Isoenzyme patterns and ultrastructural changes in tomatoes infected with potato spindle tuber viroid

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ISCENZV.ME PATTERNS AND ULTRASTRUCTURAL
CHANGES IN TOMATOES INFECTED WITH
POTATO SPINDLE TUBER VIROID

A THESIS
SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY
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FOR THE DEGREE OF MASTER OF SCIENCE

BY
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DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA
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Master of Science

of

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ABSTRACT

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Isoenzyme Patterns and Ultrastructural Changes in Tomato Plants Infected with Potato Spindle Tuber Viroid

Advisor: Dr. E. L. Stevenson

Master of Science degree conferred May, 1980

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The isoenzyme patterns of three enzymes and the ultrastructural changes of Rutgers tomato plants were determined after infection with Potato Spindle Tuber Viroid. Electrophoresis of the protein extract in polyacrylamide gels followed by staining for malate dehydrogenase, esterase, and peroxidase showed that only peroxidase was affected by the infection. Two new bands of the isoenzyme were detected in protein extracts of infected tissues. It is suggested that these bands represent the molecular symptoms of the disease.

Thin sections of diseased tissues examined with the electron microscope revealed cytopathic modifications such as the interruptions of the nuclear membrane, increased vacuolation of diseased cells, increased production of peroxisomes, the formation of paramural bodies, and the reduction of starch granules in the chloroplast.

Increased vacuolation of diseased cells suggests that PSTV infection speeds up the aging process in the tissues.
The increased production of peroxisomes might contribute to the extrasynthesis of peroxidase. The formation of paramural bodies suggests a host response to the invading pathogen and may function as a mechanism to block further spread of the pathogen to other cells. And finally it is possible that the reduction in starch granules could result from an inhibition of starch synthesis or the increased translocation of this photosynthetic product out of the chloroplast.
ACKNOWLEDGMENTS

My thanks and appreciation go to Dr. E. L. Stevenson who patiently directed this research and to Dr. J. Mayfield for all his assistance in electron microscopy work and photography.
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CHAPTER I

INTRODUCTION

Potato spindle tuber disease which is present in most potato growing areas of the world was first identified and described by Martin in 1922 in Irish cobbler potato (*Solanum tuberosum*) (O'Brien and Raymer, 1964). The causative agent of this disease was later identified as a low molecular weight RNA and subsequently the term "viroid" was proposed to describe this pathogen and other RNAs with similar properties (Diener, 1971).

This disease is easily transmitted in the field. Singh, according to Diener (1979), reported that this disease may cause up to 64% reduction in potato yield. Symptoms incited by potato spindle tuber viroid (PSTV) in potato foliage are often very difficult to detect. Study of the causal viroid was hampered by lack of a suitable indicator plant until Raymer and O'Brien, cited in Diener and Raymer (1967), observed that the virus also causes distinctive symptoms on Rutgers tomato plants. Since then Rutgers tomato has been used extensively as a test plant for indexing for PSTV infection (Fernow, 1967; Singh, 1977). Electrophoretic analysis of cellular nucleic acids on polyacrylamide gels is also used as a means of detecting PSTV infection (Morris and Smith, 1977). While this method might be very efficient, the extraction and purification procedures might limit its application.
In this study the type of changes effected at the cellular and macromolecular level associated with PSTV infection were studied. For this purpose we have chosen to examine the isozyme pattern of esterase, malate dehydrogenase and peroxidase. The analysis of isoenzymes on polyacrylamide gel electrophoresis does not require highly purified extracts (Brewer, 1970) and hence might provide a simple and quick method of detecting the molecular symptoms of this newly discovered pathogen. The combination of isoenzyme analysis along with the ultrastructural study of infected cells might help elucidate the host-pathogen interaction.
CHAPTER II

REVIEW OF THE LITERATURE

The term "viroid" was proposed by Diener (1971) to designate potato spindle tuber "virus" RNA and other RNAs with similar properties. Since then other plant diseases have been shown to be elicited by viroid. These diseases are Citrus exocortis (Semancik and Weathers, 1972; Sanger, 1972), Chrysanthemum stunt (Diener and Lawson, 1973; Hollings and Stone, 1973), Chrysanthemum chlorotic mottle (Romaine and Horst, 1975), Coconut cadang-cadang (Randles, 1975), Cucumber pale fruit (Sanger et al., 1976), and Hop stunt (Sasaki and Shikata cited in Diener, 1979). In all of these cases the causative agents of these diseases have been identified as low molecular weight RNAs of about 110,000 to 127,000 daltons (Sanger et al., 1976).

Potato spindle tuber disease was first identified and described by Martin in 1922 (O'Brien and Raymer, 1964) in Irish cobbler potato (Solanum tuberosum). Since then potato spindle tuber viroid (PSTV) has been found to be able to systemically infect 157 different plant species in twelve different families (Diener, 1979). The disease is widespread in potato growing regions of the north and northeastern United States and Canada, to some extent in the United Socialist Soviet Republic, South Africa, and Argentina (Diener, 1979).
The disease, according to Schultz and Folsom cited in O'Brien and Raymer (1964), is tuber perpetuated and can be spread in the field. The viroid can be transmitted by leaf mutilation, tuber and stem grafts (Goss, 1926), and by mechanical means (Marriam and Bonde, 1954; Easton, 1963). Earlier reports by Goss (1928, 1930) on the transmission of the spindle tuber disease by arthropod vectors are now viewed with skepticism since the test methods were not conclusive enough to rule out transmission by other means (Diener, 1979).

The ease with which PSTV is spread in the field presents a threat to certified seed potato growers. Besides, the disease is often very difficult to diagnose in potato plants since symptoms are often lacking or very obscure. Proper identification of the disease is highly dependent on the experience of the observer (Morris and Smith, 1977). Singh, according to Diener (1979), reported that this disease may cause up to 64% reduction in potato yield.

Raymer and O'Brien first reported in 1962 that Rutgers tomato *Lycopersicon esculentum* Mill reacts to PSTV with easily recognizable symptoms (Diener and Raymer, 1967). Even though a number of other hosts have since been identified, including a local lesion host, *Scopalia sinensis*, Rutgers tomato is still the most widely used test plant for PSTV due to the ease with which it can be propagated and the fact that it reacts to PSTV with easily recognizable symptoms. When
mechanically inoculated systemic symptoms appear in 2-5 weeks. These symptoms include epinasty and rugosity of the new leaves, followed by necrosis of midribs and veins of older leaves, "bunchiness" of the apical leaves, and stunting of the entire plant (O'Brien and Raymer, 1964).

Most of the symptoms associated with viroid infection also occur with viral, fungal, and bacterial infections. Hence, similar, if not identical, metabolic derangements may be responsible for symptom formation irrespective of the initial etiological factor. Hence, in this review, examples of nonviroid diseases are cited provided they help explain similar symptoms appearing as a consequence of viroid infection.

The pathological processes in virus infected plants, the origin of physiological abnormalities and how they in turn cause the characteristic external symptoms are still subjects of interest to plant physiologists. Diener (1963) has suggested that the direct or indirect effects of virus infection extend to many functional systems of the plant and virtually affect all classes of plant metabolites. The question as to which of the virus induced metabolic aberrations is the immediate cause of symptom formation is still not fully understood.

One aspect of metabolic aberration in plant infection which is intensively investigated is the change in the multiple molecular forms of enzymes (preferred isoenzymes). Such
changes have been observed not only for virus-plant infection, but also for bacterial and fungal infection. The considerable differences in the isoenzymes among tissues of most organisms, and during the life cycles, as well as changes following infection can be used clinically to detect organ damage or to study tissue differentiation and ontogeny (Brewer, 1970). In plants, isoenzyme changes or biochemical symptoms are also an aid to diagnosis of plant diseases and to an understanding of the biochemistry of infection (Reddy and Stahmann, 1972).

The importance of changes in host isoenzymes have been stressed by several authors in connection with the probable role of these changes in the regulation of metabolism of the diseased plant. Sako and Stahmann (1972) observed changes in eleven enzymes of barley susceptible to mildew and concluded that such changes are biochemical symptoms of diseases and may be the basis for increased metabolism often associated with infection. They further suggested that there are profound interactions between parasite and host at the enzyme level.

Before reviewing specific isoenzyme systems, it is necessary to note that quantitative alterations of protein in plant infection have been observed by Zaitlin and Harihara-subramanian (1972) who analyzed proteins from subcellular fractions of PSTV infected Rutgers tomato leaves by electrophoresis in sodium dodecyl sulfate containing polyacrylamide gels. They detected increased synthesis of two proteins of
molecular weights 155,000 and 195,000 daltons stimulated as a result of infection. Alteration in protein extract as well as lysed protoplast of Gynura aurantiaca accompanying citrus exocortis viroid infection were observed by Conejero and Semancik (1977) via electrophoretic analysis. Accumulation of two proteins of molecular weights 15,000 and 18,000 daltons were detected in protein extracts of infected plants. Such protein accumulation was mostly demonstrated in postribosomal preparations. These two proteins, according to the authors, reflect a quantitative alteration in host specified protein.

Although a multiplicity of plant esterases have been reported (Jooste and Moreland, 1963; Schwartz, 1964), not much is known about their physiological role. They are readily localized histochemically at places of cell division. In plant infection, very few reports of esterase activity are documented. Decreased activity of esterases has been reported in black rot disease caused by Ceratocystis in sweet potatoes (Weber and Stahmann, 1964) and also in the halo blight disease caused by Pseudomonas phaseolicola in bean, Phaseolus vulgaris (Rudolph and Stahmann, 1966). The authors suggest in both cases that such decrease in esterases activity was probably due to the catabolic tendencies of the decaying tissues. Jones and Stoddart (1971) reported the formation of additional esterase isoenzyme bands in roots of phyllody infected white clover, Trifolium repens L. They observed that in all cases infection was clearly associated
with the production of increased quantity of esterase variants in the host tissue. In a study of 16 enzymes of peas infected with *Fusarium oxysporum*, Reddy and Stahmann (1972) observed one new esterase isoenzyme band and an increase in the activity of two other bands. Sako and Stahmann (1972) detected two new esterase isoenzyme bands and an increase in the activity of two other bands in protein samples of barley leaves infected with *Erysipe graminis* f. sp. hordei. The author suggested that such changes in enzyme activities of isoenzymes are molecular symptoms of disease and may be the basis for the different metabolism of the infected host which results in the production of symptoms.

Malate dehydrogenase (MDH) is an enzyme intimately associated with cell respiration. Change in respiratory rate is one of the most observed physiological changes in plant infection and there is sufficient evidence to show that MDH is affected in the process. New forms of MDH were observed in extracts of susceptible barley leaves following mildew infection (Johnson et al., 1966). Jones and Stoddart (1971) observed higher levels of MDH in phyllody-infected white clover, *Trifolium repens* L. Sako and Stahmann (1972) observed the formation of a new band and an increase in the intensity of three MDH bands in barley leaves infected with *Erysiphe graminis* f. sp. hordei. The formation of a new MDH isoenzyme band has been reported in peas infected with *Fusarium oxysporum* f. sp. Race I (Reddy and Stahmann, 1972). Edvera
(1977), in a comparative study of the infectious disease (Blue mould) and a physiological disease of tobacco observed that some MDH isoenzymes were completely inactive while others showed reduced activity. Reduced activity of MDH subbands has also been reported for viral and chemically induced tumor extracts of hamster muscle (Prasad et al., 1972).

Peroxidase is one of the most prominent isoenzymes in plants. It is detectable in all plant tissues and probably plays a role in the oxidation of the plant hormone, indoleacetic acid. There is considerable tissue and ontogenic variations in the bands and activity and also considerable genetic variation in the system (Brewer, 1970).

The effect of pathogenic infection on peroxidase isoenzymes have been variously investigated. Farkas and Staehmann (1966) have studied the effect of southern bean mosaic virus (SBMV) infection on the peroxidase isoenzyme of bean leaves. They detected the formation of one new peroxidase isoenzyme band in protein extracts of infected leaves. Experiments with protein inhibitors such as actinomycin D, puromycin, and p-fluorophenylalanine indicated that the new isoenzyme resulted from biosynthesis and not from activation of inactive enzymes. They attributed the appearance of the new isoenzyme to accelerated senescence and to necrobiosis. Solymosy et al. (1967) have demonstrated that changes in peroxidase activity were more pronounced in lesions and areas surrounding the lesions in Phaseolus vulgaris and Nicotina
glutinosa infected with tobacco mosaic virus and alfalfa necrosis virus. Lesion formation chemically induced by the application of HgCl₂ also showed such peroxidase changes. They concluded that these changes were due to accelerated aging of tissues. Wood and Barbara (1971) found that the activity of peroxidase in leaves of cucumber infected with the W strain of cucumber mosaic virus increased with symptom severity and virus multiplication.

Other investigations have been concerned with the question as to whether the infection-induced changes in peroxidase levels are virus specific, host specific, or both. Solymosy et al. (1967) investigated this problem by inoculating 4 bean varieties (pinto, top crop, "Furi," and GN 123) as well as Nicotina glutinosa with tobacco mosaic virus (TMV), tobacco necrosis virus (TNV), and alfalfa mosaic virus in different host-virus combinations. Comparison of the changes in the peroxidase isoenzyme spectrum in the various host-virus combinations indicated that the type of change was determined mainly by the host tissue, not by the virus. Novacky and Hampton (1968) also came to a similar conclusion after studying peroxidase isoenzymes from Nicotina tabacum infected with TMV or with tobacco streak virus, from Vigna sinensis infected with cucumber mosaic virus (CMV) or tobacco ringspot virus (TRSV), from P. vulgaris infected with TRSV, SBMV, or TMV. Their results showed that changes in peroxidase isoenzymes were the same for each host regardless of the infectious particle.
Viruses cause disease by upsetting the metabolism of the cells which, in turn leads to the development by the cell of abnormal substances and conditions injurious to the functions and life of the cell. Such injuries may include development of abnormal structures and/or the degeneration of vital cell structures.

Cytopathic effects of viroid infection have been reported by Semancik and Vanderwoude (1976). Electron microscopic examination of thin sections of symptom and symptomless half-leaves of Gynura aurantiaca infected with citrus exocortis virus (CEV) revealed the plasma membrane aberrations as the primary cytopathic effect associated with infection. The presence of numerous paramural bodies or plasmalemmasomes were directly correlated with the initiation of symptoms. The authors suggested that the alterations in cell-surface properties may constitute a significant phase in viroid replication and pathogenesis.

McMullen et al. (1977) examined ultrastructural sections of young barley leaf tissues systemically infected with the ND18 strain of barley stripe mosaic virus (BSMV). They identified many paramural bodies and associated cell wall thickening as a common feature of diseased cells. Since cell wall thickening and paramural bodies were found in close association with each other, they hypothesized that these vesicles and convoluted membranes were the results of plasmalemma activity in relation to extra cell wall synthesis. Occurring
along with the paramural bodies and cell wall thickenings were bulbous-shaped "extended plasmodesmata" and extra protoplasmic sacs. They suggested in concurrence with other authors (Hiruku and Tu, 1972; Alison and Shalla, 1974) that these structures are a protective response by the plants to restrict virus translocation.
CHAPTER III

MATERIALS AND METHODS

Plant Material

Rutgers tomato (*Lycopersicum esculentum* Mill.) seeds were obtained from Park Seed Company, Greenwood, South Carolina. About 60 seeds were sown in 4-1/2 inch pots in soil mixture composed of 1/2 soil, 1/4 peat moss, 1/8 perlite and 1/8 vermiculite. Upon germination, seedlings were potted singly in 4-1/2 inch pots in the same soil mixture. Plants were adequately watered, fertilized and maintained in the greenhouse at temperatures of about 28 C during the night and 35 C during the day.

Inoculation

The source of PSTV inoculum was foliage of PSTV-infected Rutgers tomato. Infected leaf tissue was ground with a mortar and pestle in pH 7, 0.02 M chilled sodium phosphate buffer. About 2 ml of buffer was added to 5 gm of leaf tissue. Tomato seedlings were inoculated when they were about 5 days old. Inoculation was achieved by dusting the seedlings with 600 mesh Carborundum and gently applying the inoculum to the cotyledons and terminal portions of the seedlings with a cotton tipped applicator. About 5-10 minutes after inoculation, plants were rinsed with tap water (O'Brien and Raymer, 1964).
Leaf Harvesting

The first visible symptoms were observed 15-18 days following inoculation. At 15 days and 18-21 days following inoculation, the first three terminal leaves of treated and untreated plants were harvested. Five grams of each sample collected was freeze dried and stored until use.

Homogenization of Tissue

For the estimation of protein and peroxidase analysis, tissue samples (approximately 1/2 gm per 1 ml) were extracted with pH 9, 0.075 M tris-citrate buffer. Tissue was ground in a chilled mortar and pestle and the resultant homogenate brought to a final volume of 10 ml with the same buffer and centrifuged at 30,000 rpm for 30 minutes. The yellow supernatant was collected and stored at -20 C until use for protein estimation or electrophoresis.

Tissue samples used for MDH and esterase analysis were similarly extracted but tris-HCl pH 8, 0.01 M buffer was substituted for tris-citrate. Tris-HCl buffer contained 0.5 M sucrose, 0.1% ascorbic acid and 0.1% cystine hydrochloride.

Better resolution of MDH and esterase isoenzyme bands was obtained when tissue sample was extracted with tris-HCl buffer whereas more bands of peroxidase isoenzymes were resolved in samples extracted with tris-citrate buffer.

Protein Estimation

Soluble protein was precipitated by the addition of 3 ml
of cold 20% TCA to 5 ml of leaf extract to make a final concentration of 7.5% TCA. The protein was allowed to precipitate for about 30 minutes after which it was separated from the supernatant by centrifugation at 2,000 rpm for 10 minutes. The protein pellet was then dissolved in 1 ml of 1% TCA and again centrifuged at 2,000 rpm for 10 minutes. Finally, the protein precipitate was dissolved in 5 ml of 0.1 N sodium hydroxide and used for protein estimation. Protein quantity was estimated by the Lowry method using lysozyme as a protein standard (Lowry et al., 1951).

**Separation of Isoenzymes**

Isoenzymes of malate dehydrogenase, esterase, and peroxidase were separated by vertical flat-bed disc electrophoresis in a gradient pore polyacrylamide gel using an Ortec high voltage pulsing system. Gradient pore gels were prepared by layering of acrylamide solutions of varying concentrations to form an electrophoretic matrix with discoid pores of varying diameters.

With the gel casting stand in an upright position, a 59 mm layer of small pores or running gel, consisting of 8% acrylamide and 2.5% methylene bis-acrylamide, was cast. Immediately a second layer, a 9 mm layer of intermediate pore size consisting of 6% acrylamide and 2.5% methylene bis-acrylamide was carefully layered over the first layer. The second layer was immediately covered with a water layer and
allowed to polymerize for 20 minutes. After the water layer was removed, a third layer, 5 mm layer, consisting of 4.5% acrylamide and 2.5% methylene bis-acrylamide was cast, water layered and allowed to polymerize for 5 minutes. These three layers of acrylamide were buffered with tris-citrate buffer pH 9, 0.0375 M. Upon removal of the water layer, a fourth layer, a well forming layer containing 8% acrylamide and 2.5% methylene bis-acrylamide buffered with tris-citrate buffer pH 9, 0.075 M was cast. A twelve pronged well former was inserted into this layer and allowed to polymerize for at least 20 minutes. The well former was then removed and the wells rinsed with distilled water. Water was carefully removed from the wells with absorbent paper (Ortec Incorporated, 1969). The 8% acrylamide gel described above was used for the separation of peroxidase and esterase isoenzymes.

For the separation of MDH isoenzymes, a gradient pore gel of higher acrylamide concentration was required. The first layer, a running gel, contained 12% acrylamide, the second layer 9% and the third layer 5% acrylamide.

Aliquots of extract containing 200 micrograms of protein were placed in each well for the separation of peroxidase and MDH whereas aliquots of extract containing 400 micrograms of protein were necessary for the separation of esterase isoenzymes. Wells were capped with gels of composition similar to that of the well forming layer and allowed to polymerize for 20 minutes before assembling the cells in the Ortec electrophoretic tank.
Tris-borate buffer pH 9, 0.065 M was used as the electrode buffer for the separation of peroxidase and esterase isoenzymes. For the separation of MDH isoenzymes, a tris-glycine buffer pH 8.4, 0.05 M was used. The upper and lower tank buffer were of the same composition except for the addition of a tracking dye, 0.1% bromophenol blue, to the upper tank buffer. Gels were run in the buffer for 20 minutes at 75 pulses per second (pps), 5 minutes at 150 pps, 10 minutes at 225 pps and at 300 pps until the tracking dye migrated to about 2 mm of the bottom of the gels. The time required for the tracking dye to migrate to within 2 mm of the bottom of the gel was approximately 80 minutes. A current of 325 volts was applied when only one cell was run. However, when two cells were run, a current of 400 volts was applied (Ortec Incorporated, 1969). After electrophoretic separation, gels were removed from cells and isoenzymes localized by routine staining procedures.

**Localization of Isoenzymes**

The sites of esterase isoenzymes were marked by visual detection of a brown diazo dye formed by the coupling of alpha-napthol with a diazonium salt (Fast blue RR) after the alpha-napthol was liberated from alpha-napthyl acetate by the esterase activity. After equilibrating the electrophorized slab in 50 ml of pH 6.6, 0.04 M tris-chloride buffer at 37 C for 5 minutes, the buffer was poured off and a solution of substrate and complexing agent added to the slab. The
complexing agent contained 100 mg of fast blue RR in 100 ml of pH 6.6, 0.04 M tris-chloride buffer plus 4 ml of 1% alpha-napthyl acetate. Gels were incubated in the staining mixture at 37 C for 15 minutes with intermittent agitation. The reaction mixture was poured off and the reaction stopped with 100 ml of an acid alcohol mix (20 ml of 95% ethyl alcohol and 80 ml of 10% aqueous glatial acetic acid) (Allen et al., 1965). The gels were later stored in 10% acetic acid.

MDH activity was indicated by the presence of reduced nitro blue tetrazolium formed as a by-product of the oxidation of malic acid by MDH. The reaction mixture contained 0.013 gm nitro blue tetrazolium, 0.0003 gm phenazine methosulfate, 0.03 gm sodium malate and 0.013 gm nicotinamide adenine dinucleotide in 30 ml of pH 8.5, 0.1 M tris chloride buffer (E. Stevenson, personal communication). Gels were incubated in this reaction mixture at 37 C for 20 minutes in the dark and then stored in 10% acetic acid.

The sites of peroxidase activity were localized by the detection of a yellow oxidized benzidine when the substrate peroxide was reduced by peroxidases. The reaction mixture contained 90 ml of benzidine solution (0.05 gm of benzidine in 200 ml of water at 80 C), 10 ml of saturated ammonium chloride, 10 ml of 5% EDTA pH 6.0 and 0.1 ml of hydrogen peroxide. Gels were incubated in the staining mixture for 5-10 minutes and later stored in 10% acetic acid (Pearse, 1964).
Electron Microscopy

The third true leaf of healthy PSTV infected plants was used for the ultrastructural analysis. Leaf pieces about 1 mm square were fixed for 10-15 minutes at room temperature in 3% glutaraldehyde in 0.05 M cacodylate buffer pH 7.5. The tissues were then fixed for an additional 10-15 minutes at 4 C after which an equal volume of 2% osmium tetroxide and 0.05 M cacodylate was added under the hood and post fixation continued for 2 hours. Tissues were then washed in cold distilled water for one hour with at least 10 changes and left overnight in cold distilled water (Mayfield, personal communication). Dehydration was achieved by sequentially treating the tissue with 50, 70, and 95% ethanol for 10 minutes at each concentration followed by 10 minutes in 100% acetone. The tissue was then infiltrated overnight in a mixture of acetone and Spurr's media (Spurr, 1969). Infiltrated tissues were embedded in beem capsules containing Spurr's medium. Beem capsules were placed in a capsule rack and left overnight in a curing oven at 60-65 C. Tissue sections measuring from 200-300 A were cut with a glass knife using the KLB-Ultramicrotome (Ultratome III) sectioning unit. Cut sections were placed on a 200 mesh grid and stained in 2% uranyl acetate for 2-5 minutes and then in lead citrate for 5 minutes. Grids were allowed to dry on a piece of absorbent paper and viewed in the RCA EMU-4 electron microscope.
CHAPTER IV

RESULTS

Symptom Expression

Systemic symptoms characteristic of PSTV infection appeared 18-21 days postinoculation. Epinasty and rugosity were first observed on new leaves. On older leaves, midrib and vein necrosis were commonly observed. As growth progressed, diseased plants appeared stunted (about 1/3 the normal size of uninfected plants at 6 weeks postinoculation) and there was bunchiness of the apical leaves (Fig. 1).

Samples were selected from these tissues having characteristic symptoms to examine isoenzymes and ultrastructural changes accompanying viroid infection.

Protein Content

The protein content of PSTV infected Rutgers tomato plants was not grossly different from that of healthy plants. About 12.50 mg of protein were detected per gram of diseased tissue and about 12.20 mg per gram of healthy tissues.

Isoenzyme Pattern

Malate Dehydrogenase

Two minor bands of malate dehydrogenase were located at relative mobility (Rd) 0.143 and 0.357, and three major bands were located at Rd 0.457, 0.577, and 0.628 in soluble
Fig. 1. PSTV infected (left) and healthy (right) Rutgers tomato plant 6 weeks postinoculation.
protein extracts of healthy and inoculated tissues sampled prior to the expression of symptoms (Fig. 2). There was no change in the band pattern of the infected tissues following the expression of symptoms.

**Esterase**

Protein extracts sampled prior to the expression of symptoms had four isoenzyme bands located at Rd 0.214, 0.571, 0.671, and 0.714 (Fig. 3). The same bands were detected in extracts obtained after the disease symptoms were fully expressed.

**Peroxidase**

The banding patterns of peroxidase isoenzymes from healthy and infected tissues sampled 15 days postinoculation consisted of three major bands and two minor bands with Rd values of 0.285, 0.600, 0.743, 0.786, and 0.814. Two additional fast moving bands located at Rd 0.857, and 0.885 were detected in protein extracts of diseased tissues sampled 21 days postinoculation (Fig. 4). These two bands were completely absent from healthy tissues of comparable age and tissues sampled prior to the expression of any observable symptoms. Results are summarized in Tables 1 and 2.

**Cytopathic Effect of PSTV Infection**

Electron micrograph of cells from thin sections of non-infected Rutgers tomato leaves are shown in Figs. 5 and 6.
Fig. 2. Diagram and photograph of MDH isoenzymes in healthy (left half) and PSTV infected (right half) Rutgers tomato leaves 21 days postinoculation.
Fig. 3. Diagram and photograph of Esterase isoenzymes in healthy (left half) and PSTV infected (right half) Rutgers tomato leaves 21 days postinoculation.
Fig. 4. Diagram and photograph of peroxidase isoenzymes in healthy (left half) and PSTV infected (right half) Rutgers tomato leaves 21 days postinoculation.
Table 1. Isoenzyme bands of healthy and PSTV infected Rutgers tomato plants 21 days postinoculation.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Healthy</th>
<th>Infected</th>
<th>Intensity changes</th>
<th>New bands*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate dehydrogenase</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Esterase</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

*Number of isoenzyme bands detected in gels from infected plants which were not visible in extract of healthy plants.
Table 2. Relative mobility of isoenzymes in PSTV infected Rutgers tomato plants at 21 days postinoculation.

<table>
<thead>
<tr>
<th>Isoenzymes</th>
<th>Malate dehydrogenase</th>
<th>Esterase</th>
<th>Peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>0.143</td>
<td>0.214</td>
<td>0.285</td>
</tr>
<tr>
<td>E2</td>
<td>0.357</td>
<td>0.571</td>
<td>0.600</td>
</tr>
<tr>
<td>E3</td>
<td>0.457</td>
<td>0.671</td>
<td>0.743</td>
</tr>
<tr>
<td>E4</td>
<td>0.557</td>
<td>0.714</td>
<td>0.786</td>
</tr>
<tr>
<td>E5</td>
<td>0.628</td>
<td>-</td>
<td>0.814</td>
</tr>
<tr>
<td>E6</td>
<td>-</td>
<td>-</td>
<td>0.857</td>
</tr>
<tr>
<td>E7</td>
<td>-</td>
<td>-</td>
<td>0.885</td>
</tr>
</tbody>
</table>
Fig. 5. Cell of untreated Rutgers tomato leaf showing chloroplast (CH) and starch granules (SG). x 24,000.
Fig. 6. Section of untreated Rutgers tomato leaf showing the nucleus (N) surrounded by an intact nuclear membrane (NB), and mitochondrion (M). x 51,000.
Accumulation of starch granules was characteristic of healthy leaf cells. There were no modifications or irregularity of cell structures. On the contrary, cytopathic modifications were regularly observed in cells of thin sections of diseased leaves.

Among these modifications were the irregular interruptions of the nuclear membrane (Fig. 7), extensive vacuolation of diseased cells (Fig. 8) and increased production of peroxisomes (Figs. 9, 10, 11). Peroxisomes were bound by a single limiting membrane and contained a dense inner core showing a regular crystalloidal structure. Reduction in starch granules was observed in chloroplasts of diseased cells (Fig. 12).

Paramural bodies were frequently observed in cells of PSTV infected leaves (Figs. 8, 10, 11). These malformations consisted of electron dense membranous inclusions embedded in an electron-lucent matrix contiguous with the host cell wall. The membranous inclusions ranged from vesicles to tubules both of which vary in size. In some instances these structures were formed opposite one another in adjacent cells but were separated by the cell wall (Fig. 10). In other instances the paramural body and peroxisome were formed close together in the same cell (Figs. 10 and 11).
Fig. 7. PSTV infected Rutgers tomato leaf cell showing the nucleus (N), nucleolus (NU), cell wall (CW), mitochondrion (M), and chloroplast (CH). x 51,000. Arrows indicate interruptions of the nuclear membrane.
Fig. 3. Section of PSTV infected Rutgers tomato leaf cell showing vacuoles (V) and paramural body (PB). x 100,000.
Fig. 9. Section of PSTV infected Rutgers tomato leaf cell showing peroxisome (P) with rectangular crystalloid content, cell wall (CW), and starch granules (SG). x 100,000.
Fig. 10. Paramural bodies (PB) with granules occurring in adjacent cells, peroxisome (P) with rectangular crystalloid content, and cell wall (CW) in PSTV infected Rutgers tomato leaf cell. x 100,000.
Fig. 11. Adjacent cells of PSTV infected Rutgers tomato leaf showing paramural body (PB) which appear detached from the plasma membrane. Note peroxisome (P) with crystalloid content, mitochondria (M), and cell wall (CW). x 100,000.
Fig. 12. Section of PSTV infected Rutgers tomato leaf cell showing chloroplast (CH) devoid of starch granules, and cell wall (CW). x 24,000.
CHAPTER V

DISCUSSION

The protein content of the PSTV infected tomatoes was not significantly different from that of healthy tomatoes. Alteration in overall protein contents has not been reported for other viroid infections. However, Conejero and Semancik (1977) noted quantitative alteration of protein from subcellular and post ribosomal preparations of CEV infected Gynura aurantiaca. Such observations were also made by Zaitlin and Hariharasubramanian (1972) in protein from subcellular preparations of PSTV infected Rutgers tomato leaf. These alterations accordingly represent a metabolically active constituent in an abnormal developmental condition.

The isoenzyme pattern of 3 enzymes involved in the metabolism of Rutgers tomato have been detected. A summary of the results obtained with the three enzymes is given in Table 1 which lists the number of isoenzyme bands detected for each enzyme in extracts of healthy and PSTV infected Rutgers tomato leaves. Table 2 is a summary of the relative mobility of each of the isoenzymes. In extracts of healthy leaf tissues, the number of isoenzyme bands varied from 4 with esterase to 5 with MDH and peroxidase. Prior to the expression of any observable symptoms (15 days postinoculation), gel electrophoresis of protein extract from inoculated and
healthy leaf tissues were the same for each of the three enzymes. However, following the expression of symptoms only one enzyme, peroxidase, showed an increase in the number of isoenzyme bands. The bands are numbered 1, 2, 3, 4, 5, 6, 7 according to their increasing electrophoretic mobility. Bands 6 and 7 were completely absent from protein extract of the healthy leaves.

Even though a number of reports have been made on the alteration of MDH (Jones and Stoddart, 1971; Sako and Stahmann, 1972) and Esterase isoenzyme (Rudolph and Stahmann, 1966; Reddy and Stahmann, 1972) in plant infections, our observation with PSTV infected Rutgers tomato suggests that these two enzymes are not affected by the pathological process. Since the only increase was experienced with peroxidase activity, this enzyme has been chosen for further discussion.

Changes in peroxidase isoenzymes were not observed in PSTV infected tomato extracts until symptoms were fully expressed. Similar results were obtained by Solymosy and Farkas (1963) in viral infection of tobacco tissues, and by Farkas and Stahmann (1966) in SBMV infection of pinto bean. The relationship between the appearance of symptoms and changes in peroxidase content and the mechanisms involved warrants detailed discussion.

Peroxidase is one of the most prominent isoenzymes in plants. This enzyme is detectable in all plant tissues and
is known to show considerable tissue and ontogenic variation in bands and activity (Brewer, 1970). Therefore, it is reasonable to assume that the effect of pathogenic infection is easily reflected in this system. It is possible that the intracellular aberration associated with the disease development produces substances triggering the synthesis of some new peroxidase.

Generally changes in the isoenzyme banding pattern of diseased tissues have been regarded as biochemical symptoms of the disease. These changes according to Sako and Stahmann (1972) are undoubtedly important factors in producing disease symptoms. In other pathogenic infections, it has been suggested that the new peroxidase isoenzymes are formed by a triggering of enzyme protein synthesis in the host tissue in response to the pathogen (Shaw, 1963; Jones and Stoddart, 1971). Little is known about the factors involved in the triggering of these changes in enzyme synthesis. So far, two major factors—(i) accelerated aging of infected tissues and (ii) the necrobiosis associated with lesion formation—have been identified as contributing to these changes. Farkas and Stahmann (1966) demonstrated that the appearance of a new peroxidase component in bean leaves Phaseolus vulgaris infected with SBMV was clearly dependent on the progress of disease development. Virus infection according to this source speeds up aging and this leads to premature appearance of the peroxidase component. Solymosy
et al. (1967) demonstrated by gel electrophoresis of protein extract from lesions and areas immediately surrounding the lesions that lesion formation in virus infected *Phaseolus vulgaris* and *Nicotina glutinosa* resulted in the synthesis of new peroxidases. They attributed such changes to a triggering of new enzyme synthesis as a result of the necrobiosis associated with the lesion. Our investigation of isoenzyme patterns in PSTV infected Rutgers tomato plants which was done in conjunction with ultrastructural analysis of infected cells further clarifies this subject.

Increased vacuolation which is often associated with aging tissues (Esau, 1967) was observed in PSTV infected cells (Fig. 10). This suggests in essence that PSTV infection speeds up the aging process in the tissues and this might be a factor in the increased production of peroxidase as suggested earlier. Beyond this, peroxisomes contain peroxidase and the increased formation of peroxisomes in PSTV infected cells might contribute to the increased production of peroxidase isoenzymes. Peroxisomes also contain catalase together with enzymes which produce hydrogen peroxide. The action of catalase causes peroxidation of ethanol to acetaldehyde, formic acid to carbon dioxide and water or of a further mole of peroxide to oxygen and water (Roberts, Hall, and Flowers, 1976).

The accumulation of starch granules in the chloroplast was noted in all healthy leaf cells examined. Such starch
granules were highly reduced in chloroplast of diseased leaf cells. Since the accumulation of starch granules in chloro-
plast has been shown to reduce photosynthesis in some tropi-
cal plants (Hillard and West, 1969), it would seem that this
photosynthetic product is being translocated out of the in-
fected cells at a faster rate than in healthy cells to allow
for increased photosynthesis or that the synthesis of starch
is inhibited by the infection. In another viroid infection,
Lawson (1968) demonstrated that Senecio cruentus infected
with Chrysanthemum stunt virus produced more starch lesions
when grown in the winter at lower temperatures than those
grown in the summer at elevated temperatures. As our test
plants were exposed to the same environmental conditions, it
would seem that the reduction of starch granules in PSTV in-
fected plants was due to the effect of the pathogenic infec-
tion. Further investigation would be warranted in order to
clarify this relationship.

Other cytopathic modification associated with PSTV in-
fecion was the formation of paramural bodies in diseased
cells. These structures variously referred to as wall blis-
ters (Luke et al., 1966), lemmasomes or lemmasome-like bodies
(Ehrlich and Ehrlich, 1971), boundary formations (Tu and
Hiruki, 1971), or plasmalemmasomes (Semancik and Vanderwoude,
1976) have been described for diseased tissues of different
pathologic origin. Paramural bodies are often found in
cells where active synthesis is taking place. Merchant and
Robards (1968) suggested that these structures are associated with cell wall synthesis. Their distribution according to this source suggest they may be involved in enzymatic processes, possibly involving secondary modification to developing cell wall. Because of the association between paramural body and cell wall protrusions, McMullen et al. (1977) were led to suggest that these vesicles and convoluted membranes were the results of increased plasmalemma activity in relation to cell wall synthesis. Even though such alterations in physiological conditions were not observed in PSTV infected cells in relation to the formation of paramural bodies, we suggest that this malformation originated as a response of the cell to the invading pathogen and could function as a mechanism to block further spread of the pathogen to other cells.

Semancik and Vanderwoude (1976) observed the formation of paramural bodies as the primary cytopathic effect in CEV infected Gynura aurantiaca. Since this structure originated from the plasma membrane they suggested that there might be a possible alteration of the cell-surface properties and this in turn may constitute a significant phase of viroid replication. The same principle may also apply to PSTV infection since the electron micrographs of PSTV infected cells reveal also that paramural bodies originated from the plasma membrane.
CHAPTER VI

SUMMARY

The protein content detected in PSTV infected Rutgers tomato tissues was not grossly different from that detected in healthy tissues. Polyacrylamide gel electrophoresis of three enzymes, namely malate dehydrogenase, esterase, and peroxidase, showed that only the peroxidase system was affected by the infection process. Two new peroxidase bands were detected in protein extracts of PSTV infected Rutgers tomato plants. It was suggested that these new bands may represent the molecular symptoms of the disease. Alternatively they may be the result of accelerated aging of the diseased tissues and/or the increased production of peroxisomes associated with the diseased cells.

Electron microscope examination of thin sections of PSTV infected tissues revealed cytopathic modifications such as the irregular interruptions of the nuclear membrane, extensive vacuolation of diseased cells, increased production of peroxisomes, the formation of paramural bodies and the reduction of starch granules in the chloroplasts. It was suggested that (i) aging is speeded up in the disease process as evidenced by extensive vacuolation of diseased cells, (ii) increased production of peroxisomes may contribute to the extrasynthesis of peroxidase, (iii) paramural bodies may
originate as a response of the host cell to the invading pathogen and could function as a mechanism blocking further spread of the pathogen to healthy cells, and (iv) the reduction in starch granules could result from an inhibition of starch synthesis or the increased translocation of this photosynthetic product out of the chloroplasts.
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