A pectin histochemical study of the host pathogen relationship between Pyrenochaeta terrestris and Allium cepa

LeRoy Richard Cobia
*Brigham Young University - Provo*

Follow this and additional works at: [https://scholarsarchive.byu.edu/etd](https://scholarsarchive.byu.edu/etd)

**BYU ScholarsArchive Citation**

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact scholarsarchive@byu.edu, ellen_amatangelo@byu.edu.
A PECTIN HISTOCHEMICAL STUDY OF THE HOST PATHOGEN RELATIONSHIP BETWEEN PYRENOCHAETA TERRESTRIS AND ALLIUM CEPA

A Thesis
Presented to the
Department of Botany
Brigham Young University

In Partial Fulfillment
of the Requirements for the Degree of
Master of Science

by
LeRoy R. Cobia
August 1971
ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. Wilford M. Hess of the Botany and Range Science Department, Brigham Young University, for his guidance, support and encouragement in the completion of this research and manuscript.

Sincere thanks is given to Dr. Darrell J. Weber of the Botany and Range Science Department, Brigham Young University, and to Dr. Albert D. Swensen of the Chemistry Department, Brigham Young University, for their encouragement and for editing this manuscript.

Acknowledgment is also given to Dr. Dayna L. Stocks for the support from him and the Department of Botany and Range Science, Brigham Young University, during this study.

Additional thanks is also given to Mrs. Connie Swensen, Electron Microscope Laboratory Technician, and Jim V. Allen, Electron Microscopist, Brigham Young University, for their technical help.

This research was supported in part by National Science Foundation Grant GB 14161.

To my wife, Janice, I express my love and appreciation for her support and encouragement.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF MICROGRAPHS</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>2</td>
</tr>
<tr>
<td>Nomenclature</td>
<td>2</td>
</tr>
<tr>
<td>Life Cycle</td>
<td>4</td>
</tr>
<tr>
<td>Symptoms</td>
<td>5</td>
</tr>
<tr>
<td>Varieties and Resistance</td>
<td>8</td>
</tr>
<tr>
<td>Ultrastructural Work with <em>P. terrestris</em></td>
<td>9</td>
</tr>
<tr>
<td>Pectin and Pectic Enzymes</td>
<td>12</td>
</tr>
<tr>
<td>Pectilic Enzymes Produced by <em>P. terrestris</em></td>
<td>14</td>
</tr>
<tr>
<td>Pectin Histochemical Stains Used with Electron Microscopy</td>
<td>16</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>Hydroxylamine Reaction for Pectin</td>
<td>22</td>
</tr>
<tr>
<td>Ruthenium Red Stain for Pectin</td>
<td>23</td>
</tr>
<tr>
<td>Variation of Hydroxylamine for Pectin</td>
<td>24</td>
</tr>
<tr>
<td>Pectin Stain Developed During this Study</td>
<td>26</td>
</tr>
<tr>
<td>Dehydration Procedure</td>
<td>28</td>
</tr>
<tr>
<td>Embedding Procedures</td>
<td>29</td>
</tr>
<tr>
<td>Thin-Sectioning Procedures</td>
<td>31</td>
</tr>
<tr>
<td>Hydroxylamine Reaction for Pectin Post-Stain Procedure</td>
<td>32</td>
</tr>
<tr>
<td>Electron Microscopy and Photography</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>34</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>72</td>
</tr>
<tr>
<td>Hydroxylamine Reaction for Pectin Stain</td>
<td>72</td>
</tr>
<tr>
<td>Hydroxylamine Pectin Post-Stain</td>
<td>73</td>
</tr>
<tr>
<td>Ruthenium Red Stain for Pectin</td>
<td>73</td>
</tr>
<tr>
<td>Variation of Hydroxylamine Reaction for Pectin</td>
<td>74</td>
</tr>
<tr>
<td>Pectin Stain Developed During this Study</td>
<td>75</td>
</tr>
</tbody>
</table>
CONCLUSION ............................................. 82
BIBLIOGRAPHY ........................................... 84
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Oblique section of cell with hydroxylamine pectin stain showing middle lamella and a portion of non-infected onion root cell wall</td>
<td>35</td>
</tr>
<tr>
<td>2.</td>
<td>Oblique section of infected onion root tissue with hydroxylamine and the fungus</td>
<td>37</td>
</tr>
<tr>
<td>3.</td>
<td>Section of infected onion root tissue with hydroxylamine for pectin showing the host cell wall and fungus</td>
<td>38</td>
</tr>
<tr>
<td>4.</td>
<td>Section of a portion of non-infected onion root cell post stained with hydroxylamine pectin stain showing the middle lamella, cell wall and cytoplasm</td>
<td>39</td>
</tr>
<tr>
<td>5.</td>
<td>Section of a portion of two non-infected onion root cells stained with Ruthenium red for pectin showing the cell wall, middle lamella and cytoplasm</td>
<td>40</td>
</tr>
<tr>
<td>6.</td>
<td>Section of a portion of two non-infected onion root cells. Ruthenium red stained for pectin showing the cell wall, nucleus, and cytoplasm</td>
<td>42</td>
</tr>
<tr>
<td>7.</td>
<td>Section of a portion of two non-infected onion root cells showing the cell wall and pectin layering in the cell wall</td>
<td>43</td>
</tr>
<tr>
<td>8.</td>
<td>Section of non-infected control tissue showing the cell wall, mitochondria, and cytoplasm (no pectin stained)</td>
<td>44</td>
</tr>
<tr>
<td>9.</td>
<td>Section of non-infected control tissue showing the membrane around the starch grain (no pectin stained)</td>
<td>45</td>
</tr>
<tr>
<td>10.</td>
<td>Section of infected onion root tissue showing the host cell wall as the fungus is penetrating it</td>
<td>46</td>
</tr>
<tr>
<td>Page</td>
<td>Image Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>11. Section of infected onion root tissue showing the host cell wall and fungus.</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>12. Section of infected onion root tissue showing the fungus and the partially degraded host cell wall.</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>13. Section of non-infected outside onion root wall showing pectin and cellulose.</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>14. Section of non-infected root tissue showing the junction of four cell walls, and the darkly stained middle lamella.</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>15. Section of non-infected onion root tissue showing the darker stained pectin and middle lamella.</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>16. Section of non-infected onion root tissue showing the darker stained middle lamella in a primary pit field.</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>17. Section of non-infected onion root tissue showing the cell wall with no pectin stain.</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>18. Section of non-infected onion root tissue showing the cell wall with no pectin stain.</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>19. Section of infected onion root tissue four cells away from the infection front showing the dark stained middle lamella.</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>20. Section of infected onion root tissue two to three cells away from the infection front showing the middle lamella and the cellulose.</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>21. Section of infected onion root tissue at the infection front showing the host cell wall with no pectin stain.</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>22. Section of infected onion root tissue at the infection front showing the host cell wall which is being penetrated by the fungus.</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>23. Section of infected onion root tissue showing the host cell wall with the fungus in the center.</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>24. Section of infected onion root tissue showing the host cell wall and fungus cells.</td>
<td></td>
</tr>
<tr>
<td>Page</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25. Section of infected onion root tissue showing the degraded host cell wall and the fungus</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>26. Section of infected onion root tissue showing the almost completely degraded host cell wall, the fungus and the large amounts of lipid</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>27. Section of infected onion root tissue showing the fungus, and the microfibril appearance of the almost degraded host cell wall</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>28. Section of infected onion root tissue showing the fungus and the microfibril appearance of what is left of the host cell wall</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>29. Section of infected onion root tissue showing the fungus and mostly degraded host cell wall</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>30. Section of infected onion root tissue showing the fungus and the degraded host cell wall with dark and light areas</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>31. Section of infected onion root tissue showing the fungus, the degraded host cell wall, and degraded cytoplasm</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>32. Section of highly infected onion root tissue showing the remains of the host cell wall</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

Pink root has been a threat to onion growers for more than half a century. Fields highly infested with Pyrenochaeta terrestris (pink root) reduce yields sufficiently to make onion production unprofitable. Today there is still no effective commercial way of controlling the host-pathogen interaction of Pyrenochaeta terrestris and Allium cepa.

In a recent publication, Hess (1969) indicated that in order to understand the specific reasons for the different host-pathogen responses with this disease, more intense biochemical and histochemical investigations are needed. In selecting the histochemical investigation of this study, pectin was chosen as the histochemical stain. Pectin is one of the few histochemical stains used with plant tissue at the electron microscope level.

The present study was designed to investigate the involvement of pectin and pectic enzymes in the host pathogen interaction of Pyrenochaeta terrestris and Allium cepa by use of pectin histochemical stains at the electron microscope level.
**LITERATURE REVIEW**

*Pyrenochaeta terrestris* is a facultative parasite which infects a wide variety of hosts, as was mentioned by Kreutzer (1941). Onion and close relatives, however, are the most common hosts infected by *Pyrenochaeta terrestris*, and it is from this association that the common name of pink root of onion was derived.

**Nomenclature**

The description of the disease first appeared more than fifty years ago when Taubenhaus and Johnson (1917) reported about the disease which was observed in Webb County, Texas. Later Taubenhaus (1919) published a note indicating that Mally and Gilbert had done work with the disease previously, but their works were not published. However, Edgerton (1921) pointed out that the Louisiana Experiment Station had records of the disease as early as 1909.

Taubenhaus and Mally (1921) were the first investigators to publish a full account of extensive experiments on etiology and control. They named the pink root fungus for the first time as *Fusarium malli* Taub., thinking that a new species of *Fusarium* was the causal agent.
In 1924, Sideris reported that four new species and two new varieties of *Fusarium* were found to be associated with the pink root disease. Later in 1929, Sideris stated that the disease was caused by several species of *Fusarium* rather than by *Fusarium malli*.

In 1926 Hansen concluded that if root cultures are partly decayed and shrivelled, they yield a large number of *Fusaria* and many other organisms from the soil flora. However, if the root cultures are distinctly pink, turgid and still firm, the number of fungi obtained is reduced. If these same roots were immersed in a mercuric chloride solution and cultured, only one fungus was obtained and it belonged to the genus *Phoma*. Following this work, Hansen (1929) used better isolation techniques and presented evidence that *Fusarium* Spp. were not responsible for the pink-root disease indicating that *Phoma terrestris* Spp. was the cause of the pink-root disease. He mentioned that *Fusarium* acted as a secondary parasite and hastened the destruction of the host. In 1933, Du Plessis stated in his Bulletin on pink root and bulb rot of onions that *Fusarium cepae* was the causal agent of the pink-root disease.

Further morphological studies of the pink root fungus were conducted by Gornez, Walker, and Larson (1948). They collected isolates of the fungus throughout the United States. Some isolates were found to be fertile in culture and they produced pycnidia which always had Setae. Since Setae were always present on the pycnidia, the pink
root fungus was transferred from *Phoma terrestris* n. Sp., Hansen to *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker, Larson. Tims (1953) pointed out that while *Fusarium* has been eliminated as a primary cause of pink root, it could be involved in the root rotting associated with the disease.

**Life Cycle**

As a facultative parasite *Pyrenochaeta terrestris* is not confined to a single host nor does it require sporulation for survival. In 1929, Hansen reported that hyphal cells on onion roots gave rise to a thick mass of dark, thick-walled bodies resembling chlamydospores. These bodies were always apparent on infected onion roots where mature pycnidia never occurred and they acted as a carry-over or resting stage for the fungus.

After extensive morphological investigations, Hansen (1929) reported a detailed description of *Pyrenochaeta terrestris* as follows:

Pycnidia subglobose, ostiolate, papillate, dark brown to black, carbonaceous, 170 to 350 μ, may occur singly, frequently gregarious. Conidia continuous, hyaline, oblongovoid, 4.5-5 x 1.8-2.3 μ biguttulate, sessile in pycnidium, escaping as a gelatinous mass through ruptures rarely as a gelatinous cirrus through the ostiole. Mycelium septate, hyaline, guttulate 1.0-4.5, frequently anastomosing.

Hansen also reported that large amounts of free sugars seemed to be deterrent to formation of mature pycnidia which might explain the lack of them in the infected host roots.
Hansen (1929), Gorenz, Larson and Walker (1949), Kulik and Tims (1960) reported pycnidium formation of Pyrenochaeta terrestris on artificial medium. Hess (1962) also showed that when suitable environmental and nutritional conditions are used, this fungus produces pycnidia; however, this means of reproduction does not appear to be of primary importance in the life cycle of the fungus on onion.

**Symptoms**

All investigators who have previously reported studies of the fungus which causes pink root of onions, Pyrenochaeta terrestris, have reported that it causes roots to turn pink. In his work of 1919, Taubenhaus described the host-pathogen association. He reported that affected roots turn yellowish, then pink and then they dry up, and the disease was confined to the roots and not to the bulb. As fast as the old roots were affected new ones were produced, which became diseased. The bulbs used excessive energy producing additional new roots which in turn became affected, thus the bulbs failed to attain the commercial size. Diseased bulbs remained dwarfed and small throughout the season, although they appeared to be sound in every other way.

Hess (1962) also reported that the pink root fungus usually attacked the roots of onion during all stages of growth, however, the disease was more obvious on the mature
crop during hot weather. Pink coloration of the roots was the most apparent symptom. In the beginning stages onion roots turn pink or yellow one by one or all at once. They later darkened to red, purple or brownish black. As new roots were continually formed, they were attacked and killed by the fungus. The aerial leaves of badly infected plants turn white, yellow or brown and die. Scallions or bulbs are very small from plants infected early in the season.

Watson (1961) reported that inspection of propagation plants was not always sufficient to determine the presence of pink root, as pink root symptoms were not evident on all infected bulbs. The pink color was lost on the dried roots of mature bulbs and some pink-colored roots do not contain the pink root fungus *Pyrenochaeta terrestris*. There are certain red pigment-producing forms of *Fusarium* which will color onion roots.

Watson (1961) also proposed a technique for quick identification. He reported that *Pyrenochaeta terrestris* grown on a weak culture medium of wheat straw produces a pink to scarlet red color and the red pigment-producing forms of *Fusarium* will not color the culture medium of wheat straw. Hess, Vaughan, and Leach (1964) reported that the same wheat straw culture medium used with near-ultraviolet light resulted in production of pycnidia. Some isolates developed pycnidia in three or four days and all isolates formed pycnidia with Setae and spores within twelve
days. In this publication a culture medium was reported which was somewhat translucent and pycnidia developed more rapidly. Also the mycelial mat was not as thick, making it easier to identify pycnidia and identification of *Pyrenochaeta terrestris*, thus providing a means for positive identification.

Chupp and Sherf (1960) reported that *Pyrenochaeta terrestris* was not influenced much by soil wetness, except as moisture affects infection by other organisms. However, various isolates of the fungus were sensitive to temperature. Growth of *Pyrenochaeta terrestris* began at about 55° F. and infection was initiated at 60° F. The most favorable temperature for growth and infection of some strains was 75° F. For others 82° F. was the most favorable and the maximum favorable temperature for some strains was slightly above 40° F.

Gorenz, Larson, and Walker (1949) conducted optimal temperature experiments and suggested that 24° C. or 28° C. (75.3° F. or 82.4° F.) was optimum temperature for growth of the various isolates. They further pointed out that *Pyrenochaeta terrestris* grew well at all hydrogen ion levels within the range of PH 4-8. From this evidence, Chupp and Sherf (1960) postulated that if you could keep the temperature under 75° F., it would help control the disease.
Varieties and Resistance

Hansen (1929) was the first to report the variation among pink root isolates and stated that *Fusarium* was not the causal organism. In 1932, Hansen concluded that the multinucleate spores of *Pyrenochaeta terrestris* were not genetically identical. Kulik and Tims (1960), Chupp and Sherf (1960), Hess (1962) and Walker (1953) all indicated that there is a variation of pathogenicity with various isolates.

Stevenson and Jones (1953) reported that Nelwaka, Chrystal Wax and Yellow Bermuda were being used to cross with *Allium cepa* L. to produce resistant varieties. In F1 crosses of *Allium cepa* L. resistance is incompletely dominant and the number of genes is undetermined. Hess (1962) stated that onion varieties resistant to pink root in other parts of the country were not consistently resistant in Oregon.

Brandes, Cordero, and Skiles (1959) reported that the only method of control for the pink root disease seemed to be planting of resistant varieties such as Yellow Bermuda. These authors stated that no practical method has been developed to eradicate the fungus from infested soil, and the development of resistant varieties will remain important although very few satisfactory varieties are now available. Vaughan, Siemer, and Liu (1971) reported that by fumigating the soil, farmers in eastern Oregon were able to grow onions where they could not otherwise be grown.
profitably. However, they stated that fumigation in the field did not rid the soil of *Pyrenoochaeta terrestris*. It does reduce the inoculum in the first few inches of soil. At the first of the season *Pyrenoochaeta terrestris* is less severe and the onions grow more rapidly resulting in a larger bulb size at the end of the growing season. Good results were obtained with Yellow Sweet Spanish which is a moderately susceptible onion. With Southport White Globe, a very susceptible onion, no significant disease reduction or increase in size or yield was evident.

**Ultrastructural Work with Pyrenoochaeta Terrestris**

All of the ultrastructural work done on *Pyrenoochaeta terrestris* has been done by W. M. Hess. In one of his earlier papers, Hess (1967) compared frozen-etched replicas with thin sections of *Pyrenoochaeta terrestris*. He stated that the fungus used glycerol as the only carbon source and survived freezing procedures used for freeze-etching; thus the ultrastructure reflected the living state. Frozen-etched membranes had particles of various sizes and concentrations and had an overall smooth appearance. In contrast, chemically fixed membranes had particles which were difficult to distinguish and their membranes were irregular. Frozen-etched mitochondrial membranes had a rougher, more irregular appearance than other organelle membranes. Older cell walls that have been frozen-etched contain particles and microfilbrillar orientation, but
microfibrils were not visible in chemically fixed cell walls. Ground cytoplasm of chemically fixed cells had ribosomes and areas of high and low electron scattering which are not seen with freeze-etching. Cells which were fixed by glutaraldehyde-acrolein - $\text{OsSO}_4$ produced more closely resembled frozen-etched cells than cells fixed in $\text{KMNO}_4$.

*Pyrenochaeta terrestris* has typical ascomycete septal pores and Woronin bodies and the plasma membrane is continuous from cell to cell through these septal pores. Ribosomes are evident and lipid bodies appear as homogenous electron-dense spherical bodies. Frozen-etched plasma membranes have occasional irregular areas, and vesicles can often be seen outside of the plasma membrane; thus vesicles have smooth surfaces and particles.

The particle distribution differs greatly between the outer surface of the tonoplast and the plasma membrane and also varies from area to area on a tonoplast. The average particle size for both membranes averages $120\,\AA$. The nuclear pores are readily evident and measure approximately $1000\,\AA$ in diameter. It was noted that both rounded and elongated frozen-etched nuclei of *Pyrenochaeta terrestris* have smooth surfaces.

Muhlethaler, Moor and Szarkowski (1965) have suggested that the prominent particles on membrane surfaces are multi-enzyme complexes.

In 1969 Hess reported further investigations concerning the onion host and *Pyrenochaeta terrestris* on an
ultrastructure level using chemically fixed tissues. Hess used two varieties of onions, 951 and Excel. Variety 951 was commonly penetrated at the tip of the root and variety Excel 2 cm or more back from the tip. Both varieties were penetrated by dissolution of host cell walls rather than by pressure of the fungal cells. Both varieties had cells disrupted one to several cells in advance of the fungus. It was suggested that secretion products of the fungus could be causing this. Electron-dense material could be seen in both the cell wall of the host and on hyphal cells, which was possibly associated with the secretion products. To lend support to the assumption that the fungal cells entered by chemical dissolution of host cell walls, Hess grew the pink root fungus on paper which consisted primarily of gymnosperm cells. The results indicated that the cell walls were dissolved by the fungus without any evidence of physical pressure by the fungus.

It was reported that there was a large amount of lipid in the host cells and in the fungal cells growing in the host. While fungal cells grown on synthetic media did not show this lipid accumulation.

Struckmeyer, et al. (1962) reported that onions could respond to *Pyrenochaeta terrestris* by producing extensions or "pegs," which are composed of host cell wall which stretches around the hyphal tip of extending from one-eighth to one-half the width of the cell and prevents the hyphal from entering the host. Hess (1969) did not
observe these "pegs" with the Light Microscope or the Electron Microscope.

Pectin and Pectic Enzymes

The idea that pectic enzymes may be involved in diseased plant tissues was introduced by the studies of DeBary (1886) and this idea was substantiated by Jones (1909) and Brown (1915). Today, pectic enzymes are a common feature of host-pathogen interactions and their interaction in degradation of pectic constituents of cell walls in plant tissues has been reported for many diverse pathogenic agents. Pectic substances are regarded generally to be in the middle lamella. Some, however, have been found dispersed throughout the cell wall.

The composition of pectic substances is mainly of linear polymeric chains of D-galacturonic acid linked as an α1,4 glycoside and with carboxyl groups not esterfied (pectic acid) or with carboxyl groups esterfied with methanol in varying degrees (pectinic acid and pectin). Arabinose, galactose, rhamnose and xylose have also been found to make up a small fraction of pectin substances.

Hatanaka, Chitoshi, and Ozawa (1966) concluded that pectic acid is a herteropolysaccharide consisting mainly of galacturonic acid with small amounts of sugars. From the research of Conrad (1926) it was deduced that onion was 4.8 percent pectin.

Bateman and Millar (1966) reviewed the pectic enzymes. These authors stated that pectin methylesterases
are the enzymes that remove the methoxyl groups of pectin. The pectic glycosidases and lyases are enzymes that cleave the $\alpha 1,4$ glycosidic bond between adjacent uronic acid monomers in pectic substances. This is accomplished by a hydrolytic mechanism or a transeliminative mechanism. On this basis these enzymes are separated into two groups, lyases and hydrolases.

In the literature many terms have been used for pectic enzymes such as: pectinase, polygalacturonase, depolymerase, pectin depolymerase, endopolygalacturonase, exopolypgalacturonase, depolymeric polygalacturonase, liquefying polygalacturonase, saccharifying polygalacturonase, liquefying polymethylgalacturonase, pectin transeliminase, pectin lyase, and protopectinase. Many of these names have been used to describe a single type of enzyme activity. This has made a comparison of the enzymes listed above difficult.

Deuel and Stutz (1958) have divided all pectic enzymes into three classes to try to eliminate this confusion. These classes are liquefying polygalacturonases, liquefying polymethylgalacturonases, and saccharifying polygalacturonases. Liquefying indicates random cleavage and saccharifying terminal cleavage of the chain.

Nomenclature for the pectic enzymes worked with in this study are pectinesterase (which removes the methyl ester groups of pectinic acid and pectin), and endopolygalacturonase which randomly attacks pectic acids. Both of
these enzymes attack the pectic substances by hydrolysis.

Masuda (1968) postulated that auxin induced cell wall elongation was regulated by the activity of certain cell-wall degrading enzymes. So while degrading enzymes such as pectin methylesterase are breaking down cell wall substances and increasing the elasticity and plasticity between cell walls, synthesizing enzymes are resynthesizing cell wall structures. The metabolism and control of these degrading enzymes could also be involved in host pathogen interactions.

**Pectolytic Enzymes Produced by Pyrenochaeta Terrestris**

The first man to work with pectolytic enzymes associated with *Pyrenochaeta terrestris* was Horton (1964). He stated that *Pyrenochaeta terrestris* produced both pectinase and cellulase. The mycelial growth on cellulose and synthesis of cellulase did not vary much at temperatures from 15° C. to 30° C. However, mycelial grown on pectin and pectinase production showed maximum response at 20° C. Growth of *Pyrenochaeta terrestris* on pectin and glucose was similar and was far greater than that on cellulose. Horton (1964) stated that since *Pyrenochaeta terrestris* grows better on pectin than cellulose this suggested that pectinase may be of major importance in pathogenic behavior, while cellulase would be of minor importance. Keen and Horton (1966a) reported that Endo-polygalacturonase (endo-PG) and Pectinesterase (PE) were synthesized by
Pyrenochaeta terrestris in liquid cultures and in infected roots. No polygalacturonate trans-eliminase activity was found. The degree of maceration of the potato tissue was directly related to the endo-PG activity and not with PE activity which would indicate that endo-PG could be of major importance in the entrance of the fungus into the host.

In a later publication, Keen and Horton (1966b) worked with the inducers of endo-PG synthesis in liquid cultures of Pyrenochaeta terrestris. The inducers were galacturonic acid, its polymers (pectin and polypectate), and structural relatives of the polymers (mucic acid, tartronic acid, and dulcitol). It was found that on pectin the growth of the fungus and endo-PG synthesis were proportional to the pectin concentration. Keen and Horton (1966b) also reported that synthesis of endo-PG on pectin was stimulated by hexose supplements at 0.005 M and that it was repressed by supplements at 0.05 M and above. Thus, repression of induced endo-PG synthesis was not dominant at low supplement concentrations as was found for induced cellulase synthesis by the same organism by these workers. This suggests that the production of endo-PG by the parasite during pathogenesis is less repressed by host sugars than cellulase synthesis.
Pectin Histochemical Stains Used with Electron Microscopy

For many reasons progress in Electron Microscopy histochemistry has been slow. Electron stains use metals to achieve enough contrast, so development of histochemical staining procedures at the Electron Microscope level has been slow, although several good ones have been developed.

Hydroxylamine reaction with pectinic acid stain was first used as an Electron stain by Albersheim, Muhlethaler, and Frey-Wyssling, 1960. They used onion roots and iron bound to a modified pectin. The reaction forms pectic hydroxamic acids by substituting hydroxylamine for methoxyl groups of pectin. This is accomplished by a nucleophilic substitution at the carboxyl carbon of pectin which yields insoluble iron complexes upon treatment with ferric iron. The reaction was developed by Cornaz and Deuel (1954) to prepare a selective exchanger for ferric ions. Later this procedure was used by McReady and Reeve (1955) and Gee, Reeve, and McReady (1959) as an in vivo method for quantitative measurement of pectin. These same papers showed that this reaction was specific for pectin at room temperatures.

Albersheim, Muhlethaler, and Frey-Wyssling (1960) found the pectin stain quite effective at the electron-microscope level. They reported that the pectin on the immature cross-wall leads directly into the inner reacting layer of the axillary (logitudinal) wall while the more
mature transverse walls became intimately associated with the middle lamella pectin of the axillary wall. The pectin of the internal layer of the axillary wall and the transverse wall represent the residual fraction while the pectin of the middle lamella represents the hot water-soluble portion of the pectic substances. All of the electron-dense material was removed by the hot versene extraction. The reaction of the above pectin stain was quite harsh, thus the cytoplasmic constituents of the cell were almost completely destroyed.

In a later paper, Albersheim and Killias (1963) found a more effective way to preserve the cytoplasmic constituents. The tissue was fixed in 2 per cent osmic acid buffered with .02 M sodium phosphate, and the whole solution was made 1.2 per cent with respect to sucrose. This reaction was allowed to proceed for six hours at 0° C. Then the rest of the reactions were carried out in the cold. Juniper, et al. (1970) suggested that the tissue be fixed and embedded in plastic with the hydroxylamine reaction carried out as a post-stain on nickel grids.

Ruthenium red stain has not been reported for use at the Electron Microscope level for a pectin stain in plants. However, Juniper, et al. 1970, indicated its use as a pectin stain. They pointed out that Ruthenium red, in theory, stains carboxyl groups. Juniper (1970) stated in practice this means that in plant material Ruthenium stains pectin exclusively. However, many botanists have used
Ruthenium red as a stain to demonstrate pectic substances at the Light Microscope level. Leiser (1968) used it at the Light Microscope level to point out possibilities of pectin in mucilaginous sheaths on root tips.

Luft (1964, 1965, 1966a, 1966b) has developed the Ruthenium stain for use at an Electron Microscope level. He pointed out that Ruthenium red is an inorganic dye prepared synthetically which does not occur in natural form. Luft pointed out various ways of purifying Ruthenium red. Ruthenium red appears to have limited specificity as the following abstracts indicate:

Mehta, 1925, p. 985:

... ruthenium red which is generally regarded as a specific stain for pectin substances and their derivatives is found to stain in addition \( \alpha-, \beta-, \) and \( \tau- \) oxycelluloses, hemicelluloses, gums, galactans, free lignin, mannan and amylohemicallooses.

Bonner, 1936, p. 483:

This reagent Ruthenium red stains pectin very deeply and does not stain other constituents or ordinary cell walls. It, however, has two disadvantages: a) it is not completely specific for pectins, but rather for the 6th carbon atom carboxyl groups cellulose which has been subjected to mild oxidation stains with ruthenium as do other of the "plant slimes" which contain such carboxyl groups.

Preston, 1952, p. 30:

These reactions of ruthenium red are, however, given by other substances present in some cases (oxidized cellulose, cytoplasmic debris, etc.) and it is imperative therefore in all cases of doubt to check on the results by solubility tests.

Jensen, 1962, p. 193:

The pectic substances are some of the more important chemical constituents of the cell wall, yet few
good procedures exist for their localization. The standard procedure consists in staining them with Ruthenium red. This method results in staining the pectic substances red if they are present in fairly high concentration and if various ill-defined interfering substances are not present. Since the basis of the reaction is not known, it must be considered only partly specific.

Luft (1966b) reported that Ruthenium red interacted strongly with Polygalacturonic acid, citrus pectin, heparin, polyacrylic acid, and polymethacrylic acid. Agar gel strongly binds the red color which does not diffuse away in water. The substances giving a strong reaction are all polyacids of high charge density. Tani and Ametani (1971), Groniowski, Biczyskowa and Walski (1969), Gustafson, and Pihl (1967) and many other authors have used Ruthenium red to stain animal cells at the Electron Microscope level.

This study has been set up to determine the pectin distribution in the non-infected onion root (control) tissue and infected tissue, thus permitting an electron microscope study of the host pathogen interaction of P. terrestris and Allium cepa by using the pectin histochemical stains.
MATERIALS AND METHODS

Fungal mycelium of *Pyrenochaeta terrestris* was provided by Dr. W. M. Hess, Department of Botany, Brigham Young University. Dr. Hess collected these isolates of a highly virulent strain of *Pyrenochaeta terrestris* while at Oregon State. The samples were stored at 4 C. in a sterile soil tube until used. The following culturing techniques were developed by Hess (1966). *Pyrenochaeta terrestris* mycelium was transferred under aseptic conditions from the sterile tube cultures to sterile Petri dishes of low nutrient agar.  

1 The inoculated Petri dishes were left at room temperature for six days. Sprouted onion seedlings were then transferred to Petri dishes containing low nutrient agar as controls and inoculated plates. Sterile filter paper discs were then placed on top of the seeds and agar. The filter paper was kept moist with sterile distilled H$_2$O.

Onion seed varieties 'Excel' and '951' were obtained from Desert Seed Company, El Centro, California. These seeds were washed in distilled water three times and then aerated for one hour. They were then left in a solution of

1Low nutrient agar consists of twenty grams agar, three grams sodium nitrate, and one gram magnesium sulfate per liter of water. Watson (1961).
20 ml of commercial clorox and 80 ml distilled water for six minutes. Following this, the onion seeds were washed for one hour with circulating tap water after which the seeds were placed in moistened paper towels and rubber bands were placed around them. These rolled paper towels were placed in glass containers which contained distilled water in the bottom to keep the "rag dolls" moist. Within three days these seeds had sprouted and were ready to transfer to the petri dishes. The Petri dishes with low nutrient agar in them, paper towels, distilled water and filter paper discs were all autoclaved at 250 F. for twenty to forty-five minutes at fifteen pounds pressure. The inoculated and control Petri dishes were placed in a growth chamber at 1000 foot candles under continuous light at 21° C. to 22° C. (69.8° F. to 71.6° F.). They were watered with sterile distilled water once a day with a small sterile pipette. These procedures were developed by Hess (1966).

Most of the onion roots of the 951 variety were initially infected at the tip while Excel was commonly infected initially 2-3 cm back as Hess reported previously (1969). One to 2 cm pieces of the infected onion roots and control onion roots were removed with a sharp razor blade and were fixed for electron microscopy investigations.
Chemical Fixation and Staining Schedule 1.
Hydroxylamine Reaction for Pectin Stain

This reaction was prepared for electron microscope use according to Albersheim, Muhlethaler, and Frey-Wyssling (1960) and solutions one through four were prepared according to Gee, Reeve, and McReady (1959).

I. The root slices were placed in a mild fixation solution of 5 per cent formaldehyde. They were then placed in a vacuum chamber for ten to fifteen minutes to remove the air from the specimens.

II. The root slices were placed in 10 per cent, 30 per cent, 50 per cent and 60 per cent increasing concentrations of ethanol for ten to fifteen minutes each.

III. The root slices were then placed in the four solutions listed below. They were left in each solution one hour. The solutions were prepared in the following manner.

1. Fourteen gm of crystalline hydroxylamine hydrochloride dissolved in 100 ml of 60 per cent aqueous ethyl alcohol.

2. Sodium hydroxide, 14 gm dissolved in 100 ml of 60 per cent aqueous ethyl alcohol.

3. One part concentrated hydrochloric acid (specific gravity 1.188) mixed with two
parts by volume of 95 per cent aqueous ethyl alcohol.

4. Two and one-half grams of ferric chloride lumps dissolved in 100 m. of 0.1N hydrochloric acid in 60 per cent aqueous ethyl alcohol.

All solutions were in 60 per cent ethanol. The root slices were washed and allowed to remain in 60 per cent aqueous ethanol for one hour.

IV. The root slices were then further dehydrated with concentrations of 70 per cent, 95 per cent and three rinses of 100 per cent ethanol. Then three rinses of 100 per cent acetone. The root slices were then ready for embedding.

Chemical Fixation and Staining Schedule 2. Ruthenium Red Stain for Pectin Substances

The following procedures were developed by Luft (1966b).

I. Small vials of root slices were placed in cracked ice and the tissue fixed one-half to one hour in 1.5 ml of 2.5 per cent gluteraldehyde in 0.067 M cacodylate buffer with 500 ppm Ruthenium red prepared by adding the following just before using:

0.5 ml 7.5% gluteraldehyde
0.5 ml 0.2 M sodium cacodylate buffer pH 7.0-7.5
0.5 ml 1500 ppm Ruthenium red solution

II. Decant and wash slices in three changes of
0.067 M sodium cacodylate buffer of three to
five minutes each.

III. The tissues were fixed three to four hours in
1.5 ml of 1:1.67 per cent $\text{OsO}_4$ in 0.067 M sodium
 cacodylate buffer with 500 ppm Ruthenium red.
This mixture was prepared by adding the follow-
ing just before using.
0.5 ml 3-5% $\text{OsO}_4$
0.5 ml 0.2 M cacodylate buffer
0.5 ml 1500 ppm Ruthenium red
The root slices were then ready for dehydrating
and embedding.

Chemical Fixation and
Staining Schedule 3.
Variation of Hydroxylamine
Reaction for Pectin Stain

This reaction was prepared for electron microscope
use according to Albersheim, and Killias (1963) and solu-
tions one through four were prepared according to Gee,

I. The root slices were placed in 2% $\text{OsO}_4$ which
was 0.2M with respect to sodium phosphate
(pH 6.8) and 1.2% with respect to sucrose
(Millonig, 1961). The root slices were left in
this fixation for 6 hours at 0° C.
II. The root slices were placed in 10 per cent, 30 per cent, 50 per cent, and 60 per cent increasing concentrations of ethanol for ten to fifteen minutes each.

III. The root slices were then treated with the four solutions listed below, which were left in the cold while the hydroxylamine reaction for pectin was carried out. The solutions were prepared in the following manner:

1. Fourteen gm of crystalline hydroxylamine hydrochloride dissolved in 100 ml of 60 per cent aqueous ethyl alcohol.
2. Sodium hydroxide, 14 gm dissolved in 100 ml of 60 per cent aqueous ethyl alcohol.
3. One part concentrated hydrochloric acid (specific gravity 1.188) mixed with two parts by volume of 95 per cent aqueous ethyl alcohol.
4. Two and one-half grams of ferric chloride lumps dissolved in 100 ml of 0.1N hydrochloric acid in 60 per cent aqueous ethyl alcohol.

All solutions were in 60 per cent ethanol. Solutions 1 and 2 were mixed with equal parts just before the root slices were added and left in this solution one hour. The root slices were washed in solution 3 for 15 minutes and
placed in solution 4 for one hour. The root slices were washed and allowed to remain in 60 per cent aqueous ethanol for an hour.

IV. The root slices were then further dehydrated with concentrations of 70 per cent, 95 per cent and three rinses of 100 per cent ethanol. Then three rinses of 100 per cent acetone. The root slices were then ready for embedding.

**Chemical Fixation and Staining Schedule 4.**

**Pectin Stain Developed During This Study**

I. The following chemical fixation procedure was developed by Hess (1966).

1. A 2 per cent gluteraldehyde and 3 per cent acrolein fixation solution was prepared by diluting 8 per cent distilled (polyscience) gluteraldehyde 1:1 with distilled water giving a 4 per cent solution. This solution was then diluted 1:1 with 0.2M sodium cacodylate buffer (pH 7.2-7.4) yielding a 2 per cent (0.1 M) gluteraldehyde solution. For each ml of gluteraldehyde .06 ml acrolein was added. The root slices were placed in small vials, fixative was added, and the tissues were left at room temperature for about one week.

2. After the fixation period, the specimens
were washed 6-8 times over a period of one hour with sodium cacodylate buffer (0.2 M, pH 7.2-7.4) mixed with distilled water.

3. The root slices were then placed in 2 per cent $\text{OSO}_4$ solution, obtained by mixing aqueous 4 per cent $\text{OSO}_4$ and sodium cacodylate buffer (0.2 M, pH 7.2-7.4) in a 1:1 ratio. The tissue was left in this staining solution for two hours in an ice bath.

4. The root slices were washed as described in step 2 above.

II. The root slices were placed in 10 per cent, 30 per cent, 50 per cent, and 60 per cent increasing concentrations of ethanol for ten to fifteen minutes each.

III. The root slices were placed in hot methanol and concentrated HCl mixed 2:1 for one minute.

IV. The root slices were then treated with the four solutions listed below, which were left in the cold while the hydroxylamine reaction for pectin was carried out. Solutions 1 through 4 were prepared according to Gee, Reeve, and McReady (1959). The solutions were prepared in the following manner:

1. Fourteen gm of crystalline hydroxylamine hydrochloride dissolved in 100 ml of 60 per cent aqueous ethyl alcohol.
2. Sodium hydroxide, 14 gm dissolved in 100 ml of 60 per cent aqueous ethyl alcohol.

3. One part concentrated hydrochloric acid (specific gravity 1.188) mixed with two parts by volume of 95 per cent aqueous ethyl alcohol.

4. Two and one-half grams of ferric chloride lumps dissolved in 100 ml of 0.1N hydrochloric acid in 60 per cent aqueous ethyl alcohol.

All solutions were in 60 per cent ethanol. Solutions 1 and 2 were mixed with equal parts just before the root slices were added and left in this mixture one hour. The root slices were washed in solution 3 for 15 minutes and placed in solution 4 for one hour. The root slices were washed and allowed to remain in 60 per cent aqueous ethanol for one hour.

V. The root slices were then further dehydrated with concentrations of 70 per cent, 95 per cent and three rinses of 100 per cent ethanol. Then three rinses of 100 per cent acetone. The root slices were then ready for embedding.

**Dehydration Procedure**

The root slices were dehydrated by a series of five to fifteen minute rinses of ethanol in the following
concentrations: 10%, 30%, 50%, 70%, 95%. The tissues were then treated with three rinses of 100 per cent ethanol. The 100 per cent ethanol was followed by three fifteen minute rinses in 100 per cent acetone.

Embedding Procedures

Procedure I

**Plastic solution.**--Stock plastic was prepared by mixing 31.0 ml Epon 812, 40.5 ml aroldite 506, and 3.5 ml dibutyl phthalate. By mixing 6 ml of stock plastic with 6 ml of epoxy hardener DDSA (dodecenyl succinic anhydride) a dilute plastic solution was prepared. This mixture was capped and placed in a 60° C. oven for five minutes. Twenty-eight drops of DMP30 (dimethyl-amine-methyl-phenol) were then added to the warm plastic and the mixture was shaken vigorously for two minutes.

**Twenty-five per cent plastic.**--A 25 per cent plastic solution was made by adding 3 ml of the plastic solution to 9 ml of 100 per cent acetone. The root slices were immersed in this solution and left at room temperature for one hour.

**Seventy-five per cent plastic.**--Nine ml of the remaining plastic (plastic solution) was then added to 3 ml of 100 per cent acetone to give a 75 per cent plastic solution. The root slices were then removed from the 25 per cent plastic solution and placed in the 75 per cent plastic solution for one hour at room temperature.
One hundred per cent plastic.—The root slices were removed from the 75 per cent plastic solution and placed into plastic medicine vial caps with 100 per cent undiluted plastic solutions as described above. After remaining at room temperature for one hour the medicine vial caps were placed in a 60° C. oven and left over night to harden.

Procedure II

Luft's (1961) plastic mixture was prepared from two mixtures.

Solution 1.—Epon 812, 62 ml
  DDSA (dodecenyl succinic anhydride) 100 ml

Solution 2.—Epon 812, 100 ml
  MNA (methyl nadic anhydride) 89 ml.

The hardness of the plastic was determined by the amounts of solutions one and two which were added together. A medium-hard plastic, which is considered good for glass knives, was prepared by a 1 to 1 mixture of solutions one and two.

This mixture was placed in a 60° C. oven for five minutes, after which it was removed and eighteen drops of DMP-30 (dimethyl-amine-methyl-phenol) was added for each 12 ml of plastic mixture. Increasing per cents (25, 75 and 100) of this plastic solution were made as described in
procedure 1 above; also the same embedding procedures were followed.

**Thin-Sectioning Procedures**

Small pieces of embedded root slices were mounted on the ends of three-eighths by one-half inch plastic rods. The pieces of plastic were bound to the plastic rods by Eastman 910 Adhesive. According to the directions outlined in the Sorvall "Porter-Blum" ultramicrotome manual the small pieces of plastic containing the specimens were trimmed to pyramid shapes, exposing the specimen at the tip of the pyramid.

Diamond and glass knives were used in this study. Glass knives were made from commercial LKB glass strips following the procedure outlined in the directions accompanying the LKB knife breaker. The diamond knife sections were cut by Mrs. Connie Swensen, electron microscope laboratory technician, Botany Department, Brigham Young University.

Sorval, "Porter-Blum" MT-2 and Sorvall "Porter-Blum" MT-2B ultramicrotomes were used for sectioning. Speeds for sectioning were 0.56 to 0.86. The sections were 70 to 100 µ in thickness and appeared silver-gold in color when using reflected light. Thickness was determined by using a continuous interference color and thickness scale for thin sections published by the Sorvall Company. The thin sections were picked up on Formvar coated electron
microscope grids. Three-fourths of all sections were post-stained for ten to fifteen minutes with lead citrate stain (Reynolds, 1963).

Hydroxylamine Reaction for Pectin Post-Stain Procedure

The root slices were placed in 5 per cent formaldehyde as outlined in schedule 1. They were dehydrated, embedded in plastic, and then sectioned as outlined above. Once the thin-sections were put on formvar coated grids, the sections were ready for the post-staining solutions. Dilute solutions of the four solutions outlined under schedule 1 were used. The dilutions were made by adding four parts of 10 per cent aqueous ethyl alcohol to one part of the original solutions producing four dilute solutions. Solutions 1 and 2 were mixed in equal volumes just before post-staining and the nickel grids were left on the mixtures to fifteen minutes. The grids were then rinsed with 10 per cent ethanol and transferred to solution 3, diluted for one minute. The grids were then placed on solution 4 diluted for three to four minutes and they were washed with 10 per cent ethanol for one minute. The grids were then stained with lead citrate stain (Reynolds, 1963).

Electron Microscopy and Photography

Thin-sections were examined in a Hitachi model HS-7 electron microscope. Dr. W. M. Hess of the Botany Department, Brigham Young University, Provo, Utah, also
used the Hitachi HUllE electron microscope to examine and photograph some thin-sections. Photographs were taken with Kodak contrast grade, projector slides and Kodak electron microscope film. They were developed in Kodak D-11 and Kodak D-19 developers by standard procedures.

The prints were made on Kodak single weight Kodabromide paper. Kodak Dektol developer diluted 1:1 with water was used to develop the paper following standard procedures.
RESULTS

In this study various pectin stains of non-infected and infected onion cell walls were used at the electron microscope level. The stains which were used were: the hydroxylamine reaction with pectinic acid (Albersheim, Muhlethaler and Frey-Wyssling [1960]); hydroxylamine reaction with pectin used as a post-stain; Ruthenium red pectin histochemical stain; a variation of the hydroxylamine stain with prior fixation of the tissue in .2M Na₂HPO₄, 1.2% sucrose and 2% SO₄; a new pectin stain was developed during this study which involves fixing the tissues with Hess's procedure (1966), esterfying all the free COOH groups with hot CH₃OH and HCl then treating the tissue with the hydroxylamine reaction for pectin; and respective controls for the various treatments.

The hydroxylamine reaction for pectin is very harsh on the tissues (figure 1). In control and infected tissues of the host, the cell wall is all that is left after treatment (figures 1-3). In figure 1 (left side) the oblique section of the cell wall shows the darkly stained pectin in the middle lamella which resulted from pectic hydroxomic acids which were formed by hydroxylamine. This hydroxylamine was substituted for methoxyl groups of pectin forming
Fig. 1.--Oblique section of cell with Hydroxylamine pectin stain showing middle lamella (ML), and a portion of non-infected onion root cell wall (CW). X19,500.
an insoluble iron complex upon treatment with ferric iron. It is assumed that the higher stained portion of the cell wall in figure 1 is not pectin. The middle lamella is also evident in the cross section of the cell wall in the top right corner of figure 1. Both of these cell walls show a microfibrillar appearance. Figure 2 shows the fungus as it is entering the host cell wall. In this figure all of the pectin is gone and the microfibrils in the middle lamella are not as longitudinal arranged as in figure 1, especially around the tip of the fungus entering the host cell wall. In this region the microfibrils appear to be less reticulate in nature. In highly infected tissue (figure 3) most of the cell wall is dissolved and little pectin is evident. Even though the hydroxylamine reaction is harsh, some protoplasmic constituents are still left in the fungus (figure 3).

Non-infected control tissue was treated with formaldehyde, then it was dehydrated and embedded in plastic. Sections of this tissue were cut and stained by the hydroxylamine reaction (figure 4). When this procedure was used, the cytoplasm was partially preserved and the darkly stained middle lamella was also observed. However, the micrographs appear grainy (figure 4).

In theory, Ruthenium red, stains free COOH groups (Juniper, et al., 1970). Juniper stated that Ruthenium red stained pectin exclusively in plant tissue. Figure 5 shows the Ruthenium red stained middle lamella, but the
Fig. 2.--Oblique section of infected onion root tissue with hydroxylamine pectin stain showing the host cell wall (HCW), the fungus (F) and fungal cell wall (FCW). X19,500.
Fig. 3.—Section of infected onion root tissue stained with hydroxylamine for pectin showing the host cell wall (HCW), and fungus (F). X19,500.
Fig. 4.--Section of a portion of non-infected onion root cell post stained with hydroxylamine pectin stain showing the middle lamella (ML), cell wall (CW) and cytoplasm (C). X38,750.
Fig. 5.--Section of a portion of two non-infected onion root cells stained with Ruthenium Red for pectin showing the cell wall (CW), middle lamella (ML) and cytoplasm (C). Note that the middle is stained darker than the rest of the cell wall. X49,000.
tissue is not darkly stained and the middle lamella is not evident in some cell walls (figure 6), although the same stain was used. However, the cytoplasm and plasmalemma are fairly well preserved.

When onion root tissues are fixed with .2M Na₂HPO₄, 1.2% sucrose and 2% OSO₄ before treating with the hydroxylamine reaction more of the cell cytoplasm is preserved, but it is disrupted as shown in figure 7. Pectin layering is also apparent in the older cell walls when this staining procedure is used.

Tissues were treated with just .2M Na₂HPO₄, 1.2% sucrose and 2% OSO₄ before dehydration and embedding, but the hydroxylamine reaction was left out. In tissues treated in this manner, there is no pectin stain evident (figures 8-9). However, when tissues were treated with .2M Na₂HPO₄, 1.2% sucrose and 2% OSO₄ with or without hydroxylamine the cytoplasm is preserved better than when .2M Na₂HPO₄, 1.2% sucrose and 2% OSO₄ is omitted. When this procedure is used without hydroxylamine, the plasmalemma appears disrupted (figure 8-9), but the membranes surrounding the starch grains are still intact (figure 9).

Figures 10, 11, and 12 show infected onion root tissue stained with 0.2M Na₂HPO₄, 1.2% sucrose and 2% OSO₄ before treating with the hydroxylamine reaction for pectin, and there is no pectin stain apparent in these figures. Figure 10 shows the fungus as it is penetrating the host cell wall (arrows). The microfibrils of the host cell wall
Fig. 6.--Section of a portion of two non-infected onion root cells. Ruthenium Red stained for pectin showing the cell wall (CW), nucleus (N), and cytoplasm (C). X21,000. Note that the middle lamella is not stained darker than the rest of the cell wall.
Fig. 7.--Section of a portion of two non-infected onion root cells fixed in .2M Na₂HPO₄, 1.2% sucroses and 2% OSO₄ and then hydroxylamine reaction for pectin showing the cell wall (CW), and pectin layering (PL) in the wall and fragments of disrupted cytoplasm (C). X19,500. Older tissue several cells back from tip.
Fig. 8.--Section of non-infected control tissue fixed in .2M Na$_2$HPO$_4$, 1.2% sucrose and 2% OSO$_4$ with no hydroxylamine just FeCl$_3$. Note that there is no pectin layering in the cell wall (CW). Mitochondria (M), and cytoplasm (C) are evident and the plasmalemma (PM) can be seen. X19,500.
Fig. 9.--Section of non-infected control tissue fixed in .2M Na$_2$HPO$_4$, 1.2% sucrose and 2% OSO$_4$, with no hydroxylamine just FeCl$_3$ showing that the membranes surrounding the starch grains (SG) are still intact although the other membranes of the cell appear to be disrupted. X19,500.
Fig. 10.--Section of infected onion root tissue fixed in .2M Na$_2$HPO$_4$, 1.2% sucroses, and 2% OSO$_4$ and then hydroxylamine reaction for pectin showing the host cell wall (HCW) as the fungus (F) is penetrating it (arrows) and the electron transparent fungal cell walls (FCW). X19,500.
Fig. 11.--Section of infected onion root tissue fixed in .2M Na$_2$HPO$_4$, 1.2% sucrone and 2% OSO$_4$ and pectin stained by hydroxylamine reaction showing the host cell wall (HCW), fungus (F) and fungus cell wall (FCW). Note that the middle area of the host cell wall is more electron dense than the other layers of the wall. X19,500.
Fig. 12.—Section of infected onion root tissue fixed in .2M, Na$_2$HPO$_4$, 1.2% sucroses and 2% OSO$_4$ and pectin stained by hydroxylamine showing the fungus (F) and the partially degraded host cell wall (HCW). Note that the host wall has been almost completely degraded at one point. (see arrows) X19,500.
tissues next to the fungus appear to be less distinct than the microfibrils in other areas of the wall. The center of the host cell wall appears darker than the outer portion in some instances (figure 11).

When the new pectin stain developed during this study is used for non-infected onion root tissue, the pectin appears to be distributed throughout the cell wall with the greatest concentration of pectin in the darkly stained middle lamella area (figure 13-16). Figure 13 shows the microfibril appearance of the middle lamella. Even though this stain is quite harsh, some poorly preserved cytoplasm can be seen (figure 14). Also, the darkly stained pectin can be seen when sections are cut revealing the junction of the four cell walls (figure 14). Pectin also appears to be darkly stained when axillary and transverse cell walls are examined (figure 15). A primary pit field can be seen in figure 16. The pit membranes are darkly stained indicating that they are composed of middle lamella or pectin.

Figures 17 and 18 show non-infected control tissue which was stained with the new pectin stain developed during this study except hot CH₃OH and HCl were left out. When this procedure is used, pectin does not stain (figure 17-18). Figure 18 shows one of the cell walls which was stained darker by this procedure.

When the new pectin stain developed during this study was used on infected onion root tissue, the vivid, darkly stained pectin of the middle lamella area is commonly
Fig. 13.--Section of non-infected outside onion root wall fixed by Hess's procedure, treated with hot CH$_2$OH and HCl, pectin stained by Hydroxylamine. This section shows pectin (P) and cellulose (CE). X19,500.
Fig. 14.—Section of non-infected root tissue fixed by Hess's procedure treated with hot CH₃O¹H and HCl and pectin stained by Hydroxylamine showing the junction of the four cell walls. The middle lamella is darkly stained due to the presence of pectin (P). The less darkly stained cellulose (CE) is also shown, and the cytoplasm (C) is not well preserved. X19,500.
Fig. 15.--Section of non-infected onion root tissue fixed by Hess's procedure treated with hot CH$_3$OH and HCl and pectin stained by hydroxylamine showing the darker stained pectin (P) and middle lamella (ML) and the lighter areas of the wall which contain cellulose (CE). X19,500.
Fig. 16.--Section of non-infected onion root tissue fixed by Hess's procedure treated with hot CH$_3$OH and H 1 and pectin stained by Hydroxylamine showing the darker stained middle lamella (ML) in a primary pit field (PF) and the poorly preserved cytoplasm (C). X19,500.
Fig. 17.--Section of non-infected onion root tissue fixed by Hess's procedure and pectin stained by hydroxylamine without hot methanol showing the cell wall (CW) and poorly preserved cytoplasm (C). Note no pectin is depicted. X19,500.
Fig. 18.—Section of non-infected onion root tissue fixed by Hess's procedure and pectin stained by hydroxylamine without hot CH$_3$OH and HCl showing the cell wall (CW) and poorly preserved cytoplasm (C) with no pectin stain evident. X19,500.
seen (figure 19). Although the tissue shown in figure 19 is four cells away from the fungus, the middle lamella is still darkly stained, and the cytoplasm is not completely disrupted. When the infection front is only two to three cells away (figure 20), the pectin of the middle lamella is not darkly stained, but is readily distinguished. Also the cytoplasm is not completely disrupted (figure 20).

Figure 21 shows the host cell wall at the infection front when pectin is not commonly visible. Also, at the infection front the cytoplasm appears to be completely disrupted (figure 21). Figure 22 shows penetration of the host cell wall by the fungus at the infection front. Also, the electron transparent fungus cell wall and a darker more electron dense host cell wall are apparent, particularly at the sites of fungus penetration (figure 22, arrows).

Figure 23 shows the fungus growing through the center of a host cell wall. The electron transparent fungus cell wall can be seen, and around this a darker stained microfibril appearance of the host cell wall can also be seen. The outer layers of the host cell wall are commonly much less electron dense than the rest of the host cell wall (figures 21-23, 25, 28-31). With more advanced stages of cell wall degradation microfibrils become distinct (figures 23-25, 27-31). Figure 24 shows an example when one-half of the host cell wall has been completely degraded. Figures 26 and 27 show host cell walls which are almost entirely degraded. Figure 26 also shows lipid in the fungus cells.
Fig. 19.—Section of infected onion root tissue four cells away from the infection front. Fixed by Hess's procedure treated with hot CH$_3$OH and HCl, and pectin stained by hydroxylamine showing the middle lamella (ML) and less electron dense cellulose (CE). X19,500.
Fig. 20.--Section of infected onion root tissue two to three cells away from the infection front. Fixed by Hess's procedure treated with hot CH$_3$OH and HCl and pectin stained by the hydroxylamine showing the middle lamella (ML) and the cellulose (CE). X19,500.
Fig. 21.--Section of infected onion root tissue at the infection front. Fixed by Hess's procedure, treated with hot CH$_2$OH and HCl, and pectin stained by hydroxylamine showing the host cell wall (HCW). X19,500.
Fig. 22.—Section of infected onion root tissue at the infection front. Fixed by Hess's procedure, treated with hot CH₃OH and HCl, and pectin stained by hydroxylamine. Showing the host cell wall (HCW) which is being penetrated by the fungus (F) (arrows). Note the host cell walls appear to be layered. X19,500.
Fig. 23.--Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylamine showing the host cell wall (HCW) and the fungus (F) which has grown through the cell wall (FCW). X19,500.
Fig. 24.--Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylamine reaction showing the host cell wall (HCW) and fungus cells (F). X19,500.
Fig. 25.--Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylamine showing the host cell wall (HCW) and the fungus (F). X19,500.
Fig. 26. -- Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylamine showing the fungus (F), the almost completely degraded host cell wall (HCW) and the large amount of lipid (L) in the fungus. X19,500.
Fig. 27.--Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH$_3$OH and HCl and pectin stained by hydroxylamine, showing the fungus (F), and the microfibril appearance of the almost degraded host cell wall (HCW). X19,500.
When the mostly degraded host cell walls are sectioned obliquely, the electron transparent portion and the microfibrils of the host cell walls are readily distinguishable (figures 28, 30-31). Figures 28 and 29 also show the almost completely degraded host cell walls adjacent to fungal cells. In figure 29 the highly degraded host cell wall follows the contour of the fungus and a darker rod-like structure is visible in the middle of the host cell wall.

Occasionally completely disrupted cytoplasm is present adjacent to degraded host cell walls (figure 31). Figure 32 shows the most advanced degradation of the host cell wall observed during this study. The characteristic electron dense and electron transparent areas were not evident (figure 32).

It was observed that all tissues detected by pectin histochemical stains of non-infected onion root tissue were more darkly stained than similarly treated infected tissues.
Fig. 28.--Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylamine, showing the fungus (F) and the microfibril appearance of what is left of the host cell wall (HCW) which has spread over a wide area. X19,500.
Fig. 29.—Section of infected onion root tissue. Fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylationine, showing fungus (F), and mostly degraded host cell wall (HCW) which appears to follow the contour of the fungus. X19,500.
Fig. 30.--Section of infected onion root tissue fixed by Hess's procedure treated with hot CH₃OH and HCl and pectin stained by hydroxylamine, showing the fungus (F) and degraded host cell wall (HCW) tissue with distinct light and dark areas. X19,500.
Fig. 31.--Section of infected onion root tissue fixed by Hess's procedure, treated with hot CH₃OH and HCl and pectin stained by hydroxylamine, showing the fungus (F), the degraded host cell wall (HCW) tissue and degraded cytoplasm (DC) around the host cell wall tissue. X19,500.
Fig. 32.—Section of highly infected onion root tissue fixed by Hess's procedure treated with hot CH₃OH and HCl and pectin stained by hydroxylamine showing the remains of the host cell wall (see arrows). X19,500.
DISCUSSION

Hydroxylamine Reaction for Pectin Stain

As has been mentioned before, various authors have shown that the hydroxylamine reaction is specific for pectin (McReady and Reeve, 1955; Gee, Reeve and McReady, 1959; Albersheim, Muhlethaler and Frey-Wyssling, 1960). Also, the present study has shown that the hydroxylamine reaction is specific for pectin.

The intensity of the pectin stain with non-infected onion root tissue (figure 1) was similar to the results obtained by Albersheim, Muhlethaler, and Frey-Wyssling (1960). This reaction is specific for pectin which has been methyl esterated. The middle lamella pectin or water soluble pectin is highly esterfied while the residual pectin is esterfied to a lesser degree (Albersheim, Muhlethaler and Frey-Wyssling, 1960; Kooiman, 1969).

Horton (1964) reported that P. terrestris produced extra-cellular pectalytic and cellulalytic enzymes. Hess reported that pectinase may be a major factor in pathogenic behavior while cellulase has a minor role. Keen and Horton (1966) identified the pectolytic enzymes of P. terrestris as endopolygalacturonase (endo-PG) and pectinesterase (PE).
Endopolygalacturonase randomly attacks pectic acids by hydrolysis and PE which is also produced by this fungus removes the methyl ester groups of pectinic acid and pectin by hydrolysis. Since the middle lamella has completely disappeared in onion root tissue infected by this fungus (figures 2 and 3) it is assumed that the PE has desterfied this pectin forming pectic acid and endo-PG has probably degraded the pectic acid further. The appearance of a finer reticulate nature around the tip of the fungus in figure 2 possibly indicates the presence of cellulase breaking down the cellulose fibers.

**Hydroxylamine Pectin Post-Stain**

Although the hydroxylamine pectin post-stain gave positive results with a darkly stained middle lamella and partially preserved cytoplasm (figure 4), this procedure was eliminated because of the extreme graininess and poor resolution of the micrographs. The grainy appearance is often quite common when the whole hydroxylamine reaction is used as a post-stain.

**Ruthenium Red Stain for Pectin**

Juniper, et al. (1970) pointed out that in theory, Ruthenium red, stains free COOH groups. In some instances, the middle lamella stained darkly indicating the presence of free COOH groups (figure 5). In other instances, the middle lamella did not stain significantly from the other regions of the cell wall indicating that COOH groups were
not stained (figure 6). These results (figures 5-6) indicate that Ruthenium red does not stain all COOH groups the same, or that pectin does not have as many free COOH groups (figure 6) which Kertesz (1951) hypothesized.

Jensen (1962) stated that Ruthenium red stains pectin when there is a fairly high concentration of pectic substances and when various ill-defined interfering substances are not present. He further stated that this stain must be only partially specific since the reaction is unknown.

Variation of Hydroxylamine Reaction for Pectin

In older non-infected onion root tissue treated with the variation of the hydroxylamine reaction, (figure 7) pectin layering is evident. The plasma membranes of onion root tissues stained by this procedure were not preserved, although Albersheim and Killias (1963) showed that they were present when they used this procedure. Possibly the density of the cytoplasm is masking the cell membrane or membranes of tissue back from the tip disrupt more readily than membranes in meristematic cells. Albersheim and Killias (1963) also reported dense particles in the cytoplasm which were not seen during the present investigation.

The control tissues of non-infected onion roots which were processed without the hydroxylamine reaction (figure 8-9) are identical in appearance to the control
tissue of Albersheim and Killias (1963). This control tissue (figures 8-9) shows the lack of pectin stain in the cell wall when the pre-treatment of the hydroxylamine reaction is left out, indicating that this stain is specific for esterfied pectin. In infected onion root tissue (figures 10-12) fixed in OSO₄ and stained for pectin with the hydroxylamine reaction, no pectin is seen. As the fungus penetrates the host cell wall (figure 10) the host wall adjacent to the fungus appears similar to infected host walls treated with the hydroxylamine reaction without OSO₄ (figure 2). Therefore, prefixing with OSO₄ (variation of hydroxylamine reaction) preserves more cytoplasm. In some instances the host cell wall appears darker in the center with a thin layer on the sides (figure 11). This lighter layer could be the result of action of hydrolytic enzymes. It is shown in figure 12 that not only is the host cell wall degraded at the point of fungus penetration, but hydrolytic enzymes have almost entirely degraded the host cell wall in another area as is indicated by the arrows.

Pectin Stain Developed During this Study

It has been known for some time that hot CH₃OH and HCl would esterfy free COOH groups of pectin and other pyranose compounds (Morrison and Boyd, 1969). In healthy non-infected plant tissue, pectin is the predominant substance which contains free COOH groups. Once pectin has been 100 per cent methyl esterfied, the hydroxylamine
reaction will make all pectin stainable. Juniper, et al. (1970) stated that this treatment (hot CH$_3$OH and HCl) is too destructive to give satisfactory results at the electron microscope level. However, the infected and non-infected onion root tissue (figures 13-16 and 18-32) was fixed for a week (by Hess's procedure, 1966) prior to the hot CH$_3$OH and HCl treatment. Pectin is stained intensely dark when non-infected onion root tissue is stained by this procedure (figures 13-16). Also, stained pectin can be seen dispersed throughout the cell wall, although the middle lamella is much more darkly stained. In figure 13 the residual pectin can be seen. The majority of the soluble pectin has apparently been lost by the harsh reaction. Possibly some of the pectin is lost more readily because the outside cell wall receives a harsher treatment. Cell walls inside the root (figure 14) have poorly preserved cytoplasm with fully detectable soluble and residual pectin. The pit membranes (figure 16) are so darkly stained they appear to consist almost entirely of middle lamella.

Figures 17-18 show the two extremes of a control for the new pectin stain when hot CH$_3$OH and HCl are left out. It is not known why tissues not treated with hot CH$_3$OH and HCl do not react with hydroxylamine (figures 17-18). Perhaps the fixitive reacts with the methyl esters of pectin so they are unable to react with the hydroxylamine reaction. The cell walls treated with this procedure have a homogeneous appearance and the pectin does not stain.
Once this tissue was treated with hot CH$_3$OH and HCl (figure 13-18 and 19-32) probably all of the COOH groups were esterfied (thus it is assumed that all of the pectin is esterfied).

Figures 19-22 show a transition of infected tissue from four cells away to the penetration of the host cell wall by the fungus. Four cells away from the infection front (figure 19) pectinases appear to break the pectin down in layers. The pectin in the middle lamella is still intact, but the pectin in the outside cell wall has light and dark strips, thus indicating that the pectin is completely gone in the light strips and a small amount is present in the darker strips of the host cell wall. With the fungus two to three cells from the host cell wall (figure 20), the dark and light strips are not as apparent, but can still be seen. Also, the middle lamella appears much lighter than when the host cell wall is four cells away from the infection front (figure 19). This indicates that pectin has been degraded from the host cell wall in this region. Cytoplasm is still somewhat intact when the fungus is four cells away (figure 19) and two to three cells away (figure 20) from the host cell wall, but at the infection front (figures 21-22) the cytoplasm is totally disrupted, and the pectin has been totally degraded from the host cell wall by pectinases (figure 21).

Also, a faint electron transparent line on the outside of the host cell wall is easily detected in figures
22, 23, 25, and 28. It is postulated that this area is full of degrading enzymes and no free COOH groups are available to be esterfied, or this could be a host response to resist the penetration of the fungus with the cellulose cross linking forming a barrier, and thus no free COOH groups are available to be esterfied. In micrographs of infected onion root tissue where only the esterfied pectin groups are stained, (figures 2-3 and 10-12) the electron transparent area of the host cell wall is not seen, indicating that this phenomena is correlated with cellulose tissue.

The electron transparent nature of the fungus cell wall (figures 22-31) is probably due to the high amount of glucosamine or chitin and small amounts of cellulose of the fungal cell wall (Robertson, 1968) which is very resistant to this stain. As the fungus penetrates the center of the host cell wall (figure 23) darkly stained microfibrils appear around the fungus. These darkly stained microfibril areas of the host cell wall (figures 23-31) could be caused by degrading enzymes that have released free COOH groups from the cellulose. These darkly stained microfibril areas do not appear when only the esterfied pectin groups are stained (figures 2-3 and 10-12). Adjacent to this darkly stained microfibril area (figure 23), a lighter area is also seen (figures 25, 28, and 30-31) which could be caused by the degrading enzymes which have complexed with the cellulose. This area appears electron transparent because no free COOH groups are available.
Sometimes one-half of the host cell wall is degraded showing the darkly stained microfibril appearance of part of the host cell wall, and the other side of the host cell wall which remains intact (figure 24). This phenomena is probably due to the presence of the degrading enzymes on one side of the wall breaking down the cellulose and releasing free COOH groups.

As the host cell wall is degraded, it often follows the contour of the fungus cell wall (figures 26-31) which could aid in the asimulation of the degraded host cell wall tissue. At points of severe degradation (figure 32) only traces of the host cell wall are evident and a complete lack of contrast is seen.

All micrographs of non-infected tissue were stained darker than similarly stained infected tissue indicating that a large amount of cell wall was degraded in the infected tissue that complexed with methyl esters and COOH groups of pyranose rings. The dynamics of the host pathogen interactions could involve many phenomena. Masuda (1968) in his work with coleoptiles, found that IAA caused an increase in cell-wall-degrading enzymes. From this work he suggested that cell-wall-degrading enzymes play a role in initiating cell expansion. In work with leaf abscission in coleus and phaseolus LaMotte, et al. (1969) found that treatment with IAA increased pectin esterase activity. Cutler and Krusberg (1968) reported that _P. terrestris_ contains a compound with properties identical to
IAA. If onion root tissues have cell-wall-degrading enzymes, then the pathogen, *P. terrestris*, could stimulate an increase of these enzymes in the host, thus increasing the pathogenicity of the fungus.

Horton (1964) reported that pectinases may have a major factor in the pathogenic behavior of *P. terrestris*, while cellulase plays a minor role, although in the saprophytic survival of *P. terrestris* on plant debris in the soil, cellulases would be very important. Horton and Keen (1966a) observed that cellulase synthesis in *P. terrestris* is controlled by a combination of induction and repression which provides a unique sensitivity to the environmental change. Cellulose induces synthesis of cellulase, but when the concentration of glucose released from the cellulose exceeds 0.0005M cellulase synthesis is repressed (Horton and Keen, 1966b). On the other hand, these investigators reported that endo-PG synthesis of *P. terrestris* is induced by galacturonic acid, pectin, polygalacturonic acid, mucic acid, tartonic acid, and dulcitol. Endo-PG is further stimulated by hexose supplements as high as 0.005M. Horton and Keen (1966b) reported that endo-PG synthesis is reduced only by hexose concentrations of 0.05M or above, indicating that pectinases have an important role in the pathogenicity of *P. terrestris*. This also indicates that resistance of the host could be increased with high concentrations of sugars.
English, Jurale and Albersheim (1971) reported in their article on *Colletotrichum lindemuthianum* that the polysaccharide-degrading enzymes are secreted sequentially into the culture medium. Pectinase and arabinosidase were secreted first followed by B-xylosidase and cellulase, then B-glucosidase and finally gataclasidase.

While some of these same polysaccharide-degrading enzymes have not been reported in *P. terrestris*, it appears from this study that this same type of sequence is evident. Of course some alteration can be seen by the varying amounts of polysaccharide substances and the concentrations of sugars in the host tissue.

Scott, et al. (1958) in their electron microscope studies of the epidermis of *Allium cepa* reported that mucilage, which is probably composed of pectin substances, ensheaths the thin cuticle of the root. Wright and Le Tourneau (1965) reported that *P. terrestris* used L-arabinose, D-ribose, D-xylose, L-rhamnose, D-fructose, D-galactose, D-glucose, D-mannose, L-sarbose, sucrose, cellobiose, lactose, maltose, sorbitol and D-inositol as carbon sources. Four of these are important constituents of pectin compounds. From the above information it can be concluded that a fuller understanding of the pectin and pectinase host pathogen interactions of *P. terrestris* and *Allium cepa* as they relate to pathogenicity would be important in controlling pink root of onion. Thus, further work is warranted.
CONCLUSION

1. The evidence of this study indicates that pectinesterase is one of the first pectin degrading enzymes to attack the host cell wall, while endopolygalacturonase attacks the host cell wall soon after. This assumption is based on the fact that the pectin histochemical stain which stains only methyl esters is completely removed from the cell wall slightly ahead of the pectin histochemical stain which stains COOH groups.

2. It is postulated that soon after the pectinesterase has started to effect the host cell wall, endopolygalacturonase begins to degrade the desterfied pectin in layers.

3. The amount of degradation of the host cell wall by endopolygalacturonase is not known, but it is assumed that as soon as all of the stained pectin is removed from the host cell walls most of the endo-polygalacturonase activity has been completed. However, a large amount of host cell wall maceration could be due to endopolygalacturonase activity.

4. It is assumed that as soon as the pectinase has degraded most of the pectin the cellulase activity is increased with the continued degradation of the host
cell wall, and other enzymes are further released in the complete degradation of the host tissue.

5. On basis of the histochemical stain, it appears that the carbohydrate degrading enzymes are released in a sequence, pectinesterase and endo-polygalacturonase are probably released first followed by the cellulases.

6. Electron transparent areas are caused by glucosamine or chitin in the fungus. These transparent areas in the host could be due to degrading enzymes complexing with the cellulose or cellulase interlinking. It is apparent that there are no COOH groups in the electron transparent areas.
BIBLIOGRAPHY


Hess, W. M. Pink Root of Onion by Pyrenoachaeta terrestris Ph.D. dissertation, Oregon State University, Department of Plant Pathology. 1962.


Sideris, C. P. Species of Fusarium isolated from onion roots. Phytopathology 14:211-216. 1924.


A PECTIN HISTOCHEMICAL STUDY OF THE HOST PATHOGEN RELATIONSHIP BETWEEN PYRENOCHAETA TERRESTRIS AND ALLIUM CEPa

LeRoy Richard Cobia
Department of Botany
M.S. Degree, August 1971

ABSTRACT

The involvement of pectin and pectinases in the host pathogen interaction of P. terrestris and Allium cepa were investigated by use of pectin histochemical stains at the electron microscope level.

Several different pectin histochemical stains were used, but only two (the hydroxylamine reaction, and the pectin stain developed in this study) gave reliable results. It was observed that by the time the fungus has reached the host cell wall pectinesterase and endoplygalacturonase have fully removed the pectin from the host cell wall. When this has occurred, cellulases are released which continue to degrade the host cell wall. Electron transparent areas appeared where there were no COOH groups.