HYPERSENSITIVE CELL COLLAPSE INDUCED IN BELL PEPPER (*CAPSICUM ANNUUM*) BY *PSEUDOMONAS PHASEOLICOLA* AND BY AN ENDOTOXIN ISOLATED FROM THE BACTERIA

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ABSTRACT

Electron micrographs of pepper tissue infused with 10^7 and 10^8 cells/ml of Pseudomonas phaseolicola, the causal agent of the halo blight of bean, show that after 12 hours the bacterial cells aggregate in a gel-like matrix of electron translucent material on the plant cell walls facing the intercellular spaces. Within the gel matrix an electron dense amorphous material is also observed, but its occurrence depends on the inoculum concentration used to inoculate the plant. In the plant cells adjacent to the localized bacteria a plasmalemmasome first develops, followed by deposition of a wall construction, with cell collapse occurring soon afterwards. Wall constructions and cellular collapse are also observed in pepper tissue infused with a purified extract from the bacterial cells, but not from the culture broth. The purified extract contains a high molecular weight endotoxin which is capable of causing confluent cell collapse that is not accompanied by tissue browning or senescence symptoms. It is degraded by pronase, but not RNAse, and has a molecular weight of approximately 93,000. A mutant of Pseudomonas phaseolicola which is incapable of producing halo blight symptoms in its host or producing in culture the inducing principle also possesses the endotoxin which shows similar properties to that of the parent pathogenic strain.

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CHAPTER I

THE HYPERSENSITIVE REACTION IN PLANT RESISTANCE

Introduction

Incompatible reactions induced in higher organisms against potentially injurious agents form the basis for numerous types of immunological responses which serve as a natural defense of plants against pathogenic organisms. In the case of higher plants the presence of specific immune response mechanisms has not yet been demonstrated, though, in pollen-style incompatible systems, there are specific protein-protein interactions which have some of the attributes of antigen-antibody reactions. For example, plant proteins have been recently isolated from the cell walls which react specifically with enzymes of phytopathogens which degrade them (66). However, it is not known if these mechanisms are involved in the hypersensitive reaction, the most widespread expression of incompatibility in plants to phytopathogens.

Phytopathogenic bacteria can invade higher plants only passively. There they multiply in the intercellular spaces or in the conducting vessels. This milieu has sufficient of the nutrients which are necessary for proliferation of both compatible and incompatible pathogens as well as saprophytes (8, 9, 39). Despite this, the phytopathogenic bacteria are able to multiply for only a short period in plants which are not their natural host or in resistant plants where they may cause rapid necrosis (35, 37, 39, 40). On the other

hand, in the susceptible host, phytopathogenic bacteria fail to trigger the deployment of resistance mechanisms (38, 91). Though plant metabolism is altered in both host and non-host interactions, these changes are not specific since the same changes may be elicited by chemical and physical means (15). Physiologically, the difference in plant responses between the compatible and incompatible interactions appear to be one of degree rather than kind (15). Even so, resistance to the establishment of phytopathogenic bacteria is the norm in nature. The manifestation of resistance, however, which involves both static and dynamic aspects, is not absolute. The genetic complement determines the biochemical potential for resistance, but the actual expression is often variable (42, 72). This results from the wide flexibility of regulatory mechanisms which exist between the internal and external environment (72). The biochemical potential for resistance is under metabolic controls which are influenced by a multitude of factors (42). In contrast, from our current understanding of pathogenesis in plants, susceptibility appears to be a highly exacting condition and the result of an adaptation, even though for a limited time, of both host and pathogen (42). The establishment of infection involves not only overcoming plant defenses, that are independent of infection, but also overcoming altered plant metabolism which results from infection (42).

As defined by Wood (96) "resistance of plants to a disease is the extent to which a plant does not become diseased when growing in association with phytopathogens." "High and low resistance correspond to low and high susceptibility, and immunity may be considered the

uppermost level of resistance where no disease symptoms are detected" (96).

Generally, resistance is inherited as genetically dominant characters (55). These determinants direct not only the synthesis and deposition of mechanical barriers and repair mechanisms, but also regulate production of resistance factors in effective concentrations (30, 88).

In many plants, resistance factors are preformed and include such compounds as phenols, alkaloids, steroids, and glycerides. However, it is remarkable how seldom resistance operates simply because the plant contains one or more substances toxic to the pathogen (96). This is demonstrated by experiments which show that phytopathogenic bacteria begin to multiply in any plant whether susceptible, tolerant, or resistant (39). Thus preformed resistance factors appear to play a minor role in plant resistance. Rather, most expressions of resistance appear to reside in the ability of the pathogen to trigger these mechanisms in the plant, a property which is apparently lacking in saprophytes (38) and in compatible pathogens (87).

Since Ward's (94) work with Bromus spp. and the pathogen Puccinia dispersa, phytopathologists have recognized that phytopathogens in highly resistant or noncongenial plants induce characteristic, rapid, degenerative effects in the plant-cell protoplast. Concurrently, the growth of the pathogen may cease abruptly or death may ensue (38, 81). In 1915, Stakmann (80) used hypersensitivity or the hypersensitive reaction (HR), a term borrowed from animal immunity, to describe this type of interaction. The HR may be induced by phytopathogenic bacteria, fungi, both obligate and facultative, as well as by viruses (38).

The HR is recognized as the most widespread defense mechanism in higher plants (38). Though HR cannot prevent infection the hypersensitive plant cells react quickly to become substrates on which the pathogen can live for only a short time (96). The reaction is most commonly characterized by a rapid loss of membrane integrity and function and the plant cells subsequently collapse and die. This results in a localized necrosis where the pathogen is confined (30, 34, 37, 38).

Metabolic effects associated with HR can be detected by increased oxygen consumption, after only two minutes, in apple leaves infiltrated with Erwinia amylovora (59) and by immediate depolarization of transmembrane potential in tobacco with Pseudomonas pisi (58). Plant cell death has been reported to occur in potato cell within 10 minutes after penetration by incompatible Phytophthora infestans (87). Bacterially induced HR, in a number of interactions, is characterized by loss of membrane function as shown by electrolyte loss which occurs within 2-3 hours (36). Membrane disruption and disorganization has been ultrastructurally detected within 6-8 hours (7). Klement and Goodman (38) claim the reaction was irrevocably induced within 20-25 minutes after intromission with high concentrations of bacteria (ca. 10^7 to 10^8 cells/ml). However, recently, Turner and Novacky (90) found that cell collapse occurred, though not macroscopically detected, when 10^4 to 10^6 cells/ml were introduced into the plant.

The expression of HR is influenced by the nature of plant tissue (73), humidity and temperature, nutrition, age of the plant, growth regulation, day length, and stage of development (54). Under standard

conditions with a given plant and phytopathogen the rapidity with which confluent necrosis occurs is positively correlated to the inoculum concentration; however, the time required for the HR to develop is constant (38).

Cellular collapse which resembles HR may also result from treatment with certain salts, sulfhydryl compounds, and hydrolytic enzymes and HR is also accompanied by physiological responses which are commonly associated with stress (96). However, before microorganisms can induce HR they must be pathogens and the plant tissue must be highly resistant. For the HR to be expressed, genes in the plant for resistance must interact with genes for avirulence in the pathogen (18). Hypersensitivity and susceptibility both have many physiological and biochemical similarities; yet the distinct requirements for inducing the two types of responses support a model involving specificity on the part of both the plant and the pathogen. Many investigations have already substantiated the observation of Tomiyama (87), that the HR must be suppressed for the plant to become susceptible and, where both compatible and incompatible interactions are induced in the same plant, the incompatible interaction predominates (87).

To the present time, gross metabolic studies have failed to differentiate between the two responses plants show to invading pathogens. However, since cellular changes are sensitive indicators of metabolic activity, ultrastructural studies of these changes in the early interaction phases may show whether the pathogen or the host is affected first. Previous ultrastructural studies conducted on bacterially induced HR failed to show the nature of plant-pathogen interactions,

and have emphasized the degenerative effects of the bacteria on the plant cells (7, 23). Information is needed regarding the specific nature and progression of the interaction between the plant and bacterial cells and the mechanism whereby hypersensitive cell collapse is triggered. One of the most distinctive features of the incompatible interaction is shown by the restricted population growth. Most generally, an initial rapid growth phase is followed by a rapid decline (81), but in some cases the population may remain more or less static (32). The restricted growth has in some cases been related to induced substances produced by the plant. In tobacco infected with an avirulent race of Pseudomonas solanacearum (49) and in resistant pepper infected with an incompatible Xanthomonas vesicatoria, antibacterial substance(s) were detected at the time the HR appeared (11). In soybean infected with the avirulent P. glycinea and incompatible P. lachrymans, the phytoalexin hydroxyphaseolin appears to be responsible for controlling the bacterial population during the HR. Nevertheless, these studies do not explain the relationship between bacterial growth or death and hypersensitive collapse of plant cells, or if the events are initiated with the initial physiological interaction between the plant cell and the pathogen. Thus the question arises as to whether hypersensitive necrosis, which results in the resistant or incompatible interaction, is the cause or consequence of the resistant reaction. At least some of the steps of the interaction appear to be nonspecific since HR can be blocked with heat killed bacterial cells (47), by viruses (45), and by infusing subliminal concentrations of bacterial cells (60). Modification of the plant response

may be achieved with cycloheximide (65), cytokinins (57), calcium salts (12) and by prior introduction of compatible cells followed by a challenge inoculum of incompatible cells (82).

The objectives of the present investigation are to establish the nature, time and sequence of the first ultrastructural changes in bacterially induced HR and to determine if the bacterial cells themselves possess a specific inducing principle(s). This study involves the halo blight pathogen of bean P. phaseolicola (Burk) Dowson, race 2, G50 Tox^+ and a UV derived mutant of the wild type G50 tox^-, in interaction with bell pepper, Capsicum annuum. The mutant which is no longer toxigenic still retains the ability to induce HR in non-host plants. Such an investigation is warranted since the HR is the most widespread defense mechanism of plants (38) and little is known of changes induced in plants by the bacterial plant pathogens. A comparison of cellular responses of the plant to the pathogen as opposed to that induced by an HR inducing factor(s) derived from the bacterium would delineate specific metabolic activity related to HR.

Literature Review

Plant resistance

Higher plants have developed a remarkable array of biochemical mechanisms for protection against invading phytopathogens. Some of these pathways involve production of preformed substances, but many are induced. The induced substances are derived from products of both established pathways and newly instituted ones in both the host and the parasite (15, 20, 88). Disease resistance is not an absolute or

static condition (42), it depends on the existence of favorable conditions both in the plant and the external environment, as well as the physiology of the pathogen and the competing microflora (14, 42). Whether the genetic complement for biochemical resistance is expressed depends on many factors. Among them are nutrition, growth regulation, temperature, moisture, day length, light intensity, stage of development, and nature of the invaded tissue (54, 96). Thus it is not surprising that susceptibility and resistance under certain conditions are delicately balanced (42).

The possession of a highly regulated internal environment and specific defense system dependent on factors of humoral and cellular origin have not been demonstrated in plants as they have been in animals (72). The plant host requires different characteristics of the potential pathogen. For plant pathogens not only infection but also propagation involves the active penetration of mechanical barriers. These include cutin, suberin and lignin which are particularly impervious to penetration or to enzymatic attack. The bacterial pathogens, which compose the major part of animal pathogens, largely lack cell-lytic enzymes to actively invade the plant (72).

Klement and Goodman (38) classed plant defense mechanisms with reference to the bacterial diseases in two general categories: 1. preformed resistance and 2. induced resistance. Preformed factors are present before the infection and independent of it. Induced resistance is described in two classes: (a) premunity and (b) hypersensitivity. Premunity is the nonspecific, acquired immunity to a compatible interaction that may be accomplished by prior inoculation

of cell free extracts (3), killed cells (47) and other species of pathogens (47). Hypersensitivity is an induced defense reaction in resistant and noncongenial plants to phytopathogens that is characterized often by a rapid cell collapse due to the loss of membrane intregrity and function. In cases where membrane damage is initially minimized, the production of substances contributing to disease resistance, the phytoalexins, become characteristic of hypersensitive tissue (32). In reviewing fungal and virally-induced hypersensitive reaction, Muller (54) defined the HR as "encompassing all morphological and histological changes produced by an infectious agent that elicit the premature dying off (necrosis) of the infected tissue as well as the inactivation and localization of the infectious agent." However, Tomiyama, et al. (88) recently established with the facultative pathogen P. infestans in potato tubers that HR per se may not always limit the pathogen. Secondary responses and the involvement of repair mechanisms also appear to be associated with the containment.

Experimentally, HR may be delayed, accelerated or reversed by certain environmental conditions (54) or by influencing the respiration (54). Masking the visual expression of HR can be achieved with antibiotics, with growth factors (57) or with narcotics (54), and by prior infiltration of compatible pathogen before a challenge inoculum of the incompatible pathogen (82, 87). Presently many investigators agree that HR is expressed in many different ways and may represent many different phenomena. As a resistance mechanism, its stability is demonstrated by the fact that plants may repair HR even after exposure to supramaximal temperature for a short time (54).

The hypersensitive response was first described by Ward (94) when he observed rust reaction to resistant brome plants. "They turned brown and died, the destructive action of the infecting tube having killed the cells too rapidly." Gaümann (54) observed that interaction led to rapid death of host cells and resulted in local lesions where the pathogenic species was confined. The term "hypersensitivity," borrowed from animal pathology, was first used by Stakmann (80) to designate the response of plants which are sensitive beyond the norm to an organism or group of organisms. The cells of the plant in direct physiological contact with the pathogen showed very early evidence of the course of biochemical reactions that led to destruction of both directly affected and adjoining cells (72).

The intensity of expression of the HR was differential throughout the plant. The response was most acute in leaf mesophyll, lesser in stem parenchyma and was still less in the cells of the root and the petals (72). During the development of the plant, sensitivity to HR increased (54). This indicates that complex, metabolic, biochemical, and environmental interactions condition the potential for genetic expression of this defense reaction.

HR appears to be inherent in all higher plant species but depends on the pathogen's ability to trigger it, given the optimum conditions (38). Incompatible plants express the reaction more distinctly (24). In the early phase of the HR an initial normal development of the pathogen occurs which is independent of the reaction (24). To understand HR, the physiological basis for high resistance, which conditions it, has to be understood. Müller (54) reported that the HR could be

converted to the host-pathogen response by respiratory inhibitors, polyphenol oxidase inhibitors, acids of the Krebs cycle or even by lowering the temperature in some cases. This may indicate critical points where regulatory mechanisms operate.

The hypersensitive reaction induced by fungal and viral pathogens

HR interaction types in fungi

The type of HR interaction shown by fungal pathogens depends on the evolutionary development of the organism. Müller (54) has reviewed characteristic reaction types with the different fungal groups. The rust fungi, which are biotropic fungi and attack cereals, flax, corn, and beans, show a similar course of HR. In these fungi, the penetration of the pathogen is independent of the interaction type. Upon infecting the plant, both compatible and incompatible pathogens grow initially at the same rate. The range of effects on the plant varies from macroscopic appearance to extensive necrotic symptoms. Physiological breakdown is indicated by swelling of membranes, disintegration of plastids and cell nuclei, and discoloration of cells. Bhattacharya and Shaw (4) found, in the wheat rust just prior to cell collapse, new synthesis of both RNA and proteins. In the Synchytridium type HR, only the meristematic tissue was prone to infection; this resulted in the formation of tumors which collapsed and died after a few hours. Much more is known about HR in facultative biotropic fungi of the Phythophthora type. Tomiyama's group (84, 85, 88, 89) has contributed extensively to the understanding of HR in both leaves and the potato root. In the leaves, rapid necrosis of cells preceded the death of the

fungus; but in tubers the reaction was slower, mesophyll cells in resistant tubers undergoing necrosis did not completely localize the pathogen. An outer rim of cells showing intense biosynthetic activity and rapid cytoplastic streaming were necessary for repair and the sealing off of the pathogen (88). The necrotropic fungi, such as the bean pathogen Colletotrichum lindemithianum, was incapable of a compatible interaction. The speed of pathogenic destruction and the speed of counteraction by HR in the plant determined whether the pathogen could be arrested in time (54).

Hypersensitivity with virus infection is more difficult to demonstrate. Necrotic tissue which results from viral proliferation does not exceed a certain size. These are referred to as local lesions. In many cases virus may be found outside these lesions, but the necrotic tissue still contains living virus for some time. Since the size of the local lesions is positively correlated with virus quantity, HR can be demonstrated by the failure of local lesions to reach optimum size. In slow growing strains there is a regression in relative virus quantity in the lesion; this points to an inactivation mechanism (45). Farkas, Kiraly and Solomosy (19) found evidence for such a mechanism in the new synthesis of both RNA and protein in tissue surrounding the lesion (45). Ross and Israel (67) further related ultrastructural alterations to a newly synthesized defense mechanism.

The metabolic alterations in hypersensitive plants caused by fungi and viruses are remarkably similar. This is noted in the speed of the reaction and the nature of enzyme systems activated. However, in both

respects they differ from HR induced by bacteria which fail to invade the plant cell protoplast.

Interaction phases in fungal-induced HR

Many aspects of the incompatible, plant-pathogen interactions resemble those in host-pathogen combinations. Though the reaction rates of the responses may vary (54, 88), growth of the pathogen, increase in plant respiration, and loss of plant electrolytes are initially similar. However, the mechanisms involved in plant recognition of the interaction type have not been elucidated. This is a primary problem in the plant-pathogen encounter which triggers HR. When Flor (20) discovered that genes for resistance in the plant required genes for avirulence in the pathogen to induce HR, a concept of specificity was implied. Albersheim, Jones and English (1) visualized that an induction process, perhaps involving an interaction of carbohydrates of the host and of the pathogen, determines the pathogen's ability to produce degrading enzymes. This reaction would thus resemble disease inception by pneumococcus polysaccharide capsules, O-antigenic polysaccharide of E. coli to resist phagocytoses, or in recognition of bacterial cell, O-antigenic polysaccharide by bacterial viruses. With this model, plants having slight differences in wall polysaccharide would be considered distinct varieties, such as the O-antigen, distinguished the serotypes of the genus Salmonella (1). Thus variability in wall polysaccharide would determine differential resistance to pathogenic infection just as bacteria with slightly differing O-antigenic polysaccharide often differ in resistance to phage infection. However, Tomiyama (85) has expressed doubt that coat

components of different serotypes show the necessary specificity needed for the balance observed for some plant-pathogen combinations.

The recognition of plant pathogens by many plants is followed by alteration of plant metabolism resulting in the appearance of host-specific, fungitoxic substances called phytoalexins. Müller and Borger (55) found potato tuber responded to both compatible and incompatible strains of Phytophthora infestans with such substances. The phytoalexin develops more intensely in response to incompatible pathogens, but there was essentially no difference between varietal, species, or generic immunity of the plant. Though different fungi induced the same phytoalexin, they each showed a characteristic ED_{50}. Recently Varnes and Kuc (91, 92) found in 11 different cultivars of potato infected with P. infestans consistent accumulation of two terpenes shortly before necrosis occurred. These inhibitory terpenes produced in both host-pathogen and non-host-pathogen interactions were much more highly concentrated in the latter. Since different inoculum concentrations failed to affect the rate of synthesis, the investigators suggest that rates of production, not specific genetic information, are involved. HR fails to occur and the rate of accumulation of terpene is suppressed with an incompatible strain if the potato is inoculated with compatible strain 12 hours prior to the challenge. If the conditions are inversed, the disease symptoms are not prevented. This points to the problem of recognition of the pathogen by the host. These investigators favored the concept that alteration in cell metabolism of the host during the compatible response suppresses the plants' ability to respond with HR, but the converse was not possible because of the lack of genetic information to induce disease symptoms. However, it is

noteworthy that sonicated homogenates of both virulent and avirulent races could induce the HR. The view of Varnes and Kuc corresponds with that of Metlitskii and Ozerelshovskaya (51) who believe that compatible pathogens could block HR which was inherent in the plant, but only after adaptation to a specific host tissue. The unadapted pathogen would lack genetic information for such interference.

In response to infection with P. infestans potato tubers show nonspecific physiological changes that occur very rapidly when the interaction is incompatible. Within 10 minutes, plasma currents and Browian movement increased with avirulent races whereas the same responses occurred only after two hours with the virulent race (84). Increase in numbers of mitochondria and in respiration accompanied all infection, but for HR the increase was more rapid. Tomiyama (86) found that in the hypersensitive cells, the early acceleration of respiration was followed by a slower stage which was resistant to CO_2 and HCN. Concurrently, there was increased flow of CO_2 to the shikimate pathway with increased levels of both quinate and shikimate. Higher levels of these metabolites appeared to be a generalized response from increased utilization of the HMP shunt (68).

The onset of fungal induced HR in potato was also accompanied by accelerated oxidative enzyme activity (89). Higher levels of polyphenoloxidase, peroxidase, cytochrome oxidase, and IAA oxidase accompanied a rapid synthesis of phenolic compounds in the necrosing potato tissue. The oxidized products of phenols formed have been shown to inhibit phosphorylation and dehydrogenase activity of the tissue (72) and as a consequence, there was a rapid and irreversible oxidation

of the redox system (33, 72). This effect on the redox system was thought to govern resistance or susceptibility as mediated by phenolic compounds (19, 33).

As a consequence of oxidation of the redox system, plant cell respiration decreased (41, 88, 89). Phenols that were oxidized further condensed to form resins, or reacted with carbohydrates and amino acids to form brown substances, inactivated enzymes, competed with enzymes for substrates, and in general contributed to the numerous reactions associated with necrosis (41, 93).

Though some of the physiological and biochemical changes in cellular metabolism during the HR are known, it has been difficult to establish a cause-effect relationship between the two. For example, during the oxidative reactions just discussed, disruption of cell membranes is prominent. However, the question arises as to whether the oxidative changes are the cause or the result of the abberration observed. A causal relationship is supported by data from conductance measurements of Malus species infected with Venturia inaequalis. Electrolyte loss induced by this fungus increased twofold 22 hours prior to symptom development (63).

Resistance and growth regulators

It is not known whether growth regulators play a role in HR (75). Sequeria (75) reviewed growth regulation with respect to plant disease. Studies of the diseases induced by phytopathogenic bacteria where growth regulators are involved have been mainly limited to crown galls. Some investigators propose that IAA and other growth substances stimulate phenolic compounds in pathogenic infection, but others believe

that accumulations of phenolics only parallel the increased levels of IAA because they originate from common pathways (41). Thus production of IAA and phenolics would result from a general stimulation of aromatic biosynthesis (69). The problem of the actual role that the growth regulators play in plant diseases is complicated, however, by the fact some fungi and bacteria also synthesize the auxin, IAA. Though it is clear that IAA synthesis proceeds via tryptophan in some organisms, doubts remain as to involvement of tryptophan in higher plants (41).

The respiration increase observed in IAA-treated plants results indirectly from a promotion of energy-requiring processes by increasing the levels of phosphate acceptors (75). A direct relationship of IAA to an acceleration of respiration in HR has not been established.

Indirect evidence from several fungal-induced, vascular diseases indicates that IAA may prevent parasitic invasion. Resistance induced by IAA is dependent upon calcium (76). However, the relationship of IAA to resistance is apparently not a simple one since Stahmann (77) was unable to induce immunity with synthetic auxin in sweet potato infected by the fungus Ceratocystis fimbriata.

Ethylene also appears to be involved in infection of plant tissue by phytopathogens. In sweet potato infected with Ceratocystis fimbriata. Stahmann (77) implicated ethylene as one of the stimuli which moved from the area of infection to initiate metabolic changes. Since ethylene stimulates peroxidase in noninoculated tissue (77) and the enzyme increases in response to several fungi, ethylene may be involved in the HR.

Hypersensitivity induced by bacterial pathogens

Until recently, little was known about hypersensitivity induced by the bacterial phyopathogens. The lag in recognition that bacterial pathogens appeared to induce HR is related to both the nature of the necrobiotic action and the lack of development in nature of prominent lesions that could be attributed to a defense reaction of the plant. Since phytopathogenic bacteria (Pseudomonads, Xanthomonads, and some Erwinia and Agrobacteria) mainly lack cytolytic enzymes (72), they can only passively invade the plant where they are initially confined to the intercellular spaces.

In 1949, Thiers and Lester (83) found bacteria in resistant host multiply a short time and then stop growing after a short incubation period. They concluded that since no morphological barriers were apparent that the effect was due to physiological incompatibility.

When Klement and Lovrekovich (40) developed an injection infiltration method for introducing large populations of bacteria into plants, studies of experimentally induced HR developed rapidly. Initial investigations were conducted with bean pods inoculated with both pathogenic and saprophytic species of Pseudomonas and Xanthomonas (40). In bean pods phytopathogenic bacteria evoked a marked reaction which was absent in the presence of nonpathogenic bacteria and saprophytes. When visible symptoms appeared, the multiplication of the bacteria was inhibited. This was interpreted as an active response induced by the growth and metabolic activity of the pathogen. The failure of animal pathogens to grow in bean pods was interpreted as evidence for a preformed defense system which did not affect the phytopathogenic

bacteria. When two species of phytopathogenic bacteria were simultaneously injected into bean pods, growth rates were differential and the defense reaction, induced by the more rapidly growing isolate, inhibited the growth of the slower growing one (40). From these studies, Klement's group determined that the time of symptom development was independent of the amount of inoculum and is mainly regulated by the rate of growth of the specific bacterium. On the other hand, the intensity of the reaction of plant tissue depended on the concentration of the inoculum (34, 37). Tobacco infected with phytopathogenic bacteria also demonstrated responses similar to that induced in bean (34). Prior to the appearance of a rapid necrotization in plant cells the bacterial growth was inhibited. In both compatible and incompatible interactions, the same response occurred, but with the latter the growth inhibition and symptom development appeared much earlier.

From the indications of bacterial-induced HR in bean, tobacco and apple by a number of phytopathogenic bacteria, the response was not due to nutrient deficiency (2, 38, 61). This fact was indirectly concluded from the observation that multiplication of compatible bacteria and symptom development were prevented or delayed if the tissue was preloaded with incompatible or avirulent phytopathogenic bacteria. Chand and Walker (9) showed that plant sap from both susceptible and resistant plants supported equally well the compatible and incompatible pathogenic bacteria. These investigations support the hypothesis that infection by some avirulent or incompatible strains of pathogens rapidly induce the formation of substances that limit their own

growth, and, under suitable experimental conditions, also that of the virulent species (65).

Klement and Goodman (38) described the development of HR as having 3 phases. 1. Induction--the time that is necessary to trigger HR and which is independent of the host, 2. the latent period--the time following induction, when living bacterial cells are no longer needed to elicit a plant reaction, to the time just prior to tissue collapse. The latent period, which occurred independently of living bacteria, was influenced by the bacterial species and the biological activity of the plant, 3. rapid collapse of host cells. Evidence concerning the first phase is as follows: within 20 minutes after intromission of Pseudomonas syringae into tobacco the induction process was irrevocably established (64). Some factor in the intact bacteria appeared to be required for HR to occur since Hoitink (27) was unable to induce HR in soybean or susceptibility in tobacco with P. tabaci gymnoplast. However, induction was also achieved with cell free extracts of the bacteria and by a soluble protein in the wall (76). The second phase is sensitive to 37° C temperature (36). Hildebrand and Riddle (26) found preconditioning the xanthomonads at 16° C enhanced the HR, however the optimum temperature for HR induced by the pseudomonads was also the optimum temperature for preconditioning. From these observations these investigators concluded that the xanthomonads were restricted in the range of conditions under which HR was induced, while the pseudomonads were more versatile. Light did not appear to influence HR induced by the pseudomonads in tobacco (26) or in the resistance of soybean to P. glycinea (32). However, Lozano and Sequeira

(50) found that an incompatible race of P. solanacearum induced no HR in tobacco in the dark. Light was also a critical factor for the HR induced by agrobacterium, and this indicated a different system was involved in induction of HR and may be related to auxin (26).

Though many phytopathologists believe that susceptibility and hypersensitivity are not distinct phenomena (15, 42, 54), Pinkas and Novacky (65) recently found evidence to the contrary. HR was delayed 18 hours in tobacco when infused with cycloheximide from 0-3 hours after inoculation with P. pisi or E. amylovora. In contrast, disease symptoms in tobacco with P. tabaci were unaffected by this treatment. In the presence of the antibiotic the bacterial population increased over the control. Since cycloheximide strongly inhibited ^{14}C-leucine incorporation into tobacco protein, the HR expression appeared to require protein synthesis. On the other hand, a nonspecific factor from heat killed bacterial cells appear to affect both interactions in a similar manner. Recently Lozono and Sequeira (49) found that heat-killed cells of a number of different plant-pathogenic bacteria prevented the HR of a challenge inoculum, just as symptom development was prevented with heat-killed bacteria (47). Since a similar effect was achieved with several species of phytopathogenic bacteria, this indicated that a nonspecific factor was responsible for the reduction in the effects of challenge populations of both compatible and incompatible bacteria.

During the later phases of both host-pathogen and non-host-pathogen interactions, new synthesis of protein occurred, but was more concentrated in the latter. This synthesis was observed surrounding TMV lesions in

tobacco (57), around invaded cells of resistant potato infected with P. infestans, and in areas of sweet potato tissue infected with Ceratocystis fimbriata (95). Resistance appeared to be correlated to the ability to form such protein following initial infection (95).

Evidence from recent investigations suggests that the lesion caused by incompatible bacteria that is responsible for HR may be the plant cell membrane. Novacky (57) found that several cytokins, infused in tobacco leaves up to 3 hours after inoculation with the incompatible P. pisi, delayed the HR. The age of the leaf was critical for expression of the kinetin effect and younger leaves were not affected. Several investigators also observed that kinetin inhibited lesion development in halo blight of bean (28, 46). Cytokinins are known to have anti-stress effects initiated by salts, water stress, heat and cold treatment, detachment and pathogenesis (57). However, as far as inducing host resistance is concerned, the results are confusing because of the biological effects which are often opposing when different concentrations of cytokinins are used (75). The protection cytokinins confer on plants against stress resembles that for the calcium salts (57) which has stabilizing effects on cell membranes. Cytokinin is known to be indirectly responsible for the accumulation of specific solutes at the site of kinetin application.

At the ultrastructural level, bacterial-induced HR appeared to cause distinct structural damage to cell membranes. Goodman and Plurad (23) observed, that in tobacco infected with P. syringae and in apple infected with E. amylovora, the chloroplast and tonoplast membranes were most sensitive and showed disruptive effects due to the presence of the

bacteria. Ribosomes and endoplasmic reticulum were more prolific than in control tissue, but there was a reduction in the number of mitochondria. The nuclear envelope was resistant to infection, but the nucleus was somewhat aggregated. Huang, Huang and Goodman (29) demonstrated an alteration of the properties of structural proteins of thylakoid membranes of hypersensitive tobacco 3 hours after the infusion of incompatible bacteria. These investigators were not able to determine whether the changes of UV absorption, solubility, phosphate binding properties and changed electrophoretic patterns were due to altered synthesis of the protein or to denaturation.

Few studies of the biochemical and physiological nature of interactions during the bacterially induced HR have been conducted. Recently, Nemath, Klement and Farkas (56) studied the enzymes that were activated during the bacterially-induced HR in tobacco with P. syringae. Table 1 is a comparison of the enzyme changes in hypersensitive tissue in response to viral, fungal and bacterial infection. These studies indicated that the classical peroxidase-polyphenol oxidase complex, so typical of the fungal (79, 89) and viral (19, 20) induced HR, were inactive during the bacterially-induced HR in tobacco. The oxidative metabolism which accompanied the synthesis and oxidation of the phenols with viral and fungal pathogens (20, 33, 41) appeared to be activated. A factor probably contributing to this difference is the failure of bacterial cells to penetrate the plant cells (56). In vitro studies of pathogenic pseudomonads conducted by Moustafa (Darweish) and Whittenbury (52) complemented these observations. They reported that these bacteria not only failed to oxidize phenols to quinones but

TABLE 1. Enzyme changes in hypersensitive tissues (56)

Enzyme	Parasite		
	Fungus	Virus	Bacteria
Ribonuclease	+	+	(+)
Peptidase	*0	+,=	=
Glucose 6-P dehydrogenase	+	+	+
6-P-gluconate dehydrogenase	+	+	+
Polyphenoloxidase	+	+	=
Peroxidase	+	+	=
Cytochrome oxidase	+	+	=
Phenyalanine deaminase	+	+	=
Shikimate dehydrogenase	0	+	+

*not tested = equal to control + greater than control

they also reduced low concentrations of quinones by the production of 2-keto-gluconate from glucose. In contrast, Stahmann (77) found that peroxidase levels were elevated during the host-pathogen interaction of bean with Pseudomonas phaseolicola. He suggested that the level of the host peroxidase produced was influenced by the production of a bacterial catalase. From this relationship, Stahmann proposed that HR may be related to host peroxidase and bacterial catalase. Thus incompatible bacteria would produce lower levels of catalase and would not destroy the peroxides that were necessary oxidants in peroxidase-catalyzed reactions.

The consistent appearance of ribonuclease in HR induced by microorganisms was considered to be related to resistance reactions. Stahmann (77) hypothesized that the hydrolytic breakdown of nucleic acids may produce nucleotides capable of diffusing into adjoining

tissue where they combine with suppressors of the genes for resistance allowing their expression. Bhattacharya (4) found new synthesis of DNA, RNA and protein prior to the necrobiotic stage induced by wheat rust. Such processes also occurred in surrounding lesions caused by both viruses and fungi (19, 45, 72) and, in addition, the mitochrondria, endoplasmic reticulum, and membrane bound organelles increased (67).

Wall modification in certain plant-pathogen combinations occurred adjacent to the lesion (25). This indicated that containment could only be evaluated in terms of a dynamic response of healthy cells surrounding the infection sites. It appeared that cellular damage, if associated with necrobiotic processes, triggered a large variety of synthetic processes. Enzyme changes with viruses and fungi are mostly in this category (56). Nemath, et al. (56) suggested that phenol synthesis and increased polyphenoloxidase and peroxidase activity may occur during the slower reactions typical of the viruses and fungi. Presumably, in bacterially-induced HR, the cellular collapse occurred "too fast" (56) for these reactions to be observed.

Models of the hypersensitive reaction

Various physical and chemical agents as well as biologically active compounds are reported to mimic the HR in plants (38). Though rapid cell collapse may be triggered in several ways, the collapse induced by microorganisms is unique in three important respects. First, the competent inducers are pathogens. Second, the biochemical and physiological environment of the plant cells were highly resistant or non congenial to the pathogen. Finally, to induce HR, specific components of both systems are required. The latter is supported by

the fact that saprophytes and other non pathogens were unable to induce the response. Susceptible plants were incapable of responding with HR to compatible pathogens (38), and the potential for the HR was enhanced by genetic breeding for resistance (54). On the other hand, the ability of a pathogen to induce HR did not appear to be related to the degree of its virulence, but to the property determining the potential for pathogenicity (38, 84). This view is supported by the fact that if virulence is lost the potential for HR often remains (23, 62).

Explanations for the early localization or death of incompatible pathogens through the hypersensitive death of the cells, have met many difficulties. Physiological and biochemical changes, which are distinct for different plant-pathogen interaction types, are difficult to demonstrate. Most important, many models proposed from these studies fail to account for the plant's ability to recognize the specific nature of the invading pathogen.

Since Ward (94) first described the HR, opinions on the basis of differential plant responses to invading phytopathogens have varied widely. According to many investigators, nutritional and physiological factors determine the nature of the plant-pathogen relationships. If plant substrates were unsuitable for a pathogen, hypersensitive death of plant cells would presumably result from the death of the pathogen (54). Fisher and Gaümann (54) proposed that plant proteins or even carbohydrates of particular structure were a prerequisite for establishment of specific pathogens. However, neither consistent, qualitative or quantitative differences in the plant constituents could be demonstrated between susceptible and resistant plants. Lewis

(44) postulated that the right molecules in the right amounts were most important. Though patterns were discovered in metabolites derived from the plant cell environment, no specific mixture or ratio was found to be necessary for pathogenicity (8, 9). From studies of defective synthesis occurring in genetic mutants, Garber (21) proposed that pathogens required specific, small, membrange-permeable molecules such as amino acids, inorganic acids, or vitamins. The availability of such required factors in plants would then delimit the range of potential pathogens. However, Garber's hypothesis was questioned on the basis that it would be improbable that a mutant could survive competition in a natural environment. Johnson (54) proposed that genes which control HR are enzyme producers and phytopathogens could function only when they possessed the enzymatic system needed for successful parasitism. This hypothesis failed as a general condition, since dominant genes are associated with the production of enzymes and recessive genes are commonly associated with pathogenicity.

Despite these contradictions, the nutrition hypothesis is still supported by many investigators. Recently, Bogarth (5) postulated that viral confinement to local lesions may be due to the intense synthesis of the necrotic area, which may deplete the surrounding tissue of the essential nutrients necessary for the virus.

Preformed phytotoxins have been considered to be responsible for HR, but this has been difficult to prove. Many of the presumed inhibitors to phytopathogens may have resulted from preparative artifacts (54). Seldom has there been a demonstration of a correlation between production of the toxin in vivo and the sensitivity of the pathogen.

Gassner and Hassebrauk (54) postulated that a quantitative relationship existed in the ability of the plant to eliminate toxin produced by the pathogen. According to their model the resistant plants lack the ability to eliminate with sufficient speed the toxic material. However, no such specific agent in plants has been identified (54).

From investigations of potato blight caused by the facultative fungus Phythophthora infestans, Müller and Börger (55) proposed a more dynamic interpretation of the HR. Since this fungus could grow on nonliving substrate, a nutritional concept for the HR could be eliminated. In this interaction, plant cells were found to be active producers of defense factors that could contain the growth of both compatible and incompatible pathogens, as well as other organisms that grow on living tissue (55). These factors called phytoalexins were host specific, but were induced by different fungi (13), fungal metabolites (13), and antibiotics (74), as well as by stress (96). The phytoalexins, which are primarily low molecular weight compounds are believed to be the main line of plant defense involving the non host-pathogen combination (72), especially involving the fungi. However, recently Varnes and Kuc (91, 92) found two terpenes were produced in both compatible and incompatible interactions of P. infestans with potato. The terpene accumulation was greater in and correlated with the development of HR. The deployment of resistance mechanisms appeared to be controlled in tissue infected with the compatible fungus. Cruickshank (13) suggested that the condition required for resistance in the incompatible interaction was the stimulation of phytoalexin to concentrations above the threshold at

which the fungus was inhibited. However, Person, Rohringer and Samborski (64) contradict this view on the basis of the gene for gene concept. They believe that it is unlikely that genes for virulence control sensitivity to a phytoalexin. This in effect would counteract the action of all the genes increasing resistance in the plant, and thus would be improbable. They proposed instead that virulence may affect the regulation of phytoalexin production in the plant. As a model for HR, the phytoalexin theory has a weakness in two respects. These inhibitory factors have been demonstrated only in two interactions involving bacterially induced HR (11, 13). Some plants fail to produce detectable levels of phytoalexins.

The phenols, their oxidation products, and the related peroxidase-polyhenoloxidase, enzyme systems were believed by many investigators to meet many of the requirements for the HR (33, 41, 71). Since quinones can participate in deaminations, in the formation of 1, 4 addition products with sulfhydral and amino compounds, and in causing the oxidation of susceptible functional groups, key microbial enzymes and metabolites could be inactivated as result of these reactions. Similar effects could also be expected in host cells (77). Tomiyama's group (88) further proposed that the phenolic compounds resulting from HR death of host cells may function to alter the metabolism of healthy cells surrounding the infection locus thus confining the pathogen. Stahmann (77) observed that higher levels of peroxidase in resistant plants were apparently related to ethylene produced by a fungal pathogen and to the catalase in a bacterial pathogen. Even though this system may be experimentally feasible, it does not explain the

altered metabolism observed in plant cells surrounding the infection locus, which may be regulated by the pathogen. Furthermore, this model failed in three important respects. First, this system could not explain the plant's ability to differentiate between compatible and incompatible pathogens. Secondly, the system appeared to be of no consequence in bacterially induced HR (56), and thirdly it is not certain whether phenol activation precedes or follows the HR (88).

Russian phytopathologists have attempted to establish a relationship between the enzymatic equipment of both the plant and the pathogen and the immunological behavior of plants (71, 72). In this model, the enzymes that determined the nature of the changes occurring in the plant were adaptive and were considered to be controlled by the nature of the pathogen and the level of resistance in the plant. In incompatible combinations, the enzymes would be not only activated but new isoenzymes would develop. As evidence for such a model, these investigators cited formation of isoenzymes of peroxidase and oxidative enzymes possessing resistance to the toxins of the pathogen (78). Although this model emphasized the dynamic equilibrium which exists in plant-pathogen interactions, there was no consistent pattern shown in enzymes activated or the appearance of new isoenzymes. Furthermore, no specificity was demonstrated that could differentiate the interaction types.

An inclusive model to explain all aspects of plant-pathogen interactions culminating in a hypersensitive response has been difficult to construct. However, organisms must be pathogens to induce disease symptoms in host plants and avirulent pathogens to induce HR in

resistant or non host plants (38). In turn the plant must be either susceptible or highly resistant to the organism. Thus, the potential for the HR is related to genetic determinants for pathogenicity (38, 84) as well as to induced plant resistance (38).

Once the reaction is triggered, the HR is autonomous. This is supported by the ability of the plant to resume expression of HR after inactivation or after artificial reversal to expression of the symptoms of susceptibility (54).

Genetic studies of a large number of plant-pathogen interactions resulting in the HR show that the response is possible only when products of plant genes for resistance meet those of the pathogen for avirulence. Such specific requirements from both participants may indicate that the HR involves a type of immunological response on the part of both the plant and the pathogen (16). Furthermore, factors governing specificity could be related to the plant's ability to distinguish between compatible and incompatible pathogens (38). The recent discovery that homogenates from both compatible and incompatible Phytophtora infestans can induce HR in potato substantiates these observations (91). Specific factors which appear to be involved in disease susceptibility, have previously been shown to be produced during interactions between host plants and a pathogen of flax which causes rust, angular leaf spot of cotton, crown gall of sunflower and tobacco, black rot of sweet potato, and common smut of corn (10, 16). Discovered by cross titrations and precipitin tests, these factors showed common antigenic properties. Rowley and Jenkins (70) and Dineen (17), in reviewing the occurrence of common antigens in

animal systems, claimed that common antigens could influence the immunological tolerance of the host to the pathogen.

Common antigens have not been demonstrated for resistant plants and incompatible pathogens where antigenic disparity would probably be extensive. DeVay's group (16) did find cell-free antigen preparations of X. malvacearum which induced the HR in resistant cotton plants with little or no response shown for susceptible plants. The active component was a polysaccharide-protein complex. Also Sequeira, Aist, and Ainslie (76) isolated proteins from the incompatible P. pisis that were capable of both inducing and inhibiting HR in tobacco. However, the role of the antigenic substances in plants has not yet been determined since antibodies are not known to exist in plants. On the other hand, plants do possess substances showing antibody-like properties. These substances, known as the plant lectins, are found in abundance in seeds of legume plants and in smaller quantities in the leaves and also in other plant groups (6). Lectins are glycoproteins which can agglutinate specific blood groups by reacting with polysaccharides on red blood cells. The role of these antibody-like substances in plants has not been determined.

Excellent evidence of specificity in plant-pathogen interactions is observed in the production of host-specific toxins (43). Also Jones, Anderson and Albersheim (31) discovered that a group of proteins, isolated from tomato stems, bean hypocotyls and suspension-cultured sycamore, inhibit specifically the degradation of cell walls by Fusarium oxysporum polygalacturonase. A specific role determining the type interaction that is established was postulated because purified

endopolygalacturonate trans-eleminase, from the potato-rot pathogen Fusarium roseum was capable of causing necrosis in plant cells (53). The recent discovery that a cultivar specific factor from an incompatible interaction protected bean hypocotyl from disease susceptibility to Colletotrichum lindemuthianum (3) is evidence that these interactions are conditioned in a specific manner. This investigation has shown that specific factors having a genetic basis are capable of immunizing bean hypocotyls against disease susceptibility. Such specific reactions may have been already demonstrated with heat killed bacteria of species capable of inducing either disease susceptibility or the HR (47, 48). Thus, it may be concluded that at least in this aspect there is a similarity between disease resistance in plants and immunity in animals (3).

The model suggested by the foregoing evidence involves the interaction of specific factors in both plant and pathogen during the initial phases of the HR. The factor in plants may be a protein, since cycloheximide has been shown to inhibit bacterially induced HR (65). The site where plant-cell breakdown is initiated by such an active factor is unknown. However, since membrane disruption occurs in the HR, factors related to membrane synthesis and repair may be involved. As to the bacterial factor(s) involved in HR, proteins isolated from bacteria are capable of inducing HR. These proteins may be the specific factor(s) required by the pathogen for the induction of HR. Such information should warrant a further search for inducer proteins in bacterial cells.

The potential of a protein to function as a trigger for HR induction may be visualized in a number of ways. First the well-known antigenic potential of some proteins suggests a possible function in recognition of specific sites similar to the classical antigen-antibody responses in animal systems. Though antibodies have not been demonstrated in plants, the highly exacting requirements of the type shown, for example, during pollination may also be involved in plant pathogen interactions. On the other hand, a possibility exists that HR may be triggered directly by production of bacterial proteins that function as hydrolytic enzymes. Since the rapid deterioration of membrane systems is characteristic of the HR, enzymes which either cause dissolution of the cell wall components necessary for membrane integrity (38) or direct disruption of membranes, may be involved. Cellulases or pectinases might function in the former capacity while phosphatidases, lipases, or proteinases may function in the latter case. The investigation of hydrolytic potential of a bacterial protein would thus seem to be of cardinal importance.

Discussion

When products of microorganisms make biological contact with the protoplasts of higher plants, they may either repress or trigger the plant defense mechanisms or they may not produce any change. If the plant-microbe system is incompatible, the defense most commonly triggered in the plant is the hypersensitive reaction (HR). Except for reaction rate, the hypersensitivity in plant cells, induced by many microbial agents, shows biochemical and physiological changes

which are similar to susceptibility. This is shown by the initial rapid growth rate of the pathogen in both types of responses. However, in contrast to susceptibility, the hypersensitive response shows a more rapid acceleration of respiration, O_2 uptake, utilization of the hexose monophosphate shunt, activation of oxidative enzymes, synthesis of phenolics, and loss of membrane integrity with abrupt cessation of growth in the pathogen.

Genetic studies of a number of plant-pathogen combinations show evidence that HR, like susceptibility, involves gene products of both the pathogen and the plant. Such is indicated by the fact that HR is possible only when a gene product for avirulence in the pathogen encounters those of a gene for resistance in the plant. The recent discovery of common antigens in host-pathogen interactions prompted some investigators to view the HR as involving an immunological response on the part of both the plant and the pathogen (16). Such response on the part of the plant finally results in the localized death of cells which come in contact with the pathogen. A similar response may be inferred in the case of the pathogen from its ability to grow for a time on noncongenial substrates.

Such a model holds promise especially in the implied specificity whereby HR may be differentiated from susceptibility and thus perhaps from immunological tolerance. Also qualities defining pathogenicity in microorganisms may be determined. Since interchange of metabolic products appears to occur soon after contact between interacting pairs, study of early phases of HR should contribute more to understanding of this phenomenon than do the later phases as implied by other models,

discussed in the body of this paper. Examples are those involving nutritional aspects, inactivation of toxin, enzymes, and phenolics.

The recognition implied in the above model may resemble that recently studied by Jones, Anderson and Albersheim (31). These investigators found a group of proteins in walls of tomato stems, bean hypocotyls and suspension cultured sycamore cells which inhibited degradation of cell walls by a fungal polygalacturonase which caused necrosis of plant cells.

Immunological specificity in many bacteria, pathogenic to animals, involves polysaccharides. DeVay's group, in one study of plant pathogens, found a cell free antigen preparation, a polysaccharide protein complex, from X. malvacearum which induces necrosis in resistant cotton (16). Recently, Sequeira, Aist and Ainslee (76) isolated proteins from incompatible P. pisi capable of both inducing and inhibiting HR in tobacco. Since, in the plants HR has been prevented both by cycloheximide and cytokinins, the interaction sites may involve plant-membrane proteins with proteins or conjugated proteins in the pathogen.

The investigations of HR using the bacterial system offer several advantages over those with fungi and viruses. First, since these pathogens do not invade the cell protoplast, fewer products of injury are contributed to the system. Secondly, since bacteria remain in the intercellular spaces, they may be removed by centrifugation to study effects of the interaction on the pathogen. Finally, the bacteria fail to activate plant enzyme systems typically associated with stress that occurs during the fungal and virally induced HR.

On the other hand, the high concentrations of bacteria (ca. 10^7 to 10^8 cells/ml) needed to induce macroscopically visible HR lesions is considered by some investigators to indicate an overwhelming of the plant defense system. This would appear unlikely since the bacteria are killed during HR. Other investigators question its significance because a high inoculum density is required to trigger the response. However in nature, populations from HR lesions reached 10^9 to 10^{10} cells/ml (7) and microscopic evidence of localized cell collapse occurs with concentrations as low as 10^4 to 10^5 cells/ml (90).

Further investigations of the HR in plants is merited not only because the determination of the basis for recognition systems involving pathogenic relationships is important but also because such studies may contribute to the understanding of possible mechanisms which control development in plants. The antibody-like activity shown for the lectins in seeds and leaves of legumes may have such a role.

Regarding the process of HR induction, several questions need to be considered. How does the plant recognize that it is invaded by a compatible or an incompatible pathogen as opposed to a non pathogen? When is recognition triggered and what factors in the plant cell environment influence the inducing fraction in deployment of the plant defense mechanism?

The purpose of this investigation is to characterize fine structure changes in a plant-pathogen interaction during the progressive phases of the HR and to investigate the nature of a biochemical basis for induction of the HR. The model system used for this study was Capsicum annuum and Pseudomonas phaseolicola. A non-host plant was

chosen to eliminate triggering metabolic changes related to disease inception to distinguish those from the ones related to HR. Pepper was chosen because bacterial suspension can be easily infused into the leaves. The halo blight pathogen of bean was selected because a non-toxigenic mutant of a toxin-producing strain was available as a marker to determine if alteration in symptom inducing potential in the host would also alter those related to HR. The HR induced in pepper by resistant strains of its pathogen Xanthomonas vesicatoria has already been studied. Additional information is needed concerning the mechanisms of HR in plants other than tobacco, the classical test plant for the HR induction by incompatible bacterial pathogens. Two isolates of P. phaseolicola, race 2, G50 Tox^+ and G50 Tox^- were used. G50 Tox^+ is capable of producing a low molecular weight toxin in culture that is capable of reproducing halo blight symptoms in bean. The following hypotheses were investigated:

1. Tissue collapse induced in pepper by Pseudomonas phaseolicola during the hypersensitive reaction is preceded in the early phases of the interaction by deleterious effects of the plant tissue on the bacterial cells.

2. Ultrastructural changes which precede tissue breakdown in pepper are initiated by specific interactions of the bacterial cells with the plant tissue.

3. A specific inducing principle from the bacterial cells is responsible for the membrane changes initiated in the plant cell that ultimately result in tissue collapse.

Literature Cited

1. Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of cell walls in relation to infective processes. Ann. Rev. Phytopathol. 7: 171-194.

2. Averre, C. W., and A. Kelman. 1964. Severity of bacterial wilt as influenced by ratio of virulent to avirulent cells of Pseudomonas solanacearum in the inoculum. Phytopathology 54: 779-783.

3. Berard, D. F., J. Kuc, and E. B. Williams. 1972. A cultivar specific protection factor from incompatible interaction of green bean with Colletotrichium lindemuthianum. Physiol. Plant Pathol. 2: 123-127.

4. Bhattacharya, P. K., and M. Shaw. 1968. The effect of rust infection on DNA, RNA, and protein in nuclei of Khapli wheat leaves. Can. J. Bot. 46: 96-99.

5. Bogarth, R. F., and A. F. Ross. 1964. Systemic resistance induced by localized virus infection. Extent of changes in uninfected parts. Virology 24: 446-455.

6. Boyd, W. C. 1963. The lectins: their present status. Vox, Sanguinis 8: 1-32.

7. Burkowicz, A., and R. N. Goodman. 1970. Ultrastructural changes in apple leaves inoculated with a virulent or an avirulent strain of Erwinia amylovora. Phytopathol. Z. 68: 258-268.

8. Chamberlain, D. W. 1962. Reactions of resistant and susceptible soybean to Xanthomonas phaseoli var. soyensis. Plant Dis. Reptr. 46: 707-709.

9. Chand, J. N., and J. C. Walker. 1964. Relation of leaf and varietal resistance to bacterial multiplication in cucumber inoculated with Pseudomonas lachrymans. Phytopathology 54: 49-50.

10. Charudattan, R., and J. E. DeVay. 1971. Common antigens among varieties of Gossypium hirsutum and isolates of Fusarium and Verticillium species. Phytopathology 62: 230-234.

11. Chen, S. C., R. E. Stall, and A. A. Cook. 1969. Extraction and range of activity of a bacterial inhibitor from Capsicum annuum. Phytopathology 59: 112.

12. Cook, A. A., and R. E. Stall. 1971. Calcium suspression of electrolyte loss from pepper leaves inoculated with Xanthomonas vesicatoria. Phytopathology 61: 484-487.

13. Crosse, J. E. 1965. Bacterial canker of stone fruits. VI. Inhibition of leaf-scar infection of cherry by a saprophytic bacterium from the leaf surfaces. Ann. Appl. Biol. 56: 149-160.

14. Cruickshank, I. A. M. 1964. Phytoalexins. Ann. Rev. Phytopathol. 1: 351-374.

15. Day, Peter R. 1972. Genetics of Host Parasite Interactions. W. H. Freeman and Co., San Francisco, 238 p.

16. DeVay, J. E., W. C. Schnathorst, and M. S. Foda. 1967. Common antigens and host parasite interactions. In Mirocha, C. J. and I. Uritana, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Phytopathological Society, Inc., St. Paul, Minnesota, pp. 313-325.

17. Dineen, J. K. 1963. Innunological aspects of paratism. Nature 197: 268-269.

18. Farkas, G. L., Z. Kiraly, and F. Solomosy. 1960. Role of oxidative metabolism in the localization of plant viruses. Virology 12: 408-421.

19. Farkas, G. L., and M. A. Stahmann. 1966. On the nature of changes in perioxidase isoenzymes in bean leaves infected by southern bean mosiac virus. Phytopathology 56: 669-677.

20. Flor, H. H. 1955. Host-parasite interactions in flax rust. Its genetics and other implications. Phytopathology 45: 680-685.

21. Garber, E. D. 1956. A nutrition inhibition hypothesis of pathogenicity. Am. Nat. 90: 183-197.

22. Goodman, R. N. 1971. Reevaluation of the role of ammonia in the hypersensitive reaction. Phytopathology 61: 893.

23. Goodman, R. N., and S. R. Plurad. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Physiol. Plant Pathol. 1: 11-15.

24. Goodman, R. N., Z. Kiraly, and M. Zaitlin. 1967. The biochemistry and physiology of infectious plant disease. Van Nostrand Co., Princeton, New Jersey, 354 p.

25. Heath, Michele C., and I. B. Heath. 1971. Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. Physiol. Plant Pathol. 1: 277-287.

26. Hildebrand, D. C., and Beverley Riddle. 1971. Influence of environmental conditions on reactions induced by infiltration of bacteria in plant leaves. Hildgardia 41: 33-43.

27. Hoitink, H. A. J., and O. E. Bradfute. 1971. Mechanism of disease initiation by Pseudomonas tabaci. In Maas Geesteranus, H. P., ed. Proceedings of the third international conference on plant pathogenic bacteria, Wageningen, 14-21 April, 1971. Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands, pp. 55-58.

28. Hoitink, H. A. J., R. L. Pelletier, and J. G. Coulson. 1966. Toxemia of halo blight of bean. Phytopathology 1062-1065.

29. Huang, Jeng-sheng, and R. N. Goodman. 1970. The relationship of phosphatidase activity to the hypersensitive reaction in tobacco induced by bacteria. Phytopathology 60: 1020-1021.

30. Israel, H. W., and A. F. Ross. 1967. The fine structure of local lesions induced by tobacco mosiac virus in tobacco. Virology 33: 272-276.

31. Jones, T. M., A. J. Anderson, and P. Albersheim. 1972. Host-pathogen interactions. IV. Studies of the polysaccharide degrading enzyme secreted by Fusarium oxysporum f. sp. lycopersici. Physiol. Plant. Pathol. 2: 153-166.

32. Keen, N. T., and B. W. Kennedy. 1974. Hydroxyphaseollin and related isoflavanoids in the hypersensitive resistance reaction of soy beans to Pseudomonas glycinea. Physiol. Plant Pathol. 4: 173-185.

33. Kiraly, Z., and G. L. Farkas. 1962. Relation between phenol metabolism and stem rust resistance in wheat. Phytopathology 52: 657-664.

34. Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199: 299-300.

35. Klement, Z. 1963. Method of obtaining fluid from the intercellular spaces of foliage and the fluids merit as substrate for phytobacterial pathogens. Phytopathology 55: 1033-1034.

36. Klement, Z. 1971. The hypersensitive reaction of plants to bacterial infection. Acta Phytopathol. Scient. Hung 6: 115-118.

37. Klement, Z., G. L. Farkas, and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54: 474-477.

38. Klement, Z., and R. N. Goodman. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Ann. Rev. Phytopathol. 5: 17-44.

39. Klement, Z., and R. N. Goodman. 1967. The role of the living bacterial cell and the induction time in the hypersensitive reaction of tobacco plants. Phytopathol. 55: 322-333.

40. Klement, Z., and L. Lovrekovich. 1961. Defense reactions induced by phytopathogenic bacteria in bean pods. Phytopathol. Z. 41: 217-221.

41. Kosuge, Tsune. 1969. The role of phenolics in host response to infection. Ann. Rev. of Phytopathol. 7: 195-222.

42. Kuc, J. 1968. Biochemical control of disease resistance in plants. World Rev. Pest Control 7: 42-55.

43. Kuo, M. S., C. O. Yoder, and R. P. Scheffer. 1970. Comparative specificity of the toxins of Helminthosporium carbonum and Helminthosporium victorae. Phytopathology 60: 365-368.

44. Lewis, Ralph W. 1953. An outline of the balance hypothesis of parasitism. Am. Nat. 87: 273-281.

45. Lobenstein, G. 1972. Localization and induced resistance in virus infected plants. Ann. Rev. Phytopathol. 10: 177-206.

46. Lovrekovich, L., and G. L. Farkas. 1963. Kinetin as an antagonist of toxic effect of Pseudomonas tabaci. Nature 196: 710.

47. Lovrekovich, L., G. L. Farkas. 1965. Induced protection against wildfire disease in tobacco leaves treated with heat killed bacteria. Nature 205. 823-824.

48. Lovrekovich, L., H. Lovrekovich, and R. N. Goodman. 1970. Ammonia as a necrotoxin in hypersensitive reaction caused by bacteria in tobacco leaves. Can. J. Bot. 48: 167-171.

49. Lozano, J. C., and Luis Sequeira. 1970. Prevention of the hypersensitive reaction in tobacco leaves by heat killed bacterial cells. Phytopathology 60: 675-679.

50. Lozano, J. C., and L. Sequeira. 1970. Differentiation of races of Pseudomonas solanacearum by a leaf infiltration technique. Phytopathology 60: 833-838.

51. Metlikskii, L. V., and R. Ozerelskovskaya. 1968. Plant immunity. Biochemical aspects of plant resistance to parasitic fungi. Plenum Press, New York, New York, 114 p.

52. Moustafa (Darweish), F. A., and R. Whittenbury. 1971. Properties which appear to allow phytopathogenic pseudomonads to counteract plant defense mechanisms. Phytopathol. Z. 67: 214-224.

53. Mullen, J. M., and D. F. Bateman. 1971. Production of an endopolygalacturonate trans-eliminase by a potato dry rot pathogen, Fusarium roseum avenaceum in culture and disease tissue. Physiol. Plant Pathol. 1: 363-373.

54. Muller, K. O. 1959. Hypersensitivity. In Horsfall, J. G., and A. E. Dimond, eds., Plant pathology 1. Academic Press, New York, pp. 470-519.

55. Muller, K. O., and H. Borger. 1940. Experimentalle Untersuchungen uber die Phythophthora-Resistenz der Kartoffel; zuglich ein Beitrag zum Problem der "erworbenen Resistenz" in Pflanzenreich. Arb. biol. Reichsenstalt. Land-u. Fortwirtach. Berlin-Dahlem 23: 189-231.

56. Nemath, J., Z. Klement, and G. L. Farkas. 1968. An enzymological study of the hypersensitive reaction induced by Pseudomonas syringae in tobacco leaf tissue. Phytopathol. Z. 65: 267-278.

57. Novacky, A. 1972. Suppression of the bacterially induced hypersensitive reaction to cytokinins. Physiol. Plant Pathol. 2: 101-104.

58. Novacky, A. 1974. Transmembrane potential in bacterial hypersensitivity. Sixty-sixth annual meeting. American Phytopathological Society, August 11-15, 1974, Vancouver, Canada.

59. Novacky, A., G. Acedo, and R. N. Goodman. 1973. Prevention of bacterially induced hypersensitive reaction by living bacteria. Physiol. Plant Pathol. 3: 133-136.

60. Novacky, A., and R. N. Goodman. 1973. Prevention of bacterially induced hypersensitive reaction by living incompatible bacteria, Pseudomonas pisi. Phytopathol. 62: 780-781.

61. Omer, M. E., and R. K. S. Wood. 1969. Growth of Pseudomonas phaseolicola in susceptible and resistant bean plants. Ann. Appl. Biol. 63: 103-116.

62. Patil, S. S., A. C. Hayward, and R. Emmons. 1974. An ultra-violet-induced nontoxigenic mutant of Pseudomonas phaseolicola of altered pathogenicity. Phytopathology 64: 590-595.

63. Pellizzari, E. D., J. Kuc, and E. B. Williams. 1970. The hypersensitive reaction in Malus species: Changes in leakage of lectrolytes from apple leaves after innoculation with Venturia inaequalis. Phytopathology 60: 373-376.

64. Person, C., D. J. Samborski, and R. Rohringer. 1962. The gene for gene concept. Nature 194: 561-562.

65. Pinkas, Y., and A. Novacky. 1971. The differentiation between bacteria hypersensitive reaction and pathogenesis by use of cycloheximide. Phytopathology 61: 906.

66. Ries, Stephen H., and Peter Albersheim. 1973. Purification of a protease secreted by Colletotrichium lindemuthianum. Phytopathology 63: 625-629.

67. Rohringer, R., and D. J. Samborski. 1967. Aromatic compounds in the host-parasite interactions. Ann. Rev. Phytopathol. 7: 77-86.

68. Rohringer, R., D. J. Samborski, and C. O. Person. 1961. Ribonuclease activity in rusted wheat leaves. Can. J. Bot. 39: 77-84.

69. Ross, A. Frank, and H. W. Israel. 1970. Use of heat treatment in the study of acquired resistance to tobacco mosiac virus in hypersensitive tobacco. Phytopathology 60: 755-770.

70. Rowley, D., and C. R. Jenkins. 1962. Antigenic cross reaction between host and parasite as a possible cause of pathogenicity. Nature 193: 151-154.

71. Rubin, B. A., and V. A. Arkenova. 1957. The participation of the polyphenolase system in the defense reaction of potato against Phytophthora infestans. Biokhimiya 22: 202-208.

72. Rubin, B. A., and Ye. V. Artsikhovskaya. 1963. Biochemistry and physiology of plant immunity. The Macmillan Company, New York, 358 p.

73. Rubin, B. A., and E. V. Arcichovakaia. 1967. The biochemical and physiological background of plant immunity. Phytopathol. Z. 58: 101-102.

74. Schwochau, M. E., and L. A. Hadwiger. 1968. Stimulation of pisatin production in Pisum sativum by Actinomycin D and other compounds. Arch. Biochem. Biophys. 126: 731-733.

75. Sequeira, L. 1963. Growth regulators in plant disease. Ann. Rev. Phytopathol. 1: 5-30.

76. Sequeira, L., S. Aist, and V. Ainslie. 1972. Prevention of the hypersensitive reaction in tobacco by proteinaceous constituents of Pseudomonas solanacearum. Phytopathology 62: 536-541.

77. Stahmann, M. A. 1967. Influence of host-parasite interactions on proteins, enzymes and resistance. In Mirocha, C. J., and I Uritani, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Phytopathological Society. St. Paul, Minnesota, pp. 357-364.

78. Stahmann, M. A., B. G. Clare, and W. Woodbury. 1966. Increased disease resistance and enzyme activity induced by ethylene and ethylene production by black rot infected sweet potato tissue. Plant Physiol. 41: 1505-1512.

79. Stahmann, M. A., I. Uritani, and K. Tomiyama. 1960. Changes in potato proteins induced by fungus infection. Phytopathology 50: 655.

80. Stakmann, E. C. 1914. A study in cereal rust, physiological races. U. Minn. Agri. Exper. St. Bull. 138.

81. Stall, R. E., and A. A. Cook. 1966. Multiplication of Xanthomonas vesicatoria and lesion development in resistant and susceptible pepper leaves. Phytopathology 58: 1584-1587.

82. Stall, R. E., J. A. Bartz, and A. A. Cook. 1974. Decreased hypersensitivity to Xanthomonads in pepper after inoculation with virulent cells of Xanthomonas vesicatoria. Phytopathology 64: 731-735.

83. Thiers, H. D., and M. B. Lester. 1949. Histological studies of bacterial blight infection of the cotton plant. Phytopathology 39: 499-500.

84. Tomiyama, K. 1956. Cell physiological studies on the resistance of potato plants to Phytophthora infestans. IV. On the movement of cytoplasm of the host cell induced by invasion of Phytophthora infestans. Ann. Path. Soc. Japan 21: 54-62.

85. Tomiyama, K. 1960. Some factors affecting the death of hypersensitive cells infected by Phythophthora infestans. Phytopathol. Z. 39: 134-138.

86. Tomiyama, K. 1963. Physiology and biochemistry of disease resistance in plants. Ann. Rev. Phytopathol. 1: 295-324.

87. Tomiyama, K. 1966. Double infection by a compatible race of Phythophthora infestans of a potato cell which had been previously infected by a compatible race. Ann. Phytopathol. Soc. Japan 32: 181-185.

88. Tomiyama, K., R. Sakai, T. Sakuma, and N. Ishizaka. 1967. The role of polyphenols in the defense reaction in plants induced by infection. In Mirocha, C. J., and I. Uritani, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Pathological Society, St. Paul, Minnesota, pp. 165-179.

89. Tomiyama, K., and M. A. Stahmann. 1964. Alteration of oxidative enzymes in potato tuber tissue by infection with Phythophthora infestans. Plant Physiol. 39: 483-490.

90. Turner, John G., and Anton Novacky. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64: 885-890.

91. Uritani, I., T. Asahi, T. Minamikawa, H. Hyodo, K. Oshima, and M. Kojima. 1967. The relation of metabolic changes in infected plants to changes in enzyme activity. In Mirocha, C., and I. Uritani, eds, The dynamic role of molecular constituents in plant-parasite interaction. American Pathological Society, St. Paul, Minnesota, pp. 342-355.

92. Uritani, I., and M. A. Stahmann. 1961. Changes in nitrogen metabolism in sweet potato with black rot. Plant Physiol. 36: 770-782.

93. Varns, J. L., and J. Kuc. 1971. Suppression of risitin and phytotuberin accumulation and hypersensitive response in potato by compatible races of Phythophthora infestans. Phytopathology 61: 178-181.

94. Walker, J. C., and M. A. Stahmann. 1955. Chemical nature of disease resistance in plants. Ann. Rev. of Plant Physiol. 6: 351-366.

95. Ward, A. M. 1902. On the relations between host and parasite in the Bromes and their brown rust, Puccinia dispersa (Erikas) Ann. Bot. London 16: 233-315.

96. Wood, R. K. S. 1967. Physiological plant pathology. Blackwell Scientific Publications, Oxford, England, 570 p.

CHAPTER II

HYPERSENSITIVE CELL COLLAPSE INDUCED IN BELL PEPPER, *CAPSICUM ANNUUM* BY *PSEUDOMONAS PHASEOLICOLA*

Abstract

In leaves of *Capsicum annuum* the hypersensitive reaction is induced within 12-18 hours after infiltration with 10^7 -10^8 cells/ml of (race 2), of a toxin producing bacterial pathogen of bean, *Pseudomonas phaseolicola*, G50 Tox^+, or a non toxin producing mutant, G50 Tox^-. Ultrastructure studies of plant tissue prior to cell collapse show that the plant cells, having the bacteria aggregated in an electron translucent gel matrix on the cell wall, are the first to show deviation from normal appearance. This is first exemplified by invagination of the plasmalemma with formation of a plasmalemmasome. Subsequent cellular changes suggest intensified synthesis in the plant cell. This is associated with increased density of ribosomes, endoplasmic reticulum and dictyosomes which appear at the infection site. Cellular disruption is first observed as disorganization of the chloroplast and later as the loss of structural integrity of other cell organelles. At infection sites of tissue infused with 10^7 cells/ml, a wall deposit forms between the cell wall and the invaginated plasmalemma.

In the intercellular spaces of pepper tissue, the two isolates are differentially affected by the hypersensitive reaction (HR). Eighteen hours after infiltration into leaf tissue, the free bacteria show wall

lesions which suggest loss of structural integrity. These lesions appear twice as frequently in G50 Tox^+ as in G50 Tox^-, when the inoculum density is 10^8 cells/ml. If the inoculum concentration is 10^7 cells/ml the inverse occurs. However, those bacteria, enveloped in the gel matrix in which an electron dense amorphous material is observed, appear to be unaffected by the plant. If the inoculum concentration used is 10^8 cells/ml, cell division for each isolate remains constant at both 12 and 18 hours, but is 3-fold greater in favor of G50 Tox^-. With the lower inoculum concentration the division rate is equal for both isolates.

Four developments associated with bacterially induced HR are reported for the first time: a localization reaction in leaves involving the bacteria, wall lesions in the free bacterial population, and, in the plant cell, the formation of plasmalemmasomes and wall deposits at the infection sites.

Introduction

Numerous hypotheses have been formulated to explain the rapid hypersensitive collapse of plant cells which occurs during interactions involving incompatible plant-pathogen systems. Those investigated most extensively were nutritional imbalance (11, 25), toxic products or hydrolytic enzymes secreted by the pathogen (13, 17, 23), production of phytoalexins or activation of the polyphenoloxidase and peroxidase enzyme systems in the plant (22, 27, 32, 35) or the appearance of new isoenzymes (33). However, Flor's (9) discovery that gene products for resistance in the plant must encounter gene products

for avirulence in the pathogen to induce hypersensitive reaction (HR) imply a concept of specificity in host-parasite interactions. Though this concept was extended by some investigators (1) to involve specific plant substrates required by the pathogen, others visualized the involvement of a specific immunological response on the part of both the plant and the pathogen (3, 7). Information is needed to reconstruct the specific nature and progression of the interaction of the pathogen with the plant cell in incompatible plant-pathogen systems.

Few ultrastructural studies have been conducted on bacterially-induced HR (2, 13). They have emphasized the degenerative effects of bacteria on the plant cells. Nothing has been reported concerning the effect of the incompatible plant environment on the bacteria themselves to relate these changes to potential effects on the ultrastructure of the plant cell. To determine which partner is affected first may give clues as to the nature of HR.

The object of this investigation was to establish the nature and time sequence of the early ultrastructural changes of the plant-bacterial interaction between Capsicum annuum, L. and the bean pathogen Pseudomonas phaseolicola (Burk.) Dowson, race 2, G50 Tox^{+} and a UV-derived mutant, G50 Tox^{-}, derived from the wild type. The mutant shows altered pathogenicity to its natural host and fails to produce its characteristic toxin in culture (30). It was included in this study as a potential genetic marker to determine if specific cellular changes induced in pepper during the HR may be related to its altered pathogenicity. For such observations, the mutant may be valuable since

its growth potential and nutritional requirements appear to be unaffected (30). Quantiative determinations of the effect of the plant environment on the bacteria were made by removing the bacteria from the leaf intercellular spaces at various time intervals and observing them with electron microscopy using negative staining. In complementary studies, the effects of bacteria on the plant cells at various time intervals after infection were followed in ultra-thin sections of the plant tissue. Four models are proposed to explain the nature of the interaction between the plant tissue and the bacterial cells.

Materials and Methods

Pseudomonas phaseolicola G50 Tox^+ and the mutant G50 Tox^- were cultured on YDC slants. Liquid shake cultures containing Watanabe's medium (39) were inoculated with the two isolates and grown at 19° C at 150 rpm (Gyratory shaker) for 12-14 hours. The suspension was centrifuged at 3000 g, washed twice, and resuspended in 0.85% saline solution or deionized water. The final suspension was adjusted to 10^7 and 10^8 cells/ml. Inoculum suspension of the non-pathogens listed in Table 1 were similarly prepared from new slants of Nutrient agar. Leaf infiltration was performed using a hypodermic syringe with a fine needle. Approximately 2 centimeter areas of leaf tissue were inoculated in a Latin Square plot design to randomize the effects of leaf age and inoculum concentration. The host plants Capsicum annuum (Yolo wonder) were grown in a controlled, growth chamber set to a 12 hour day/night cycle at a relative humidity of 70-75% with a 28° C day/24° C night

temperature. Most of the investigations were performed with 3-5 month old plants, but plants 6-7 months old were used for complementary studies.

Samples prepared for evaluating the effect of the plant on the bacteria (approximately 75 and 35 1 cm discs for 10^7 and 10^8 cells/ml respectively) were harvested at 3, 6, 9, 12 and 18 hrs and put in cold 0.1M phosphate buffer at pH 7.2. The tissue discs were washed and infiltrated in vacuo with sterile 0.85% saline. After blotting the excess fluid with filter paper, the discs were centrifuged (4° C) at 1100 g to remove the intercellular fluid, and which was recentrifuged to sediment the bacteria. These bacteria, negatively stained according to Fuerst and Hayward (10), were observed by electron microscopy and observed for dividing or non dividing cells on the basis of the presence or absence of wall constrictions, and as cells showing irregular structure or deformations. Bacterial ultrastructure was studied with thin sections of cells from the intercellular fluid, prepared by the method of Kellengberger, Ryter and Sechaud (20).

For ultrastructure studies of infected tissue, leaf tissue samples, 1 mm square, were excised under cold phosphate buffer and transferred immediately to Karnovsky's glutaraldehyde fixative (18) buffered with 0.5M phosphate buffer at pH 7.2 for 2 hours. The sections were then washed in 4 changes of the buffer during 2 hours and were post fixed in 2% osmium for 2 hours. Dehydration was performed with a graded series of acetone. After three changes of propylene oxide, the tissue was embedded in Epon 812 and sectioned with a diamond knife. The thin sections were stained on coated grids with 2% aqueous uranyl

acetate for 15 minutes at 25° C, followed by lead citrate for 3 minutes (31). All electron microscopy was performed on an Hitache HS-85 electron microscope. Electron micrographs of the two isolates interacting with pepper at different bacterial concentrations, times, and tissue maturity were observed. Twenty-five different subjective criteria were used to determine if qualitative or quantitative variability between the two isolates could be detected. The criteria not only included observations of ultrastructural alterations related to synthesis and/or degeneration, but also the frequency that the changes occurred.

The significance of host effects on the bacteria was determined by testing the null hypothesis of no difference with the "t" test using the standard error of difference between percentages.

Results

Effect of selected bacterial isolates on pepper tissue. On infiltration of 10^8 cells/ml of Pseudomonas phaseolicola G50 Tox^+ and G50 Tox^- into fully expanded leaves of young pepper plants (prior to blooming) the first macroscopic indication of the interaction is a silvery-like sheen observed on the underside of the leaf 8-9 hours after inoculation. Within 12-18 hours, extensive areas of cellular collapse occur where the leaf tissue becomes tissue-thin and water congested. Afterwards the total area of collapsed tissue increases until it becomes confluent. Within 24 hours tissue browning occurs and the infused area dries out. Surrounding the infused zone, the leaf tissue becomes chlorotic and leaf abscission occurs soon

afterwards. When 10^6 cells/ml are introduced, no macroscopic cellular collapse is evident even after 48 hours. The expression of the hypersensitive reaction (HR) is reduced or completely blocked if prior to infiltration of bacterial cells the leaf shows evidence of chlorosis, senescence or pathogenic attack. It is delayed 6-8 hours in older plants (after fruit set).

To determine if hypersensitive cell collapse caused by pathogenic bacteria is a unique property of these organisms, selected bacterial strains were tested in pepper at a concentration of 10^8 cells/ml. Among those tested were gram negative bacteria, both capsulated and non capsulated forms, gram positive species, as well as a saprophyte (Table 1). Control leaves were infused with sterile deionized water. Even with repeated experiments using 10^9 cells/ml as inoculum density, the nonpathogens failed to induce tissue necrosis or HR in pepper leaves. On the other hand, the bacterial, plant pathogens induce HR in tobacco, and the non toxigenic mutant induces the same response in chinese yam, an alternate host of P. phaseolicola (Hayward, unpublished).

Effects of pepper-leaf tissue on plant-pathogenic bacteria and on a saprophyte. The bacterial cells were removed from inoculated pepper discs 12 and 18 hours after infusing the tissue with 10^7 or 10^8 cells/ml with either P. phaseolicola G50 Tox^+ or G50 Tox^- or P. florescens, a saprophyte. The cells were observed with electron microscopy using negative staining and counted for cells showing morphological alterations, normal appearing cells, or dividing cells. Lysed cells were not counted.

Since the saprophyte causes no apparent tissue lesions, this bacterium was used to determine if saprophytes survive the interaction with the plant. After 3 hours, the cells of P. florescens show dividing cells; however, it is difficult to determine if these represented cells carried over in division stages from the culture medium. After 6 hours of incubation all the cells recovered were completely lysed.

In contrast to this even after 18 hours, flagellated P. phaseolicola were recovered by low speed centrifugation from the intercellular spaces (Table 2). All of the bacteria had normal appearance until 9 hours. After 12 hours, malformations appeared as blebs in the wall of the bacterial cells and apparently resulted from a structural weakening of wall components (Fig. 1). When the incubation was increased to 18 hours, the frequency of cells with wall blebs increased and the number of these deformations per cell also increased. When either isolate was infused at a density of 10^7 cells/ml, the division rates for both isolates were similar at 18 hours (Table 2). Similar division rates were also observed for in vitro and in vivo culture (30). The rate of bleb formation at the same inoculum density in the mutant was 2-fold greater than the wild type, G50 Tox^+ (Table 2). However, when the inoculum concentration was increased to 10^8 cells/ml, the interaction at 18 hours differed. In this case the division rate was 5-fold in favor of the mutant, while the frequency of bleb formation was one-half that of the toxigenic strain (Table 1). Even at 12 hours the multiplication rate of the mutant exceeded that of the toxigenic strain, at which time the frequency of wall blebs in the two isolates was the same. Cell division and bleb formation were not mutually exclusive events, since dividing cells often possessed wall blebs.

Ultrastructure of the interactions of Pseudomonas phaseolicola with Capsicum annuum. Thin sections of young pepper leaves sampled at 12, 18, and 24 hours show that the bacterial cells are progressively aggregated within an electron translucent matrix having an electron opaque boundary on the free surface of the plant cell wall (Figs. 4-5). This matrix is referred to as the localization gel. Plant cells harboring the localized bacteria are the first ones to show evidence of disorganization in response to the interaction (Figs. 4-5). Until 9 hours no cellular changes are apparent either in the bacteria or plant cells and the bacteria remain free and flagellated within the intercellular spaces. Even though flagellated bacteria were removed from the leaf intercellular spaces at 18 hours, the localized bacteria lack flagella.

The inoculum concentration apparently influences the nature of the balance established between the localized bacteria and the plant cells. At 18 hours, if a 10^7 inoculum concentration is used, the progressive changes related to both the bacteria and the plant cells at the site of the localization reaction are relatively homogenous in the infused tissue. In contrast, when the inoculum density was 10^8 cells/ml, a wide variability was noted in the progression of the interactions between the plant cell and bacteria. For both G50 Tox^+ and G50 Tox^- the changes occurring at either inoculum concentration appear to be very similar.

Negatively stained cells removed from the intercellular spaces of pepper at 12 and 18 hours are shown in Figures 1, 2, 3. The two types of cells observed at an 18 hour sampling appear to be normal

cells and cells possessing wall blebs, e.g., cells having bulges in the cell walls into which the cellular contents often protruded (Fig. 1). These blebs developed randomly and were observed both in dividing and non dividing cells (Figs. 2, 3).

In the initial phases of the localization reaction the bacteria, minus flagella, appear to be suspended within a fluid-like, electron-translucent substance having greater staining density on the boundary facing the intercellular spaces (not shown). At this stage, the electron opaque boundary was tightly appressed to the aggregated, bacterial cells and to the surface of the cell wall. A primary response of the plant cells to this phase of the localization was the development of a plasmalemmasome between the cell wall and the invagination of the plasmalemma (see later electron micrographs). The structural integrity of the plant cell wall appears to be intact, but staining density is often variable (Figs. 4-5). Though the primary components of the plant cell wall are normally electron translucent in electron microscopy, in these preparations the walls sometimes were dense staining. A glancing section through the plant cell in Figure 4 indicates that the wall distal and proximal to the localized bacteria has similar staining density.

The configuration of the electron-opaque layer surrounding the bacteria in Figure 5 suggests that it bounds a substance with a gel-like consistancy, that is apparent both by the separated orientation of the bacterial cells and by the tension of this material on the curved surface of the plant cell. Though the composition of the gel is unknown its electron translucent property in these preparations

may suggest that it is a polysaccharide. In further reference to this material it will be called the localization gel. The phase of development of the localization gel shown in Figure 5 is the one most frequently observed in the 12 hour samples. The bacterial cells, though lacking flagella, at this time have normal appearance with centrally dispersed fibrillar DNA, peripheral distribution of ribosomes, often dispersed with polyribosomes, and have an undulating trilaminar wall (Figs. 4-5).

Bacterial cells removed from leaf tissue after 12 hours of incubation show only a few widely dispersed wall blebs (Fig. 6). Formation of these blebs may be responsible for the loss of structural integrity of lysing bacteria observed in the thin sections of the electron translucent localization gel (see later micrographs). However, these structures were poorly defined after preparative procedures for thin sectioning were performed.

Preceding lysis, characteristic changes occur (Figure 7). In the bacterium on the left, masses of electron-dense amorphous material occur among the polyribosomes located within dense peripheral ribosomes. The cell on the right shows a greater distribution of this electron-dense amorphous material. In the latter cell, an unbounded electron-translucent zone occurs which resembles the lysogenous space which forms in response to chemical stress (16). Prior to lysis these spaces accumulate in large numbers in P. phaseolicola. However, the bacteria in the localization gel which are surrounded by dense deposits of the electron-dense amporphous material show normal morphology.

A section taken at the 12 hour sampling demonstrates more distinctly the gel-like properties of the localization gel (Fig. 8). In this case where the electron opaque outer portion is ruptured in preparation for electron microscopy, opaque fibrillar material and electron dense amorphous material occur. The adjacent plant cell wall where the bacterial cells are aggregated, appears to be intact and lacks evidence of solubilization. In the plant cell, within the space between the plant cell wall and the invaginated plasmalemma, the plasmalemmasome is present and in the protoplast intense synthetic activity is apparent by the dense ribosomes and the development of the endoplasmic reticulum. The chloroplast, shown in Figure 8, which is one of the first plant organelles to be affected due to the HR, shows no abnormalities as compared to the control (Fig. 9). The first indications (not shown) of disorganization occur in the chloroplast lamellae; the starch grains show abnormal osmiophilia, and the electron opaque lipid globules become less frequent.

The rapid cellular changes which occur in young pepper tissue undergoing the HR make it difficult to follow the intermediate phases between initiation of the response and subsequent cellular collapse. However, in preliminary investigations it was observed that in older tissue, at fruit set, the HR was delayed 6-8 hours. Thin sections of this tissue showed that not only a delay in cellular changes occurs, but also that there is a corresponding delay in the localization process. This was evident from the large accumulation of bacterial cells before the localization reaction occurs (Fig. 10).

In this case some of the bacterial cells appear to be lysing and possess a large number of lysogenous spaces. The damage to the adjacent plant cell chloroplast was variable, showing lamellar disorganization as well as formation of crystals (not shown). However, in this tissue the development of the plasmalemmasome could be followed (Fig. 10). It appears first (Fig. 10) as a deep invagination of the plasmalemma into the cytoplasm with the formation of tubular structures containing some cytoplasm and endoplasmic reticulum. An electron translucent amorphous material surrounds these tubules, and the spacial relationship suggests this material is deposited by the tubules. Electron opaque lipid deposits (5) are prominent in the older tissue and are found surrounded by rough and smooth endoplasmic reticulum (Fig. 11). These structures were not observed in young tissue. Prior to ultrastructural disorganization in the chloroplast, cytoplasmic organization becomes very complex at the infection site (Fig. 11). Mitochrondria with dilated cristae, dictysomes, lipid bodies surrounded with rough endoplasmic reticulum, and deep tubules into the cytoplasm are sites of synthetic activity at this time (Fig. 11). Within the localization gel, some electron dense amorphous material accumulates, along with fragments that appear to be a lysed bacterium (Fig. 11). As progressive cellular deterioration and vesiculation of the cytoplasm occurs, the electron translucent amorphous material surrounding the tubules accumulate near the plant cell wall (Fig. 12). From its staining properties, it appears to be the same material that is involved in the formation of wall deposits of young pepper tissue (see later). A high magnification of a section of the

older tissue also shows deposits of electron opaque fibrillar material in the space between the wall and the invaginated plasmalemma (Fig. 13). The dictyosomes which were often observed at the infection site after the localization gel is formed may be responsible for the synthesis of this material (Fig. 11). In this section the electron dense amorphous material located on the bacterial cell surface appears to peel off into the surrounding localization gel and is visible only within the thin, dense-staining boundary layer of the localization gel.

The localization gel of the 12 hour samplings contains only small deposits of the electron dense amorphous material (not shown). The quantity of this material did not increase in the 18 hour samples if an inoculum density of 10^7 cells/ml was used (Fig. 14). In contrast, if concentration of 10^8 cells/ml was infused, most of the reaction sites showed that this substance filled the entire gel matrix, though a few sites having the 12-hour configuration remained. It is uncertain if the localization gel itself is responsible for the bacterial lysis observed, or if it results from a plant secreted substance(s). However, bacterial cells which have normal appearance are always found when the entire gel matrix is filled with the electron dense amorphous material.

In contrast to the interaction of younger and older tissue infused with 10^8 cells/ml inoculum density, young tissue infused with 10^7 cells/ml demonstrates a response to the localized bacteria which involves a massive deposition of the newly synthesized translucent amorphous material (Figs. 14-17). This material, previously

described, accumulates between the plant cell wall and the invaginated plasmalemma (Fig. 14). The localization gel in this tissue contains only 2-3 bacteria, but a single bacterium was commonly found (Fig. 16). All of the bacteria at 10^7 cells/ml inoculum concentration appear to be moribund since they are flattened or angular and have coagulated DNA within the expanded mucleoid surrounded with condensed peripheral ribosomes. Synthesis of the electron-dense, amorphous material appears to be aborted since only a small amount of this material is present. The wall deposits in the plant cell (Fig. 15) were composed of small quantity of fibrillar material and large quantities of the electron translucent amorphous material in which was entrapped membranous material (Fig. 15). The primary component of these deposits appears to be callose, β 1,3 glucan, since the infection sites fluoresce bright yellow with fluorescence microscopy after staining with Aniline blue (10) (see Chapter III). After the plasmalemma pulls away from the cell wall the structural integrity of these wall-deposits remains intact (Fig. 16).

The development of the interaction phases in the tissue infused with 10^7 cells/ml is more homogenous than in tissue using the higher inoculum density. The affected plant cells at this concentration are either collapsed or very necrotic at the 18 hour sampling (Fig. 17).

In the 18 hour samples of tissue infused with 10^8 cells/ml a differential in the development of the localization gel was observed and the affected cell showed a corresponding degree of damage. The development phase most commonly observed shows that the gel matrix is completely filled with electron dense amorphous material that

appears to be of bacterial origin (Fig. 18). In close proximity to the phase of interaction shown in Figure 18 were other phases where the localization gel had little or none of this material (Fig. 4), and the plant cell showed little or no disruption. The plant cell adjoining the localization gel such as illustrated in Figure 18 often, but not always, was distended into the space of the invaginated plasmalemma. Loose fibrillar material which appears in the space of the invaginated plasmalemma (Fig. 19) and where dictysomes were observed (Fig. 13) may contribute structural elements to the distended wall. If the wall distention results from solubilization of the wall, it should be noted that disruption appears to be greater in the plant cell wall distal than proximal to the localization gel (Fig. 19). The bacteria within the mature localization gel remain remarkably intact and appear to be surrounded by a non staining zone. It is uncertain whether its appearance is due to unutilized nutrients or due to shrinkage during preparation for electron microscopy.

After evaluating the interaction phases of the two isolates of P. phaseolicola at different sampling times using 25 different criteria for synthesis and/or degradation, no qualitative differences between the two isolates were found. The plasmalemma is initially affected by the presence of the bacteria followed subsequently by chloroplast disorganization. Changes in other cellular organelles were discerned only in the later phases, just prior to cell collapse. In most cases the mitochrondria appear to be resistant to the bacterial infection, but degeneration was first evident with loss of stroma material. Only in a few very necrotic cells did dense staining masses

appear in the vacuole. They resemble phenols reported in other plant-pathogen interactions. Though 12 hour samples show that the plant cell walls have variable staining-density, they appear to be virtually intact at the time the localization reaction is first observed. Dense staining materials are present in the wall at the interaction sites at 18 hours and in addition some encrusting material is also evident; this could be the electron dense amorphous material produced by the free bacterial cells. Solubilization and/or synthesis of plant cell walls often, but not always, occurs in cells adjacent to the localization reaction when 10^8 cells/ml are introduced, and disruption and erosion of outer wall layers is observed in the 24 hour samples.

Discussion

Hildebrand and Riddle (16) experimentally established that most bacterial plant pathogens of the Pseudomonaceae, many of the Xanthomonaceae, and Agrobacteriaceae were capable of inducing the HR in tobacco. The different genera produced characteristic lesions, although specific environmental conditions for optimal expression in every case were not the same.

In the interaction between pepper and P. phaseolicola, conditions which maintain the plant in an optimal vegetative condition appear to facilitate optimum expression of HR. When the reproductive cycle is initiated, the HR expression is both delayed and the localization reaction inhibited as compared to the young vegetative plant. Under optimal conditions that favor expression of the HR with P. phaseolicola, P. florescens and other members of the Enterobacteriaceae fail to

induce necrosis in pepper tissue even when the inoculum density is 10^9 cells/ml. However, high molecular weight substances (endotoxins) from E. coli, P. florescens as well as P. phaseolicola induce cellular collapse in pepper (see Chapter III), but the specific activity of the endotoxin from P. phaseolicola was more than 2000-fold greater than those derived from nonpathogens. This may explain why no necrosis occurs in pepper tissue even though complete lysis of P. florescens occurs within 6 hours.

The basis for plant recognition of the incompatible pathogen and the relationship of this recognition to subsequent hypersensitive cell collapse has not been established. Information that is available does not indicate that HR results from a single universal reaction. Even in pepper, HR may result by different mechanisms. This is indicated by the difference in the pattern of electrolyte loss which results when pepper is infected by either of two incompatible strains of Xanthomonas vesicatoria and is exposed to the same environmental and chemical parameters (6). Also Stall, Bartz and Cook (37) have shown that when isolates of compatible and incompatible Xanthomonas vesicatoria are mixed and infused into pepper, HR is the determining reaction even when the population is 9:1 in favor of the compatible pathogen. If a primary inoculum of the compatible isolate is followed by a challenge inoculum of the incompatible isolate, HR is attenuated. The same is true if the challenge inoculum is another species of the xanthomonads. However, if the challenge inoculum was a pseudomonad, full expression of the HR occurs and the process of recognition was unaltered. These findings may suggest that recognition

of different incompatible pathogens is also related to different mechanisms. In soybean infected with incompatible P. glycinea, phytoalexin production rather than membrane disruption occurs during HR (19).

Alterations in the normal ultrastructural appearance of plant and bacterial cells occur in the pepper-P. phaseolicola system shortly before the first macroscopic evidence of cell collapse is visible. Twelve hours after the introduction of the bacteria disorganization of plant cells and wall blebs in bacterial cells occur. The significance to HR of the observed alterations and of the sequence of cellular changes which follow can be determined by comparing the changes with those of other host-pathogen interactions. Though other ultrastructural studies of pepper with bacterial pathogens have not been done, a study has been done on the halo blight lesions of bean caused by P. phaseolicola. The bacteria found in the water soaked zones of these lesions, from whence antibacterial substances have been isolated (29), develop wall malformations similar to the wall blebs found during the HR in pepper (24). Materials similar to the electron dense amorphous material described in pepper were also reported to occur on the cell walls of bean. However, no localization reaction or wall deposits were reported in the host of this pathogen. Though extensive leakage of metabolites, accumulation of organic compounds and partial destruction of cell walls and cell organelles occur, bean cells do not collapse until after several days (34). There are three lesions induced in pepper by P. phaseolicola that do not occur in bean. These are cells prone to collapse, a localization reaction, and wall

deposits. Pepper tissue, in response to this organism, shows both HR and synthesis of massive wall deposits, while in immune cowpea infected with rust (15) these events are mutually exclusive and are interpreted to reflect mechanisms which block the advancing fungus. Since bacterial pathogens remain in the intercellular spaces, the occurrence of wall deposits may instead reflect effects of injury to the plant cell (5).

A hypothesis relating the changes in ultrastructure of the bacterial cells to subsequent, hypersensitive cell-collapse in pepper is difficult to propose since variability of interactions occurs with the two inoculum densities. Three observations favor using the results obtained with the lower density as a basis for formulating a hypothesis. First the growth rate in pepper for the two bacterial isolates is similar, which corresponds with results obtained in bean leaves and in culture (30); second, at the lower concentration the tissue response to the infused bacteria is more homogeneous; and third, Turner and Novacky (38, found a linear relationship between the number of collapsed cells and the introduced bacteria in tobacco when the population was 10^3 - 10^5 cells/ml. A departure from linearity occurred with inoculum concentrations above 10^7 cells/ml. Often phytopathologists have observed that plant resistance can be overcome by high inoculum densities (33). In the present investigation, increasing the inoculum density from 10^7 to 10^8 cells/ml resulted in an increase in wall lesions in the free population of bacterial cells. However, the localized population show a decrease in disruptive effects of the plant with increase of the inoculum concentration. Specific

ultrastructural changes observed in plant cells appear to correspond to the development of a localization reaction. This suggests that the two events are related, and may be most important for relating changes in the bacterial population to hypersensitive cell collapse.

There are at least 4 models which may explain how the ultrastructural changes in the bacterial cells are related to the hypersensitive response in plant tissue. First, inhibition of growth of the bacterial cells or damage to them may cause production or release of toxic substances responsible for plant cell collapse. This model was also suggested by Kiraly, Barna and Ersek (21) to explain experimental findings using 3 nonrelated, fungal, host-parasite systems. These investigators found that even compatible pathogens, when killed or damaged by appropriate antibiotic treatment of infected tissue, produced a hypersensitive necrosis rather than typical, symptomatic lesions. From these observations cell collapse was considered to result when the damaged pathogen released an endotoxin. By this interpretation, the HR was an unknown resistance mechanism responsible for the damage to the invading fungus.

Damage and/or inhibition of growth of bacterial cells in pepper does not appear until after 12 hours. Though bacterial cells which appeared to be moribund are localized on plant cells undergoing HR, plant cell degeneration is also consistently observed in cells adjacent to localized bacteria showing normal morphology. The latter observations were made when an inoculum density of 10^8 cells/ml were used and where the gel matrix is filled with the electron dense amorphous material. At this concentration, dividing cells were

observed in the electron-translucent gel matrix, but it may be possible that inhibition of further growth would be a sufficient stimulus to cause release of toxic substance(s).

I present two observations against this model: First, an endotoxin capable of causing cell collapse in pepper has been isolated from log phase cells of P. phaseolicola (see Chapter III). This toxin requires the same time to induce the cell collapse as do the bacterial cells, and release of a hypothetical toxin due to stress on the bacteria would not account for a lag of 12 hours. Second, incompatible X. phaseoli in pepper causes cell collapse whether or not the bacterial cells are killed (36).

The three remaining models consider the same requirement necessary for the isolated endotoxin from P. phaseolicola (see Chapter III) to cause cellular collapse after release from the bacterial cells. The second and third models would propose that soon after the bacteria encounter the plant cell the endotoxin is released from the bacteria possibly by the enzyme action of the plant. The released endotoxin may interfere with membrane synthesis or membrane repair mechanisms or may even stimulate the production of plant substances causing membrane degeneration. This mechanism would presume that the ultrastructural changes in plant cells associated with the localized bacteria constitute changes induced in cells already destined for collapse. The homogeneity of response shown when 10^7 cells/ml inoculum concentration is used supports this view. In this case homogeneity of the response would suggest the stimulus was simultaneous. This aspect of models two and three would agree with (a) observations with other bacteria in pepper, (b) the

initiation of cellular collapse being dependent on the initial, introduced population and not the final moribund population, (c) cellular collapse occurring in plant cells having either moribund or normal appearing bacteria in the gel matrix, and (d) in many plants the damaged pathogen is unable to cause hypersensitive cell collapse. Models two and three would differ only in the explanation of the basis of the disparity observed between the interaction phases in tissues infused with 10^8 or 10^7 cells/ml. The second model proposes that plant tissues, on initial infusion of a high inoculum density of bacteria, may be affected physiologically so that the cells are unable to recognize the aggregated bacteria. The differences in interaction phases at the high inoculum concentration would thus result because of the differential ability of the cells themselves to recover the facility of recognition. According to the third model, the aggregated bacteria themselves would directly affect the plant-cell production of the localization gel. When 10^7 cells/ml inoculum concentrations are used, the plant tissue is capable of consistently responding with a localization reaction even to a single bacterium. Though similar effects would finally result if either model is operative, they would differ as to the nature of the population of the bacteria inducing the change. In the former case, the infused population would determine the response whereas in the latter one the population already affected by the plant environment would determine the secondary alterations in plant cell ultrastructure.

A fourth model is proposed whereby the HR results directly from the release of endotoxin from bacterial cells during the onset of the

localization reaction. At this phase bacterial cells were observed in division stages in the fluid-like gel matrix, when 10^8 cells/ml are introduced. Since threshold levels of the HR-inducing principle would be present in a single bacterium, additional triggering potential would be redundant. The fourth model is favored by involving fewer steps. However, there is a 12 hour lag between the time required for the isolated endotoxin to induce the HR and the time of the localization reaction. The fourth model may not provide sufficient time for the cell collapse to be precipitated. This may not be important if the endotoxin encounters problems in reaching the active site.

TABLE 1. Effect of selected bacterial strains on Capsicum annuum

Klebsiella pneumoniae	-[a]
Enterobacter aerogenes	-
Escherichia coli	-
Pseudomonas aeruginosa	-
Pseudomonas florescens	-
Pseudomonas phaseolicola G50 (Tox^+)	+[b]
Pseudomonas phaseolicola G50 (Tox^-)	+
Staphylococcus aureus	-
Bacillus subtilis	-

[a] no macroscopic lesions

[b] macroscopic tissue collapse

TABLE 2. Effects of inoculum concentration and incubation time in pepper on populations of Pseudomonas phaseolicola G50 Tox^+ and G50 Tox^- at 12 and 18 hours

Isolate	Inoculum density	Percentage dividing cells	Percentage non dividing cells	Percentage cells with wall blebs	Total number of bacteria counted
G50 Tox^+ (12 hr)	10^8	8	84	8	100
G50 Tox^- (12 hr)	10^8	18	72	10	109
G50 Tox^+ (18 hr)	10^7	15	68	12	194
G50 Tox^+ (18 hr)	10^8	3	52	45	175
G50 Tox^- (18 hr)	10^7	15	60	25	140
G50 Tox^- (18 hr)	10^8	17	60	23	197

List of Abbreviations Used in Figures

B--bacteria

Ch-chloroplast

c--callose

D--dictyosome

ER-endoplasmic reticulum

IS-intercellular space

L--lipid body

LG-localization gel

PL-plasmalemma

PS-plasmalemmasome

M--mitochondria

V--vacuole

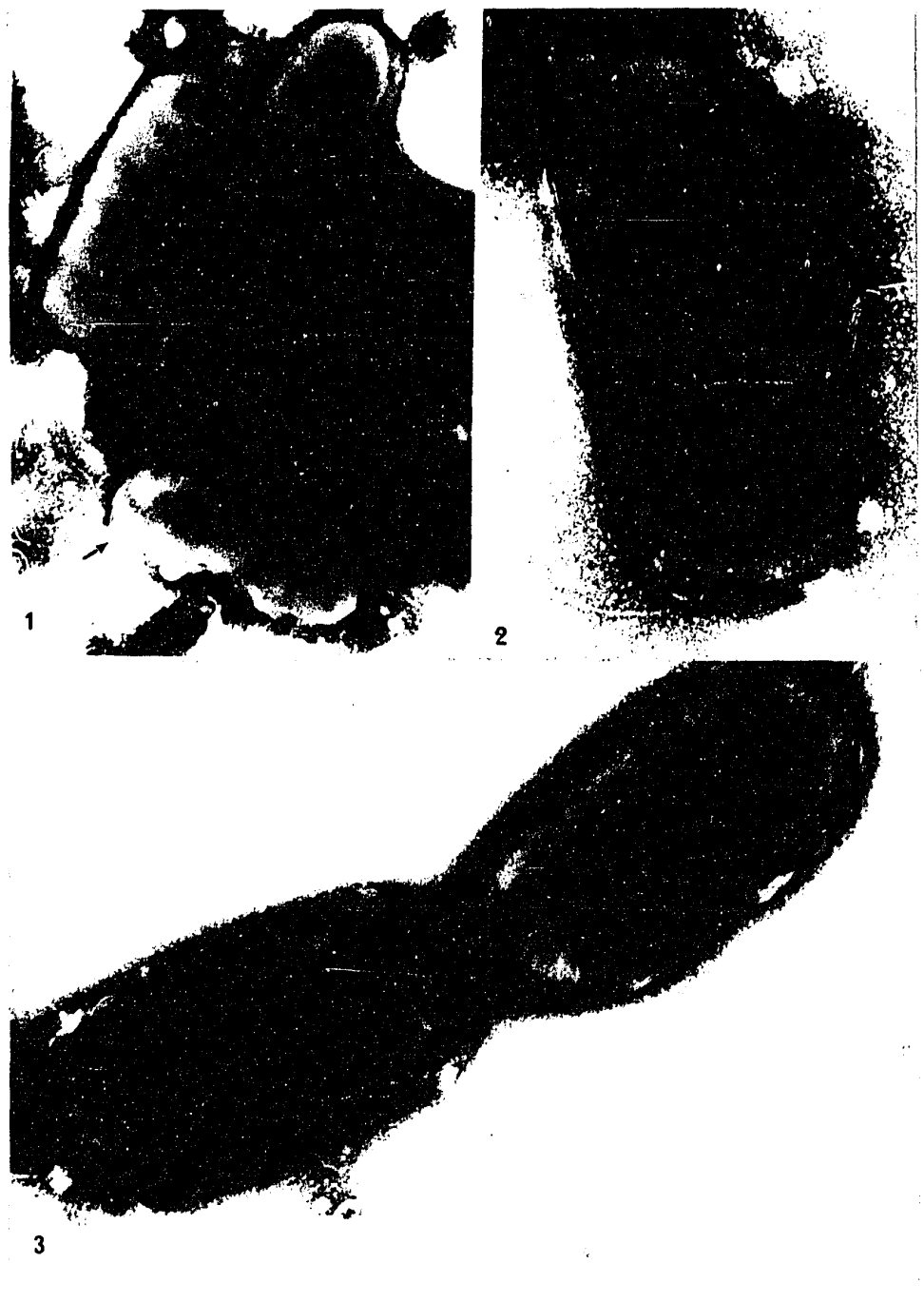
WD-wall deposit

W--plant cell wall

Fig. 1. P. phaseolicola G50 Tox$^+$, 10^8 cells/ml, from the intercellular spaces of C. annuum at 18 h. Note bleb formation in the lower cell (arrow) (x16,000).

Fig. 2. P. phaseolicola, G50 Tox$^+$, 10^8 cells/ml, from the intercellular spaces of C. annuum at 12 h. showing wall separation at the site of the bleb (x45,000).

Fig. 3. P. phaseolicola, G50 Tox$^+$, 10^8 cells/ml, from the intercellular spaces of C. annuum, after 18 h. showing the distribution of wall blebs (arrows) (x39,600).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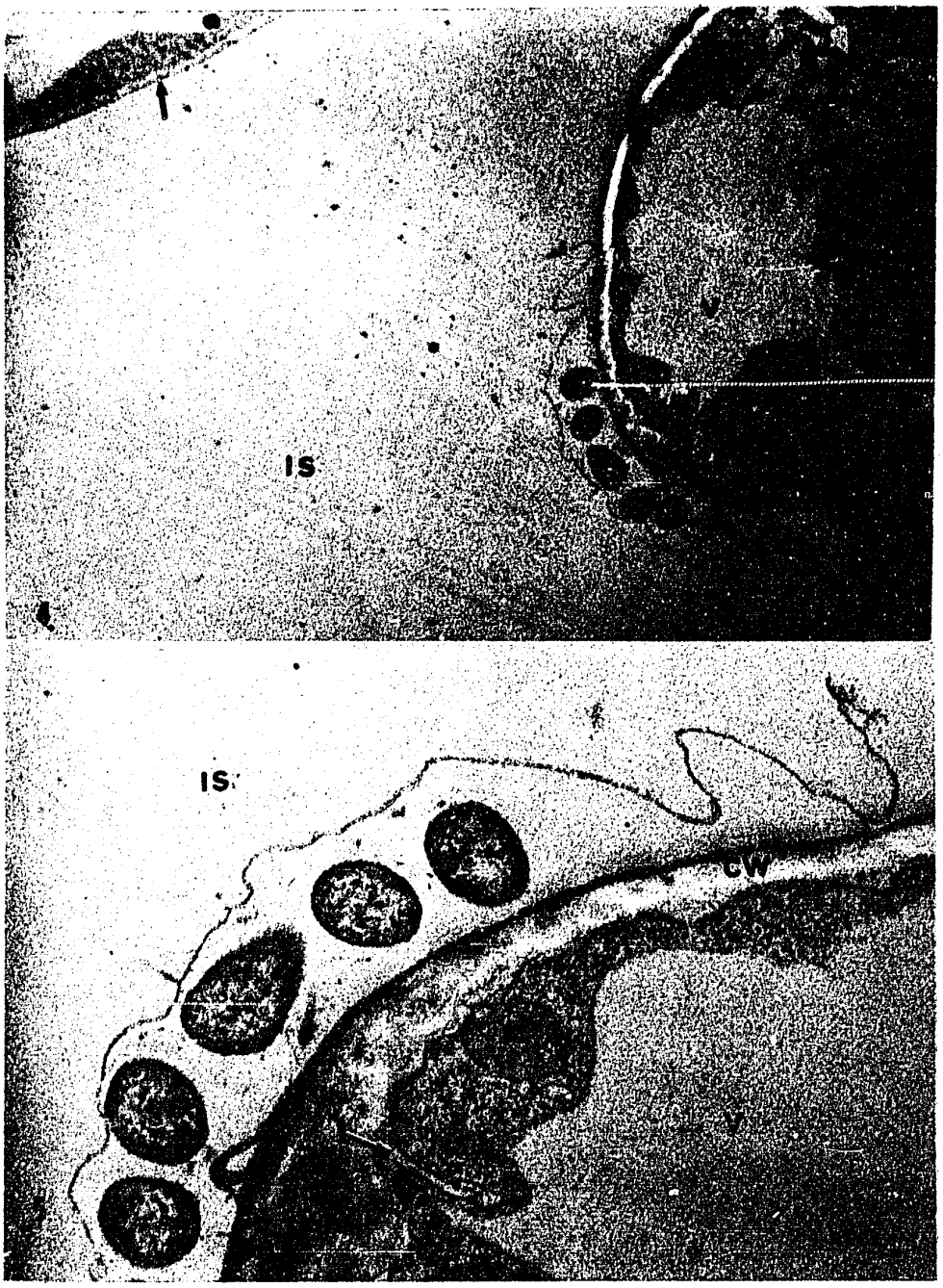
3

Fig. 4. C. annuum, young tissue, 18 h. after inoculation with P. phaseolicola, 10^8 cells/ml, G50 Tox^+, showing an early configuration of the localization gel. Note the density of plant wall stain is similar both proximal and distal to the localized bacteria. (x6,400).

Fig. 5. The same section as above shows the plasmalemma pulling away from the cell wall at the infection site. (x16,740).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CW

Fig. 6. P. phaseolicola, G50 Tox$^+$, 10^8 cells/ml, at 12 h. show fewer blebs (arrow) per cell than at 18 h. (x7,600).

Fig. 7. A thin section of P. phaseolicola, G50 Tox$^+$, in the intercellular space of C. annuum at 18 h. Note the accumulation of electron opaque material (arrow at lower left). In cells undergoing the localization reaction both this material and an electron translucent material (arrow at upper right) accumulate in bacterial cells. (x37,000).

Fig. 8. C. annuum, 12 h. after inoculation with P. phaseolicola, G50 Tox$^+$, 10^8 cells/ml. Note the dividing bacterium (arrow) inside the electron opaque boundary of the localization gel (LG) which is broken. A second arrow shows fibrillar material where the bacteria lay in the gel matrix. Adjacent in the plant cell, plasmalemmasomes (PS) develop early in the process of localization of the bacteria. (x11,800).


6
7
LG
PS
8

Fig. 9. C. annuum, 18 hours after infusing of deionized water into the intercellular spaces. (x11,000).

Fig. 10. C. annuum, older tissue, 24 h. after inoculation with P. phaseolicola, G50 Tox^+, 10^8 cells/ml. Extensive invagination and evagination of the plasmalemma form plasmalemmasomes (PS) which contain tubules (arrow) surrounded by electron translucent callose deposits. Note the osmiophilia of the cell wall. (x8,600).

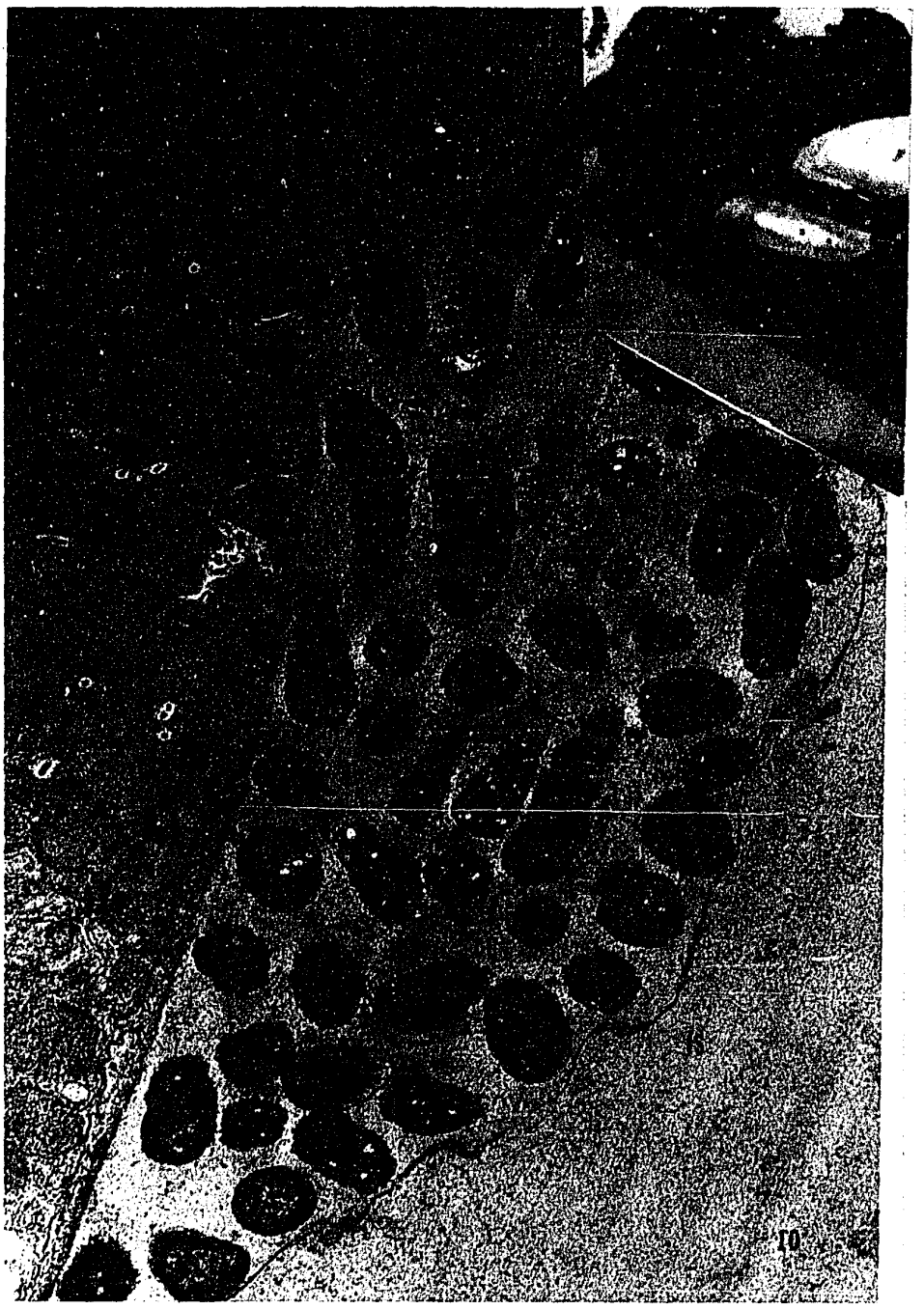

Fig. 11. C. annuum, older tissue, 24 h. after inoculation with P. phaseolicola, G50 Tox$^-$, 10^8 cells/ml. At the lower right is the edge of the grid and an adjacent artifact. Electron dense amorphous material is present both inside and surrounding the bacteria localized inside the localization gel. Near the evagination of the plasmalemma (plasmalemmasomes) in the plant cell dictysomes (D), undilated endoplasmic reticulum (ER) and lipid bodies (L) appear. At this stage no disruption is observed in either mitochondrial or chloroplast membranes. (x11,170).

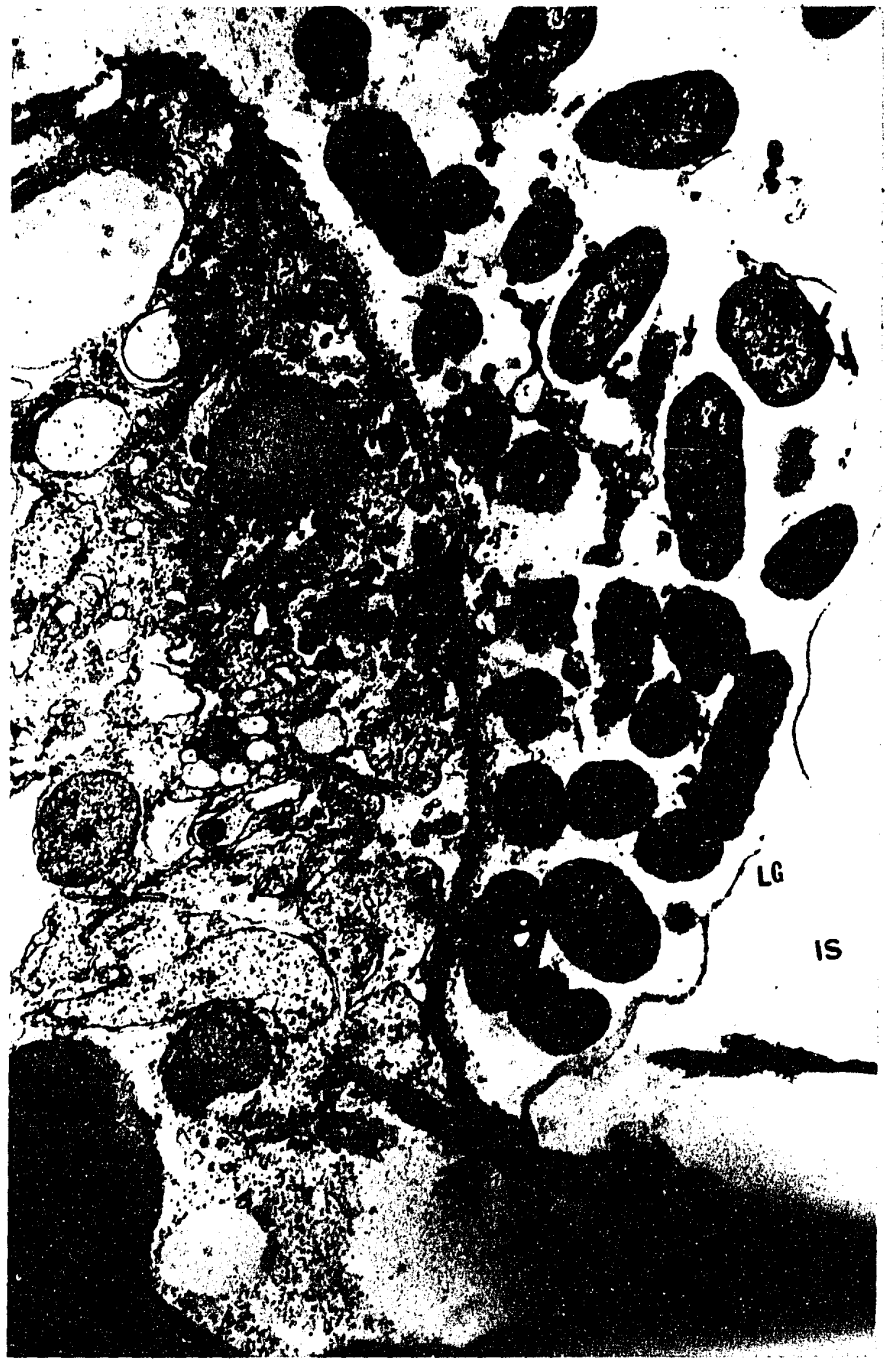
LG
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Fig. 12. C. annuum, older tissue, 24 h. after inoculation with P. phaseolicola, G50 Tox^+, 10^8 cells/ml. Note callose deposits in evaginations of the plasmalemma.

Fig. 13. C. annuum, older tissue, 24 h. after inoculation with P. phaseolicola, G50 Tox^-, 10^8 cells/ml showing the plasmalemmasomes (PS). The tubular structures at the lower left are composed of the evaginated plasmalemma, with cytoplasm, and some endoplasmic reticulum. At the upper right (arrow) note the electron density of the tripartitate tubular membranes. Dense fibrillar deposits are observed at the upper right (arrow). Electron dense amorphous material occur both inside and on the surface of the bacterium (arrow). (x49,500).

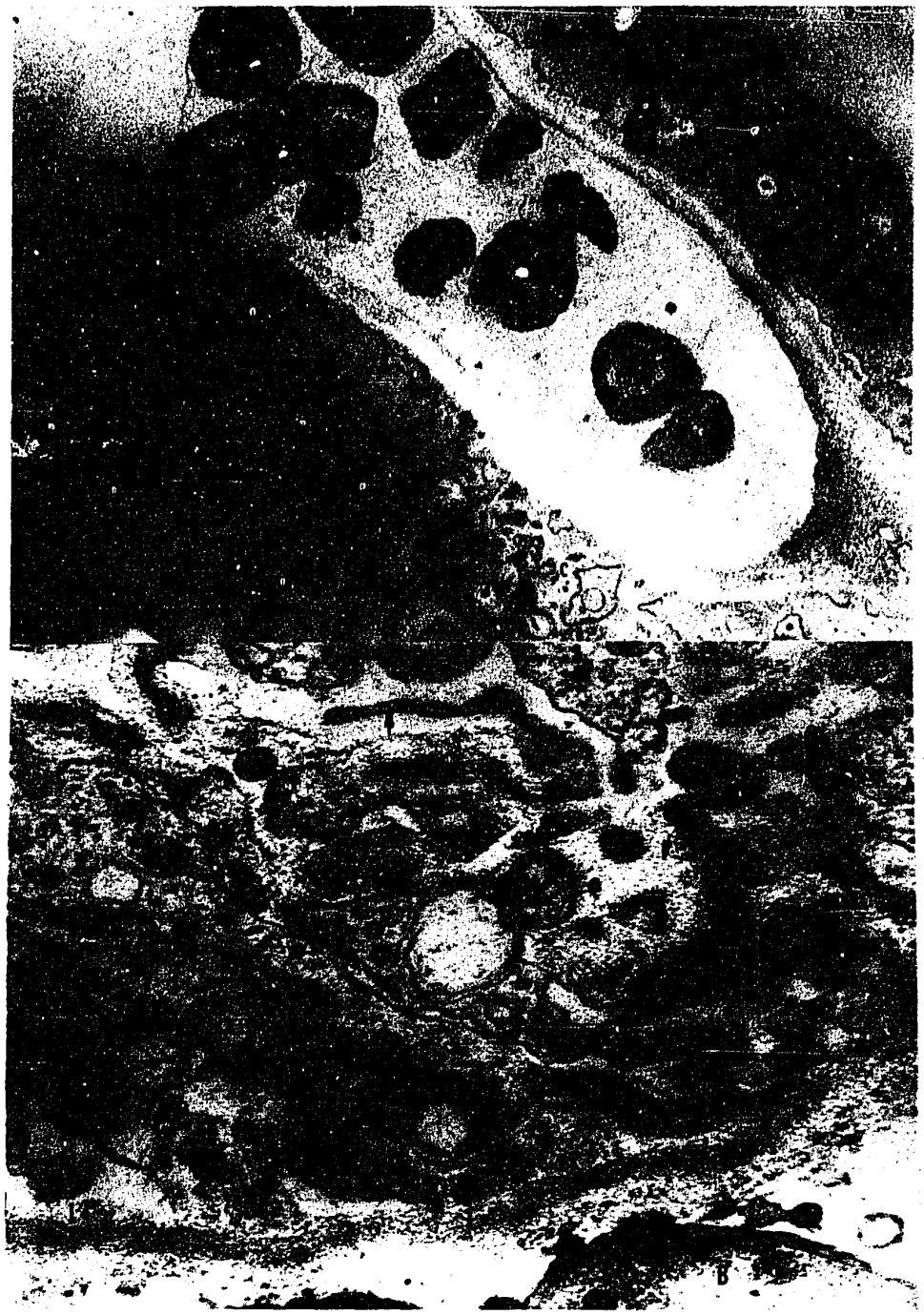
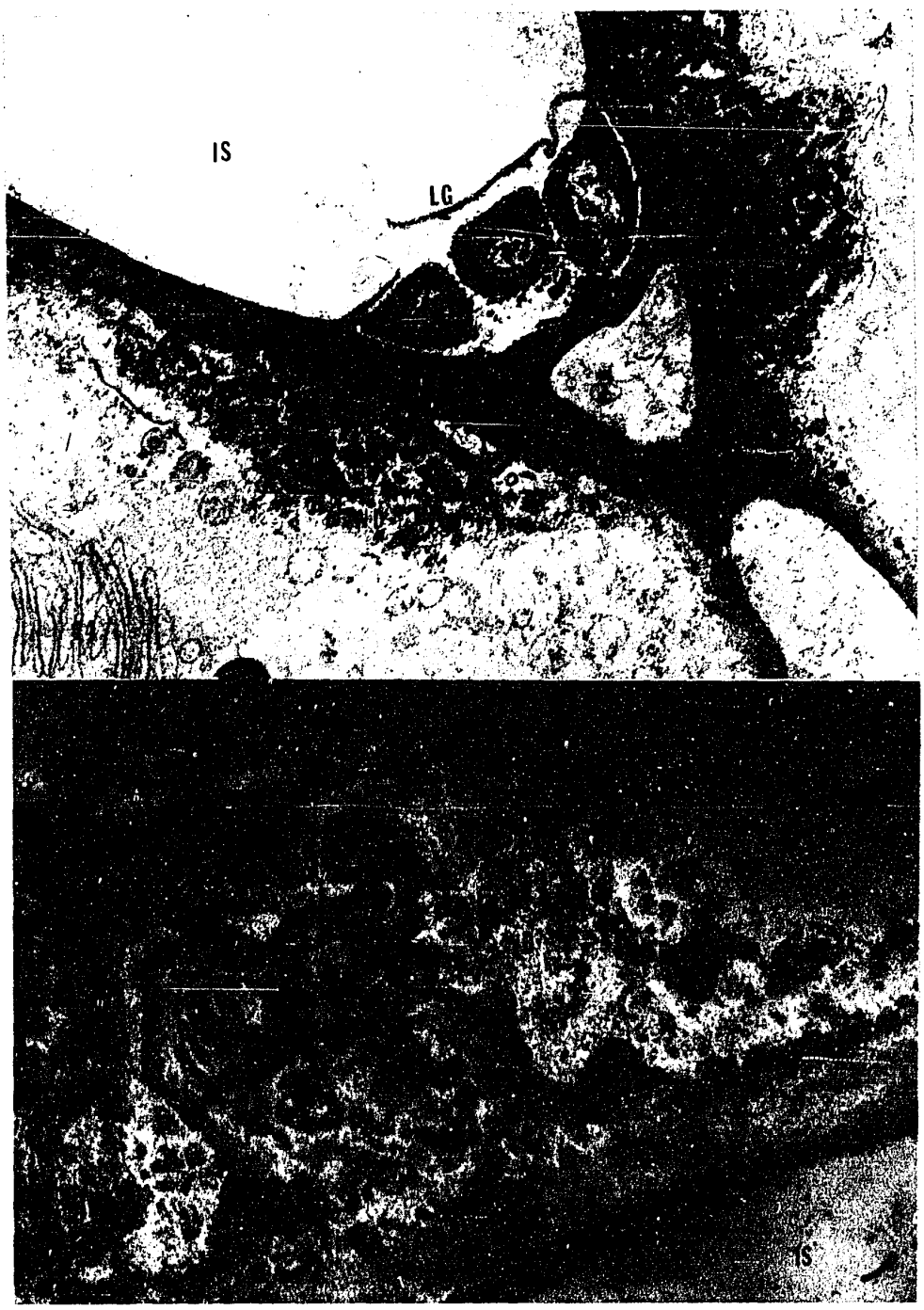


Fig. 14. C. annuum, young tissue, 18 h. after inoculation with P. phaseolicola, G50 Tox$^-$, 10^7 cells/ml showing a collapsed cell. The wall deposit (WD) contains membrane fragments entrapped in both electron opaque and electron translucent materials. Inside the localization gel only a small amount of electron dense amorphous material surrounds the bacterial cells, as compared to that found at 10^8 cells/ml. The bacteria have expanded nucleoids with coagulated DNA. (x19,200).

Fig. 15. Wall deposit in C. annuum, young tissue, inoculated with the G50 Tox$^-$, 10^7 cells/ml, where extensive membrane material (opposing arrows) is surrounded by callose deposits. (x25,700).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LG
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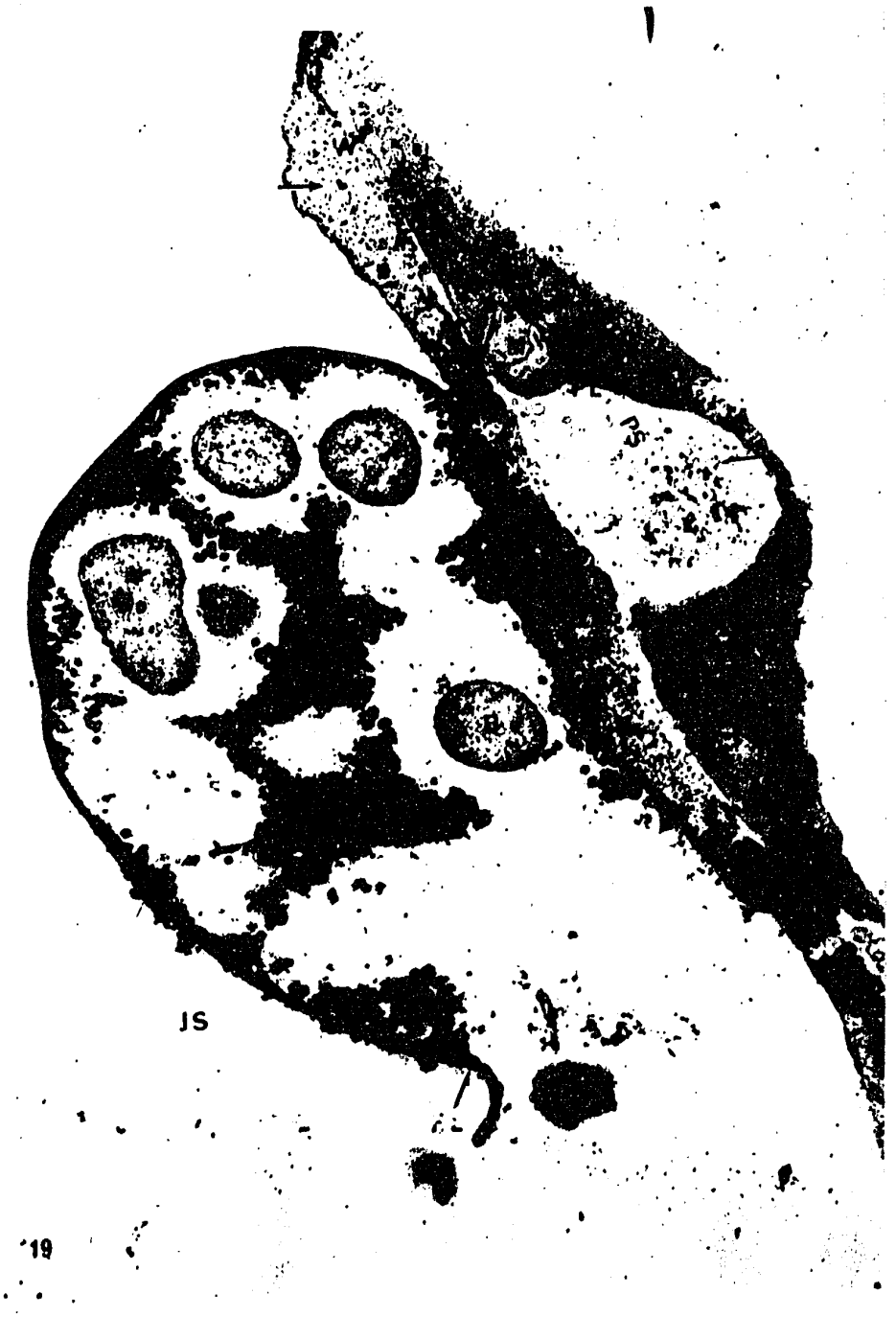
Fig. 16. Wall deposit (WD), in a younger plant of C. annuum inoculated with 10^7 cells/ml of G50 Tox$^-$. This structure is still attached to the plant cell wall though the protoplast has collapsed. (x15,780).

Fig. 17. A stage in wall deposition where convoluted tubules occur in new wall materials of C. annuum after inoculation of G50 Tox$^-$, 10^8 cells/ml. Note the density of the ribosomes in the adjacent cytoplasm surrounding the endoplasmic reticulum (ER). (x24,360).

Fig. 18. A localization gel formed in C. annuum with 10^8 cells/ml of P. phaseolicola at 18 h. Note the structural integrity of the bacteria. Adjacent to the localization gel in the plant cell deposits of fibrillar material occur between the plasmalemma and the wall (arrow). (x26,000).


18
15

Fig. 19. A localization gel surrounding P. phaseolicola G50 Tox^+, in young tissue of C. annuum at 18 h., 10^8 cells/ml. The orientation of the bacteria suggests the presence of electron translucent material adjacent to bacteria which is surrounded by electron dense amorphous material. Note fragmentation of the plant cell wall at the upper left. (x23,150).


JS
19

Literature Cited

1. Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of cell walls in relation to infective processes. Ann. Rev. Phytopathol. 7: 171-194.

2. Burkowicz, A., and R. N. Goodman. 1970. Ultrastructural changes in apple leaves inoculated with a virulent or an avirulent strain of Erwinia amylovora. Phytopathol. Z. 68: 258-268.

3. Charudattan, R., and J. E. DeVay. 1971. Common antigens among varieties of Gossypium hirsutum and isolates of Fusarium and Verticillium species. Phytopathology 62: 23 -234.

4. Chen, S. C., R. E. Stall, and A. A. Cook. 1969. Extraction and range of activity of a bacterial inhibitor from Capsicum annuum. Phytopathology 59: 112.

5. Clowes, F. A. L., and B. E. Juniper. 1968. Plant cells. Blackwell Scientific Publications, Ltd., Oxford and Edinburgh, 546 p.

6. Cook, A. A. 1973. Characterization of the hypersensitive reaction in Capsicum annuum induced by tomato strain of Xanthomonas vesicatoria. Phytopathology 63: 915-918.

7. DeVay, J. E., W. C. Schnathorst, and M. S. Foda. 1967. Common antigens and host parasite interactions. In Mirocha, C. J. and I. Uritana, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Phytopathological Society, Inc., St. Paul, Minnesota, pp. 313-325.

8. Faulkner, G., W. C. Kimmins, and R. G. Brown. 1972. The use of flurochromes for the identification of β (1,3) glucans. Can. J. Bot. 51: 1503-1504.

9. Flor, H. H. 1955. Host-parasite interactions in flax rust. Its genetics and other implications. Phytopathology 45: 680-685.

10. Fuerst, J. A., and A. C. Hayward. 1969. Surface appendages similar to fimbrae (pili) on Pseudomonas species. J. Gen. Microbiol. 58: 227-237.

11. Garber, E. D. 1956. A nutrition inhibition hypothesis of pathogenicity. Am. Nat. 90: 183-187.

12. Goodman, R. N. 1971. Reevaluation of the role of ammonia in the hypersensitive reaction. Phytopathology 61: 893.

13. Goodman, R. N., and S. R. Plurad. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Physiol. Plant Pathol. 1: 11-15.

14. Heath, Michele C., and I. B. Heath. 1971. Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. Physiol. Plant Pathol. 1: 277-287.

15. Hildebrand, D. C., and Beverley Riddle. 1971. Influence of environmental conditions on reactions induced by infiltration of bacteria in plant leaves. Hildgardia 41: 33-43.

16. Horino, Osamu. 1973. Ultrastructure of Xanthomonas oryzae and its morphological changes by chemical treatment. Ann. Phytopathol. Soc. Japan 39: 14-26.

17. Huang, Jeng-sheng, and R. N. Goodman. 1970. The relationship of phosphatidase activity to the hypersensitive reaction in tobacco induced by bacteria. Phytopathology 60: 1020-1021.

18. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27: 137A.

19. Keen, N. T., and B. W. Kennedy. 1974. Hydroxyphaseollin and related isoflavnoids in the hypersensitive resistance reaction of soy beans to Pseudomonas glycinea. Physiol. Plant Pathol. 4: 173-185.

20. Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscope study of DNA containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4: 671-676.

21. Kiraly, Z., B. Barna, and T. Ersek. 1972. Hypersensitivity as consequence, not the cause of plant resistance to infection. Nature 239: 456-457.

22. Kiraly, Z., and G. L. Farkas. 1962. Relation between phenol metabolism and stem rust resistance in wheat. Phytopathology 52: 657-664.

23. Klement, Z., and R. N. Goodman. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Ann. Rev. Phytopathol. 5: 17-44.

24. Le Normand, M., A. Coleno, J. P. Gourret, and H. Fernandez-Arias. 1971. Etude de la graisse du haricot et de agent Pseudomonas phaseolicola (Burk.) Dowson. Ann. Phytopathol. 3: 390-418.

25. Lewis, Ralph W. 1953. An outline of the balance hypothesis of parasitism. Am. Nat. 87: 273-281.

26. Lovrekovich, L., and H. Lovrekovich. 1970. Tissue necrosis in tobacco caused by a saprophytic bacterium. Phytopathology 60: 1279-1280.

27. Mullen, J. M., and D. F. Bateman. 1971. Production of an endopolygalacturonate trans-eliminase by a potato dry rot pathogen, Fusarium roseum avenaceum in culture and disease tissue. Physiol. Plant Pathol. 1: 363-373.

28. Novacky, A., G. Acedo, and R. N. Goodman. 1973. Prevention of bacterially induced hypersensitive reaction by living bacteria. Physiol. Plant Pathol. 3: 133-136.

29. O'Brien, Francis, and R. K. S. Wood. 1973. Antibacterial substances in hypersensitive response induced by bacteria. Nature 242: 532-533.

30. Patil, S. S., A. C. Hayward, and R. Emmons. 1974. An ultraviolet-induced nontoxigenic mutant of Pseudomonas phaseolicola of altered pathogenicity. Phytopathology 64: 590-595.

31. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

32. Rubin, B. A., and V. A. Arkenova. 1957. The participation of the polyphenolase system in the defense reaction of potato against Phytophthora infestans. Biokhimiya 22: 202-208.

33. Rubin, B. A., and Ye. V. Artsikhovskaya. 1963. Biochemistry and physiology of plant immunity. The Macmillan Company, New York, 358 p.

34. Rudolph, K. 1971. Reactions of the host plant to specific pathogens. In Maas Geesteranus, H. P., ed., Proceedings of the third international conference on plant pathogenic bacteria, April 14-21, 1971, Wageningen, The Netherlands, pp. 179-190.

35. Rudolph, K., and M. A. Stahmann. 1964. Interaction of peroxidase and catalases between Phaseolus vulgaris and Pseudomonas phaseolicola (halo blight of bean) Nature 204: 474-475.

36. Sasser, Myron, A. K. Andrews, and Z. U. Doganay. 1974. Inhibition of photosynthesis diminishes antibacterial action of pepper plants. Phytopathology 64: 770-772.

37. Stall, R. E., J. A. Bartz, and A. A. Cook. 1974. Decreased hypersensitivity to Xanthomonads in pepper after inoculation with virulent cells of Xanthomonas vesicatoria. Phytopathology 64: 731-735.

38. Turner, John G., and Anton Novacky. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64: 885-890.

39. Varns, J. L., J. Kuc, and E. B. Williams. 1971. Terpenoid accumulation as a biochemical response of the potato tuber to Phythophthora infestans. Phytopathology 61: 174-177.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF AN ENDOTOXIN FROM *PSEUDOMONAS PHASEOLICOLA* WHICH INDUCES CELL COLLAPSE IN BELL PEPPER (*CAPSICUM ANNUUM*)

Abstract

A high molecular weight endotoxin capable of causing cell collapse in bell pepper (*Capsicum annuum*) was purified from bacterial cells of two isolates of *Pseudomonas phaseolicola*, G50 Tox^+ and G50 Tox^- respectively, the toxin and non toxin producing isolates of the causal agent of halo blight of bean (*Phaseolus vulgaris* L.). The endotoxin from either isolate has similar chemical and biological properties. A molecular weight determination of the endotoxin from G50 Tox^- indicates a molecular weight of approximately 93,000. At concentrations as low as 2 µg/ml, confluent necrosis is induced in pepper without browning, senescence symptoms or leaf abscission. The endotoxin is thermostable, and is degraded by pronase, but not RNAse. The lesion induced in pepper by the endotoxin requires the same time to develop as the lesions induced by the bacterial cells.

Introduction

The hypersensitive reaction (HR), classically associated with plant resistance, and exemplified by rapid cell collapse when an incompatible pathogen is introduced into non host or resistant plants, is mimicked by crude extracts obtained from the pathogen (14, 16). So far, however, only in a single case has a HR-inducing principle

been purified (5). Apart from inducing HR, this purified material exhibits pectin transeliminase activity. From the information available concerning induction mechanisms involved in HR, the reaction does not appear to utilize a universal mechanism. In pepper, Cook (1) found the HR induced by two different, incompatible strains of Xanthomonas vesicatoria was affected differentially when subjected to the same physical and chemical treatment. Since it appears that the HR is suppressed during the host-pathogen interactions, it is important to understand how the reaction is triggered in this, the most widespread mechanism of plant resistance.

The purpose of this investigation was to determine if discrete, inducing principles which induce HR in pepper can be isolated from cultures of Pseudomonas phaseolicola, G50 Tox^+ and G50 Tox^-, the causal agent of halo blight of bean. This organism produces a low molecular weight exotoxin in culture which has halo blight inducing potential in bean. The UV-derived, nontoxigenic mutant was included in this study to determine if alterations in genetic characters affecting halo blight symptoms would also affect the properties of the HR inducing principle(s). Information is available concerning HR induced by Xanthomonas vesicatoria in pepper. Studies using this plant would extend progress in understanding HR in systems other than those using tobacco, the classical indicator for testing the HR inducing potential of pathogenic bacteria.

Materials and Methods

Assay of fractions from cultures of P. phaseolicola for HR inducing potential was routinely performed by infusing test suspensions in 0.025 M phosphate buffer into pepper leaves with a hypodermic syringe. Biological activity was arbitrarily graded by the degree of tissue collapse which occurred in the infused zone.

Log phase cells of P. phaseolicola, G50 Tox^+ and G50 Tox^- were collected from Watanabe's medium (18) by centrifugation at 6000 g for 20 minutes. Bacterial cells were subjected to either sonication or osmotic shock (11).

Cells (30-50 gms) subjected to sonication were washed three times in 0.025 M phosphate buffer, pH 6.8, and resuspended in 5 gm/20 ml of a medium composed of the same buffer, 0.5% sodium ascorbate and 0.5M sucrose. Sonication was performed at high frequency with a Bronwill Biosonik III probe at 80% power source, for 6 minutes in 15 second bursts while cooling the medium in an acetone and ice bath. Cellular fragments were removed first by two successive centrifugations at 27,000 g for 40 minutes and then filtering the supernatant through 0.45 μm Millipore filter. Release of wall derived fractions by osmotic shock was performed by the procedure of Neu and Chou (11). The cells were washed with 0.01 M Tris buffer, pH 7.3, at 4° C and suspended in 20% sucrose-0.03 M Tris buffer, pH 7.3, with 0.1 mM EDTA for 10 minutes. The cells removed by centrifugation were resuspended in 0.05 mM $MgCl_2$ at 4° C for 5-10 minutes. After centrifugation the supernatant was concentrated with Aquacide II.

A precipitate was obtained from the supernatant prepared both by sonication and osmotic shock by adding $(NH_4)_2SO_4$ to a final concentration of 50%. This precipitate was collected by centrifugation, dissolved in 0.025 M phosphate buffer, pH 6.8, and dialyzed against several changes of the same buffer for 24 hours. After heat precipitation over a boiling water bath for 3-7 minutes, the supernatant was again precipitated with protamine sulfate. After centrifugation the resulting supernatant was dialyzed in the above buffer for 24 hours with several changes and then subjected to gel filtration on Sephadex G-25 (2.5 x 35 cm) previously equilibrated with the same buffer. The activity which emerged in the void volume is referred to as the crude extract. The crude extract was applied to Sephadex G-200 (2.5 x 44.5 cm) equilibrated with 0.025 M, pH 6.8, phosphate buffer. This yielded several protein peaks as determined by Lowry's method (10), but only two of them caused tissue collapse in pepper. One was a fast-moving Peak I activity and the other a slower-moving Peak II fraction. Since only the Peak II fraction caused pepper tissue collapse corresponding to protein concentration, it was chosen for further purification. However, the yield is very low and its biological activity is rapidly lost. Approximately 13 mg of Peak II protein was recovered from 100 gm of bacterial cells. After dialysis of the Peak II fraction against 0.01 M, pH 7.5, phosphate buffer it was loaded on a DEAE-cellulose column (1.0 x 17 cm) equilibrated with the same buffer. The column was washed with deionized water and chromatographed with a linear gradient of 0.01-0.025 M, pH 7.5, phosphate buffer.

Protein determinations were made using the method of Lowry et al. (10) with bovine serum albumin as the standard.

Pronase digestion was performed at 37° C in phosphate buffer (pH 7.5). The ratio of pronase concentration used to endotoxin concentration was 2:1 (i.e., 50 µg/ml pronase/25 µg/ml endotoxin).

Estimation of molecular weight was performed on a calibrated Sephadex G-200 column (2.5 x 44.5 cm) in pH 6.8 buffer using a flow rate of 0.3 ml/min. (3). Molecular weight of standard proteins used were peroxidase 3.98×10^4, alkaline phosphatase 1.00×10^5, β amylase 1.52×10^5 and urease 2.43×10^5.

Isolated cells from various plant tissues were prepared using the procedures of Jensen, Francki and Zaitlin (6) and were labelled either with methyl-D-^{14}C-glucose, ^{14}C-glucose or $NaH^{14}CO_3$. Leakage of ^{14}C-intermediates were detected after removing the cells by Millipore filter and counting the supernatant in Bray's solution with a Packard Model 2420 scintillation counter. The pepper discs were exposed to $^{14}CO_2$ or 14 C-glucose and infiltrated in vacuo with the active Peak II fractions. After appropriate incubation intervals the discs were infiltrated with water and centrifuged for 30 minutes at 1000 g. The fluid from the intercellular spaces was counted in Bray's solution. In experiments testing incorporation of the active fraction Peak II into isolated bean cells after labelling with ^{14}C-glucose, $^{14}CO_2$ was collected in Hyamine (Rohm and Haas) and counted in Bray's solution.

Results

Isolation of an inducing principle which causes cellular collapse in pepper from two isolates of Pseudomonas phaseolicola. As shown in Table 1 the culture medium failed to produce any evidence of inducing principle(s) capable of causing cell collapse. However, two active components were precipitated by 50% satuation with $(NH_4)_2SO_4$ of the soluble sonicates of the bacterial cells. One of these components which appeared in the osmotic shock fluid was selected for further purification. According to Neu and Chou (11), the latter procedure presumably does not kill the bacteria. However, since the quantity of active material recovered by this method is roughly 10% of that recovered by osmotic shock, sonication was routinely used. The tissue lesions caused by the material recovered after 50% satuation of soluble sonicates with $(NH_4)_2SO_4$ were identical in appearance to those produced by the bacteria. The time required for the lesion to develop was also the same. However, the intensity of the cell collapse induced in pepper increased after precipitation of the soluble fraction over a boiling water bath (3-10 minutes). It appeared that some substance(s) which interfered with factor(s) causing hypersensitive cell collapse was inactivated by this treatment, since after gel filtration of both heat treated and non-treated samples the activity was recovered at the same elution volume. Subsequently, 3-5 minutes of heat precipitation was used in the purification procedures.

Ammonium sulfate fractionations of soluble sonicates of two non-pathogens E. coli and P. florescens were also performed. Both non-pathogens yielded biologically active fractions causing cell collapse

in pepper in the same saturation range as did P. phaseolicola. However, in contrast to the active fractions obtained from P. phaseolicola isolates, these substances were heat labile and required more than a 2000-fold greater protein concentrations to cause tissue collapse. The high molecular weight substances from E. coli did not yield active fractions after gel filtration.

Since high molecular weight components of bacterial origin were capable of causing cell collapse in pepper, naturally occurring compounds of high molecular weight were tested for potential toxicity to pepper tissue (Table 3). The substances tested included nucleic acids, proteins, polysaccharides, bacterial endotoxins, peptides and pectinase enzymes. None of these at the designated concentrations caused visible necrosis. Macerozyme (Kanematsu-Goshu, Ltd., Tokyo, Japan), the pectinase enzymes derived from Aspergillus oryzae, also failed to induce any lesions at concentrations to 10 mg/ml.

Properties of the crude extract of P. phaseolicola G50 Tox^+ and G50 Tox^- shown in Table 4 represent the stage of purification just prior to gel filtration with Sephadex G-200. The active components appear to be high molecular weight substances having unusual stability to heat and pH change. When this crude extract was subjected to Sephadex G-100, the active components emerged near the void volume. When the same extract was subjected to Sephadex G-200, the activity eluted as a fast-moving component, Peak I (Fig. 1) which showed no correspondence in activity to protein concentration, and a slower-moving component, Peak II, which showed a correspondence with a protein peak. The biological activity of Peak II was lost within 30 minutes

with pronase hydrolysis (Table 4), and this fraction was chosen for further purification. Though heat stable, Peak II failed to retain biological activity beyond 2 days in storage at 0° C. Both lyophilization and concentration greatly reduced the activity so that active material was never recovered when Peak II was recycled Sephadex G-200. When the crude extract was chromatographed on DEAE-cellulose (1.0 x 17 cm) equilibrated with 0.025 M potassium phosphate buffer, pH 7.5, loaded in the same buffer and eluted stepwise with 0.025, 0.1, 0.2, 0.3, and 0.4 M of the above buffer, all the activity was recovered in the 0.025 M buffer eluate. When this fraction was concentrated and eluted from Sephadex G-200, cell collapse was detected only in the elution volume of Peak II activity. However, the protein concentration was too low to obtain an elution profile by Lowry's method. The central peak fraction of Peak II activity from several runs on Sephadex G-200 was concentrated, and subjected to gradient elution on DEAE-cellulose using 0.01-0.025 M phosphate buffer, pH 7.5. This concentrate showed 4 closely-eluting protein peaks (Fig. 3). However, since the peak had already lost biological activity, it was impossible to determine which of the eluted peaks caused cell collapse in pepper. Biologically active products were never recovered either with preparative or quantitative procedures using disc gel electrophoresis. These procedures were carried out according to the method of Davis (2) using 6% and 7% polyacrilamide gel concentrations and a discontinuous buffer system of Tris-glycine (pH 8.8).

The biological life of Peak II is not extended significantly by freezing, by several buffer systems, using BSA for osmotic stabilization

and a buffering effect, reducing agents, dilute alcohol or by recombining with post-fraction, slow-moving peaks. Both lyophilization and concentration using the Amicon centriflo cone and Aquacide II greatly reduce the activity compared with nontreated fractions having the same protein concentration.

The ratio of the elution volume of the Peak II active fraction to the elution of blue dextran (Ve/Vo) was 2.309. On a column calibrated with proteins of known molecular weight, the Peak II fraction eluted near alkaline phosphatase and has a molecular weight of approximately 93,000 (Fig. 5).

Since pepper tissue was shown to lose electrolytes within 3 hours after infusion of Xanthomonas vesicatoria (17), Peak II fractions from P. phaseolicola were tested for the ability to cause leakage of ^{14}C-intermediates from isolated cells of tobacco, bean and passion fruit labelled with methyl-D-^{14}C-glucose, ^{14}C-glucose or $^{14}CO_2$ or from pepper discs labelled with $^{14}CO_2$ or ^{14}C-glucose. Though the active, Peak II fractions caused confluent collapse of the leaf tissue of intact pepper plants, neither the labelled cells nor the pepper discs showed any significant loss of ^{14}C-intermediates above the control for a period of 6 hours. Evidence showing the Peak II fraction is active in this system, at least initially, is suggested by the rapid increase followed by a decline in $^{14}CO_2$ evolved from bean cells, after incorporation of ^{14}C-glucose. This indicates that the faction was transported across the cell membranes (Fig. 5).

The best assay for Peak II activity proved to be the intact plant. The fractions as they eluted from Sephadex G-200 caused tissue

collapse corresponding to protein concentration of the fractions in the protein peak (Fig. 2). Diluting or concentrating these fractions caused inconsistent responses which made end-point dilution studies meaningless. Pepper seedlings, fresh water and marine algae, blood cells, or beet disc were tested for potential assay material, but all proved to be ineffective.

The appearance of the lesion induced in pepper progressively changes through purification steps. The fraction precipitated with $(NH_4)_2SO_4$ produces symptoms identical to the intact bacterial cells. With heat precipitation chlorosis surrounding the lesions was reduced and leaf abscission was delayed. At this step of purification the crude extract, like the bacterial cells, induces callose formation in pepper tissue as indicated by the bright yellow fluorescence observed with UV fluorescence microscopy after staining with Aniline Blue (4) (Figs. 6, 7). The principle responsible for causing the browning response and senescence is removed with Peak I on Sephadex G-200. Since neither cellular browning nor early membrane leakage (to 6 hours) is observed with Peak II activity, these responses which occur during HR may result from complex interaction of plant and bacterial cells.

Discussion

Sonicated cells of the two isolates of Pseudomonas phaseolicola, race 2, G50 Tox^+ and G50 Tox^-, each yield 2 active components which elute at the same place from Sephadex G-200 that are capable of causing cell collapse in pepper. Though by definition the active fractions are endotoxins, toxic substances not released into the culture medium,

Peak II could be associated with the cell membrane, cell wall or the periplasmic space.

Two observations suggest that Peak II endotoxin may be a proteinaceous compound. These are: first, the loss of biological activity with pronase digestion (Table 4) and second, the correspondence of biological activity to the protein peak eluted from Sephadex G-200 (Fig. 2). Though the molecular weight of 93,000 is near the size of many enzymes, its thermostability suggests the presence of a non-proteinaceous component.

Sequeira, Aist and Ainslie (15), and Sleesman, Perley and Hoitink (16) have reported that proteinaceous substances isolated from P. solanacearum and P. tabaci and P. pisi were capable of causing cell collapse in tobacco, but no further information has been reported concerning their purification. Recently, Gardner and Kado (6) induced cell collapse in tobacco with a protein isolated from Erwinia rubrifaciens which also showed pectin transeliminase activity (5). However, Macerozyme which contains the pectic enzymes derived from Aspergillus oryzae required 10 mg/ml before tissue collapse in pepper was induced. In contrast, only 2 µg/ml of the endotoxin was required to cause confluent collapse. Keen and Williams (7) found a lipopolysaccharide from P. lachrymans, the causal agent of angular wilt of cucumber, had noncatalytic protease activity, but the endotoxin from P. phaseolicola failed to cleave Azacol (12) indicating that it has no proteolytic activity under the conditions used here.

The endotoxin from P. phaseolicola failed to cause tissue necrosis in tobacco, tomato, passion fruit and a number of dicotyledonous plants

growing in their natural environment. Though this endotoxin appears to be specific, it has not been determined if environmental parameters or problems of transport may be involved in the failure of lesion formation in other plants. However in tobacco, lesions were not formed when the leaves were infused with the endotoxin under conditions that are conducive to lesion production when the leaves are infiltrated with intact bacteria. BSA did not inhibit lesion formation in pepper when infused both prior to or after introduction of the endotoxin. BSA and other foreign substances prevent the expression of the HR in tobacco (9).

Recently Kiraly, Barna and Ersek (8) have claimed that both hypersensitive, tissue necrosis and phytoalexin production occur in an infected plant as the result of an already-damaged or dying pathogen and are not causally related to plant resistance. This conclusion was based on the finding that killing the pathogen in infected tissue with antibiotics in 3 different host-pathogen systems caused tissue necrosis in each instance. Phytoalexin production was also found in the tested compatible system. Although heat killed cells of P. phaseolicola are capable of causing inconsistent flecks in pepper tissue, it is not known if this is the same activity exhibited by the purified endotoxin. Ultrastructural studies of pepper leaves infected with P. phaseolicola show that the first visible disruptive effects on the bacteria occur 12 hours after infusion and tissue collapse occurs within 18 hours (see Chapter II). The time required for tissue collapse induced by the endotoxin is also 18 hours. If some factor does not delay the

endotoxin in reaching the active site, it appears that death of bacterial cells is not required to induce cellular collapse in the pepper P. phaseolicola interaction. This is consistent with the observation of Sasser, Andrews and Doganay (13) who showed that in an incompatible system involving pepper and Xanthomonas phaseoli cell collapse occurred regardless of whether or not the bacteria were dead. The basis for this conclusion was the observation that inhibition of antibacterial substances either in the dark or by infusing 3-(p-chlorophenyl)-1, 1-dimethyl urea to prevent photosynthesis allowed population growth comparable to that observed in the compatible pepper X. vesicatoria system. In either case whether the production of antibacterial substance was blocked or not pepper tissue collapse occurred within the same time period. Thus, tissue collapse was not causally related to the production of antibacterial substances. In terms of a resistance mechanism the cell collapse in the P. phaseolicola-pepper system may be related to the physical delimitation of the pathogen by the death of plant tissue.

TABLE 1. Effects of ammonium sulfate fractionation of the culture broth and bacterial cells of G50 Tox^+ and G50 Tox^- on induction of the hypersensitive reaction in pepper

Source	% $(NH_4)_2SO_4$ fraction	HR response
Culture filtrate	20	-
	30	-
	40	-
	50	-
	60	-
	80	-
	90-100	-
Cells subjected to osmotic shock treatment	20	+
	30	+
	40	+
	50	+
	60	-
	80-100	-
Sonicated cells	20	+
	30	+
	40	+
	50	+
	60-70	-
	80-100	-

TABLE 2. Effect of ammonium sulfate fractionation of soluble sonicates derived from nonpathogens on the development of the hypersensitive reaction in pepper

Bacteria	% $(NH_4)_2SO_4$ fraction	HR response
Escherichia coli	20	+
	40	+
	60	-
	80-100	-
Pseudomonas florescens	20	+
	40	+
	60	-
	80-100	-

TABLE 3. Effect of selected naturally occurring high molecular weight substance as potential inducers of cellular necrosis in pepper

Molecules of biological origin	Concentration	Tissue necrosis
DNA (sperm)	10 mg/ml	-
E. coli LPS W (endotoxin)	3 mg/ml	-
Salmonella typhimurium (endotoxin)	3 mg/ml	-
Bovine serum albumin (Fraction IV)	25 mg/ml	-
Gelatin hydrolysate	10 mg/ml	-
Casein hydrolysate	10 mg/ml	-
Dextrans 40,000 MW	25 mg/ml	-
Dextrans 4,000 MW	25 mg/ml	-
Pectinase Macerozyme	10 mg/ml	-

TABLE 4. Properties of a crude extract[1] from P. phaseolicola isolates, G50 Tox$^+$ and G50 Tox$^-$

1. Non dialyzable
2. Retained by Amicon Centriflo Cone (Molecular exclusion limit 50,000)
3. Fractionated on Sephadex G-200
4. Stable to pH adjustments 5-9 (followed by dialysis)
5. Withstands freezing
6. Biological activity enhanced more than 2-fold by boiling 15 minutes
7. Biological activity retained after autoclaving 15 minutes at 15 psi
8. Biological activity retained by the crude extract for as long as 3 weeks, but the active fraction from Sephadex G-200 in phosphate buffer retains activity for only 2-3 days at 4° C. (Fractions eluted from G-200 are also heat stable.)
9. Biological activity of eluants from Sephadex G-200 are reduced by concentration
10. Biological activity is completely lost within 4 hours when subjected to enzymatic digestion with pronase
11. No reduction of biological activity was detected after 5 hours when subjected to RNAse digestion

[1]Crude extract refers to the purification step just prior to fractionation on G-200.

Fig. 1. Fractionation of Pseudomonas phaseolicola G50 Tox$^-$ crude extract on Sephadex G-200

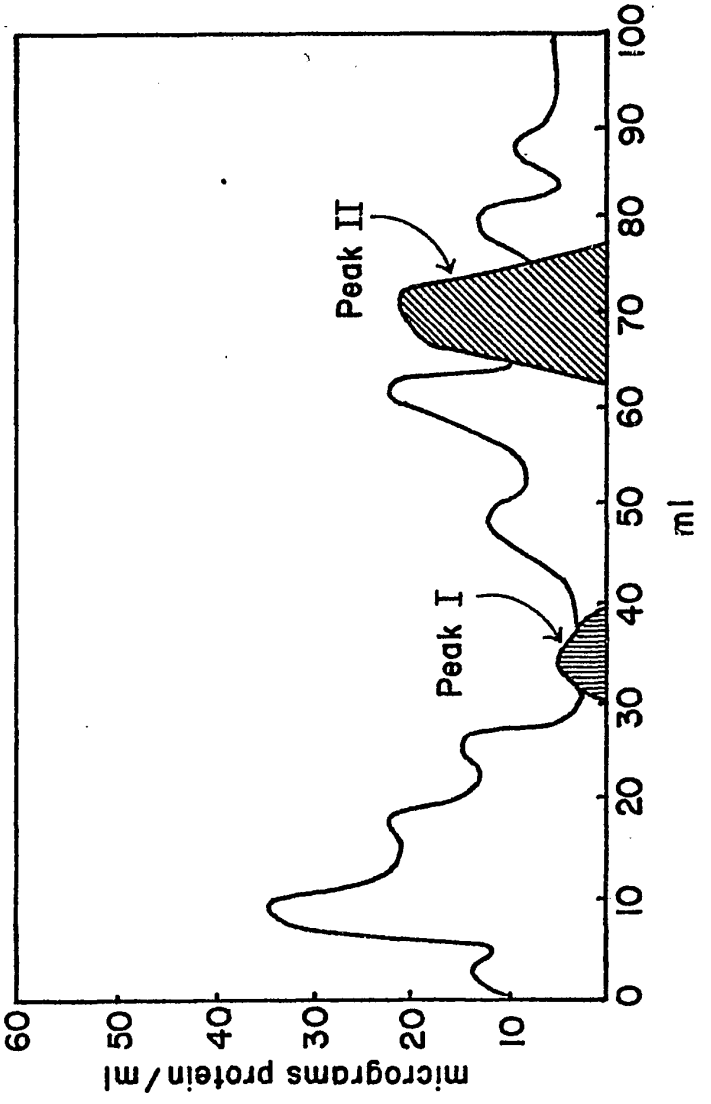

Peak II
Peak I
ml
micrograms protein / ml
0
10
20
30
40
50
60
70
80
90
100
10
20
30
40
50
60

Fig. 2. Tissue collapse induced in pepper by active fractions of Peak II. The fractions were inoculated serially from right top to bottom and left top to bottom

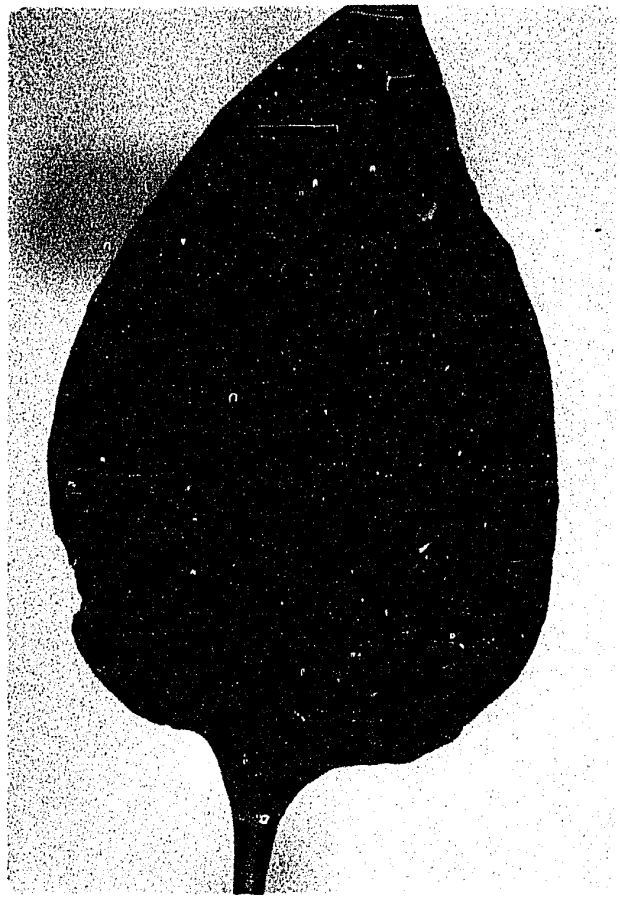


Fig. 3. Elution profile of Peak II endotoxin from Sephadex G-200 by gradient elution from DEAE-cellulose

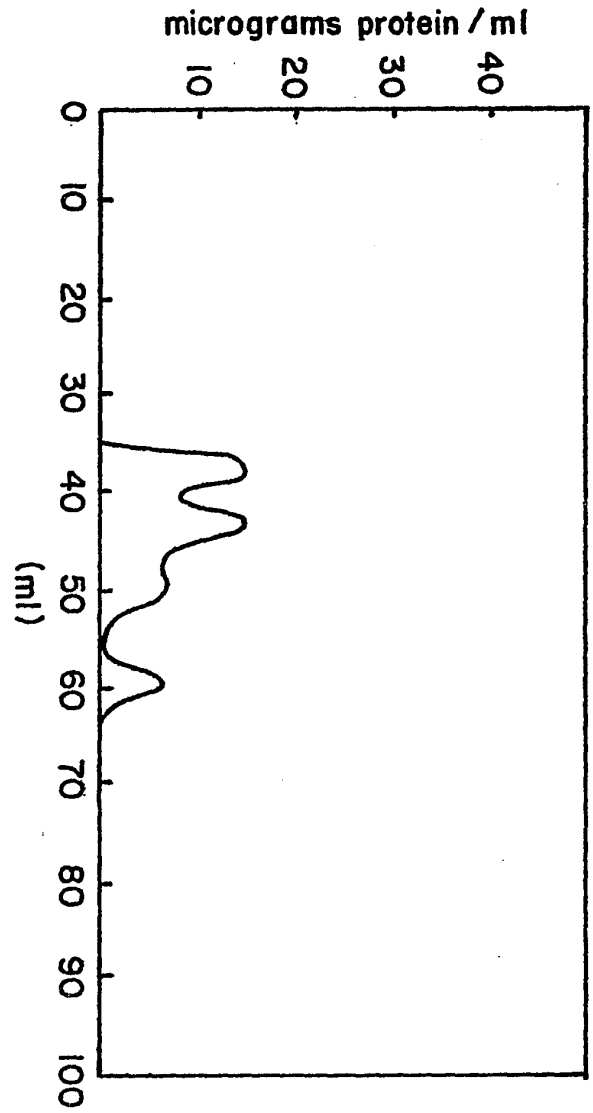

micrograms protein / ml
10
20
30
40
0
10
20
30
40
50
60
70
80
90
100
(ml)

Fig. 4. Molecular weight estimation of Peak II endotoxin with gel filtration on a calibrated column of Sephadex G-200 (2.5 x 44.5 cm) flow rate 0.3 ml/min

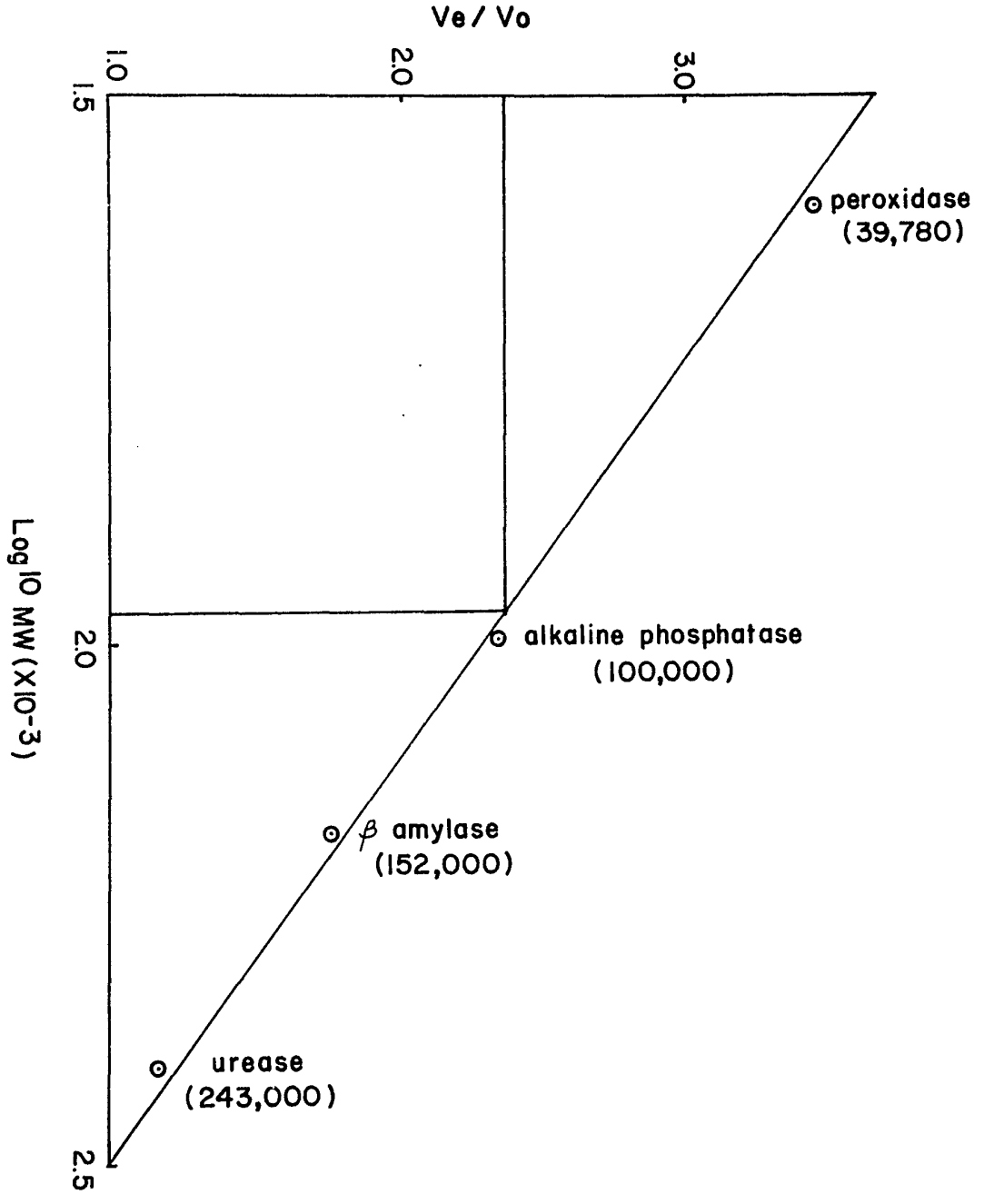

Ve / Vo
1.0
2.0
3.0
1.5
2.0
2.5
Log10 MW (X10-3)
peroxidase
(39,780)
alkaline phosphatase
(100,000)
β amylase
(152,000)
urease
(243,000)

Fig. 5. Effect of active Peak II endotoxin from Pseudomonas phaseolicola G50 Tox⁻ on $^{14}CO_2$ evolution after 15 minutes of glucose-^{14}C incorporation into isolated bean cells

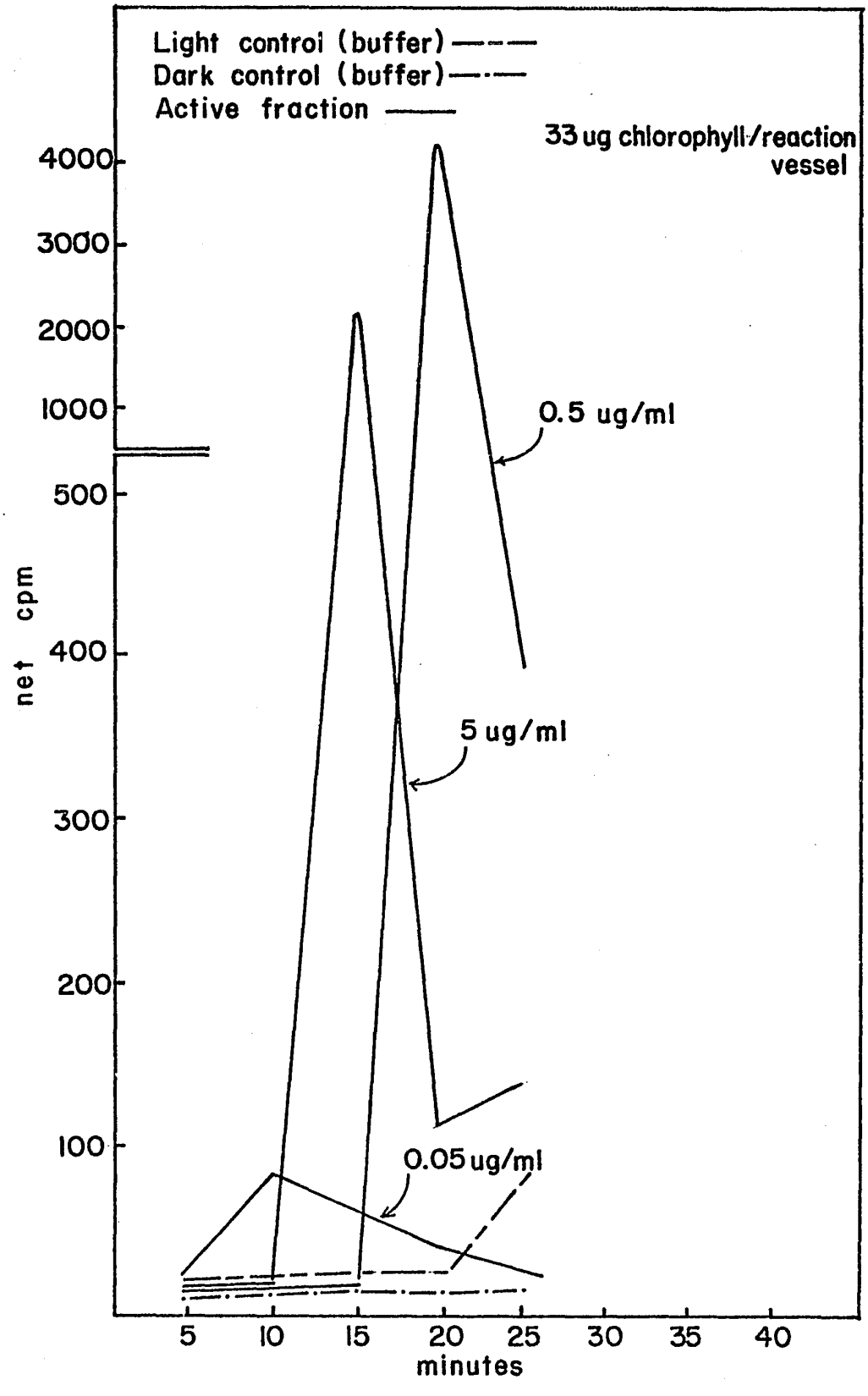
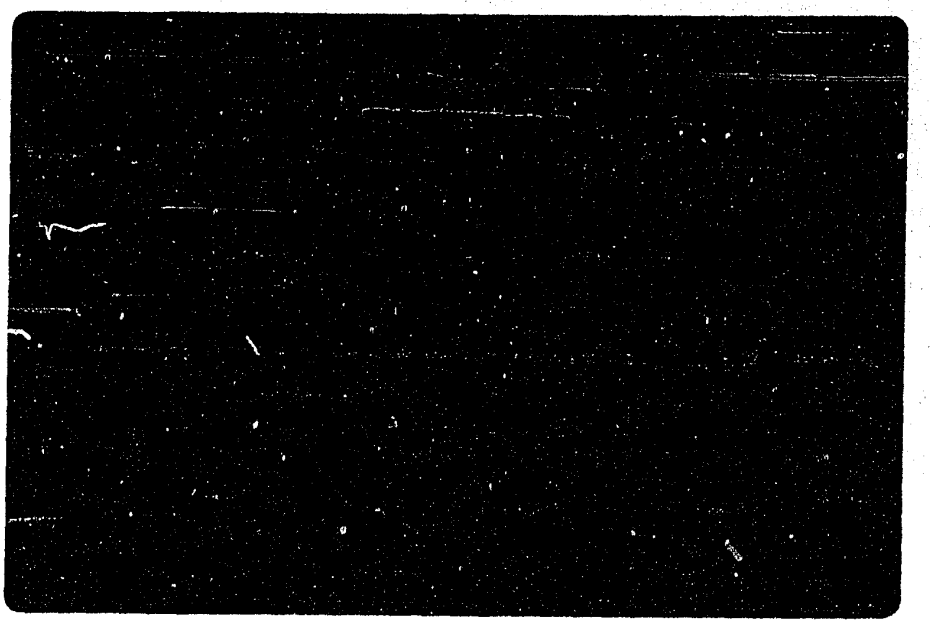
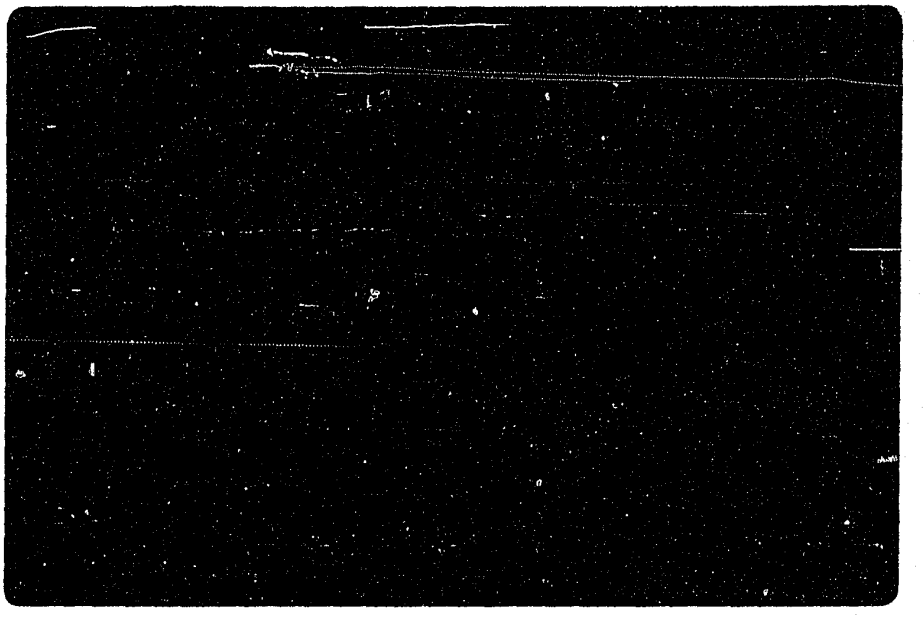

Light control (buffer)
Dark control (buffer)
Active fraction
33 ug chlorophyll/reaction vessel
0.5 ug/ml
5 ug/ml
0.05 ug/ml
net cpm
4000
3000
2000
1000
500
400
300
200
100
5
10
15
20
25
30
35
40
minutes

Fig. 6. Fluorescing material shown in pepper after inoculation of Pseudomonas phaseolicola

Fig. 7. Fluorescing material shown in pepper after inoculation with the crude extract from Pseudomonas phaseolicola





Literature Cited

1. Cook, A. A., and R. E. Stall. 1971. Calcium suspression of electrolyte loss from pepper leaves inoculated with Xanthomonas vesicatoria. Phytopathology 61: 484-487.

2. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. 121: 404-427.

3. Determann, Helmut. 1969. Gel chromotography, Second edition. Springer-Verlag, New York, Inc., New York, 202 p.

4. Faulkner, G., W. C. Kimmins, and R. G. Brown. 1972. The use of flurochromes for the identification of β (1,3) glucans. Can. J. Bot. 51: 1503-1504.

5. Gardner, J. M., and C. I. Kado. 1972. Induction of the hypersensitive reaction in tobacco with specific high-molecular weight substances derived from the osmotic shock fluid of Erwinia rubrifaciens. Phytopathology 62: 759.

6. Jensen, R. G., R. I. B. Francki, and M. Zaitlin. 1971. Metabolism of separated leaf cells. I. Preparation of photosynthetically active cells from tobacco. Plant Physiol. 48: 9-13.

7. Keen, N. T., P. H. Williams, and J. C. Walker. 1967. Characterization of a protease produced by Pseudomonas lachrymans. Phytopathology 57: 257-262.

8. Kiraly, Z., B. Barna, and T. Ersek. 1972. Hypersensitivity as consequence, not the cause of plant resistance to infection, Nature 239: 456-457.

9. Klement, Z. 1971. The hypersensitive reaction of plants to bacterial infection. Acta Phytopathol. Scient. Hung. 6: 115-118.

10. Lowry, O. H., N. J. Rosebrough, A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

11. Neu, Harold C., and James Chou. 1967. Release of surface enzymes in Enterobacteriaceae by osmotic shock. J. Bacteriol. 94: 1934-1945.

12. Ries, Stephen H., and Peter Albersheim. 1973. Purification of a protease secreted by Colletotrichium lindemuthianum. Phytopathology 63: 625-629.

13. Sasser, Myron, A. K. Andrews, and Z. U. Doganay. 1974. Inhibition of photosynthesis diminishes antibacterial action of pepper plants. Phytopathology 64: 770-772.

14. Sequeira, L. 1963. Growth regulators in plant disease. Ann. Rev. Phytopathol. 1: 5-30.

15. Sequeira, L., S. Aist, and V. Ainslie. 1972. Prevention of the hypersensitive reaction in tobacco by proteinaceous constituents of Pseudomonas solanacearum. Phytopathology 62: 536-541.

16. Sleesman, H. C., J. E. Perley, and H. A. J. Hoitink. 1970. Susceptible and hypersensitive reactions in tobacco and their prevention by cell free extractions of Pseudomonas tabaci and Pseudomonas glycinea. Phytopathology 60: 1314.

17. Stall, R. E., and A. A. Cook. 1966. Multiplication of Xanthomonas vesicatoria and lesion development in resistant and susceptible pepper leaves. Phytopathology 58: 1584-1587.

18. Watanabe, Minoru. 1963. Studies on the nutritional physiology of Xanthomonas oryzae. Ann. Path. Soc. Japan 28: 201-208.

CHAPTER IV

CONCLUSIONS

Both bacterial cells and a crude endotoxin, isolated from the two isolates of Pseudomonas phaseolicola, G50 Tox^+ and G50 Tox^-, induced in pepper callose-containing, wall deposits and hypersensitive cell collapse followed by tissue browning, senescence symptoms, and leaf abscission. The time required for cellular collapse to occur was the same for the crude extract as for the bacterial cells. Purification of the endotoxin removed the factor(s) responsible for tissue browning, senescence and abscission. Thus the leaf tissue remained green while the time required for cell collapse to occur was unchanged. Though further research will be needed to determine if the purified endotoxin has a role in hypersensitive collapse in vivo, such a role may be indicated by its unusual properties, high specific activity, high molecular weight, and the fact that BSA, when inoculated into pepper tissue prior to or after the endotoxin is injected, does not inhibit HR.

The HR induced in tobacco by Pseudomonas phaseolicola is inhibited by BSA and other foreign proteins (4). Since the endotoxin, under the conditions tested, failed to cause cell collapse in tobacco, it is not known if plant pathogenic bacteria possess more than one factor that may cause HR in different plant species. There is evidence that, in a single, incompatible, plant species, different mechanisms are involved in HR when triggered by different pathotypes of the same bacterial species. Cook (1) found that HR triggered in pepper by either

a tomato or a pepper strain of Xanthomonas vesicatoria was differentially affected when subjected to different environmental and chemical treatments. At least in pepper, a single universal mechanism is not involved in induction of HR. Correspondingly, it is conceivable that different, HR-inducing factors may be detected in a single, plant-pathogenic bacteria depending on the plant used. However, a discrete number of genetic determinants would be expected depending on the latitude of tolerance involved for both plant and pathogen.

In some cases, factors which induce HR appear to be specific. This was shown in resistant apple to the fungus Venturia inaequalis, the causal agent of apple blight. Raa and Sijpesteijn (6) found strong evidence that the fungus secreted a compound which was selectively toxic to resistant apples and caused HR, whereas the susceptible varieties were not affected by the toxic principle. In non host plants, such specificity would not be expected. However, an explanation is needed for the inability of BSA to inhibit the HR in pepper induced by the endotoxin.

If the endotoxin is responsible for induction of HR by P. phaseolicola, in vivo, it must be inferred that the factor is released at some time during the interaction with pepper tissue. The similarity of time-lapse required for lesion development by both the endotoxin and by the bacterial cells suggests this may occur soon after the bacteria encounter the plant cells. However, if the endotoxin is delayed in reaching the active site in the plant cell, this may occur when the localization reaction is initiated. That bacterial-cell death is not required for release of such a factor is indicated by bacterial cells

showing normal appearance during HR induced in pepper both by Xanthomonas phaseoli (8) and Pseudomonas phaseolicola (2). The time-lag required for the endotoxin to cause cell collapse in pepper may indicate that plant cell synthesis is affected. It is possible that the endotoxin interferes with plant, cell-membrane repair or synthesis or may activate a substance in the plant which casues membrane breakdown.

The present investigation has not established if the endotoxins of the two isolates of P. phaseolicola are the same, but several of their properties show that they are qualitatively similar. Further studies will be needed to determine if the endotoxins are also affected when genetic determinants responsible for disease symptoms are altered.

The HR observed in pepper suggests a dynamic process. If the reaction is triggered soon after the bacterial cells contact the plant cells, then the model inferring involvement of toxic products, degradative enzymes, and of changes in oxidative metabolism associated with later phases of the interaction, may be precluded. These phenomena may be secondary and thus not causally related to HR. Also, since the death of the bacteria does not appear to have cause-effect relationships to HR in pepper, phytoalexins may be excluded from consideration. Further study is needed to establish if the endotoxin can cause cell collapse in other plant species. The failure to observe cell collapse in other plants tested may be due to problems in transport. Thus, at the present state of the study, it cannot be determined if the endotoxin may act as a specific bacterial factor that reacts with a specific plant factor.

This investigation has demonstrated that similar plant cell modifications occur during the bacterially induced HR as was reported for HR induced by a virus (5). The modifications include formation of plasmalemmasome and wall deposits (see Chapter II). Recently, a localization reaction involving bacterial pathogens has been demonstrated in the vascular tissue (3, 4). Further study of the isolated endotoxin may provide an insight into the metabolic pathways involved in cell collapse caused by HR.

Literature Cited

1. Cook, A. A. 1973. Characterization of the hypersensitive reaction in Capsicum annuum induced by tomato strain of Xanthomonas vesicatoria. Phytopathology 63: 915-918.

2. Goodman, R. N. 1974. Induced resistance and hypersensitive reaction to bacterial infection. In Induction mechanisms of biochemical and cytological responses in diseased plants. Proceedings of post congress Osaka Symposium on phytopathology 9-11 September 1974 Suita, Osaka, Japan.

3. Horino, Osamu. 1974. Induction of bacterial leaf blight resistance by incompatible strains of Xanthomonas oryzae. In Induction mechanisms of biochemical and cytological responses in diseased plants. Proceedings of post congress Osaka Symposium on phytopathology 9-11 September 1974 Suita, Osaka, Japan.

4. Klement, Z. 1971. The hypersensitive reaction of plants to bacterial infection. Acta Phytopathol. Scient. Hung 6: 115-118.

5. Lobenstein, G. 1972. Localization and induced resistance in virus infected plants. Ann. Rev. Phytopathol. 10: 177-206.

6. Raa, J., and A. Kaars Sijpesteijn. 1968. A biochemical mechanism of natural resistance of apples to Venturia inaequalis. Neth. J. Plant Pathol. 74: 229-231.

7. Sasser, Myron, A. K. Andrews, and Z. U. Doganay. 1974. Inhibition of photosynthesis diminishes antibacterial action of pepper plants. Phytopathology 64: 770-772.

BIBLIOGRAPHY

Albersheim, P., T. M. Jones and P. D. English. 1969. Biochemistry of cell walls in relation to infective processes. Ann. Rev. Phytopathol. 7: 171-194.

Averre, C. W., and A. Kelman. 1964. Severity of bacterial wilt as influenced by ratio of virulent to avirulent cells of Pseudomonas solancearum in the inoculum. Phytopathology 54: 779-783.

Berard, D. F., J. Kuc, and E. B. Williams. 1972. A cultivar specific protection factor from incompatible interaction of green bean with Colletotrichium lindemuthianum. Physiol. Plant Pathol. 2: 123-127.

Bhattacharya, P. K., and M. Shaw. 1968. The effect of rust infection on DNA, RNA, and protein in nuclei of Khapli wheat leaves. Can. J. Bot. 46: 96-99.

Bogarth, R. F., and A. F. Ross. 1964. Systemic resistance induced by localized virus infection. Extent of changes in uninfected parts. Virology 24: 446-455.

Boyd, W. C. 1963. The lectins: their present status. Vox, Sanguinis 8: 1-32.

Burkowicz, A., and R. N. Goodman. 1970. Ultrastructural changes in apple leaves inoculated with a virulent or an avirulent strain of Erwinia amylovora. Phytopathol. Z. 68: 258-268.

Chamberlain, D. W. 1962. Reaction of resistant and susceptible soybean to Xanthomonas phaseoli var. soyensis. Plant Dis. Reptr. 46: 707-709.

Chand, J. N., and J. C. Walker. 1964. Relation of leaf and varietal resistance to bacterial multiplication in cucumber inoculated with Pseudomonas lachrymans. Phytopathology 54: 49-50.

Charudattan, R., and J. E. DeVay. 1971. Common antigens among varieties of Gossypium hirsutum and isolates of Fusarium and Verticillium species. Phytopathology 62: 230-234.

Chen, S. C., R. E. Stall, and A. A. Cook. 1969. Extraction and range of activity of a bacterial inhibitor from Capsicum annuum. Phytopathology 59: 112.

Clowes, F. A. L., and B. E. Juniper. 1968. Plant cells. Blackwell Scientific Publications, Ltd., Oxford and Edinburgh, 546 p.

Cook, A. A. 1973. Characterization of the hypersensitive reaction in Capsicum annuum induced by tomato strain of Xanthomonas vesicatoria. Phytopathology 63: 915-918.

Cook, A. A., and R. E. Stall. 1971. Calcium suspression of electrolyte loss from pepper leaves inoculated with Xanthomonas vesicatoria. Phytopathology 61: 484-487.

Corden, M. E., and A. E. Dimond. 1959. The effect of growth regulating substances on disease resistance and plant growth. Phytopathology 49: 68-72.

Crosse, J. E. 1965. Bacterial canker of stone fruits. VI. Inhibition of leaf-scar infection of cherry by a saprophytic bacterium from the leaf surfaces. Ann. Appl. Biol. 56: 149-160.

Cruickshank, I. A. M. 1964. Phytoalexins. Ann. Rev. Phytopathol. 1: 351-374.

Daley, J. M., L. R. Krupka, and A. A. Bell. 1962. Influence of hormones on respiratory metabolism of healthy and rust affected tissues. Plant Physiol. 37: 130-134.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. 121: 404-427.

Day, Peter R. 1972. Genetics of Host Parasite Interactions. W. H. Freeman and Co., San Francisco, 238 p.

Determann, Helmut. 1969. Gel chromotography, Second edition. Springer-Verlag, New York, Inc., New York, 202 p.

DeVay, J. E., W. C. Schnathorst, and M. S. Foda. 1967. Common antigens and host parasite interactions. In Mirocha, C. J. and I. Uritana, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Phytopathological Society, Inc., St. Paul, Minnesota, pp. 313-325.

Dineen, J. K. 1963. Immunological aspects of paratism. Nature 197: 268-269.

Doubly, J. A., H. H. Flor, and C. O. Clagett. 1960. Relation of antigens of Melapsora lini and Linum usitatissium to resistance and susceptibility. Science 13: 229.

Ercolani, G. L. 1970. Bacterial wilt of tomato. IV. The interaction between virulent and avirulent strains of Corynebacterium michiganese (E. F. Sm.) Jens. in vivo. Phytopathol. medit. 9: 151-159.

Farkas, G. L., Z. Kiraly, and F. Solomosy. 1960. Role of oxidative metabolism in the localization of plant viruses. Virology 12: 408-421.

Farkas, G. L., and M. A. Stahmann. 1966. On the nature of changes in perioxidase isoenzymes in bean leaves infected by southern bean mosiac virus. Phytopathology 56: 669-677.

Faulkner, G., W. C. Kimmins, and R. G. Brown. 1972. The use of flurochromes for the identification of β (1,3) glucans. Can. J. Bot. 51: 1503-1504.

Flor, H. H. 1955. Host-parasite interactions in flax rust. Its genetics and other implications. Phytopathology 45: 680-685.

Fuerst, J. A., and A. C. Hayward. 1969. Surface appendages similar to fimbrae (pili) on Pseudomonas species. J. Gen. Microbiol. 58: 227-237.

Galston, A. W., and W. K. Purves. 1960. The mechanism of action of auxin. Ann. Rev. of Plant Physiol. 11: 239-276.

Garber, E. D. 1956. A nutrition inhibition hypothesis of pathogenicity. Am. Nat. 90: 183-197.

Gardner, J. M., and C. I. Kado. 1972. Induction of the hypersensitive reaction in tobacco with specific high-molecular weight substances derived from the osmotic shock fluid of Erwinia rubrifaciens. Phytopathology 62: 759.

Goodman, R. N. 1968. The hypersensitive reaction in tobacco as a reflection of changes in host cell permeability. Phytopathology 58: 872-873.

Goodman, R. N. 1971. Reevaluation of the role of ammonia in the hypersensitive reaction. Phytopathology 61: 893.

Goodman, R. N. 1974. Induced resistance and hypersensitive reaction to bacterial infection. In Induction mechanisms of biochemical and cytological responses in diseased plants. Proceedings of post congress Osaka Symposium on phytopathology 9-11 September 1974 Suita, Osaka, Japan.

Goodman, R. N., and S. R. Plurad. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Physiol. Plant Pathol. 1: 11-15.

Goodman, R. N., Z. Kiraly, and M. Zaitlin. 1967. The biochemistry and physiology of infectious plant disease. Van Nostrand Co., Princeton, New Jersey, 354 p.

Heath, Michele C., and I. B. Heath. 1971. Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. Physiol. Plant Pathol. 1: 277-287.

Hildebrand, D. C., and Beverley Riddle. 1971. Influence of environmental conditions on reactions induced by infiltration of bacteria in plant leaves. Hildgardia 41: 33-43.

Hoitink, H. A. J., and O. E. Bradfute. 1971. Mechanism of disease initiation by Pseudomonas tabaci. In Maas Geesteranus, H. P., ed., Proceedings of the third international conference on plant pathogenic bacteria, Wageningen, 14-21 April, 1971. Centre for Agricultural Publishing and Docjmentation, Wageningen, The Netherlands, pp. 55-58.

Hoitink, H. A. J., R. L. Pelletier, and J. G. Coulson. 1966. Toxemia of halo blight of bean. Phytopathology 1062-1065.

Horino, Osamu. 1973. Ultrastructure of Xanthomonas oryzae and its morphological changes by chemical treatment. Ann. Phytopathol. Soc. Japan 39: 14-26.

Horino, Osamu. 1974. Induction of bacterial leaf blight resistance by incompatible strains of Xanthomonas oryzae. In Induction mechanisms of biochemical and cytological responses in diseased plants. Proceedings of post congress Osaka Symposium on phytopathology 9-11 September 1974 Suita, Osaka, Japan.

Huang, Jeng-sheng, and R. N. Goodman. 1970. The relationship of phosphatidase activity to the hypersensitive reaction in tobacco induced by bacteria. Phytopathology 60: 1020-1021.

Huang, Jeng-sheng, Pi-yu Huang, and Robert N. Goodman. 1973. Reconstitution of membrane-like structures with structural proteins and lipids isolated from tobacco thylakoid membranes. Am. J. Bot. 60: 80-85.

Israel, H. W., and A. F. Ross. 1967. The fine structure of local lesions induced by tobacco mosiac virus in tobacco. Virology 33: 272-276.

Jensen, R. G., R. I. B. Francki, and M. Zaitlin. 1971. Metabolism of separated leaf cells. I. Preparation of photosynthetically active cells from tobacco. Plant Physiol. 48: 9-13.

Jones, T. M., A. J. Anderson, and P. Albersheim. 1972. Host-pathogen interactions. IV. Studies of the polysaccharide degrading enzyme secreted by Fusarium oxysporum f. sp. lycopersici. Physiol. Plant Pathol. 2: 153-166.

Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27: 137A.

Keen, N. T., and B. W. Kennedy. 1974. Hydroxyphaseollin and related isoflavanoids in the hypersensitive resistance reaction of soy beans to Pseudomonas glycinea. Physiol. Plant Pathol. 4: 173-185.

Keen, N. T., P. H. Williams, and J. C. Walker. 1967. Characterization of a protease produced by Pseudomonas lachrymans. Phytopathology 57: 257-262.

Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscope study of DNA containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4: 671-676.

Kelman, A., and L. Sequeira. 1972. Resistance in plants to bacteria. Proc. Roy. Soc. Lond. B. 181: 247-266.

Kiraly, Z. 1974. Tissue necrosis and plant disease resistance. Sixty-sixth annual meeting, American Phytopathological Society, August 11-15, 1974, Vancouver, Canada.

Kiraly, Z., B. Barna, and T. Ersek. 1972. Hypersensitivity as consequence, not the cause of plant resistance to infection. Nature 239: 456-457.

Kiraly, Z., and G. L. Farkas. 1962. Relation between phenol metabolism and stem rust resistance in wheat. Phytopathology 52: 657-664.

Klement, Z. 1963. Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199: 299-300.

Klement, Z. 1963. Method of obtaining fluid from the intercellular spaces of foliage and the fluids merit as substrate for phytobacterial pathogens. Phytopathology 55: 1033-1034.

Klement, Z. 1971. The hypersensitive reaction of plants to bacterial infection. Acta Phytopathol. Scient. Hung. 6: 115-118.

Klement, Z., G. L. Farkas, and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54: 474-477.

Klement, Z., and R. N. Goodman. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Ann. Rev. Phytopathol. 5: 17-44.

Klement, Z., and R. N. Goodman. 1967. The role of the living bacterial cell and the induction time in the hypersensitive reaction of tobacco plants. Phytopathol. 55: 322-333.

Klement, Z., and L. Lovrekovich. 1961. Defense reactions induced by phytopathogenic bacteria in bean pods. Phytopathol. Z. 41: 217-221.

Kosuge, Tsune. 1969. The role of phenolics in host response to infection. Ann. Rev. of Phytopathol. 7: 195-222.

Kuc, J. 1968. Biochemical control of disease resistance in plants. World Rev. Pest Control 7: 42-55.

Kuo, M. S., C. O. Yoder, and R. P. Scheffer. 1970. Comparative specificity of the toxins of Helminthosporium carbonum and Helminthosporium victorae. Phytopathology 60: 365-368.

Le Normand, M., A. Coleno, J. P. Gourret, and H. Fernandez-Arias. 1971. Etude de la graisse du haricot et de agent Pseudomonas phaseolicola (Burk.) Dowson. Ann. Phytopathol. 3: 390-418.

Lewis, Ralph W. 1953. An outline of the balance hypothesis of parasitism. Am. Nat. 87: 273-281.

Lobenstein, G. 1972. Localization and induced resistance in virus infected plants. Ann. Rev. Phytopathol. 10: 177-206.

Lovrekovich, L., and G. L. Farkas. 1963. Kinetin as an antagonist of toxic effect of Pseudomonas tabaci. Nature 196: 710.

Lovrekovich, L., G. L. Farkas. 1965. Induced protection against wildfire disease in tobacco leaves treated with heat killed bacteria. Nature 205: 823-824.

Lovrekovich, L., and H. Lovrekovich. 1970. Tissue necrosis in tobacco caused by a saprophytic bacterium. Phytopathology 60: 1279-1280.

Lovrekovich, L., H. Lovrekovich, and R. N. Goodman. 1970. Ammonia as a necrotoxin in hypersensitive reaction caused by bacteria in tobacco leaves. Can. J. Bot. 48: 167-171.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lozano, J. C., and Luis Sequeira. 1970. Prevention of the hypersensitive reaction in tobacco leaves by heat killed bacterial cells. Phytopathology 60: 675-679.

Lozano, J. C., and L. Sequeira. 1970. Differentiation of races of Pseudomonas solanacearum by a leaf infiltration technique. Phytopathology 60: 833-838.

Metlikskii, L. V., and R. Ozerelskovskaya. 1968. Plant immunity. Biochemical aspects of plant resistance to parasitic fungi. Plenum Press, New York, New York, 114 p.

Misaghi, Iraj, and R. G. Grogan. 1969. Nutritional and biochemical comparisons of plant pathogenic and saprophytic pseudomonads. Phytopathology 59: 1436-1450.

Moustafa (Darweish), F. A., and R. Whittenbury. 1971. Properties which appear to allow phytopathogenic pseudomonads to counteract plant defense mechanisms. Phytopathol. Z. 67: 214-224.

Mullen, J. M., and D. F. Bateman. 1971. Production of an endopolygalacturonate trans-eliminase by a potato dry rot pathogen, Fusarium roseum avenaceum in culture and disease tissue. Physiol. Plant Pathol. 1: 363-373.

Muller, K. O. 1959. Hypersensitivity. In Horsfall, J. G., and A. E. Dimond, eds., Plant pathology 1. Academic Press, New York , pp. 470-519.

Muller, K. O., and H. Borger. 1940. Experimentalle Untersuchungen uber die Phythophthora-Resistenz der Kartoffel; zuglich ein Beitrag zum Problem der "erworbenen Resistenz" in Pflanzenreich. Arb. biol. Reichsenstalt. Land-u. Fortwirtach. Berlin-Dahlem 23: 189-231.

Nemath, J., Z. Klement, and G. L. Farkas. 1968. An enzymological study of the hypersensitive reaction induced by Pseudomonas syringae in tobacco leaf tissue. Phytopathol. Z. 65: 267-278.

Neu, Harold C., and James Chou. 1967. Release of surface enzymes in Enterobacteriaceae by osmotic shock. J. Bacteriol. 94: 1934-1945.

Novacky, A. 1972. Suppression of the bacterially induced hypersensitive reaction to cytokinins. Physiol. Plant Pathol. 2: 101-104.

Novacky, A. 1974. Transmembrane potential in bacterial hypersensitivity. Sixty-sixth annual meeting. American Phytopathological Society, August 11-15, 1974, Vancouver, Canada.

Novacky, A., G. Acedo, and R. N. Goodman. 1973. Prevention of bacterially induced hypersensitive reaction by living bacteria. Physiol. Plant Pathol. 3: 133-136.

Novacky, A., and R. N. Goodman. 1973. Prevention of bacterially induced hypersensitive reaction by living incompatible bacteria, Pseudomonas pisi. Phytopathol. 62: 780-781.

O'Brien, Francis, and R. K. S. Wood. 1973. Antibacterial substances in hypersensitive response induced by bacteria. Nature 242: 532-533.

Omer, M. E., and R. K. S. Wood. 1969. Growth of Pseudomonas phaseolicola in susceptible and resistant bean plants. Ann. Appl. Biol. 63: 103-116.

Patel, P. N., and J. C. Walker. 1963. Changes in free amino acid and amide content of resistant and susceptible beans after infection with the halo blight organism. Phytopathology 53: 522-528.

Patil, Suresh S. 1974. Toxins produced by phytopathogenic bacteria. Ann. Rev. Phytopathol. 12: 259-279.

Patil, S. S., A. C. Hayward, and R. Emmons. 1974. An ultraviolet-induced nontoxigenic mutant of Pseudomonas phaseolicola of altered pathogenicity. Phytopathology 64: 590-595.

Pellizzari, E. D., J. Kuc, and E. B. Williams. 1970. The hypersensitive reaction in Malus species: Changes in leakage of lectrolytes from apple leaves after innoculation with Venturia inaequalis. Phytopathology 60: 373-376.

Person, C., D. J. Samborski, and R. Rohringer. 1962. The gene for gene concept. Nature 194: 561-562.

Pinkas, Y., and A. Novacky. 1971. The differentiation between bacteria hypersensitive reaction and pathogenesis by use of cycloheximide. Phytopathology 61: 906.

Raa, J., and A. Kaars Sijpesteijn. 1968. A biochemical mechanism of natural resistance of apples to Venturia inaequalis. Neth. J. Plant Pathol. 74: 229-231.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Ries, Stephen H., and Peter Albersheim. 1973. Purification of a protease secreted by Colletotrichium lindemuthianum. Phytopathology 63: 625-629.

Rohringer, R., and D. J. Samborski. 1967. Aromatic compounds in the host-parasite interactions. Ann. Rev. Phytopathol. 7: 77-86.

Rohringer, R., D. J. Samborski, and C. O. Person. 1961. Ribonuclease activity in rusted wheat leaves. Can. J. Bot. 39: 77-84.

Ross, A. Frank, and H. W. Israel. 1970. Use of heat treatment in the study of acquired resistance to tobacco mosiac virus in hypersensitive tobacco. Phytopathology 60: 755-770.

Rowley, D., and C. R. Jenkins. 1962. Antigenic cross reaction between host and parasite as a possible cause of pathogenicity. Nature 193: 151-154.

Rubin, B. A., and V. A. Arkenova. 1957. The participation of the polyphenolase system in the defense reaction of potato against Phytophthora infestans. Biokhimiya 22: 202-208.

Rubin, B. A., and Ye. V. Artsikhovskaya. 1963. Biochemistry and physiology of plant immunity. The Macmillan Company, New York, 358 p.

Rubin, B. A., and E. V. Arcichovakaia. 1967. The biochemical and physiological background of plant immunity. Phytopathol. Z. 58: 101-102.

Rudolph, K. 1964. Analysis of proteins and enzymes of the interaction between Phaseolus vulgaris and Pseudomonas phaseolicola (halo blight of bean) Phytopathology 54: 904.

Rudolph, K. 1971. Reactions of the host plant to specific pathogens. In Maas Geesteranus, H. P., ed., Proceedings of the third international conference on plant pathogenic bacteria, April 14-21, 1971, Wageningen, The Netherlands, pp. 179-190.

Rudolph, K., and M. A. Stahmann. 1964. Interaction of peroxidase and catalases between Phaseolus vulgaris and Pseudomonas phaseolicola (halo blight of bean) Nature 204: 474-475.

Sakuma, T., and K. Tomiyama. 1967. The role of phenolic compounds in the resistance of potato tuber tissue to infection by Phytophthora infestans. Ann. Path. Soc. Japan 33: 48-58.

Sands, D. C., M. N. Shroth, and D. C. Hildebrand. 1970. Taxonomy of phytopathogenic pseudomonads. J. Bacteriol. 101: 9-23.

Sands, D. C., and M. L. Zucker. 1971. Impaired amino acid biosynthesis in phytopathogenic pseudomonads. Phytopathology 61: 90.

Sasser, Myron, A. K. Andrews, and Z. U. Doganay. 1974. Inhibition of photosynthesis diminishes antibacterial action of pepper plants. Phytopathology 64: 770-772.

Schnathorst, W. C., and J. E. DeVay. 1963. Common antigens in Xanthomonas malvacearum and Gossypium hirsutum and their possible relationship to host specificity and disease resistance. Phytopathology 53: 1142.

Schwochau, M. E., and L. A. Hadwiger. 1968. Stimulation of pisatin production in Pisum sativum by Actinomycin D and other compounds. Arch. Biochem. Biophys. 126: 731-733.

Sequeira, L. 1963. Growth regulators in plant disease. Ann. Rev. Phytopathol. 1: 5-30.

Sequeira, L., S. Aist, and V. Ainslie. 1972. Prevention of the hypersensitive reaction in tobacco by proteinaceous constituents of Pseudomonas solanacearum. Phytopathology 62: 536-541.

Shepard, James F. 1970. The potential significance of potato hemagglutinins (lectins) in serodiagnosis. Phytopathology 60: 1623-1625.

Sleesman, H. C., J. E. Perley, and H. A. J. Hoitink. 1970. Susceptible and hypersensitive reactions in tobacco and their prevention by cell free extractions of Pseudomonas tabaci and Pseudomonas glycinea. Phytopathology 60: 1314.

Solomosy, F., and G. L. Farkas. 1962. Simultaneous activation of pentose phosphate shunt enzymes in a virus-infected local lesion host plant. Nature 195: 835.

Stahmann, M. A. 1967. Influence of host-parasite interactions on proteins, enzymes and resistance. In Mirocha, C. J., and I. Uritani, eds. The dynamic role of molecular constituents in plant-parasite interaction. American Phytopathological Society. St. Paul, Minnesota, pp. 357-369.

Stahmann, M. A., B. G. Clare, and W. Woodbury. 1966. Increased disease resistance and enzyme activity induced by ethylene and ethylene production by black rot infected sweet potato tissue. Plant Physiol. 41: 1505-1512.

Stahmann, M. A., I. Uritani, and K. Tomiyama. 1960. Changes in potato proteins induced by fungus infection. Phytopathology 50: 655.

Stakmann, E. C. 1914. A study in cereal rust, physiological races. U. Minn. Agri. Exper. St. Bull. 138.

Stall, R. E., and A. A. Cook. 1966. Multiplication of Xanthomonas vesicatoria and lesion development in resistant and susceptible pepper leaves. Phytopathology 58: 1584-1587.

Stall, R. E., J. A. Bartz, and A. A. Cook. 1974. Decreased hypersensitivity to Xanthomonads in pepper after inoculation with virulent cells of Xanthomonas vesicatoria. Phytopathology 64: 731-735.

Tani, T., H. Yamamoto, T. Onae, and N. Naito. 1974. Primary recognition and following expression of resistance in oat leaves hypersensitively responding to the crown rust fungus. In Proceedings of post congress Osaka symposium on phytopathology. 9-11, September, Suita, Osaka, Japan

Thiers, H. D., and M. B. Lester. 1949. Histological studies of bacterial blight infection of the cotton plant. Phytopathology 39: 499-500.

Tomiyama, K. 1956. Cell physiological studies on the resistance of potato plants to Phytophthora infestans. IV. On the movement of cytoplasm of the host cell induced by invasion of Phytophthora infestans. Ann. Path. Soc. Japan 21: 54-62.

Tomiyama, K. 1960. Some factors affecting the death of hypersensitive cells infected by Phythophthora infestans. Phytopathol. Z. 39: 134-138.

Tomiyama, K. 1963. Physiology and biochemistry of disease resistance in plants. Ann. Rev. Phytopathol. 1: 295-324.

Tomiyama, K. 1966. Double infection by a compatible race of Phythophthora infestans of a potato cell which had been previously infected by a compatible race. Ann. Phytopathol. Soc. Japan 32: 181-185.

Tomiyama, K., R. Sakai, T. Sakuma, and N. Ishizaka. 1967. The role of polyphenols in the defense reaction in plants induced by infection. In Mirocha, C. J., and I. Uritani, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Pathological Society, St. Paul, Minnesota, pp. 165-179.

Tomiyama, K., and M. A. Stahmann. 1964. Alteration of oxidative enzymes in potato tuber tissue by infection with Phythophthora infestans. Plant Physiol. 39: 483-490.

Turner, John G., and Anton Novacky. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64: 885-890.

Uritani, I., T. Asahi, T. Minamikawa, H. Hyodo, K. Oshima, and M. Kojima. 1967. The relation of metabolic changes in infected plants to changes in enzyme activity. In Mirocha, C., and I. Uritani, eds., The dynamic role of molecular constituents in plant-parasite interaction. American Pathological Society, St. Paul, Minnesota, pp. 342-355.

Uritani, I., and M. A. Stahmann. 1961. Changes in nitrogen metabolism in sweet potato with black rot. Plant Physiol. 36: 770-782.

Varns, J. L., and J. Kuc. 1971. Suppression of risitin and phytotuberin accumulation and hypersensitive response in potato by compatible races of Phythophthora infestans. Phytopathology 61: 178-181.

Varns, J. L., J. Kuc, and E. B. Williams. 1971. Terpenoid accumulation as a biochemical response of the potato tuber to Phythophthora infestans. Phytopathology 61: 174-177.

Walker, J. C., and M. A. Stahmann. 1955. Chemical nature of disease resistance in plants. Ann. Rev. of Plant Physiol. 6: 351-366.

Ward, A. M. 1902. On the relations between host and parasite in the Bromes and their brown rust, Puccinia dispersa (Erikas) Ann. Bot. London 16: 233-315.

Watanabe, Minoru. 1963. Studies on the nutritional physiology of Xanthomonas oryzae. Ann. Path. Soc. Japan 28: 201-208.

Wimalajeewa, D. L. S., and J. E. DeVay. 1971. The occurrence and characterization of a common antigen relationship between Ustilago maydis and Zea mays. Physiol. Plant Pathol. 1: 523-535.

Wood, R. K. S. 1967. Physiological plant pathology. Blackwell Scientific Publications, Oxford, England, 570 p.