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## **Presymptomatic detection of Fusarium wilt of tomato by electrical measurement as related to pectic enzyme production.**

Frank Lawrence Caruso  
*University of Massachusetts Amherst*

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PRESYMPTOMATIC DETECTION OF FUSARIUM WILT OF TOMATO  
BY ELECTRICAL MEASUREMENT AS RELATED  
TO PECTIC ENZYME PRODUCTION

FRANK L. CARUSO

B.S.

Gettysburg College

Gettysburg, Pennsylvania

Thesis submitted to the Graduate Faculty in partial  
fulfillment of the requirement for the degree of

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Department of Plant Pathology

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September 1974

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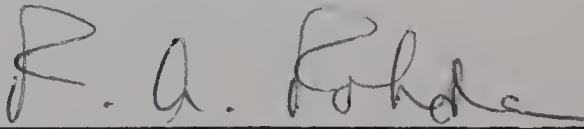
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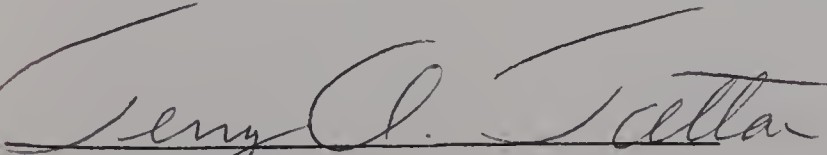
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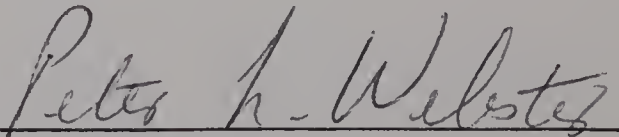
Frank L. Caruso

Approved as to style and content by:

  
(Chairman of Committee)

  
(Head of Department)

  
(Member)

  
(Member)

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## INTRODUCTION

Fusarium wilt of tomato is a major soil-borne disease of field and greenhouse tomatoes (67). A more thorough understanding of the mechanisms involved in vascular wilt disease is needed. The use of non-destructive electrical measurements that are sensitive to diseased conditions in plants may allow the detection of presymptomatic host expression of vascular wilt disease. Permeability changes have been measured with changes in the electrical conductivity of living plant tissues (16). Alterations in electrical measurements have been detected beyond infected areas of potato tubers (31) and rotted or decayed tissues (23, 24, 65). The roots of diseased plants were observed to undergo electrolyte leakage (27) and changes in electrical measurement (20).

Endopolygalacturonase has been implicated as an important component of Fusarium wilt of tomato (15, 49, 67). This enzyme is an important agent of maceration (3, 6, 63) and electrolyte leakage (43).

The purpose of this work was to relate changes in permeability of root tissue as measured by alterations in electrical resistance, and to correlate polygalacturonase activity to Fusarium wilt progression in tomato.

## LITERATURE REVIEW

Fusarium wilt of tomato was first reported in Massachusetts in 1911 (62). Since that time it has remained the major soil-borne disease of field and greenhouse tomatoes. The pathogen, Fusarium oxysporum Schl. f. sp. lycopersici (Sacc.) Snyder & Han., invades the epidermis or hairs of the fibrous root system of the host, eventually reaching the xylem vessels. In young plants, earliest symptoms are clearing of the veinlets and epinasty of the petioles. The lower leaves may simultaneously become chlorotic. As these leaves wither and die, vascular cells within turn brown. In compatible host-parasite situations, plants will wilt and death may result. Plants able to withstand mortality may be severely stunted and have greatly reduced fruit yield.

The physiology of fusarial wilt has been extensively studied. The most drastic consequence of infection is a vast alteration in the water relations of the vascular system. The mechanism responsible for this stress has been debated by advocates of two schools of thought. Advocates of the "toxin theory" assert that the leaves wilt as a result of an increase in the permeability of parenchymatous cells so that osmotic efficiency is lost. Researchers supporting the "plugging theory" believe that leaves wilt because they receive less water than they transpire as the resistance to flow of water through xylem becomes very high (17).

Proponents of the toxin theory have existed since 1919 when Brandes noted that "the fungus tissue in the lumina of vessels is not



present in quantity sufficient to cause serious obstruction to the passage of water" (11). Most of the foundation of this theory was established by Gäumann and his associates as the first fusarial toxin lycomarasmin was isolated in 1944 (25). The compound was purified from cultures of the fungus and has never been found in infected plants. Hence, it is not a vivotoxin. Scheffer and Walker (57) reported that it does not cause vascular browning or plugging of the vessels, and the water economy of tissues is not effected in similar fashion to the infection process itself. Lycomarasmin was discovered to be associated with cell walls and was found to be liberated only after autolysis of the fungus (52).

The second wilting toxin, fusaric acid, was described in 1952 (26). Fusaric acid has been isolated from wilt-diseased plants (41), but its primary role in pathogenesis has been questioned. Collins and Scheffer (13) believed it was not responsible for the stimulated respiration characteristic of infected plants. In addition, Kuo and Scheffer (42) noted the dissimilarity between symptoms of the toxin versus those seen in infected plants. Fusaric acid had no apparent effect on the conductive ability of the xylem. The toxin decreases respiration and mitochondrial activity, whereas these processes occur in higher rates during infection. In a series of ultraviolet mutants of *F. oxysporum* f. sp. *lycopersici*, Kuo and Scheffer (42) found no correlation between ability to produce fusaric acid and pathogenicity. The toxin competitively inhibits the action of polyphenol oxidase, yet in the diseased plant, activity of this enzyme is vastly increased (47).

The evidence implicating toxins as a primary cause of wilting is not convincing. It consists first of the demonstration that a collapse in water economy occurs when excised tomato shoots are allowed to take up the toxins, and second, of the demonstration that these toxins alter the water permeability of plant protoplasts. Morphological symptoms produced by high doses do not coincide with those associated with the disease. A collapse in the water economy and wilting may be expected after the application of sufficiently great amounts of any translocatable non-specific toxic substance. Low doses of lycomarasmin produce an increase in transpiration but no general breakdown. Observed increases in the water permeability of the protoplasts cannot be emphasized as increases in water and solute permeability are to be expected as death approaches and the osmotic system breaks down (45). The toxins are able to cause a disturbance in physiological processes, but may not be implicated as the primary cause of wilting.

The plugging theory is based on a number of factors which collectively contribute to the vascular dysfunction of the host. Chambers and Corden (12) noticed a frequency of vessel collapse and distortion, especially in petiolar bundles. A proliferation of the xylem parenchyma cells is caused by increased levels of indoleacetic acid and these cells put great pressure on the vessel elements.

Higher levels of growth regulators also cause the conductive elements to become hypertrophic. Development of abundant tyloses reduces the effective radius available for water transport, although the host can usually use an alternate pathway. Such is the case for

Verticillium-infected hops which only show mild wilting despite the presence of large numbers of tyloses (64).

Plugging of tracheal elements by mycelium, bud cells, microconidia and macroconidia also contributes to vascular dysfunction. Waggoner and Dimond (66) found mycelium was able to reduce vessel flow efficiency. Bud cells and spores would be dislodged from mycelium and transported through conductive elements to become lodged against end walls or at narrow constrictions. These propagative cells were observed to germinate and aid in the systemic spread of the pathogen (7). The extent of the interference to flow was contributory rather causal.

Wilting of diseased plants may also be a result of an altered viscosity of the tracheal fluid, although it appears to be the same in infected as in healthy tomato plants (66). Fungal pathogens are not able to secrete extracellular polysaccharides as readily as are bacterial organisms such as Pseudomonas solanacearum (37).

High molecular weight compounds such as pectins, gels, gums, polymerized melanoid pigments and polysaccharides can pass readily in solution through open conductive elements but are not able to pass through cell wall openings. These polysaccharides and glycopeptides may have the most pronounced effect on the water economy of the host plant. Portions of them may accumulate in the pit membranes of vessels which act as ultrafilters. This entrapment will reduce the lateral and longitudinal flow of water through the vascular system (18). Smaller molecules transported to leaves will disrupt water transfer from veins

and accumulate in the parenchymatous cell membranes where tissue damage will occur as marginal drying (26).

Pectolytic and cellulolytic enzymes secreted extracellularly by the pathogen have been shown to be an important part of the plugging hypothesis. Pierson, et al. (55) histologically proved plugs to be of pectinaceous material by means of ruthenium red staining. Pectin methyl esterase (PME) and depolymerase (DP or PG) were suspected as being responsible. Gothoskar et al. (29) and Waggoner and Dimond (67) found PME and DP in culture filtrates and suspected a definitive role in wilting. Polygalacturonase (PG) and PME were demonstrated in much larger amounts in infected stem juice than in uninoculated controls (15). Much higher PG activity was found in susceptible tomato stems than stems of resistant varieties. Because PG activity correlated well with the onset and development of disease symptoms, it was supposed that PG was an important entity in pathogenesis by this fungus (49). Endopolygalacturonate trans-eliminase (PGTE or pectin lyase) is produced by F. oxysporum but its relation to wilt production remains unanswered (58). Cellulase has also been found in the culture filtrate and it may have a minor role in vascular dysfunction (38).

Since PME does not split the pectin molecule, but merely demethylates carboxyl side chains, it is unlikely that it contributes directly to formation of pectin-containing plugs. The demethylated pectin molecule is, however, more subject to be cleaved by endo-PG than the original form. As host tissue is macerated and pectic acid is hydrolyzed, reducing sugars and other substances are released into the vascular stream. Such processes would eventually lead to pectin gels.

Other indirect evidence has implicated endo-PG as a focal point of the disease syndrome. Rufianic acid, which inhibits both PG and PME, reduces severity of wilt symptoms when applied to infected plants (18). Production of PG is repressed by glucose and its derivatives, and when glucose is introduced into vascular bundles of inoculated plants, less PG is present in diseased tissues and wilting symptoms are reduced (54). Calcium deficiency increases the susceptibility of tomato plants to fusarial wilt, despite prior treatment with indoleacetic acid. Severity of wilt was increased by deficient calcium nutrition after infection, and decreased if the deficiency occurred before infection. Calcium is a known inhibitor of PG and may influence wilt disease by interfering with decomposition of pectic substances within the host (14). Calcium pectate, which is present in the plant receiving a normal calcium supply, is more resistant to attack by PG than is pectic acid. Plants treated with indoleacetic acid tend to contain a larger amount of pectates and hence, resistance may result.

Endo-PG has also been considered an important agent in tissue maceration. In maceration of tissue by Rhizoctonia solani, PG was shown to have a primary role and cellulase a secondary role. Upon extraction from cultures, PG in the absence of PME and cellulase was able to macerate potato tissue (2). The macerating enzymes of Rhizopus spp. (63) and Sclerotium rolfsii (6) were also indicated to be PG.

Polygalacturonase is very important in the degradation of the cell wall during infection. Sunflower stem tissue infected with S. sclerotiorum contained large amounts of PG and the galacturonide

content of infected tissue was only one-fourth that of healthy tissue (34). Approximately 80% of the galacturonic acid present in tomato stem cell walls is removed in six hours of enzymolysis with dialyzed Fusarium oxysporum culture filtrates. Upon inhibition of the PG with isolated proteins, cell wall degradation by the mixture of fungal enzymes in the dialyzed filtrate was prevented (39).

A 100% greater rate of electrolyte leakage was detectable in mung bean hypocotyls 14-18 hr after inoculation with R. solani. Symptoms did not develop until 20-24 hr and endo-PG was identified as the permeability-altering factor (43). Highly purified endo-PGTE induced electrolyte loss prior to maceration and cellular death in potato tissue (48). These phenomena have been monitored through various electrical monitoring techniques. Such methods can measure changes in electrical conductivity which is dependent on increased ion leakage (27). These methods have been employed recently by plant physiologists (28, 32, 33, 35, 51, 69).

Electrical resistance (the reciprocal of electrical conductance) and electrical impedance have been used to study cellular death (51), frost hardiness (28, 32), and in studies related to membranes (33, 69). Damage to cell membranes is clearly associated with a drop in electrical resistance (ER) at a given temperature. Changes in ER within living tissue reflect the physiological state of the tissue itself even if membrane damage does not result (51). Variable low frequency sine wave current was found to be conducted chiefly in the channels of the cell walls of potato tubers, the roots and stems of

alfalfa, and similar plant tissue (35). Such measurement of the corresponding impedance were used to detect the state of water relations in these channels (35).

There has been limited use of these electrical techniques in research of plant diseases. The capacitance of immature potato tubers infected with potato virus X was lower than that achieved for corresponding control tubers (30). Changes of low frequency (1 Kilohertz) conductance were detected 4 mm in advance of the hyphal tips of Phytophthora infestans and Pythium ultimum on potato tubers (31). Changes in permeability, indicated by a release of sodium and potassium ions, was detected in the roots of tobacco etch virus--infected Tabasco pepper plants 24-48 hr before wilt symptoms occurred (27). An altered permeability of cell boundaries tends to allow these particular mobile ions to escape from the cell more readily. Electrolyte leakage was shown measured through electrical measurement to be a more accurate index of disease severity in Texas male-sterile cytoplasm and N cytoplasm inbreds inoculated with Helminthosporium maydis race T (24). Differences in impedance have also been detected in the roots of apple trees affected by the proliferation disease mycoplasma (20). Degree of resistance in progressive stages of fungal and bacterial discoloration and decay in living trees was correlated primarily with the concentration of mobile potassium and calcium ions (65).

Observations of witloof chicory tissue infected by soft rot bacteria correlated well with this thesis problem. Electrical conductance of the leaf midrib tissue was found to be substantially increased in the rotted area. The conductance increased before symptom

development and in advance of the margin of visible lesions. Tissue injury was the result of the action of pectolytic enzymes produced by the pathogens, as a mutant non-pectolytic strain of Pseudomonas marginalis caused neither soft rot nor conductance changes (23).



## MATERIALS AND METHODS

## PATHOGEN ISOLATES:

Three wild strains of Fusarium oxysporum f. sp. lycopersici, all isolated from diseased tomato plants, were procured from the Massachusetts Suburban Experiment Station in Waltham. A fourth strain, Wellman R-5-6Q, was acquired from the American Type Culture Collection in Rockville, Maryland. All strains were maintained on potato dextrose agar slants at 25°C.

## LIQUID CULTURES:

A liquid culture medium containing, per liter, 0.36 g  $MgSO_4$ , 1.64 g KCl, 1.09 g  $KH_2PO_4$ , 8.41 g  $Ca(NO_3)_2$  and 20.0 g sucrose (1) were divided into 250 ml portions and autoclaved at 15 psi and 250°C for 20 min. Flasks were seeded with a 1-ml spore suspension of F. oxysporum from the stock cultures and agitated on a rotary shaker at 150 rpm. This suspension was utilized as inoculum.

A nutrient medium containing 1.5% citrus pectin N.F. (Nutritional Biochemicals Corp.) as a carbon source (67) was employed for enzyme production. Cultures were shaken at 150 rpm.

## ENZYME PREPARATION:

PG was partially purified from 4-5 day-old liquid culture filtrates. Ammonium sulfate was added to the filtrate to precipitate the proteins selectively at 50% and 90% saturation. After centrifugation, pellets were resuspended in 3 ml distilled water and dialyzed for 1.5 hr

the bath being changed every 10 min. The dialyzed solution was lyophilized and stored at  $-20^{\circ}\text{C}$ . A 1.5 x 8.5 cm column of diethylaminoethyl (DEAE) cellulose ( $\text{Cl}^-$  form) equilibrated at  $5^{\circ}\text{C}$  with several volumes of 0.01 M citrate buffer, pH 4.5, was used for further purification. The lyophilized 50-90%  $(\text{NH}_4)_2\text{SO}_4$  protein fraction was dissolved in 2 ml 0.01 M citrate buffer, pH 4.5, and applied to the column. Fourteen 5 ml fractions were eluted with the same buffer and collected. Fractions containing PG were stored at  $-20^{\circ}\text{C}$ .

#### HOST INOCULATION:

Tomato plants (*Lycopersicon esculentum* Mill.) of the susceptible variety Bonny Best were grown in a sterilized mixture of soil and sand in a greenhouse and inoculated at eight weeks after seeding or when plants had sufficient sized taproots. Inoculation was done by placing plants in a solution of 100 ml spore suspension added to 200 ml of half-strength Hoagland's nutrient solution. The pH of the solution had been adjusted to 6.5 by addition of 0.1N NaOH prior to the addition of spores. Plants used in diffusive resistance determination were inoculated by the root dip technique after mechanical wounding to the roots (57).

#### ENZYME ANALYSIS:

The loss in viscosity of a 1.5% pectin substrate was used to determine polygalacturonase activity (4). Size 300 Will. viscometers were run at  $30^{\circ}\text{C}$  with 5 ml substrate and 1 ml test solution at pH 4.5, 6.5 and 8.0. Autoclaved controls were also examined.

The presence of other enzymes in the DEAE cellulose fractions was checked at pH 4.5 and 6.5. Presence of pectin methyl esterase was determined using the method of Smith (60) which utilized bromthymol blue as an acid indicator. A 0.6 ml portion of partially purified culture filtrate was added to 7.4 ml of 0.5% pectin substrate plus 2 drops bromthymol blue and incubated for 24 hr. PME activity was also monitored by determining the increase in acidity of a 1% pectin solution in 0.1N NaCl (2). Two ml of partially purified enzyme was added to 25 ml substrate with pH adjusted to 6.0. The reaction was run at room temperature for 30 min. Presence of cellulase was determined by loss in viscosity of 1.2% carboxymethyl cellulose (9). PGTE was assayed by the periodate thiobarbituric acid method (56) in which the activity of the enzyme is indicated by the formation of a red chromagen which shows a maximum absorption at 548 nm. The reaction mixture contained 0.1 ml of 0.6% (w/v) sodium polypectate in 0.05M Tris-HCl buffer (pH 8.0) plus  $10^{-4}$  M  $\text{CaCl}_2$  and 0.1 ml partially purified culture filtrate. The reaction mixture was incubated for 12 hr.

The cup-plate method of Doery et al. (19) was used for phosphatidase detection using 1% soybean lecithin, and for protease detection using 1% gelatine. Peroxidase was assayed by the method of Maehly and Chance (46) which measured the decomposition of hydrogen peroxide with o-dianisidine in methyl alcohol and 6 ml of 3% hydrogen peroxide at pH values of 4.5 and 6.5. Rate of color development was measured on a Beckman DB-G grating spectrophotometer at 460 nm at room temperature. Addition of 1% catechol confirmed the presence or absence

of polyphenol oxidase (69). All protein determinations were done with the colorimetric assay of Lowry et al. (44).

#### ELECTRICAL MEASUREMENTS:

An apparatus developed by Skutt et al. (59) that delivered a constant current pulse of  $0.5 \mu\text{A}$  for 0.5 ms, with an interval of 10 ms between pulses was used to take all electrical measurements. The resulting voltage pulse was then converted to the electrical resistance and was read in Kilo (K) ohms. Subdermal platinum needle electrodes, 1 cm long and 0.5 mm in diameter were inserted 1 cm apart with the ends protruding from the other side of taproots 2 mm in diameter. The usual region of measurement was 5-6 cm below the soil level. During measurement of electrical resistance, electrodes and wire leads were connected directly to the resistance meter terminals to avoid polarization. Plants were initially measured in 200 ml of half-strength Hoagland's solution, and after the addition of 100 ml Fusarium spore suspension of each of the four isolates (Fig. 1,2). Readings were monitored until symptom development. It was necessary to remove plants from the solution and to dip the roots in distilled water before making resistance measurements. Autoclaved spore solutions were added as controls in addition to plants without spore supplement.

#### ENZYME TRIALS:

Sections of the taproot, 2 cm long and 2 mm in diameter were placed in small B.P.I. Syracuse dishes (Fig. 3). Electrodes were inserted 1 cm apart and the changes in electrical resistance were

monitored in the following preparations: partially purified PG from culture filtrates run through a millipore filter, pectinol (Rohm and Haas Co.), pectinase (Nutritional Biochemicals Corp.) endo-PGTE from Erwinia carotovera (partially purified by DEAE cellulose column chromatography) in pH 8.0 Tris-HCl buffer, pectin methyl esterase (Nutritional Biochemicals Corp.), phospholipase A (Sigma Chemical Co.) and buffers at pH 4.5 and 8.0. One-ml enzyme was used for each trial. Roots were washed in sterile distilled water. Readings were monitored at 30 min intervals, and later at 2 min intervals. Highly purified PGTE by isoelectric focusing (48) was utilized in this latter trial, as was PG with the addition of  $10^{-4}$  M  $\text{Ca}^{++}$ .

#### DIFFUSIVE RESISTANCE:

The diffusive resistance of leaves to water vapor loss was determined with a porometer of the type described by Kanemasu et al. (40). Six plants were measured 46 hr prior to inoculation. At this time five of the six plants were inoculated by F56 and measurements were taken until the appearance of symptoms. A leaf from the third set of leaves was monitored on each plant.

## RESULTS

## STRAIN PATHOGENICITY:

Cultures of Fusarium oxysporum f. sp. lycopersici obtained from the Massachusetts Suburban Experiment Station were designated F56, F59 and F60. Upon root dip inoculation, isolates F56 and F59 caused epinasty and chlorosis within 48 hr after inoculation; leaves had withered by 60 hr and in 50% of the trials plants died within 96 hr. Some vascular browning was evident at 48 hr. Isolate F60 was not as virulent; epinasty and chlorosis did not appear until 72 hr. Plants were able to overcome infection in 75% of the cases, although stunting was a consequence after recovery. Vascular browning became evident at the same stage as the F56 and F59 isolates, but was never as pronounced. The isolate purchased from the American Type Culture Collection was designated as F1. It appeared to be only mildly virulent, as plants very often developed no symptoms at all and serious wilting never occurred.

The amount of endo-PG activity in these strains seemed to generally support the finding of Paquin and Coulombe (53) (Table 1, Fig. 4,5). The mildly virulent F1 strain produced far less endo-PG than the other three virulent strains.

## ELECTRICAL RESISTANCE OF INTACT PLANT STEMS:

Several methods were employed in attempts to relate electrical resistance measurements with vascular wilt disease. After root dip inoculation, plants were positioned adjacent to a ringstand with clamps

attached. Each clamp contained two stainless steel pins which were set apart to avoid contact. These pins were inserted through the plant stem at 6 cm above soil level and were 2.5 cm apart. Wire leads were connected to a device that measured electrical resistance to a constant current pulse as described by Skutt et al. (59) and ER was read in Kiloohms.

There was no discernible difference between the ER of stems of control and inoculated plants, and much variation in single plants was observed. Distance between pins were varied and measurements were made at different locations on the stem.

A group of seven plants were replanted; five were inoculated and two served as controls. Plants were removed from the soil and electrical resistance was measured by inserting stainless steel insect pins (size 00) 1 cm apart at 5, 4, 3, 2, and 1 cm above the soil level, at the soil level, and 1 cm below the soil level. Measurements were taken on a microohmmeter as described previously (59). A second set of stainless steel probes (13 mm long and 2 mm wide) were also utilized as electrodes. These electrodes were insulated by a plastic handle which allowed rapid and easy measurement. One plant was used for each of five subsequent days. After completion of measurements, the stem was slit to examine for vascular browning. Although browning was evident by 50 hr after inoculation, no variation in ER in the stems could be detected.

#### ELECTRICAL RESISTANCE OF INTACT PLANT ROOTS:

Subdermal platinum electrodes were substituted and measurements

were concentrated on the taproot from the soil level to 6 cm below soil level. A decrease in ER was noted in the plants inoculated by isolate F56 at 6 cm below (44 Kohms at 24 hr, 84 Kohms at 30 hr, 89 Kohms at 49 hr) and 5 cm below (29 Kohms at 24 hr, 30 Kohms at 30 hr, 63 Kohms at 49 hr). ER remained constant in the upper regions of the taproot, as it did in the entire roots of control plants. It was necessary to group plants which had uniform sized taproots, because ER varies widely depending upon the diameter of the root. In addition, the smaller feeder roots were brushed away from the electrodes to eliminate fluctuation of the needle on the meter.

Inherent differences between individual plants made it necessary to devise a model for monitoring single plants throughout the infection process. A pH of 6.5 does not cause any damage to tomato plants, so Hoagland's solution was adjusted to this pH for use in the trials. Upon measurement, roots had to be washed in distilled water to eliminate the presence of any ions present in the solution which might lower the ER and affect root measurements. Before the addition of inoculum, plants were monitored for 24 hr in Hoagland's solution to insure that readings were constant.

An immediate drop in electrical resistance was noticed within 2 hr after inoculation by all strains (Fig. 6). Readings continued to drop at a steady rate after an initial rapid decrease within 12 hr after inoculation. Since measurements were only taken periodically, any polarization effects of the electrodes were negligible. Foliar symptoms had appeared by 22 hr after inoculation except for isolated F1, in which symptoms occurred by 28 hr. Maceration of the roots was



evident in the virulent strains 22 hr after inoculation and the experiment had to be terminated because probes would not remain in position thereafter.

Rate of ER decrease seemed to correlate well with PG production by these isolates (Table 1, Fig. 6). After 22 hr, F1 had caused a drop in ER of 36 Kohms, far less than the respective ER decreases for F56, F60, F59 (137 Kohms, 94 Kohms, 87 Kohms). Addition of autoclaved spore suspensions and plants remaining solely in Hoagland's solution resulted in an increase in ER.

Upon the addition of the spore suspensions of all the Fusarium isolates, the pH of the medium was changed from 6.5 to 6.0. Plant roots monitored in 0.01 M citrate buffer, pH 6, resulted in a slight decrease in ER.

Because isolate F56 produced the most severe symptoms, caused the greatest drop in ER and had a high PG activity at both pH 4.5 and 6.5, all further tests were conducted using partially purified enzyme from this strain.

#### ENZYME ANALYSIS:

PG precipitated in the 50-90% ammonium sulfate saturation range, and significant activity occurred at pH 4.5 and pH 6.5 (Table 1). Suppression of activity at both pH values was achieved by the addition of  $10^{-4}$  Ca<sup>++</sup> similar to results obtained by Bateman (4).

The enzyme was eluted in the buffer void by DEAE column chromatography and was shown to possess high activity as shown by viscometry. Relative activity was shown to be 625 units.

Qualitative analysis for PME activity in DEAE fractions showed no change in indicator color after 24 hr incubation of the enzyme reaction mixture at either pH. No increase in acidity was observed upon incubation with a 1% pectin solution for 30 min.

No significant PGTE activity was apparent after 12 hr of incubation, with or without the addition of  $\text{Ca}^{++}$  in reaction mixtures. Cup-plates detected negligible phosphatidase activity and no protease activity.

Peroxidase was absent, as no absorbance changes were noted at 460 nm. The addition of 1% catechol to reaction mixtures caused no change in color; hence, no polyphenol oxidase.

Cellulase activity was fairly high, as relative activity of 170.69 units and 63.32 units (50) were detected via viscometry at pH 4.5 and 6.5 respectively.

PG was very stable, as very little activity was lost through storage at  $-20^{\circ}\text{C}$ .

#### EFFECT OF ENZYMES ON THE ELECTRICAL RESISTANCE OF EXCISED ROOTS:

A drop in ER was observed in excised taproots immersed in pectinol, pectinase, PGTE and partially purified PG from Fusarium culture filtrates (Fig. 7). Phosphalipase A (50 ug dissolved in 1 ml distilled  $\text{H}_2\text{O}$ ) and PME (1 mg dissolved in 1 ml distilled  $\text{H}_2\text{O}$ ) affected an increase in ER, whereas solutions of distilled  $\text{H}_2\text{O}$  and 0.01 M citrate buffer pH 4.5 did not appreciably change the ER. At the end of 90 min maceration had occurred only in roots in the commercial preparation of pectinol and pectinase solutions.

Upon reducing the interval to 2 min, it was found that these changes in root ER occurred within the first 2 min immersed in the enzymes (Fig. 8). Highly purified PGTE (substituted for partially purified PGTE) was able to affect the typical drop in ER. Rate of decrease was rapid until values tended to level off after 20 min. The addition of  $\text{Ca}^{++}$  to the PG reaction tended to cause a much reduced drop in ER. No maceration had occurred in any roots by 20 min.

#### DIFFUSIVE RESISTANCE (DR):

Results were similar to those of Duniway (21) in which the DR of the leaves of inoculated plants was consistently as high or higher than that of healthy leaves (Table 2). It was noticed that DR increased substantially in inoculated plant leaves just prior to and after symptom development. During the course of the experiment, plants were kept at greenhouse conditions, with fairly constant relative humidity (70%) and temperature (25-26°C).

TABLE I

PG activity of various isolates,  
as measured in RVU.

Isolate	PG activity, RVU <sup>a</sup>		
	pH 4.5	pH 6.5	pH 8.0
F1	<u>          </u> <sup>b</sup>	19.88	<u>          </u>
F56	564.97	469.48	65.53
F59	145.77	189.75	43.48
F60	333.33	684.93	68.96

<sup>a</sup>RVU Relative Viscometric Units defined as 1,000 times the reciprocal of the time required for 1-50% reduction in viscosity of a mixture of 5 ml pectin N.F. substrate and 1 ml enzyme (from 50-90%  $(\text{NH}_4)_2\text{SO}_4$  precipitation) incubated at 30°C (50).

<sup>b</sup>Enzyme did not result in 50% reduction in viscosity.

TABLE 2

Diffusive resistance of tomato plants before and after inoculation  
by isolate F56

<u>Time (hr)</u>	Diffusive resistance (sec cm <sup>-1</sup> )					
	<u>Plant 1</u>	<u>Plant 2</u>	<u>Plant 3</u>	<u>Plant 4</u>	<u>Plant 5</u>	<u>Plant 6</u>
0	4.0	2.4	10.2	8.1	11.3	8.2
3	3.4	2.8	4.3	5.3	18.6	8.8
9	3.8	5.0	9.5	7.2	20.1	9.9
22	6.0	8.1	4.0	2.1	15.3	3.4
25	4.6	8.0	7.9	5.2	13.1	5.6
28	3.6	7.6	12.1	7.9	18.8	7.8
32	4.0	6.8	9.0	10.1	15.6	8.8
46	Plants 1-5 inoculated with F56; plant 6 left as a control					
49	4.8	4.5	9.9	9.6	18.3	5.1
54	3.3	6.2	10.5	10.2	11.5	8.2
60	4.9	5.8	10.8	7.8	9.5	7.0
70	25.6	9.5	24.9	40.3	36.3	7.9
72	Extensive wilting for all inoculated plants					

Fig. 1 Tomato plants in 200 ml half-strength Hoagland's solution. A 100-ml spore suspension of F56 was added to the beaker on the left, 100 ml pectinol was added to the beaker on the right. Symptoms shown occurred at 24 hr.



EPINASTY (24 hrs)

LEAF CURL +  
SCORCH CAUSED  
BY FERTICOL (24 hrs)

Fig. 2 Platinum electrodes inserted 1 cm apart in the main taproot as monitored by the pulse resistance meter



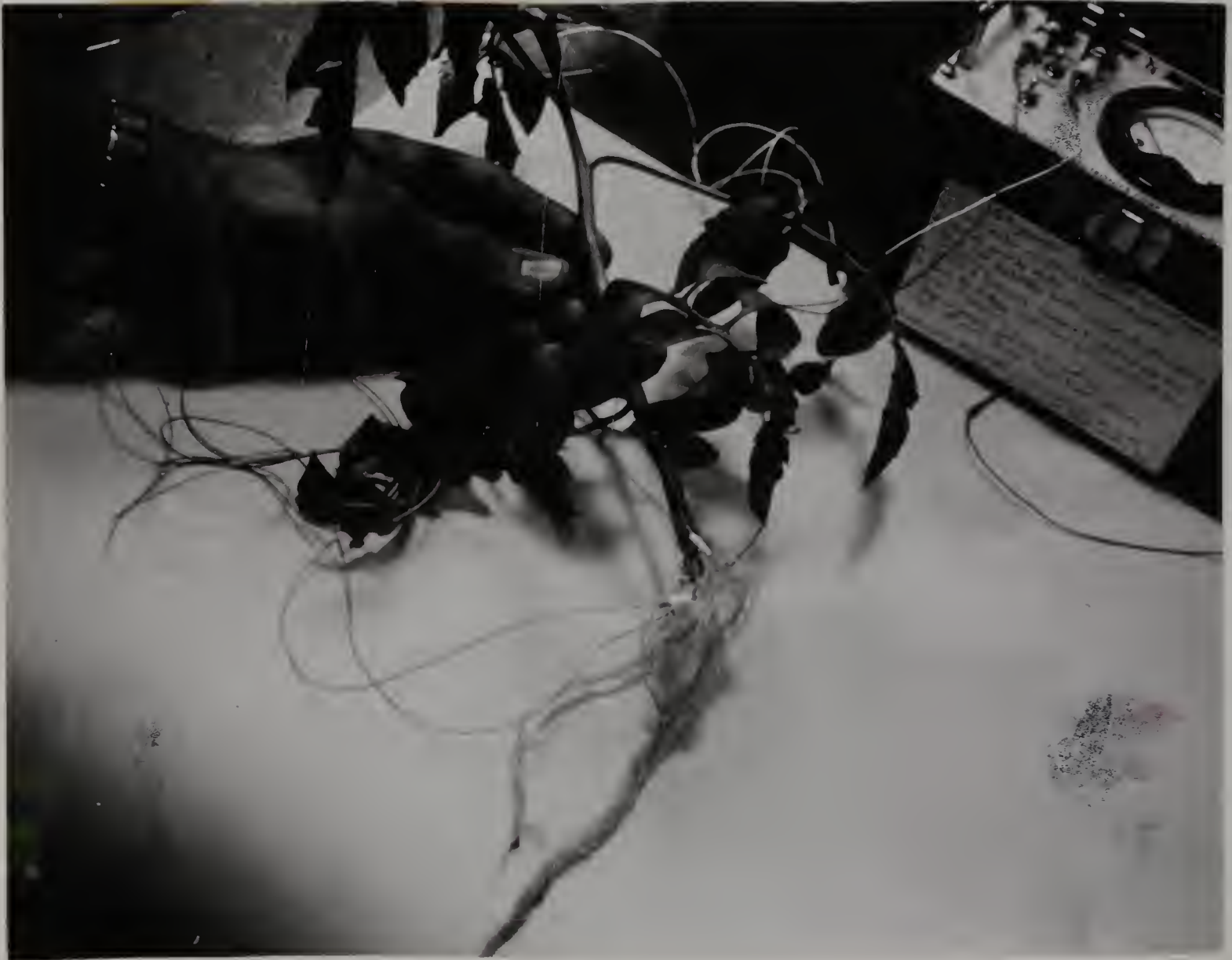


Fig. 3 Electrodes inserted in an excised taproot of a tomato plant and immersed in 1-ml of enzyme.



Fig. 4 Endopolygalacturonase activity of four F. oxysporum f. sp. lycopersici isolates as indicated by change in viscosity of a 1.5% pectin substrate at pH 4.5.

Fig. 5 Endopolygalacturonase activity of four F. oxysporum f. sp. lycopersici isolates as indicated by change in viscosity of a 1.5% pectin substrate at pH 6.5.

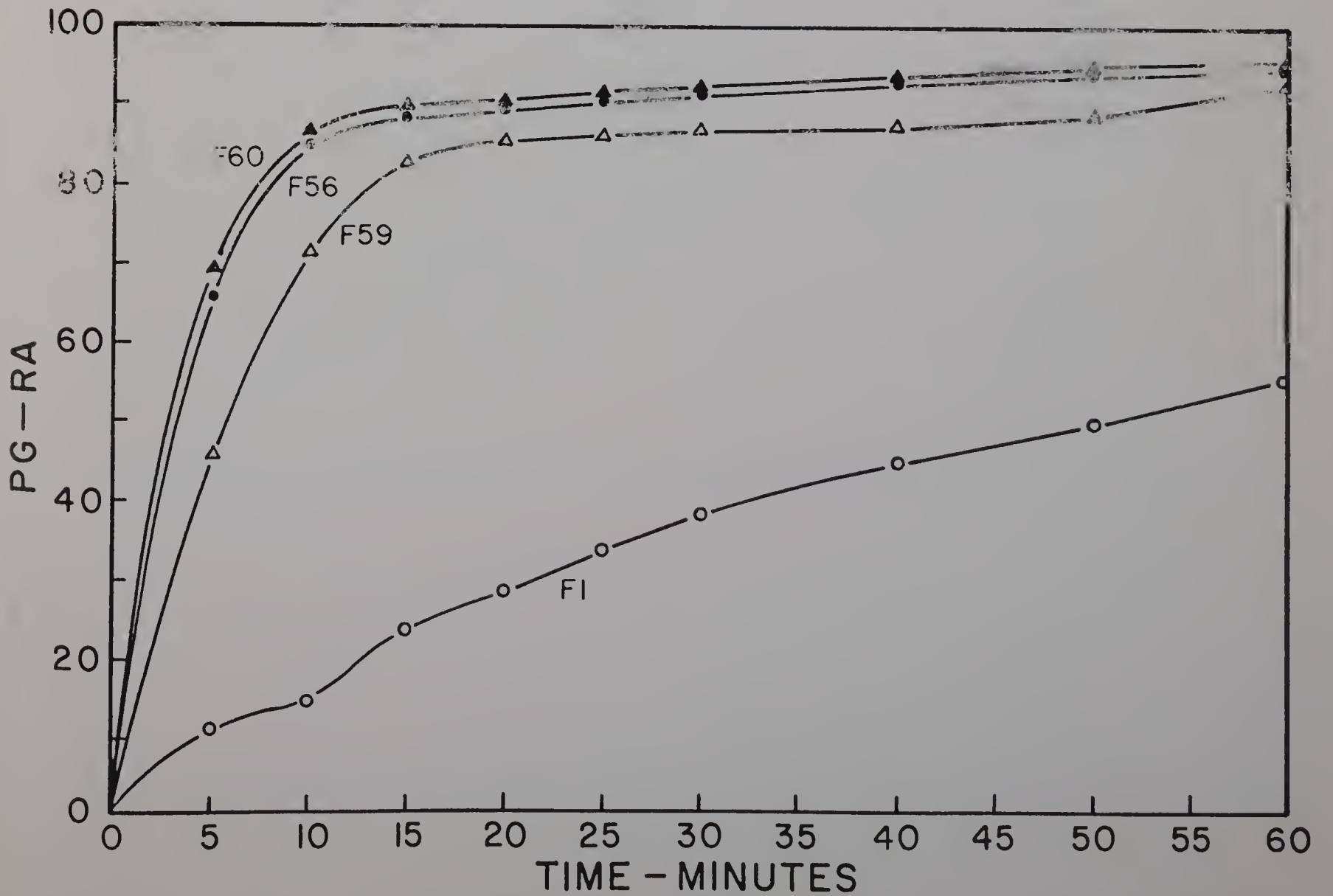
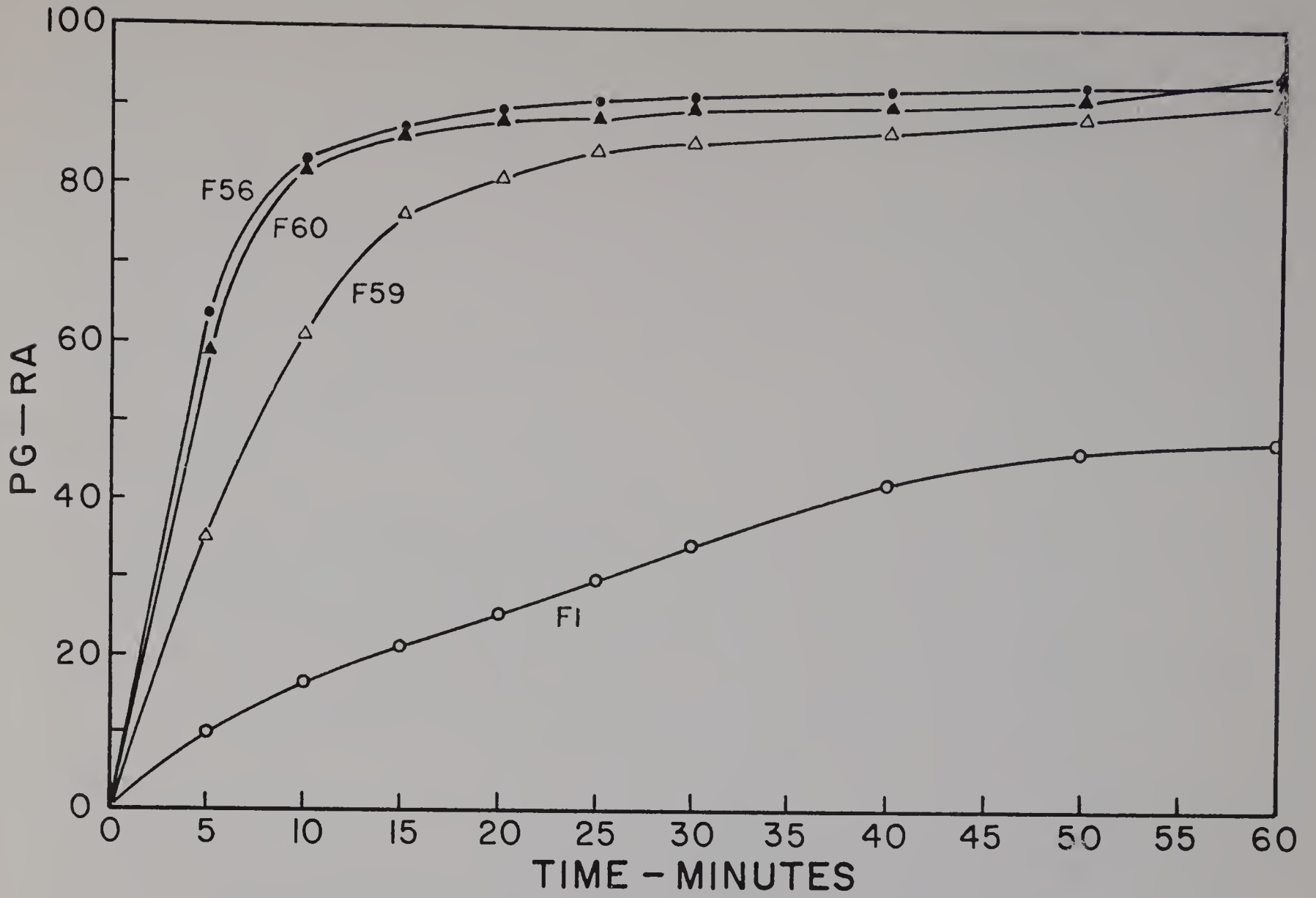


Fig. 6 Respective increase or decrease in electrical resistance in roots of intact tomato plants measured 5 cm below the soil level. Plants were immersed in 200 ml half-strength Hoagland's solution and 100 ml of the following: ● F56; ◻ F60; ▲ F59; ○ F1; ■ autoclaved F56; ▲ Hoagland's solution. Bars indicate the standard error of the mean.

Fig. 7 Respective increase or decrease in electrical resistance in excised roots of tomato plants placed in various enzyme and control solutions. Arrows indicate the time at which maceration was first detected. ◻ partially purified PGTE (activity defined in reference 48); ○ pectinol (highly active); ▲ PG (partially purified from F56 by DEAE cellulose); ● pectinase (one unit liberated 1.0 u mole of galacturonic acid from polygalacturonic acid per min at pH 4.0 at 25°C); ■ 0.01 M citrate buffer, pH 4.5; ◻ distilled H<sub>2</sub>O; ▼ phosphalipase A (one unit hydrolyzed 1.0 u mole of L-alpha-lecithin to lysolecithin and fatty acid per min at pH 8.5 at 37°C); ▼ PME (one unit hydrolyzed 1.0 u mole of methanol from pectin per min at pH 7.5 at 30°C).

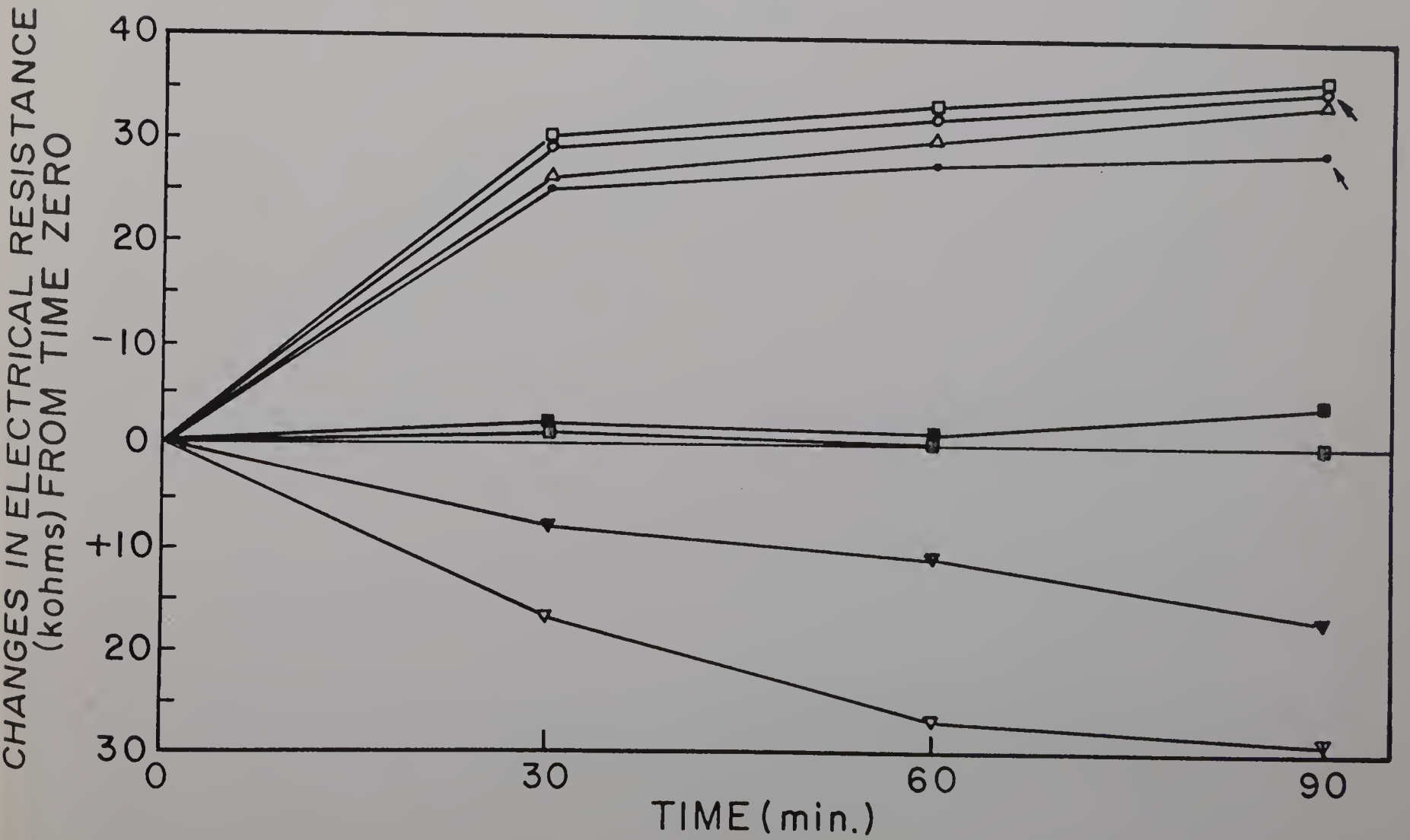
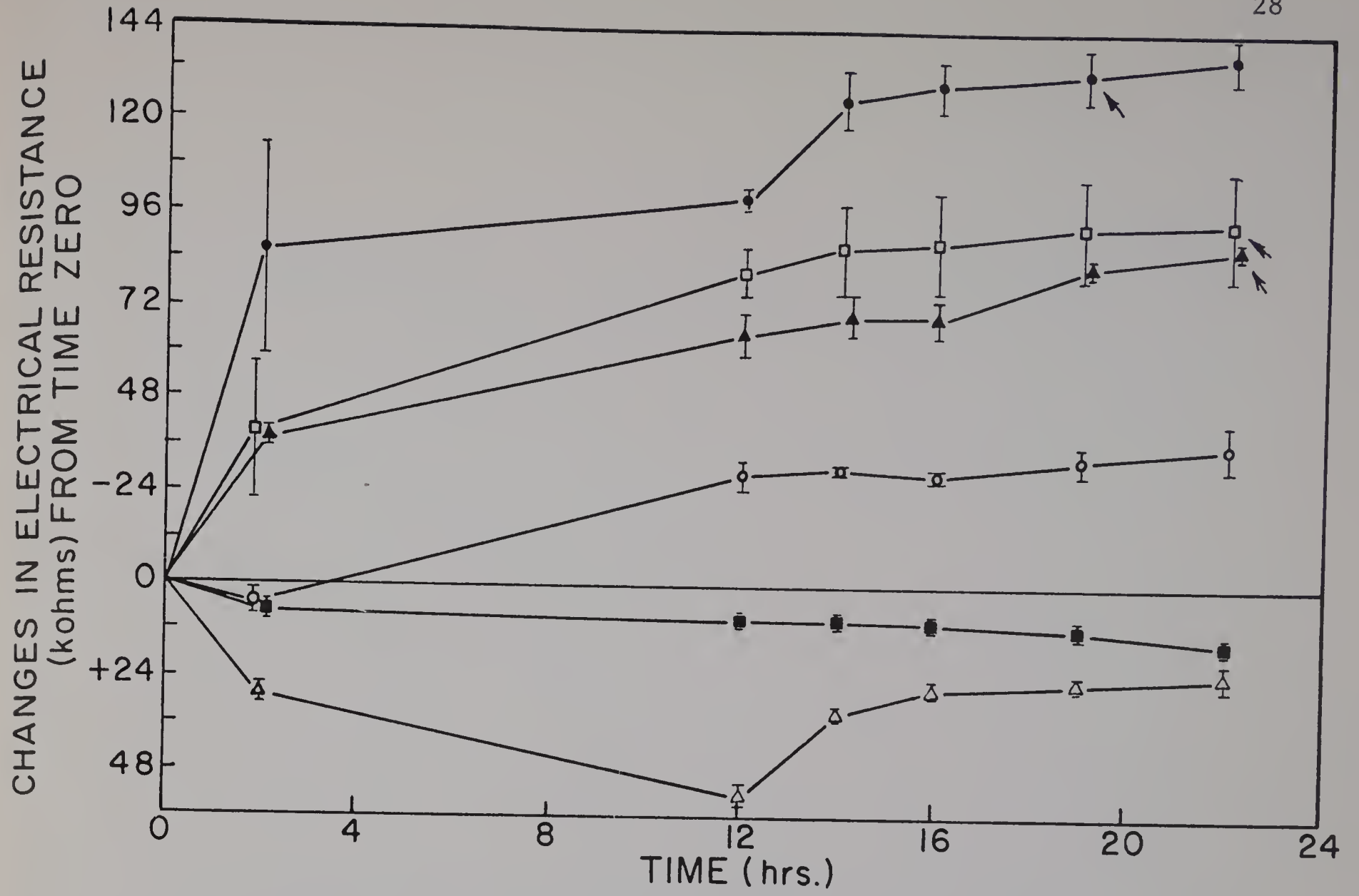
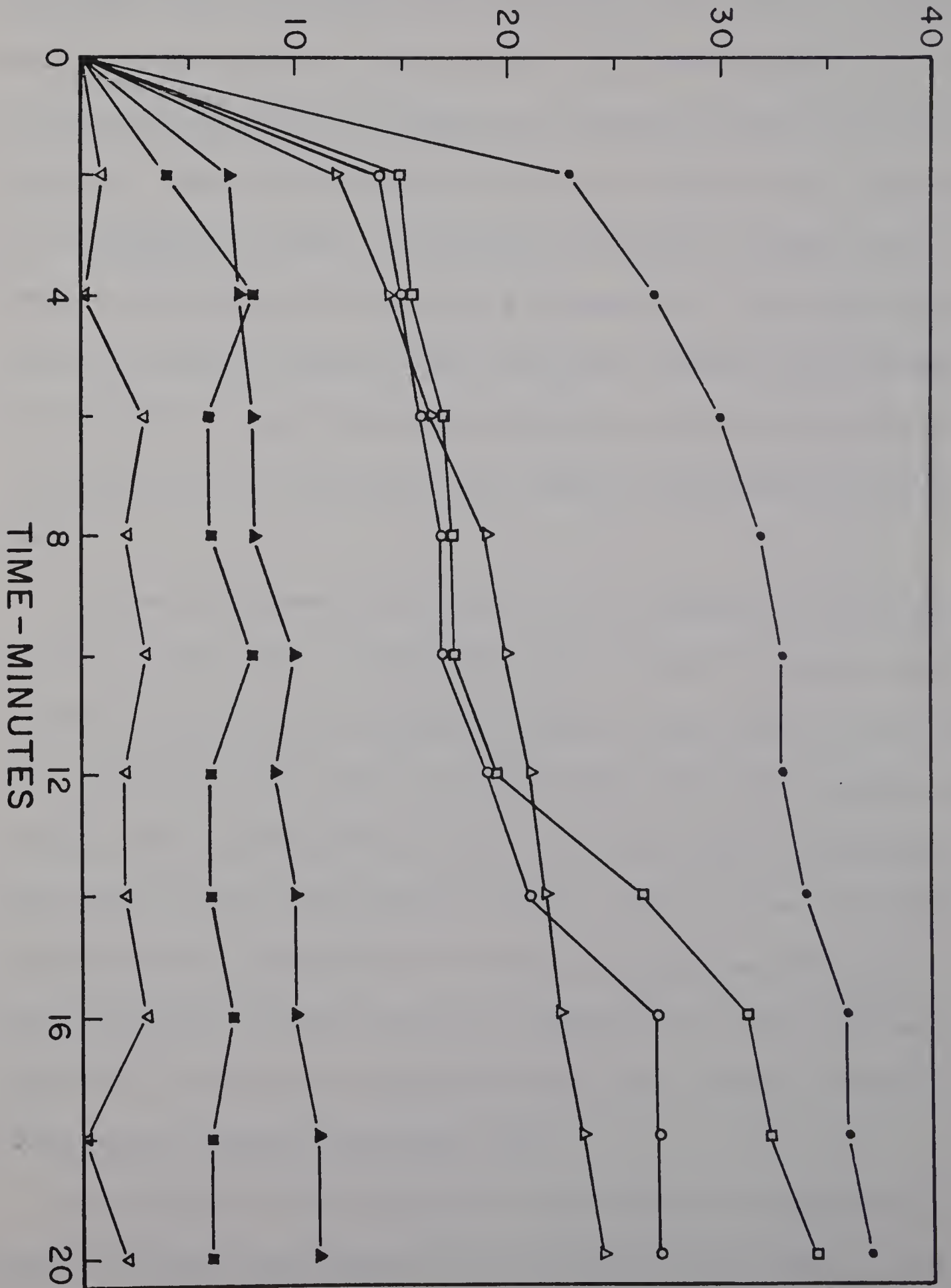


Fig. 8 Respective decrease in electrical resistance in excised roots of tomato plants placed in solutions. ● pectinase; ■ PGTE (purified 300X by isoëlectric focusing - 48); ○ pectinol; ▲ PG (F56); ▲ PG &  $10^{-4}$ M  $\text{Ca}^{++}$ ; ■ 0.01 M citrate buffer pH 4.5; ▼ 0.01 M Tris buffer pH 8.5.



DECREASE IN ELECTRICAL RESISTANCE  
(kohms) FROM TIME ZERO



## DISCUSSION

Decrease in electrical resistance of tomato taproots were correlated with the progression of Fusarium wilt disease. Changes in electrical resistance in these tissues could be detected 2 hr following inoculation and 17 hr before the appearance of the first foliar symptoms. Magnitude of changes in ER were related to the pathogenicity of the Fusarium isolate. Electrical resistance of tomato stems, however, was unchanged throughout pathogenesis. These findings are similar to those of Dostalek (20) who found a significant decrease in the impedance of apple roots associated with the presence of the proliferation disease mycoplasma, but found no differences in apple shoots.

Changes in ER have been shown to be an indicator of the condition of plant tissues (22). Mobile ions carry electric current through the vascular fluids, but ion content is higher inside the cell than outside (61). Inorganic ions escape from the cells due to the permeability increase upon disintegration of the cell wall and/or plasmalemma. These mobile ions, especially  $K^+$  and  $Ca^{++}$ , leak to the intercellular spaces where the concentration of ions increases and resistance decreases (65). ER and electrical conductivity to low frequency signals as used in these experiments have been related to mobile ion concentration between electrodes (65).

In addition to the increase of electrolytes in intercellular spaces resulting from exosmosis from injured cells, there is also the possibility that the hydrolysis of extracellular pectic substances by

the release of pectolytic enzymes is responsible for the release of bound ions to further decrease electrical resistance of tissue (23). Extracellular pectic substances are believed to exist in bound form, such as calcium and magnesium pectates (10).  $\text{Ca}^{++}$  would contribute to the decrease in resistance, whereas  $\text{Mg}^{++}$  usually exists in a hydrated form too large to carry electrons at a rate affecting the resistance.

Polygalacturonase has been implicated as an important factor in successful pathogenesis of Fusarium (15, 49). The amount of PG activity correlated with the rate of symptom development of the four isolates of Fusarium used in these experiments. In addition, PG activity was also shown to be related to the changes in ER during the progression of vascular wilt in intact plants. As early as 2 hr after inoculation this enzyme has caused changes in the permeability of the taproot.

PG has been shown to be the earliest secreted enzyme by Fusarium in growth medium containing isolated cell walls of tomato varieties (39). Hydrolysis of random bonds between galacturonic acid residues liberates large molecular fragments from the primary cell walls and middle lamellae which form consequent gels. If the host-parasite situation is susceptible, the pathogen will break down these gels further to prevent occlusion of systemic spread (8).  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  are freed as the salt bridges between uronic acid carboxyls and adjacent uronide polymers are broken (5). PG is important early in pathogenesis and is not present in high quantities later in infection due to the increase in available  $\text{Ca}^{++}$  which inhibits enzyme activity.

The early secretion of PME may aid PG in demethylating substrates to more favorable pectic acid substrates (5), although PME has no effect on ER of root tissue.

The critical nature of PG was shown by the detection of decreases in ER within 2 min after treatment. Decreases in ER for partially purified PG from Fusarium cultures paralleled those of highly concentrated pectic enzyme solutions. Especially noteworthy is the fact that partially purified PG paralleled highly purified PGTE which contained no detectable contaminants. All enzyme solutions effect electrolyte loss prior to maceration. Maceration occurred in 1.5 hr (pectinol, pectinase), 6 hr (PGTE) and 20 hr (PG from Fusarium) respectively. Reasons for an increase in ER for PME and phosphatidase A are unclear. These enzymes may cause the roots to take up additional ions from the bathing solution thereby causing ER to rise. Their role is obviously not that of loss of selective permeability and electrolyte loss. PME produced by Fusarium is not a significant factor in maceration (3), but may be a predisposal agent for the PG substrate.

Diffusive resistance was always found to remain at a fairly constant rate similar to that of healthy plants until 1-2 hr prior to wilt symptom development. It was apparent that an adequate amount of pectic substance degradation was necessary to form occlusions which would cause water stress. Results confirmed the findings of Duniway (21), in that a change in transpirational behavior is not responsible for stress through excessive water loss.

It was concluded from this study that pectic enzymes may play an important role in early progression of Fusarium wilt of tomato. The amount of production of endopolygalacturonase by the fungus was

correlated with the relative virulence of the pathogen and severity of symptomatic expression. Wilting of leaves may be a result of water loss by obstruction partially caused by enzymatic breakdown of the host, rather than a marked increase in cell permeability and subsequent solute loss. No significant transpirational effects were shown to occur until wilting was eminent. The measurement of ER allowed for a presymptomatic detection of vascular wilt disease and implicated pectic enzymes as causal agents in electrolyte loss.

## SUMMARY

Of four isolates of Fusarium oxysporum f. sp. lycopersici, three were found to be virulent and the remaining isolate was found to be only mildly virulent. Upon isolation of endopolygalacturonase from each isolate by ammonium sulfate saturation, production of enzyme correlated with the degree of virulence of each isolate.

Measurement of electrical resistance in the stems of inoculated tomato was found to be no different from that of uninoculated plants. Changes in ER were detectable, however, in the main taproot at 6 cm below the soil level. An immediate drop in ER was noticed in plant roots inoculated separately by all strains within 2 hr after inoculation. These drops in ER preceded symptom development by 20 hr. Rate of ER decrease correlated well with the production of PG by these isolates: the higher the PG activity, the greater the decrease in ER.

Partially purified PG by DEAE cellulose column chromatography caused a drop in ER in excised tomato taproot sections within 2 min after treatment. Decreases in ER paralleled those of roots immersed in commercial pectinases and highly purified endo-PGTE.

The diffusive resistance of the leaves of inoculated plants was the same or slightly higher than that of healthy plants; DR increased substantially in inoculated plant leaves just prior to and after symptom development.

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