of culturable mycelium in resistant (WR-7) and susceptible (Bonny Best) tomato plants measured at intervals 5 to 22 days after *Fusarium* inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after Inoculation</th>
<th>5</th>
<th>10</th>
<th>14</th>
<th>18</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
<td>WR-7 BB</td>
</tr>
<tr>
<td><em>Fusarium</em></td>
<td>1^2</td>
<td></td>
<td>360</td>
<td>1000</td>
<td>21</td>
<td>1440</td>
</tr>
<tr>
<td><em>Fusarium and Cephalosporium</em></td>
<td>2</td>
<td></td>
<td>9</td>
<td>560</td>
<td>204</td>
<td>2880</td>
</tr>
</tbody>
</table>

^1Values are the number of *Fusarium* colonies present in 1 ml when 20 mm of tomato stem were macerated with 50 ml sterile water.  
^2All values are an average of two samples each replicated three times. Standard error is approximately ± 10 per cent.

In Bonny Best tomato plants inoculated with only *Fusarium*, the PME activity increased until the fourteenth day after inoculation (Fig. 1). After a decrease on the eighteenth day, there was another increase on the twenty-second day. In plants inoculated with both organisms there was no rapid increase in PME activity until the fourteenth and eighteenth days. This increase was followed by a very large decrease on the twenty-second day. In the resistant WR-7 plants there was a small increase on the tenth day followed by a rapid decrease which lowered the PME activity below that of the uninoculated checks. Since the PME activity of the checks has been subtracted from that of the inoculated plants there was negative amount of enzyme at the fourteenth, eighteenth, and twenty-second days after inoculation. Preinoculation with *Cephalosporium* caused a slight decrease in PME on the fifth and tenth days in the WR-7 plants. The amount of culturable
Fig. 1.—Development of PME activity with time after inoculation in resistant and susceptible tomato plants. The values of uninoculated checks have been subtracted from all values.
mycelium was greater in the Bonny Best plants inoculated with only *Fusarium* than in Bonny Best plants inoculated with both organisms, at all times except the fourteenth day after inoculation (Table 6). At this time there was twice as much culturable mycelium in plants inoculated with *Fusarium* plus *Cephalosporium*. In these plants the amount of culturable mycelium decreased rapidly at the eighteenth and twenty-second days. In the plants with *Fusarium* only there was an increase on the eighteenth day and a decrease on the twenty-second day. On the tenth day after inoculation there was the largest amount of culturable mycelium in the WR-7 plants inoculated with *Fusarium* alone, while in plants inoculated with both organisms there was more culturable mycelium on the fourteenth day after inoculation. In both treatments the amount of mycelium in WR-7 plants was greatly reduced by the eighteenth and twenty-second days after inoculation.

The extent of vascular discoloration present in the tomato stems is represented in Figure 2. In susceptible Bonny Best tomato plants inoculated with only *Fusarium* there was a marked increase at the tenth day and the amount of discoloration continued to increase at a decreasing rate throughout the experiment. In Bonny Best plants inoculated with both *Cephalosporium* and *Fusarium* there was only slight discoloration at the tenth day. There was a large increase on the fourteenth day and a decrease at the twenty-second day. In resistant WR-7 tomato plants, vascular discoloration was always less than 15 per cent of the height of the plant. Pre-inoculation with *Cephalosporium* delayed the
Fig. 2.--Development of vascular discoloration with time after inoculation in resistant and susceptible tomato plants. Discoloration expressed as percentage of total height of plant.

- Fusarium
- Fusarium and Cephalosporium
- Bonny Best
- WR-7
increase of vascular discoloration until the fourteenth day as compared to the increase on the tenth day in plants inoculated with Fusarium alone.

In Bonny Best plants there was a direct relationship between the amount of polyphenol oxidase in the stem and the extent of vascular discoloration (Fig. 3). In plants inoculated with Fusarium alone, there was a large increase on the tenth day and the activity continued to increase throughout the experiment. When plants were inoculated with both organisms, there was a large increase on the fourteenth day and a decrease on the twenty-second day. In WR-7 plants there were only slight changes in polyphenol oxidase activity during the course of the experiment (Fig. 4), but the greatest increase in the plants inoculated with only Fusarium was on the tenth day when the amount of culturable mycelium in these plants was also the greatest. Except on the twenty-second day in WR-7 plants, there was always less polyphenol oxidase in plants inoculated with Fusarium plus Cephalosporium. There was little change in polyphenol oxidase in WR-7 and Bonny Best plants which were not inoculated with Fusarium.

Polygalacturonase was not detected in any of the plants during these experiments. It is believed that there was some substance present in the stem tissues which inactivated this enzyme before its activity could be measured.
Fig. 3.—Development of polyphenol oxidase with time after inoculation in susceptible (Bonny Best) tomato plants.

Fig. 4.—Development of polyphenol oxidase with time after inoculation in resistant (WR-7) tomato plants.
DISCUSSION

During the ten years of research which began with the first isolation of Cephalosporium from a "healthy" tomato plant previously inoculated with Fusarium, there have been several possible mechanisms proposed to explain the Cephalosporium-Fusarium-tomato plant relationship. Chisler (4) discussed these in regard to his own research, and concluded that the Cephalosporium produces various metabolites within the tomato plant which interfere with the metabolism of Fusarium. This results in prevention or inhibition of toxin production by Fusarium.

It seems equally feasible that one of the in vivo metabolites of Cephalosporium could be a pectic enzyme inhibitor which could inactivate the pectic enzymes produced by the Fusarium and prevent Fusarium wilt symptom development in this manner. This research was designed to investigate this phase of the Cephalosporium-Fusarium-tomato plant relationship.

The selection of a Cephalosporium isolate for this research work was based on several experiments designed to compare the effectiveness of isolates 13, 16, and 88 in preventing Fusarium wilt of tomato as measured by vascular discoloration. Isolate 13 was most effective in these experiments so it was used in all further research. This confirms the work of Mwanza (31), who found that isolate 13 was most effective in controlling Fusarium wilt of Bonny
Best tomato plants when the plants were inoculated with *Fusarium* isolate C. Phillips (33) also found isolate 13 one of the more effective strains of *Cephalosporium*.

Chisler (4) reported that a *Cephalosporium* conidia-water suspension was not effective in reducing vascular discoloration while both whole cultures and culture filtrates were effective. Under the experimental conditions of the present research the reverse was true. Menon (29) and Phillips (33) have also reported that *Cephalosporium* conidia-water suspensions were effective in reducing vascular discoloration. Chisler (4) was using 18 to 21-day-old cultures of *Cephalosporium* so there may have been metabolites present in the cultures which enhanced the *Cephalosporium* effect.

Chisler (3) demonstrated that agitating the *Cephalosporium* culture in which the roots of tomato plants were suspended increased the effectiveness of the treatment. Phillips (33) and Mwanza (29) also used this method but neither offered an explanation for its increased effectiveness, however, Mwanza did report that *Cephalosporium* was reisolated more frequently from plants treated in this manner than from plants just placed in the *Cephalosporium*. In this research dipping the plants in the *Cephalosporium* almost doubled the decrease in vascular discoloration. Aerating the *Cephalosporium* suspension did not have the same effect so it was concluded that the enhancement due to dipping was not due to increased aeration. Perhaps the enhancement was due
to more conidia adhering to the roots and lower stem surfaces but this aspect was not investigated.

The aeration of the root systems during *Cephalosporium* inoculation did have several profound effects on the *Cephalosporium-Fusarium-tomato* plant relationship, even though it did not enhance the *Cephalosporium* effect. The increased amount of mycelium in the aerated plants was undoubtedly due to increased root injury which provided more possible points for the *Fusarium* to invade the root systems. However, in the aerated plants inoculated with only *Fusarium* there was a significant decrease in the PME activity even though the amount of mycelium was about the same. In the aerated plants inoculated with *Fusarium* plus *Cephalosporium* there was also a suppression of PME activity for 300 per cent increase in culturable mycelium produced only a 40 per cent increase in PME. The aeration had little effect on the PME produced by the host for there was little change in the checks. These results indicated that the aeration prior to inoculation facilitated the entrance of the *Fusarium* but it inhibited *in vivo* PME production by the *Fusarium*. The *Cephalosporium* was still effective in the aerated plants but to a lesser degree due to the increased amounts of *Fusarium* mycelium present in the aerated plants. The marked changes in PME activity were not matched by similar changes in vascular discoloration and polyphenol-oxidase activity. This suggested the presence of separate systems in the diseased plant. This experiment also illustrated
the supreme importance of antecedent environment in determining the effectiveness of Cephalosporium.

Twenty-five days after inoculation there was more PME and polyphenol-oxidase activity in stems of diseased Bonny Best tomato plants than in uninoculated checks. There was also a large amount of mycelium in the stems and extensive vascular discoloration. Wood (44) in his review of the possible roles of pectic and celluolytic enzymes in plant diseases suggested that the Fusarium enters the plant through the roots, grows extensively in the vascular system and then as pectic enzymes degrade host tissues, the fungus spreads throughout the plant. Pectin methylesterase is important in this process for the demethylated pectin is more readily degraded by the other pectic enzymes. The demethylated pectin molecules and high molecular weight subunits resulting from pectin degradation can react with metallic ions forming gels inside the vascular system. It is also assumed that there is an active endo-polygalacturonase produced in the tomato plant, however none was found in this research. Wood (45), and Matta and Dimond (26) also were unable to find this enzyme in diseased plants although it is produced in large quantities in culture. Deese and Stahmann (9) found a 400 per cent increase in depolymerase activity in diseased plants by measuring the increase in reducing groups in expressed stem juice. Matta and Dimond (29) have suggested that their failure to find any activity might be due to the methods used, for they were measuring activity as a reduction in viscosity of a pectin solution to which the stem juice was added. In this
research both methods were tried without success. It is felt that the viscosity assay should be the more sensitive test for endo-polygalacturonase (syn. depolymerase) due to the structure of the pectin molecule. In a study of the hydrolysis of pectin by purified fungal polygalacturonase, Rahman and Joslyn (34) concluded that the polygalacturonase degraded the pectin molecule randomly, and at low levels of activity, hydrolysis appeared to be limited to depolymerization without releasing any galacturonic acid. There was a large decrease in viscosity before any appreciable increase in reducing power occurred. Wood (45) was unable to demonstrate any polygalacturonase activity in xylem exudates or stem juice from tomato plants with Verticillium wilt. He suggested that it is unlikely that the enzyme is not produced in the plant for the fungus would grow and produce the enzyme when cultured on xylem exudates. He believed that it is either produced in undetectable small quantities or that it is inactivated soon after it is produced. Another possibility is that it is produced in the stem in small quantities and then transported to other parts of the plants, such as the leaves, before it has an active role in pathogenesis. Matta and Dimond (26) failed to find a polygalacturonase inhibitor in stem juice of tomato plants, but Wood (45) reported slow inactivation of polygalacturonase when it was in contact with tomato leaf and stem tissue, and Deverall and Wood (11) demonstrated that polygalacturonase of Botrytis sp. was inactivated by leaf extracts of the bean plant. Further research
will undoubtedly determine why polygalacturonase is not found regularly in the diseased tomato plants.

The high levels of pectin methylesterase in diseased tomato stems have been reported previously by Deese and Stahmann (9) and Matta and Dimond (26). This increase was believed to be fungal pectin methylesterase when measured at pH 5.5 because tomato pectin methylesterase has a pH optimum at 7.0 while that of fungal pectin methylesterase is pH 5.5. The absence of any increase in pectin methylesterase activity in resistant tomato stems was also noted by Deese and Stahmann (9). The increased polyphenol oxidase activity in diseased plants is considered a reaction of the host to the presence of the pathogen. Deverall (12) and Deverall and Wood (11) discussed the activation of polyphenol oxidase by pectin and products of pectin hydrolysis. If this activation occurs in the diseased tomato stem then polyphenol oxidase activity would be an indirect measurement of pectic enzyme activity. Polyphenol oxidase is believed to be the important enzyme in production of the melanin pigments of vascular browning and in these experiments the extent of vascular discoloration was directly related to the amount of polyphenol oxidase activity in the stem juice.

In order to obtain a clearer picture of what metabolic changes were taking place in the tomato plant during symptom development, samples were examined at regular intervals during the 22 days following Fusarium inoculation. The increase of PME, with time, in Bonny Best tomato inoculated with only Fusarium was
not unexpected because of the high activity 25 days after inoculation. It was apparent that the fungus was increasing inside the tomato, and as the amount of mycelium in the plant increased, so did the amount of PME. This is true for all times except 18 days after inoculation when there was a decrease in PME without a decrease in Fusarium mycelium. When the standard error of the points is considered, the decrease may only be a plateau region of the curve. PME activity should decrease also when the fungus moves from the vascular system into surrounding tissues which are rich in glucose for PME production is dependent on a pectic substrate. The decrease in the quantity of mycelium at the twenty-second day is undoubtedly due to the method of sampling. The samples for determining culturable mycelium were removed from the lower region of the stem in all experiments. Twenty-two days after inoculation the tissue in this region was starting to lignify so the amount of carbohydrate readily available to the Fusarium was decreasing and the Fusarium was dying in the lower stem.

In the Bonny Best plants inoculated with both Fusarium and Cephalosporium the amount of Fusarium was increasing in the plant but PME activity was suppressed until the eighteenth day. The large quantity of Fusarium mycelium in the plant on the fourteenth day did not produce a large quantity of PME so it appeared as if PME production by Fusarium was inhibited at this time. The large increase in PME at the eighteenth day suggested that any inhibition that was present had been nullified, but the inhibition was present again on the twenty-second day.
The vascular discoloration and polyphenol oxidase level in the plants inoculated with both organisms did not increase until five days after they had increased in plants inoculated with Fusarium alone. This suggested that disease development was less rapid and less severe in plants inoculated with both organisms. Because of this time lag in disease development the defenses of the host plant were not broken down and a few plants escaped the final stage of Fusarium wilt which is death.

In the resistant WR-7 plants there was very little culturable mycelium at any time, and the amount in the plants at the tenth and fourteenth days was not maintained. In previous research (20, 40) microscopic examination of Fusarium-infected tomato stems revealed that in resistant plants the Fusarium was confined to the primary xylem vessels. This would indicate that there was either an inhibitor or an inadequate supply of carbohydrate in the stems of the resistant plants. The PME activity increased at the tenth day but after that time there was less activity in the Fusarium inoculated plants than in the uninoculated checks. This was believed to be evidence of an active resistance mechanism in the WR-7 plants which was initiated by the presence of the Fusarium. Deese and Stahmann (9) have reported similar findings in their research with resistant and susceptible tomato plants.

Talboys (41) has developed a concept of the host-parasite relationship in the Verticillium wilt disease which may also apply to the Fusarium wilt diseases. He suggested that all the
factors which determine the frequency and intensity of wilt development operate before the fungus enters the vascular system of the host. Under this concept the severity of disease is directly proportional to the amount of fungus in the plant. This seems to be only partially valid in the case of WR-7 tomato plants and Bonny Best tomato plants inoculated with Fusarium plus Cephalosporium, for in both cases the amount of Fusarium decreased after the fourteenth day. This suggests an active resistance factor is operating inside the tomato stem which is inhibiting the growth of the Fusarium. It is not likely that this resistance factor is only a pectic inhibitor as used by Grossman (17, 18) to control Fusarium wilt, for the polyphenol oxidase activity and, in turn, vascular browning were also decreasing. Perhaps a more detailed investigation would demonstrate that the pectic enzymes are being inhibited first in the plant and then a food relationship is causing a decrease in the amount of fungus in the vascular system which then affects the level of polyphenol oxidase and vascular browning. However, the data in this research does not suggest such a mechanism nor does it rule out the possibility that Cephalosporium is inhibiting in vivo toxin production by the Fusarium. The decrease in amount of culturable mycelium from the high level present on the fourteenth day does suggest that the Fusarium itself was inhibited and not just toxin production. In the plants inoculated with Fusarium plus Cephalosporium there was a general absence of wilt symptoms at the twenty-second day so it appeared that the amount of Fusarium in the plants was approaching a level that the tomato plant could tolerate.
CONCLUSIONS

Under the conditions of this research, conidial suspensions of *Cephalosporium* isolate 13 were more effective than conidial suspensions of isolates 16 and 88 in controlling vascular discoloration of tomato plant caused by *Fusarium oxysporum f. lycopersici*.

Continuously dipping the roots of the tomato plants in the *Cephalosporium* conidial suspensions prior to *Fusarium* inoculation was more effective in mitigating *Fusarium* wilt symptom development than soaking the roots in the *Cephalosporium* conidial suspension without repeated dipping. Aerating the conidial suspension in which the plant roots were soaking did not have the same effect so it was postulated that the additional benefit of dipping was not due to aeration alone. The aeration of the roots increased the amount of culturable mycelium in the plants and decreased the amount of fungal pectin methylesterase (PME).

PME activity increased rapidly on the tenth, fourteenth, and twenty-second day in Bonny Best tomato plants inoculated with *Fusarium*. In plants which had been inoculated previously with *Cephalosporium*, this increase did not occur until the fourteenth and eighteenth day, and on the twenty-second day there was a large decrease. The amount of culturable mycelium in the diseased plants followed a different pattern. In the Bonny Best plants
inoculated with both organisms, the most culturable mycelium was present on the fourteenth day after inoculation. This amount then decreased on the eighteenth and twenty-second day. In the Bonny Best plants inoculated with only Fusarium, the most culturable mycelium was present on the eighteenth day after inoculation. In resistant WR-7 tomato plants there were only slight changes in PME activity. However, from the fourteenth day until the end of the experiment there was less PME in the inoculated plants than in the uninoculated checks. This inhibition of PME indicated an active resistance mechanism in these plants. The polyphenol oxidase activity was closely related to the extent of vascular browning in the stems.

No measurable amount of polygalacturonase was detected in the plants during any of the experiments.

It appears that the mechanism of Cephalosporium action is more complex than just in vivo inhibition of pectic enzymes. Although there was evidence that this was happening in the plant there were other effects, such as decreasing amounts of culturable mycelium and decreased vascular browning, which cannot be completely explained by a decrease in pectic enzyme activity.
LITERATURE CITED


AUTOBIOGRAPHY

I, John E. Rodebaugh, was born in Geneva, Ohio, June 28, 1938. I received my secondary-school education in the public schools at Ashtabula, Ohio. I attended Case Institute of Technology from 1956 to 1958. I completed my undergraduate training in botany and plant pathology at The Ohio State University, obtaining the Bachelor of Science degree in 1960. In December, 1962, I was granted the Master of Science degree. During the past four years, while completing the requirements for the Master of Science degree and the Doctor of Philosophy degree in botany and plant pathology, I have held a research fellowship from the Ohio Florists' Association for whom I have maintained a disease clinic.

I have accepted a position as the Plant Pathologist of The Soil and Plant Laboratory, Inc., at Orange, California.