CALCIUM NUTRITION OF A BLIGHT-SUSCEPTIBLE COTTON AND ITS EFFECTS ON INTERACTIONS WITH *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*

By

Paul Wilm II

Bachelor of Arts

Blackburn College

Carlinville, Illinois

1986

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 1988

CALCIUM NUTRITION OF A BLIGHT-SUSCEPTIBLE COTTON AND ITS EFFECTS ON INTERACTIONS WITH *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*

Thesis Approved:

Paul E Richardson
Thesis Adviser

Carol Bender

Margaret Pierce

Glenn W. Todd

Norman N. Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

The author expresses his appreciation to his major advisor, Dr. Paul E. Richardson, for his unfailing and vital support throughout the entire study.

Gratitude is also extended to Dr. Margaret Essenberg for the unrestricted use of her laboratory. Thanks also go to Dr. Margaret Pierce for her invaluable advice and provoking questions during the inception of this project.

Additional thanks are given to Dr. Glenn Todd for assistance, especially in procurement of a growth chamber and in dealings with other departments. Thanks are also in order for Dr. Carol Bender for backround information relating to this project.

TABLE OF CONTENTS

TABLE

LIST OF FIGURES

INTRODUCTION

This investigation is composed of a single manuscript written in format acceptable to Phytopathology and entitled "Calcium Nutrition of Blight-Susceptible Cotton and Effects on Interactions with Xanthomonas campestris pv. malvacearum". Research efforts are directed at possible differences in the ability of the bacterium to incite a diseased state in a susceptible line of cotton, WbM (4.0), which has been grown at two levels of calcium availability.

Approval for presenting the thesis in this manner is based upon the Graduate College's policy of accepting a thesis written in manuscript form and is subject to the Graduate College's approval of the major professor's request for a waiver of the standard format.

CALCIUM NUTRITION OF A BLIGHT-SUSCEPTIBLE COTTON AND ITS EFFECTS ON INTERACTIONS WITH *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*

Paul Wilm, P.E. Richardson, M. Pierce, M. Essenberg and C. Bender. Graduate Student and assistant professors. Senior and second authors, Department of Botany; third author and fourth authors,Department of Biochemistry; fifth author, Department of Plant Pathology, Oklahoma State University, Stillwater, Oklahoma 74078;

ABSTRACT

Leaves of susceptible cotton WbM (4.0) were inoculated with *Xanthomonas campestris* pv. *malvacearum*. Plants were grown in either 0.1 mM or 10 mM calcium nutrient solution. Macroscopically, inoculated leaves of cotton receiving low levels of calcium became water-soaked while leaves of plants receiving high levels of calcium were dry and chlorotic. Bacterial growth curves showed insignificant differences between treatments except at day five . Changes at the

light microscope level were studied at three and five days post-inoculation. Bacteria were aggregated near host cells of cotton receiving the higher level of calcium, but not in the other treatment. Ultrastructural studies were conducted up to five days and demonstrated what appeared to be increased levels of damage in plants receiving the higher level of calcium compared to plants receiving a low levelof calcium.

INTRODUCTION

Cotton has been cultivated for at least 5000 years. Its origins were in the Indus Valley region of India, where old world cottons Gossypium arboreum and G. herbaceum were known. Peoples of the Western Hemisphere, in the American southwest and Peru, utilized G. barbadense and G. hirsutum as a fiber source. G. hirsutum is now the major cotton species grown in the United States (49). The monoculture of productive cotton lines led to the selection and multiplication of various pathogens of cotton. One of the most important of these is Xanthomonas campestris pv. malvacearum (Smith) Dye et al. (12), the cotton blight organism (Xcm).

Bacterial blight of cotton (BBC), caused by the blight organism, was first described in 1891 by Atkinson (3), and was originally referred to as black rust of cotton. This name was in reference to the color of infected parts of the host. The host response to BBC varies in different locations depending upon the climate, cultivar grown and

race of Xcm present. These variable responses resulted in a number of names which were used to describe the disease including; angular leaf spot, blackarm, bract spot and vein blight, as well as the currently used term, bacterial blight (51).

Currently, BBC occurs worldwide wherever cotton is grown, and local outbreaks have accounted for major losses. In the U.S., significant economic losses due to BBC started to occur in 1945 (49). It was soon recognized that genetic resistance was the best solution to reducing losses due to BBC and that sanitary measures could lower the inoculum load (51). Presently, lines of cotton are available which range from highly blight susceptible to near blight immune.

Exactly how Xcm is able to colonize and parasitize the host plant is not known. Pectic and cellulolytic enzymes have been considered to be involved after it was shown that supernatant, composed of nutrient broth containing pectin in which Xcm had been cultured, could macerate potato discs (50). More recent studies have shown a host reaction in which necrotic host cells appear after inoculation by filtrates of nutrient broth containing pectin in which Xcm had been cultered. Pectinase activity was detected in the filtrate (25, 48). Though the mechanisms of pathogenesis are unknown, two basic types of host-pathogen interactions are seen. Either a resistant (incompatible) or susceptible (compatible) response will

occur in the host upon inoculation with bacteria, often resulting in loss of the infected part (5).

The incompatible response of cotton to Xcm is characterized by necrotic brown cell clusters made up of collapsed host cells and by desiccation and necrosis of inoculated areas. This is the usual reaction pattern typical of the hypersensitive response (HR) (14, 19, 28). Accompanying the HR is a rapid loss of electrolytes in resistant hosts (11, 19). Histological observations of the immune response in cotton shows that damage to the host includes chloroplast degradation and tissue shrinkage within six hours post-inoculation, followed by desiccation and necrosis. Bacterial colonies were localized around the necrotized host cells (7).

Electron microscopic studies have extended observations of the incompatible reaction to the ultrastructural level. Chloroplasts show disruption and loss of membrane integrity as well as vesiculation of the ER membranes and plasmalemma (31, 43). Envelopment of bacteria may also be seen and is associated with necrosis of host cells, a phenomenon not seen in compatible reactions of cotton Ac 44 and Xcm (8).

The importance of envelopment has been questioned (15), though it has been proposed as a mechanism for immobilizing bacteria in incompatible reactions (18) or as present only during incompatible responses (2). An opposed view is that incompatible responses do not depend upon envelopment, and

that some enveloping structures may occur in the absence of bacteria (15, 24). In addition to structural responses, the incompatible reaction also involves metabolic reactions.

In cotton, production of Xcm growth inhibiting compounds has been observed in resistant lines. There is evidence that cotton phytoalexins are concentrated in leaf areas containing infection sites and resultant necrotic cells and that these areas are associated with limited bacterial growth (39). The resistant response, unlike the susceptible response, usually occurs within several hours of inoculation.

The susceptible response of most plants has a longer incubation period before changes are seen either macroscopically or microscopically. Macroscopically, electrolyte leakage was thought to be the primary cause of water soaking in susceptible beans inoculated with Pseudomonas syringae pv. phaseolicola (43). In susceptible cotton, leaves inoculated with Xcm showed continued growth of bacteria for several days post-inoculation accompanied by spread of bacteria to new parts of leaves and watersoaking (7). In this histopathological study, it was demonstrated that bacteria were densely distributed throughout intercellular spaces of mesopohyll, with no apparent host reaction for several days post-inoculation.

Al-Mousawi et al. (1) have characterized the compatible response between a susceptible cotton, Ac 44, and Xcm at the ultrastructural level. Up to 12 hours post

inoculation, host cells appeared normal. At two days post-inoculation, vesicles were seen between host cell wall and the plasma membrane. At three days post-inoculation, chloroplasts began to lose their integrity while other organelles appeared to be unaffected. Five days following inoculation, chloroplasts and nuclei stained densely and cell walls appeared to be loosening. Whether cell wall loosening was due to autolysis or to the effects of Xcm was not known. By day six bacteria were present intracellularly. These alterations were similar to those seen in other compatible interactions of bacteria with their plant hosts (20, 43).

The genetic constitution of the host plant appears to be most important in disease resistance, but environmental factors may contribute to the progress of the disease. In the case of BBC, humidity, soil moisture, temperature, light and nutrition are all known to influence disease progress and severity. High humidity is known to favor initiation of bacterial blight, though after introduction of bacteria was made into leaf mesophyll, disease progress was little influenced by external relative humidity (47). Studies by Massey (34) indicated that BBC could induce lesions with a shorter incubation time when plants were subjected to a high humidity of 60-75%. High temperatures favored disease development and shortened the incubation period (42). Combinations of high temperature and high humidity afforded maximal disease development (47). High day

temperatures (30^0 C) and low night temperatures (19^0 C) were found to favor disease development in all cultivars of cotton, and increase severity of symptoms (6). Higher soil moisture content is correlated with more rapid disease development (50).

Light has induced various responses to BBC in cotton. Infection rate was higher on leaves inoculated in the dark as compared to those in light (51). Higher light intensity levels have also been shown to increase susceptiblilty to Xcm (44). It was shown that resistant guar, the host of X. cyamopsidis, showed a susceptible response when it was grown under short day cycles (8 hrs) instead of long day cycles (16 hrs) (38). Inoculated leaves of susceptible cotton varieties grown in continuous dark did not show the usual water soaking reaction, but instead were dried and collapsed. Resistant cultivars grown in the dark after inoculation of leaves showed the usual cell disruption asssociated with HR, but bacterial levels increased above those normally found in resistant plants (36).

Plant nutrition also affects progress of disease symptoms. In cotton, higher nitrogen levels in the growth medium were shown to increase susceptibility in resistant cultivars while higher phosphorus seemed to inhibit disease intensity (9).

Calcium is thought to be quite important in disease resistance through its involvement both in wall components and possibly as part of a pathogen recognition signal (27).

Calcium is a divalent cation which is readily taken up by the roots and transported to leaves of most plants at rates which correlate positively to the level of plant transpiration (22). An adequate supply of the element will allow growth of meristems and continued accumulation in older leaves, while insufficient levels will cause meristem necrosis due to inability of the plant to relocate calcium (33). Calcium is most often found as a precipitate in vacuoles and mitochondria, bound in salts of organic acids or phosphate (10). There are two other main locations of calcium, both are apoplastic. One is the outer surface of the plasmalemma where calcium stabilizes membrane structure by bridging phospholipids and proteins. This is a role no other cation is known to perform (32). Calcium resulted in reduced or reversed ion efflux caused by acidic efflux promotion solutions (40). It may have a role in maintaining membrane potentials due to its ability to activate an ATP-ase powered potassium pump (26). Calcium may have several roles in warding off membrane damage and leakiness.

Calcium is associated with pectins and hemicelluloses in middle lamella . It is the major cation in the protein-pectin "cement", acting as a crosslinking ligand (10). Ginzburg (17) demonstrated the role of calcium in giving cohesion to the protein-pectin complex holding plant cells together. In fungal diseases, increased levels of calcium have been found to reduce disease severity in soybean (37) and bean (3). In the case of bean, increased calcium

appears to result in host tissues being more resistant to enzyme degradation.

In addition to structural effects, calcium has several essential functions in cell metabolism. These include enzyme activation, reaction to external stimuli and amplitude modulation of enzyme reactions. Calcium may operate by direct coupling to the protein response element or by binding to the enzyme modulator calmodulin. Since cytoplasmic levels of calcium are usually very low (0.1 uM), a small influx of external calcium into the cell is seen as having a large effect (27). The ion channel defense model has been proposed to explain the means by which calcium may be a direct link, which couples stimulus to response. Electrolyte leakage of plant cells is usually thought to result from cellular damage caused by wounding or pathogen activity. It may, however, be part of a recognition event. The ion channel defense model (16), proposes that transmembrane proteins can receive signals of host or pathogen origin, and then allow leakage. It is thought that calcium may effect the outcome of these events.

Other models have implicated calcium in regards to defense response involving callose (27) and phytoalexins (29, 46). It was demonstrated that in plant suspension tissue cultures which were challenged with an incompatible pathogen, plant cells were only able to synthesize callose or phytoalexins when calcium was present in the external growth medium.

In this study, calcium nutrition of host plants was hypothesized to have one or more of the following effects on the interaction of the host plant with Xcm. It was thought that increased calcium might make the host cell walls more resistant to degradation by polygalacturonases and inhibit breakdown of host tissues. Increased calcium may stabilize host membranes, lessening electrolyte leakage and loss of turgor which leads to cell collapse. Alternatively, plants receiving lower amounts of calcium may exhibit increased electrolyte leakage due to less stable membranes, and allow faster host cell collapse and death as in the HR. Finally, calcium may have some direct effect upon the pathogen itself or its disease inducing metabolites.

MATERIALS AND METHODS

Host growth environment. Seeds of Westburn M (4.0) (WbM 4.0), a bacterial-blight susceptible line of cotton, were scarified by immersion in concentrated sulfuric acid at 50^{o}C for 10 minutes (14). Seeds were then rinsed in distilled water and wrapped in moist germination paper. One week after germination, seedlings were placed in nutrient solution prepared from Hyponex 15-30-15 plant food (Hyponex Corporation, Fort Wayne, Indiana). After lateral roots had developed, plants were randomly divided into 2 groups of 5 plants each. One group was placed in 10 mM $Ca(NO_3)_2$ solution and the other in 0.1mM $Ca(NO_3)_2$ solution. In order to make nitrate equally available to both groups of plants, 19.8 mM

$NaNO_3$ was supplied in 0.1 mM $Ca(NO_3)_2$ solution. The nutrient solution was otherwise prepared as by Guinn (21). Plants were grown hydroponically in individual glass jars in aerated nutrient solutions. Solutions were replaced every 5 days. Plants were grown in a growth chamber with a light intensity of 350 uE/M^2/s. Day length was 14 hrs at 30^0C $^0\pm$1 C and 50% relative humidity with nights at 19^0C $\pm1^0$C and 90% relative humidity. At 4 weeks, the first set of foliage leaves used in this study had fully expanded.

Bacterial culture and inoculation methods. The inoculum source was a culture of Xanthomonas campestris pv. malvacearum race 3 stored in a cryogenic freezer. Frozen bacteria were added to nutrient broth the day before use. Bacteria in broth solution were diluted to 1 x 10^8 cells/ml with sterile, $CaCO_3$- saturated water to prepare inoculum for use in host plants. Approximately 3-4 hrs after the start of the day cycle, a syringe fitted with rubber tubing instead of a needle and containing inoculum solution was used to infiltrate pre-marked areas on abaxial surfaces of host leaves. Inoculation volume was approximately 0.2 ml for each site. Distilled, $CaCO_3$-saturated water controls were used for each treatment group. Bacterial population levels in plants were determined from inoculated leaves using the method of Morgham et al. (36). Two leaf discs from each of the first two mature leaves were taken at 0, 1, 3, 5 and 9 days for bacterial growth determination. On each sampled day, ten independent samples from each treatment

were compared using Student's T-test. Samples from the same inoculated leaves were collected at 12 hrs, 1, 3, and 5 days and processed for electron microscopy (EM).

Tissue preparation and sectioning. Leaf sections of five different plants from each treatment, approximately 1 mm^2 in area, were excised and fixed in 4% glutaraldehyde/0.1 M potassium-phosphate buffer fixative for 2 hours. Leaf tissue was rinsed and post fixed in 2% OsO_4 for 4 hrs., dehydrated in H_20/ethanol series, rinsed in transition solvent propylene oxide and embedded in firm formulation Spurr's resin (45). Tissue was thick-sectioned on a Sorvall MT-2 ultramicrotome. Thick sections were mounted on glass slides and stained with toluidine blue. Areas of interest were photographed using a Nikon photomicroscope. Areas of interest of thick sections were selected for thin sectioning. Silver or gold colored sections were collected on copper grids and stained with 0.5% uranyl acetate and 0.4% lead citrate. Thin sections from a minimum of five different host leaves, all sectioned at least three times, were examined in a Jeol CX-100 TEM at 80 kv.

Calcium analysis. At the termination of the experiment, inoculated leaves from each plant were collected and dried separately in a Virtis Consol 12 freeze-dryer. Leaves from individual plants were ground to a powder and divided into two 0.250 g samples. Samples were digested in perchloric-nitric acid solution and analyzed for calcium

content on a Perkin-Elmer Atomic Absorption Spectrophotometer.

RESULTS

Plants receiving higher levels of calcium had slightly larger leaves than plants supplied with lower levels of calcium, though plant maturity did not appear different in different treatments.

Infiltration of bacteria into the leaf mesophyll of plants of both calcium nutrient regimes resulted in similar initial Xcm populations at time 0 and at days 1, 3 and 9 (Figure 1). Only day 5 population growth differences were significant at the 5% level but not the 1% level. Growth yield determinations were made by dividing day 9 populations by their corresponding day 0 populations. The results showed plants supplied with 10 mM calcium did not have significantly lower yields of bacteria than plants supplied with 0.1 mM calcium (Table 1).

Leaves collected from plants of both treatments were analysed and found to contain statistically significant different amounts of calcium. In leaves of plants supplied with 10 mM calcium, calcium constituted 9% of dry weight while plants supplied with 0.1 mM calcium contained 0.5% calcium on a dry weight basis.

Macroscopically, plant responses between treatments were similar until days 4-6. Plants supplied with 0.1 mM calcium showed signs of water-soaking, while 10 mM calcium-supplied plants evidenced little water-soaking. Instead,

inoculated regions of these plants had become dry and russeted in appearance. These differences in appearance persisted until day 9, when the experiment was terminated (figure 2). By day 8, the dry areas of plants supplied 10 mM calcium had slight water-soaked fringes, with slight water-soaking along the path of some minor veins. Inoculated areas of plants supplied with 0.1 mM calcium were either confluently water-soaked or showed slight desiccation.

Microscopic changes observed in thick sections of host leaves from both treatments were similar. Three day post-inoculation plants which had been supplied with 10 mM calcium (Fig. 3 and 4) and 0.1 mM calcium supplied plants (Fig. 5 and 6) had little visible damage at the light microscope level. Differences in distribution of bacteria were apparent. Bacteria of 0.1 mM plants were not visible, however, bacteria in 10 mM calcium-supplied plants appeared to be clustered near host cells (Fig. 4).

Thick sections of five day post-inoculation leaves exhibited cell damage consisting of collapsed and distorted walls in both 10 mM (Fig. 7 and 8) and 0.1 mM (Fig. 9 and 10) calcium-supplied plants. As in thick sections from three-day-sampled leaves, bacteria were not apparent in intercellular spaces of hosts supplied with the lower level of calcium. Micrographs made from host plants provided with the higher amount of calcium had aggregated bacteria associated with host tissue damage (Fig. 7).

Ultrastructural changes in host tissues were also similar in both treatments. Micrographs from 10 mM calcium-supplied plants which were sampled at twelve hours post-inoculation had mesophyll cells (Fig. 11) similar to those of controls (Fig. 12). Some host cell walls exhibited slight distortion (Fig. 13). Electron opaque material was occasionally observed between a bacterium and host cell wall (Fig. 14).

Twelve hours post-inoculation mesophyll of 0.1 mM calcium supplied plants (Fig. 15) appeared similar to that of controls (Fig. 16). Some bacteria were surrounded by an electron opaque material (Fig. 17) of apparent host origin, while occasional bacteria were found appressed against the host cell wall in similar material (Fig. 18). Organelles and plasma membrane appeared as in controls.

After one day post-inoculation, vesiculation was observed between host cell wall and chloroplasts of higher calcium-supplied plants (Fig. 19) and lower calcium-supplied plants (Fig. 20). Mesophyll cell organelles of both treatments appeared intact.

At three days post-inoculation, ultrastructural differences were observed in micrographs made from host tissue of plants of the two treatments. Host cells of plants supplied with the lower level of calcium were intact, and the cell walls had fibrillar material on intercellular surfaces (Fig. 21, 23 and 24). Plasma membranes were broken (Fig. 21) and appeared as if they were segmented

(Fig.22). Mitochondria still appeared to have outer membranes and discernable cristae (Fig. 21 and 23). Chloroplasts were rounded and had undergone degenerative changes. Granal and stromal lamellae were separated (Fig. 21) and plastoglobuli had become larger (Fig.23) and more numerous (Fig. 24).

At three days post-inoculation, higher calcium level supplied host mesophyll cells were found to have chloroplast degeneration. Chloroplast lamellae began to degenerate and margins became ill defined (Fig. 25, 26 and 27). Vesicles were present between chloroplasts and cell walls (Fig. 25). Cell walls appeared disrupted (Fig. 26). As in lower calcium level supplied plants, mitochondria had observable cristae (Fig. 26). However, the tonoplast was disrupted in some host cells (Fig. 27). At low magnification, some aggregations of bacteria could be found in an apparent matrix along host cell walls as had been observed in thick sectioned host tissue (Fig. 28).

Final stages of host degeneration were observed to be similar in both treatments. Five day post-inoculation lower calcium supplied host tissue was distorted and collapsed (Fig. 29 and 30). Chloroplast inner membrane structure had completely disintegrated (Fig.31) or was ill defined (Fig. 32). Starch grains often appeared to be stained darkly and plastoglobuli were numerous (Fig. 32). Cytoplasm was condensed, but mitochondria were still seen with visible cristae. The tonoplast was observed to be

highly vesiculated and disrupted (Fig. 30 and 32).

At five days post-inoculation in plants supplied with 10 mM $Ca(NO_3)_2$, host tissue damage was very great, as evidenced by broken cell walls (Fig. 33 and 34), and bacteria were present intracellularly and in large spaces formed by cell wall breakdown (Fig. 34 and 35). Most chloroplasts were degenerated, with lost membrane structure (Fig. 34 and 35). Chloroplasts did not contain starch grains and ruptured tonoplasts were observed. However, mitochondria were still found to have intact membrane structures, as did nuclei when present (Fig. 36).

DISCUSSION

Severe damage to host mesophyll cells was evident in the interaction of Xanthomonas campestris pv. malvacearum with both 0.1 mM and 10 mM calcium supplied as $Ca(NO_3)_2$ to a susceptible cultivar of G. hirsutum. Differences in host leaf accumulation of calcium was probably due to the different availabilities of calcium. However, differences in transpiration may have influenced the results, since plants supplied with higher levels of calcium transpired more water than plants supplied with low levels of calcium. These results are comparable to those of Loneragan and Snowball (30) in which a correlation was observed between calcium supplied and amount accumulated in shoots. They found hydroponically grown plants accumulated 1.5% of dry weight as calcium when supplied with 0.1 mM calcium and up to 7.6% calcium dry weight when supplied with 1 mM calcium.

In field grown plants, calcium can account for 4-5 % of dry weight (13).

Final growth yields of Xanthomonas campestris pv. malvacearum (Table 1) in cotton from both treatments were not significantly different. Differences were observed in macroscopic reactions of hosts from different treatments. Cotton supplied with a higher level of calcium never became confluently water-soaked despite bacterial populations greater than 1 x $10^{8}/cm^{2}$ leaf. Inoculated areas were dry and russeted, similar to descriptions of the reactions of Ac 44 inoculated with compatible bacteria and grown in darkness (36). Differences in ability of Xcm exopolysaccharide slime (EPS) to retain water may be responsible. EPS is hygroscopic (23) and could account for water-soaking seen in normal compatible interactions (1, 26, 33, 51). The higher level of calcium available to the host may have affected the ability of Xcm to produce EPS effective in inducing water-soaking. Al-Mousawi et al. (1) proposed that EPS is a substance which helps provide a favorable environment for bacteria, and is abundantly produced in compatible interactions. Xanthan gum, the polysaccharide produced by Xanthomonas campestris, is stable and compatible with many salts (41). Usually, the solubility of polysaccharides is lowered by salt solutions. Cations, especially calcium, have been found to chelate polysaccharide chains in xanthan gum, and properties of xanthan gum seem to depend upon its conformation, which in

turn may affect its hydration characteristics *in planta*. Xanthan gum adheres to plant cell walls, and calcium may have affected the properties of host cell walls causing the aggregation seen in high calcium supplied plants.

Alternatively, plants supplied with more calcium may have had more stable membranes which did not allow electrolyte leakage and water-soaking. This may have had the effect of sealing off the inoculated areas from the rest of the leaf. Light microscopy revealed that bacteria were not homogeneously dispersed in host mesophyll of high calcium plants, but were often found aggregated along the perimeters of host cells. This was not found in water soaked areas of lower calcium-supplied leaves, and may be due to a lack of fluid media within host mesophyll of plants supplied with more calcium.

Ultrastructural examination revealed a pattern of damage similar to that reported by others investigating compatible plant host interactions with pathogens (1, 20, 23, 43). Early damage included vesicles seen near plasma membranes followed by degenerating chloroplasts in hosts from both treatments, as was previously reported for susceptible cotton (1). Plasma membranes were disrupted in both treatments. Though not evident in this experiment, plasma membranes can be made more stable by various treatments involving elevated levels of calcium (40). Host cells in later stages of disease development had collapsed and distorted cell walls in both treatments, with extensive

disruption of host cell cytoplasm and vacuoles. More extensive damage was found in cotton mesophyll of plants supplied higher levels of calcium and included broken cell walls and completely degenerated chloroplasts. This extent of damage was not found in low calcium-supplied plants.

Xcm is thought to produce enzymes important in the disease process (25, 48, 50). In other systems where enzymes are important factors in pathogenesis, higher calcium levels can augment resistance. This has been demonstrated in bean (4) and in potato (35). In interactions with E. carotovora, the proportion of calcium binding cell wall material of tubers, polygalacturonate, rises as calcium availability increases. Disease indices of tubers grown on elevated levels of calcium are lower (35).

In conclusion, higher calcium levels in the growth medium do not seem to increase resistance of cotton to Xcm. The high amount of ultrastructural damage seen in plants grown on higher levels of calcium could be due to the localization of bacteria along host cell walls. If damage is correlated to concentration of bacteria along cell walls of mesophyll, then more extensive damage would be expected in high calcium grown plants as compared to low calcium grown plants where bacteria were plentiful though not aggregated.

LITERATURE CITED

1. Al-Mousawi, A.H., Richardson, P.E.,Essenberg, M., & Johnson, W.M. 1982. Ultrastructural studies of a

compatible interaction between Xanthomonas campestris pv. malvacearum and cotton. Phytopathology 72:1222-1230.

2. Al-Mousawi, A.H., Richardson, P.E., Essenberg, M., & Johnson, W.M. 1983. Specificity of the envelopment of bacteria and other particles in cotton cotyledons. Phytopathology 73:484-489.

3. Atkinson, G.F. 1891. The black rust of cotton. Ala. Agr. Exp. Sta. Bul. no. 27.

4. Bateman, D.F., & Lumsden, R.D. 1965. Relation of calcium content and nature of the pectic substances in bean hypocotyls of different ages to susceptiblity to an isolate of Rhizoctonia solani. Phytopathology 55:734-738.

5. Bird, L.S. 1959. Loss measurement caused by the bacterial blight disease of cotton. Phytopathology 49:315.

6. Brinkerhoff, L.A. & Presley, J.T. 1967. Effect of four day and night temperature regimes on bacterial blight reactions on immune, resistant, and susceptible strains of upland cotton. Phytopathology 57:47-51.

7. Cason, E.T., Jr., Richardson, P.E. Brinkerhoff, L.A., & Gholson, R.K. 1977. Histopathology of immune and susceptible cotton cultivars inoculated with Xanthomonas

malvacearum. Phytopathology 67:195-198.

8. Cason, E.T., Jr., Richardson, P.E., Essenberg, M.K., Brinkerhoff, L.A., Johnson, W.M., & Venere, R.J. 1978. Ultrastructural cell wall alterations in immune cotton leaves inoculated with Xanthomonas malvacearum. Phytopathology 68:1015-1021.

9. Chopra, B.L. & Virk, J.S. 1980. Integrated control of bacterial blight of cotton. Indian Phytopathology 33:157.

10. Clarkson, D.T. & Hanson, J.B. 1980. The mineral nutrition of higher plants. Annual Review of Plant Physiology 31:239-298.

11. Cook, A.A. & Stall, R.E. 1968. Effect of Xanthomonas vesicatoria on loss of electrolytes from leaves of Capsicum annuum. Phytopathology 58:617-619.

12. Dye, D.W., Bradbury, J.F., Goto, M., Hayward, A.C., Lelliot, R.A. & Schroth, M.N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Reveiw of Plant Pathology 59:153-168.

13. Epstein, E. 1972. Mineral nutrition of plants: principles and perspectives. pp.117-120. Wiley: New York.

14. Essenberg, M., Cason, E.T., Jr., Hamilton, B.,

Brinkerhoff, L.A., Gholson, R.K., & Richardson, P.E. 1979. Single cell colonies of Xanthomonas malvacearum in susceptible and immune cotton leaves and the local resistant response to colonies in immune leaves. Physiological Plant Pathology 15:53-68.

15. Fett, W.F., & Jones, S.B. 1982. Role of bacterial immobilization in race-specific resistance of soybean to Pseudomonas syringea pv. glycinea. Phytopathology 72:488-492.

16. Gabriel, D.W., Loschke, D.C., & Rolfe, B.G. 1988. Gene-for-Gene:. The ion channel defense model. To appear in Molecular Plant-Microbe Interactions. Proceedings of 4 th Internationl Symposium, Desh Pal Verma and Rafael Palacios, eds. APS Press..

17. Ginzburg, B.Z. 1961. Evidence for a protein gel structure crosslinked by metal cations in the intercellular cement of plant tissue. Journal of Experimental Botany 12:85-107.

18. Goodman, R.N., Huang, P-Y., & White, J.A. 1976. Ultrastructural evidence for immobilization of an incompatible bacterium, Pseudomonas pisi, in tobacco leaf tissue. Phytopathology 66:754-764.

19. Goodman, R.N. & Plurad, S.B. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria.

Physiological Plant Pathology 1:11-15.

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

21. Guinn, G. 1974. Abscission of cotton floral buds and bolls as influenced by factors affecting photosynthesis and respiration. Crop Science 14:291-293.

22. Hanger,B.C. 1979. The movement of calcium in plants. Communications in Soil Sciences and Plant Analysis. 10:171-193.

23. Harper, S., Zewdie, N., Brown, I.R. & Mansfield, J.W. 1987. Histological, physiological and genetical studies of the responses of leaves and pods of Phaseolus vulgaris to three races of Pseudomonas syringae pv. phaseolicola and to Pseudomonas syringae pv. coronafaciens. Physiological and Molecular Plant Pathology 31:153-172.

24. Hildebrand, D.C., Alosi, M.C. & Schroth, M.N. 1980. Physical entrapment of pseudomonas in bean leaves by films formed at air-water interfaces. Phytopathology 70:98-109.

25. Hopper, D.G., Venere, R.J., Brinkerhoff, L.A., & Gholson, R.K. 1975. Necrosis induction in cotton.

Phytopathology 65:206-213.

26. Jones, R.G.W. & Lunt, O.R. 1967. The function of calcium in plants. Botanical Review 33:407-426.

27. Kauss, H. 1987. Some aspects of calcium-dependent regulation in plant metabolism. Annual Review of Plant Physiology 38:47-72.

28. Klement, Z. & Goodman, R.N. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Annual Review of Phytopathology 5:17-44.

29. Kurosaki, F., Tsurusama, Y., Nishi, A. 1987. The elicitation of phytoalexins by Ca2+ and cyclic AMP in carrot cells. Phytochemistry 26:1919-1923.

30. Loneragan, J.F. & Snowball, K. 1969. Calcium requirements of plants. Australian Journal of Agricultural Research 20:465-478.

31. Lyon, F. & Wood, R.K.S. 1976. The hypersensitive reaction and other responses of bean leaves to bacteria. Annals of Botany 40:479-491.

32. Marshner, H. 1986. Mineral nutrition of higher plants. Academic Press. Austin.

33. Marschner, H. 1974. Calcium nutrition of higher plants. Netherlands Journal of Agricultural Science 22:275-282.

34. Massey, R.E. 1934. Studies on blackarm disease of cotton. Empire Cotton Growing Review 11:188-198.

35. Mcguire, R.G. and Kelman, A. 1986. Calcium in potato tuber cell walls in relation to tissue maceration by Erwinia carotovora pv. atroseptica. Phytopathology 76:401-406.

36. Morgham, A.T., Richardson, P.E., Essenberg, M. & Cover, E.C. 1988. Effects of continuous dark upon ultrastructure, bacterial populations and accumulation of phytoalexins during interactions between Xanthomonas campestris pv. malvacearum and bacterial blight susceptible and resistant cotton. Physiological and Molecular Plant Pathology 32:141-162.

37. Muchovej, M.C. & Muchovej, J.J. 1982. Calcium supression of sclerotium induced twin stem abnormality of soybean. Soil Science 134:181-184.

38. Orellana, R.G. & Thomas, C.A. 1968. Light and nitrogen affect reaction of guar to bacterial blight caused by Xanthomonas cyamopsidas. Phytopathology 58:250-251.

39. Pierce,M. & Essenberg, M. 1987. Localization of phytoalexins in fluorescent mesophyll cells isolated from bacterial blight-infected cotton cotyledons and seperated from other cells by fluorescence-activated cell sorting. Physiological and Molecular Plant

Pathology 31:273-290.

40. Poovaiah, B.W. & Leopold, A.C. 1976. Effects of inorganic salts on tissue permeability. Plant Physiology 58:182-185.

41. Sandford, P.A. & Baird, J. 1983. Industrial utilization of polysaccharides. in The Polysaccharides. ed. G. O. Aspinall. pp.412-472. Academic Press, New York.

42. Shalyshkina, V.I. 1938. Preliminary data on the length of the incubation period of the leaf form of cotton blackarm (Bacterium malvacearum). Reveiw of Applied Mycology 17:439.

43. Sigee, D.C. & Epton, H.A.S. 1976. Ultrastructural changes in resistant and susceptible varieties of Phaseolus vulgaris following artificial inoculation with Pseudomonas phaseolicola. Physiological Plant Pathology. 9:1-8.

44. Smith, M.A. & Kennedy, B.W. 1970. Effect of light on reactions of soybean to Psuedomonas glycinea. Phytopathology 60:723-725.

45. Spurr, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research 26:31-43.

46. Stab, M.R. & Ebel, J. 1987. Effects of Ca2+ on

phytoalexin induction by fungal elicitor in soybean cells. Archives of Biochemistry and Biophysics 257:416-423.

47. Stoughton, R.H. 1933. Influence of environmental conditions on the development of the angular leaf spot disease of cotton. Annals of Applied Biology 20:590-611.

48. Venere, R.J., Brinkerhoff, L.A. & Gholson, R.K. 1984. Pectic enzyme: An elicitor of necrosis in cotton inoculated with bacteria. Proceedings of the Oklahoma Academy of Science 64:1-7.

49. Verma, J.D. 1986. Bacterial Blight of Cotton. pp.1-181. CRC Press,Inc. Boca Raton.

50. Verma, J.P. & Singh, R.P. 1970. Pectic and cellulolytic enzymes of Xanthomaonas malvacearum, the incitant of bacterial blight of cotton. Current Science 40:21-22.

51. Wickens, G.M. 1953. Bacterial blight of cotton. Empire Cotton Growing Review 30:81-103.

Table 1. 1, Relative growth yields of bacteria in host tissue 9 days post inoculation. Values are ± 95% of estimated true mean.

Figure 1. 1, Logarithmic plot of bacterial growth in host leaves. Error bars show the 95% confidence interval for values shown.

Figure 2. 2, Macroscopically differentiable disease reactions between (A) host leaves of plants grown in 0.1 mM calcium solutions and plants grown in 10 mM calcium solutions (B).

Figures 3-4. Third day post-inoculation thick section of mesophyll 10 mM calcium supplied plants. 3, Host cells appear normal, bacteria are aggregated in intercellular space (arrows) (X1900). 4, Border of bacterial aggregate in 10 mM calcium supplied plants (X1760). AB = aggregated bacteria.

Figures 5-6. Third day post-inoculation thick section of mesophyll 0.1 mM calcium supplied plants. 5, Host cells appear normal, no bacteria are noticeable intracellularly (X960). 6, Mesophyll unaltered in appearance (X1500). E = epidermal cells, Is = intercellular space, PP = palisade parenchyma.

Figures 7-8. Fifth day post-inoculation of 10 mM calcium supplied leaves. 7, Broken palisade cell with large aggregation of bacteria (X1800). 8, Mesophyll showing

distorted cell walls (arrows) of palisades parenchyma (X1100). AB = aggregated bacteria, CC = collapsed cell, Cw = cell wall, PP = palisade parenchyma.

Figures 9-10. Fifth day post-inoculation thick section demonstrating open space after dissolution of cell in lower calcium supplied leaf (X1500). 10, Similar section with open space and distorted host cells, no apparent bacteria (X1400). Cc = collapsed cell, E = epidermal cell, Is = intercellular space, PP = palisade parenchyma.

Figures 11-12. Twelfth hr post-inoculation of 10 mM calcium supplied plants. 11, Bacteria near host cell, cytoplasm and host cell structure appear as in control (X59,700). 12, Control post-inoculation of 10 mM calcium supplied plants. Host cell wall, membranes and organelles appear intact (X17.800). B = bacterium, C = chloroplast, Cw = cell wall, Er = endoplasmic reticulum, Is = intercellular space, P = plastoglobuli, Pl = plasmalemma, S = starch.

Figures 13-14. Twelfth hr post-inoculation of cotton mesophyll cells of 10 mM calcium supplied plants. 13, Host cell exhibiting disruption of outer cell wall. Ribosomes present in cytoplasm (X57,000). 14, Electron opaque material present between bacteria and host cell (15,100). B = bacterium, C = chloroplast, Cw = cell wall, F = fibrillar material, Is = intercellular space, Mb = microbody, r = ribosomes.

Figures 15-16. Twelfth hr post-inoculation of 0.1 mM calcium supplied plants. 15, Bacteria have electron opaque material around perimeter. Host cell appears as in control, except for slight membrane disruption (X51,900). 16, Control, cellular structure and organelles appear intact (X28,700). B = bacterium, C = chloroplast, Cw = cell wall, F = fibrillar material, Is = Intercellular space, M = mitochondrion, P = plastoglobuli, Pl = plasmalemma, S = starch.

Figures 17-18. Twelfth hr post-inoculation of 0.1 mM calcium supplied plants. 17, Bacterium trapped intercellularly in material of apparently at least partial host origin (X55,700). 18, Twelfth hr post-inoculation of 0.1 mM calcium supplied plants. Bacteria appressed against host cell wall in light staining material (X29,400). B = bacterium, C = chloroplast, F = fibrillar material, Is = intercellular space, P = plastoglobuli, S = starch.

Figures 19-20. 19, Twenty-four hr post-inoculation high calcium supplied host plant mesophyll. Vesicle formation (arrows) between chloroplast and plasma membrane (X34,200). 20, Twenty-four hr post-inoculation if low calcium host mesophyll. Vesicles present (arrows) in host cytoplasm. Fibrillar material present between bacterium and host cell wall (X48,100). B = bacterium, C = chloroplast, Ve = vesicles.

Figures 21-22. Third day post-inoculation low calcium

supplied plants. 21, Plasma membrane is broken, cytoplasm is condensed. Chloroplast membranes are seperated while mitochondrial membranes are intact (X26,600). 22, Plasma membrane appears segmented in cross section (X96,000). B = bacterium, C = chloroplast, Cw = cell wall, Is = intercellular space, M = mitochondrion, Pl = plasmalemma.

Figures 23-24. Third day post-inoculation low calcium supplied plants. 23, Lamellae occupy less volume while plastoglobuli have increased in size. Vesicle has formed on chloroplast. Mitochondrion has intact outer membranes and cristae (X16,500). 24, Chloroplasts have become rounded with numerous plastoglobuli and starch grains (9,900). B = bacterium, D = dictyosome, M = mitochondrion, P = plastoglobuli, S = starch, Ve = vesicle.

Figures 25-26. Third day post-inoculation of high calcium supplied plants. 25, Chloroplasts have less distinct lamellae. Vesicles present between chloroplast and plasma membrane (X32,000). 26, Cell wall appears distorted. Mitochondria have distinct outer membranes and cristae (X22,600). B = bacterium, C = chloroplast, Cw = cell wall, M = mitochondrion.

Figures 27-28. Third day post-inoculation of high calcium supplied plants. 27, Chloroplast membrane system is indistinct. Tonoplast is ruptured (X22,000). 28, Aggregation of bacteria along host cell wall (arrows), as was observed in thick sectioned material (X3800). AB =

aggregated bacteria, B = bacteria, C = chloroplast, T = tonoplast.

Figures 29-30. Fifth day post-inoculation of low calcium supplied plants. 29, Distorted and broken host cell (x2,900). 30, Highly collapsed host cell with vesiculated vacuole and indistinct chloroplasts (X4,700). B = bactrium, C = chloroplast, Cc = collapsed cell.

Figures 31-32. Fifth day post-inoculation of low calcium supplied plants. 31, Bacterium present intracellularly near disintegrated chloroplast (X20,000). 32, Fifth day post-inoculation of low calcium plant. Chloroplast contains dark staining starch grains. Cytoplasm appears condensed and disrupted and contains may vesicles (X6,400). B = bacterium, C = chloroplast, P = plastoglobuli, S = starch, Ve = vesicles.

Figures 33-34. Fifth day post-inoculation of high calcium supplied plants. 33, Bacteria collected around pieces of broken cell walls (X9,600). 34, Chloroplasts are rounded and extensively degraded. Cell walls are broken and seperated (X3,900). B = bacterium, C = chloroplast, Cw = cell wall, M = mitochondrion.

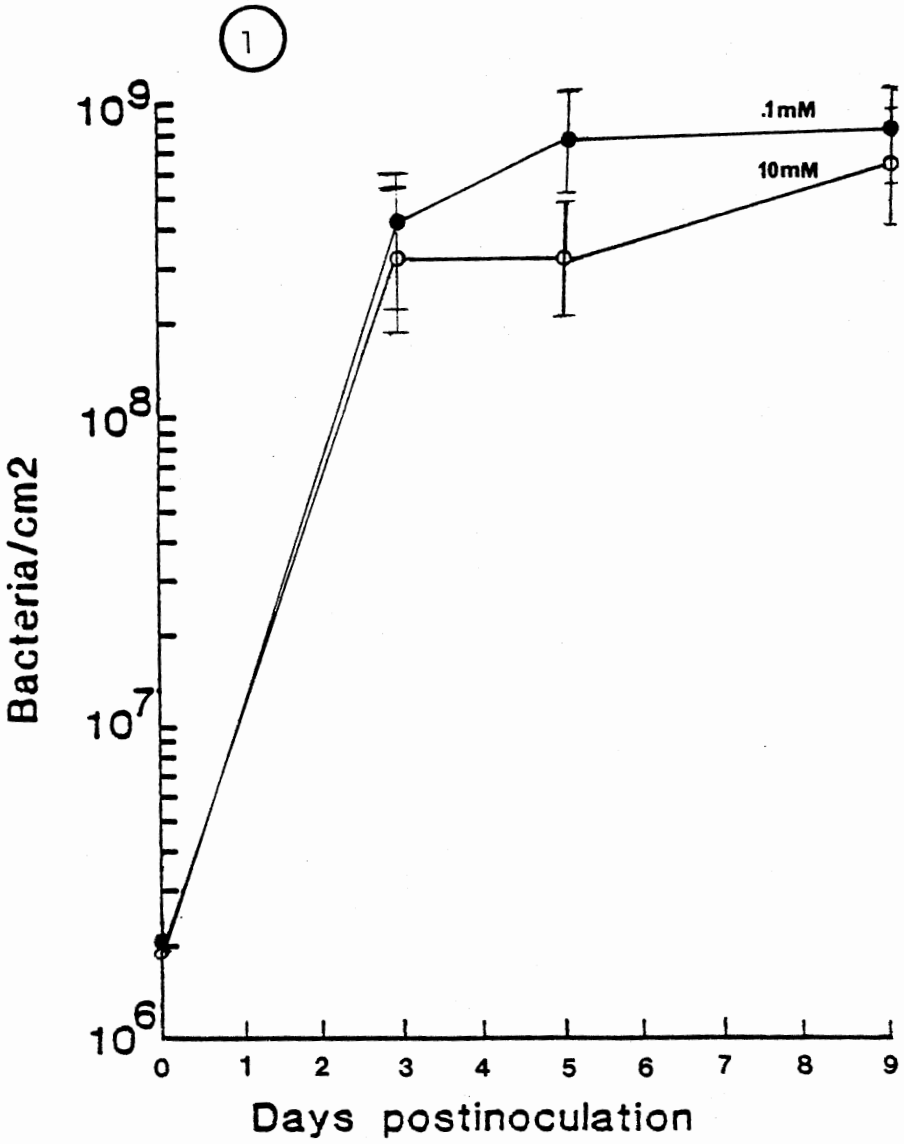
Figures 35-36. Fifth day post-inoculation of high calcium supplied plants. 35, Some chloroplasts exhibit complete loss of membrane structure (X12,200). 36, Intact chloroplasts did not contain starch. Condensed cytoplasm

was observed, though mitochondria retained membrane structure, as did nucleus (X7,600).

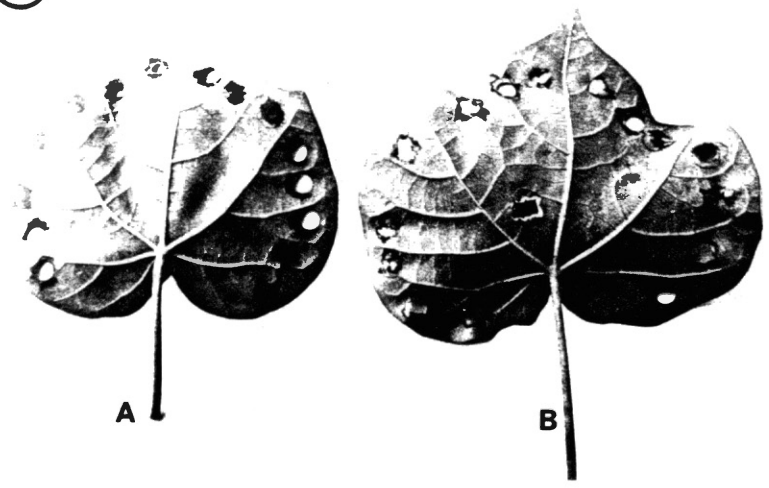
TABLE 1

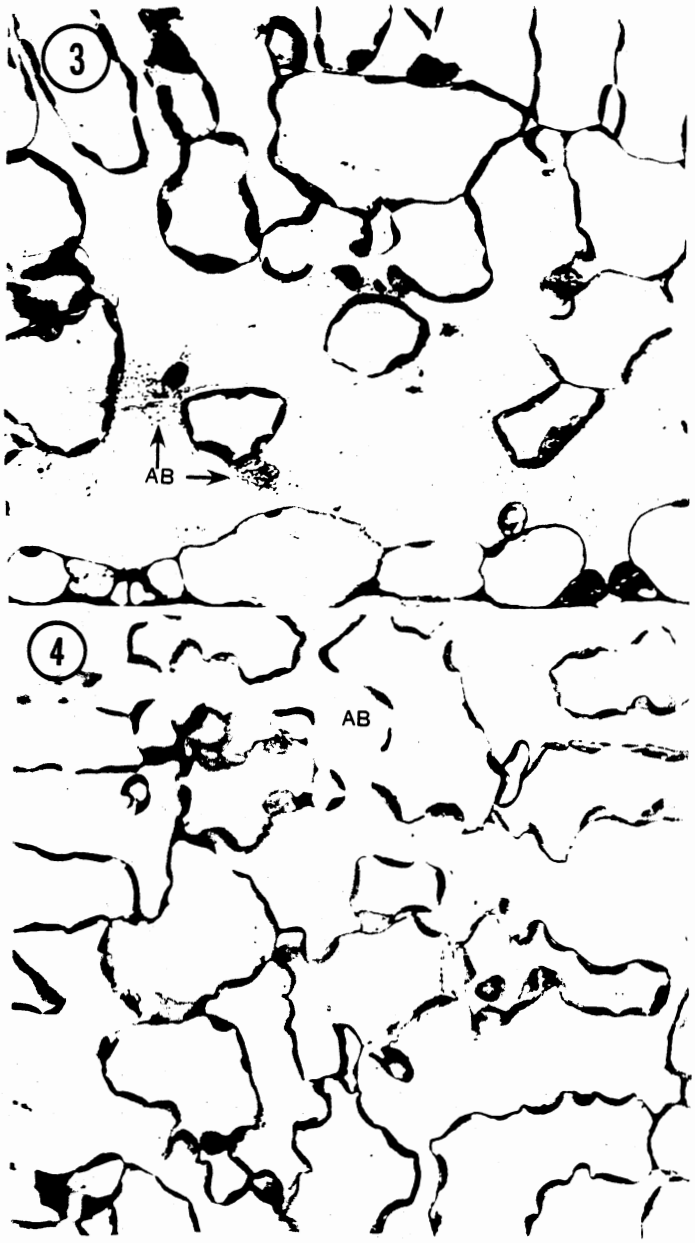
Xcm GROWTH YIELD

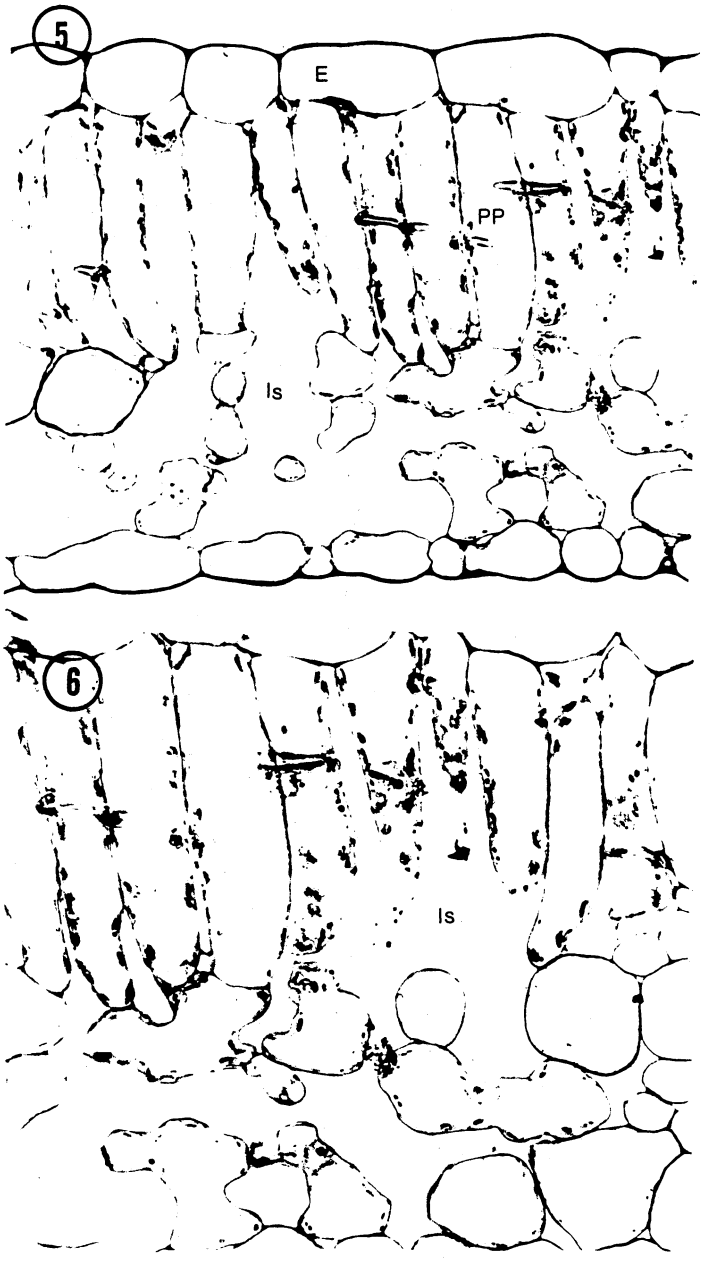
Calcium Concentration of Nutrient Solution as Nitrate	*Xcm* Growth Yield
0.1 mM	830 ± 431
10 mM	582 ± 355

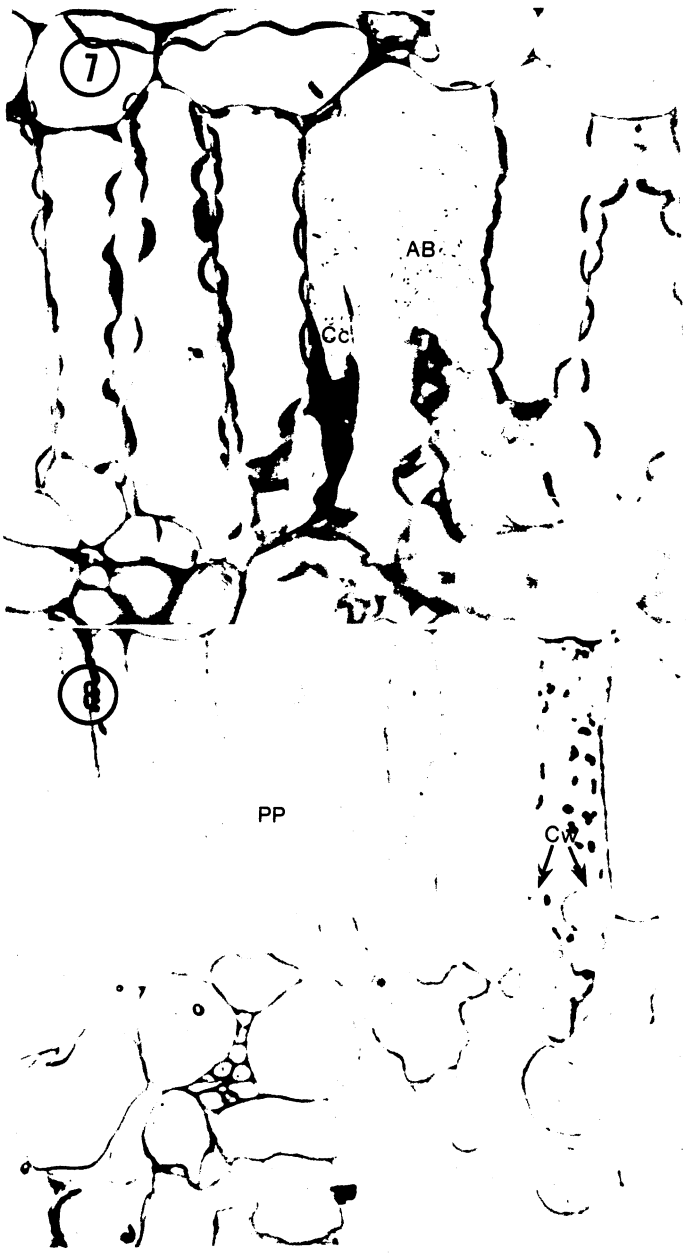

1
.1mM
10mM
10^9
10^8
10^7
10^6
Bacteria/cm2
0 1 2 3 4 5 6 7 8 9
Days postinoculation

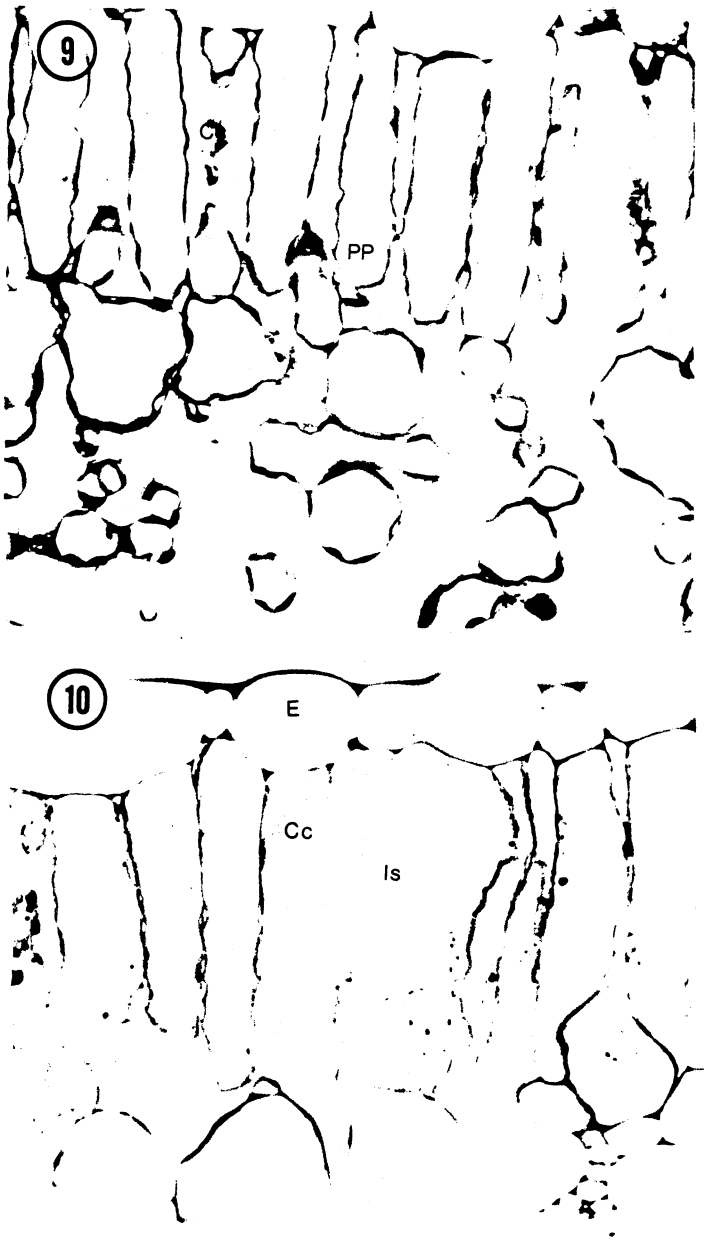
(2)

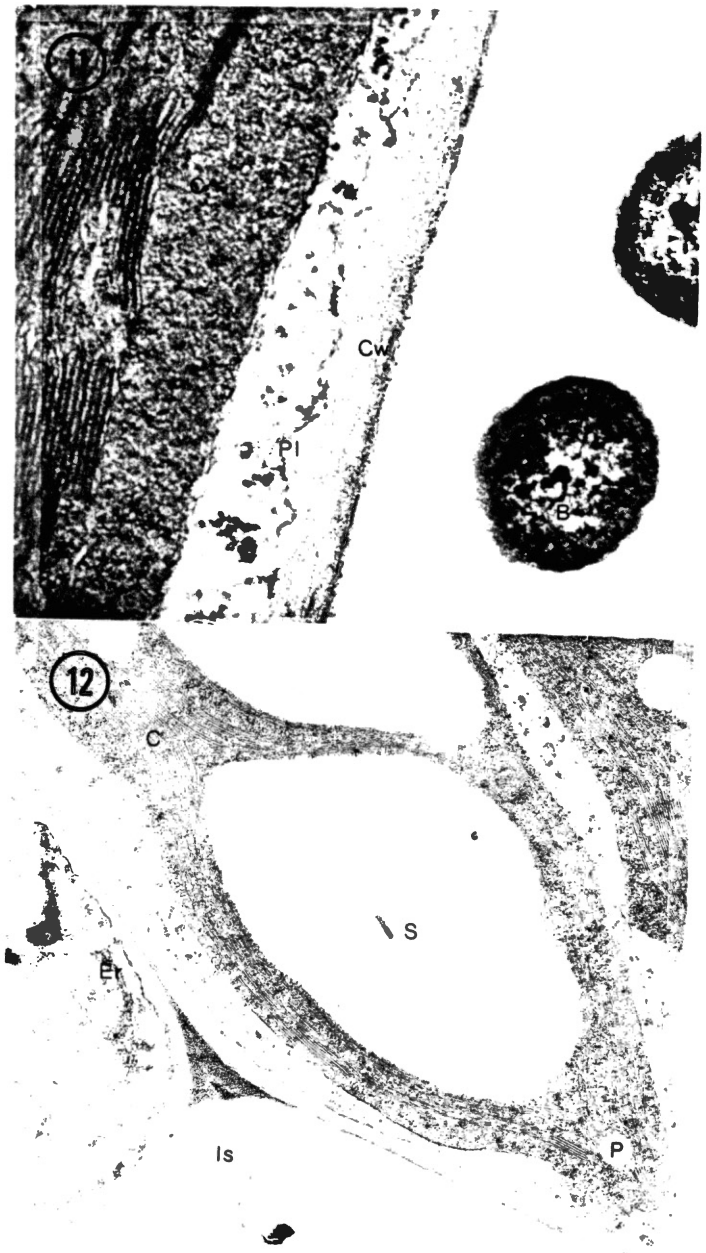


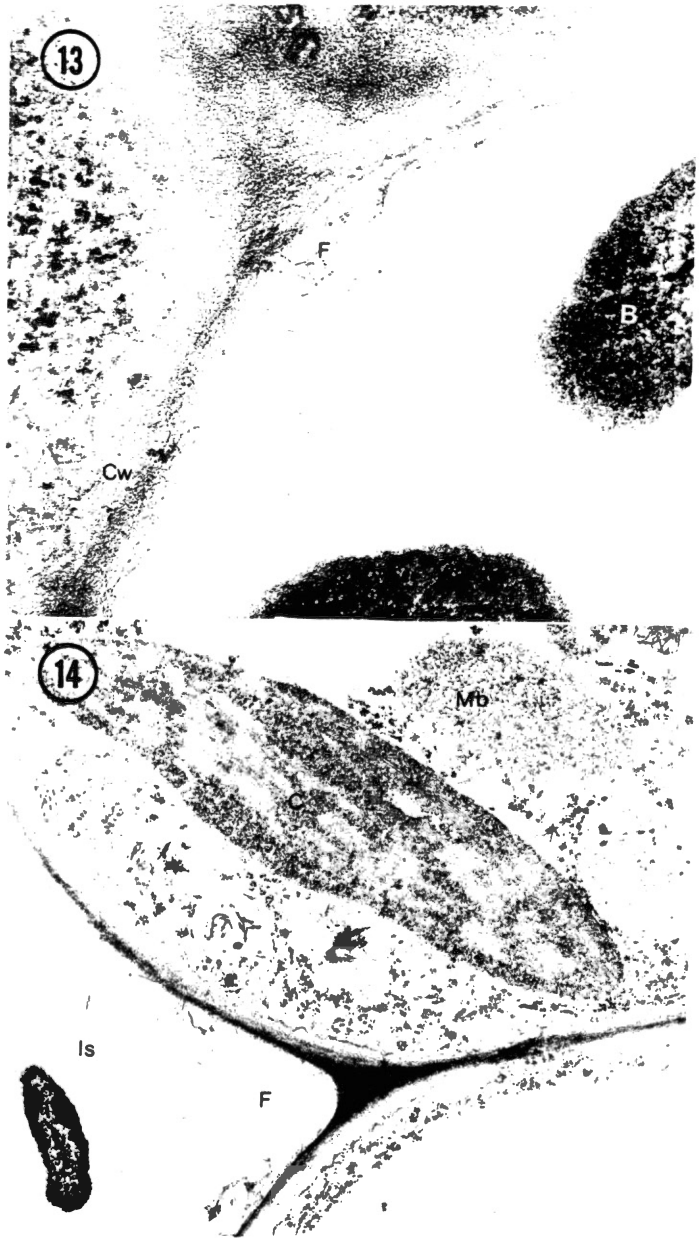

3
AB
4
AB

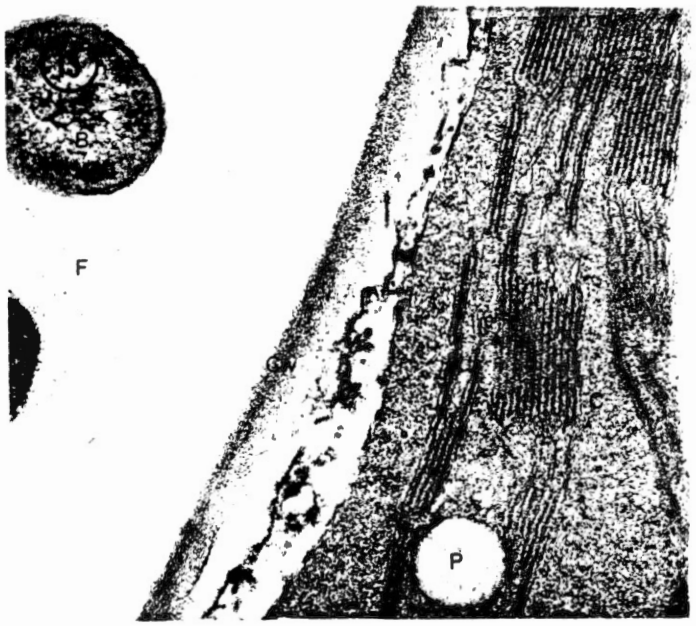

5
E
PP
Is
6
Is


7
AB
Cc
8
PP
CW

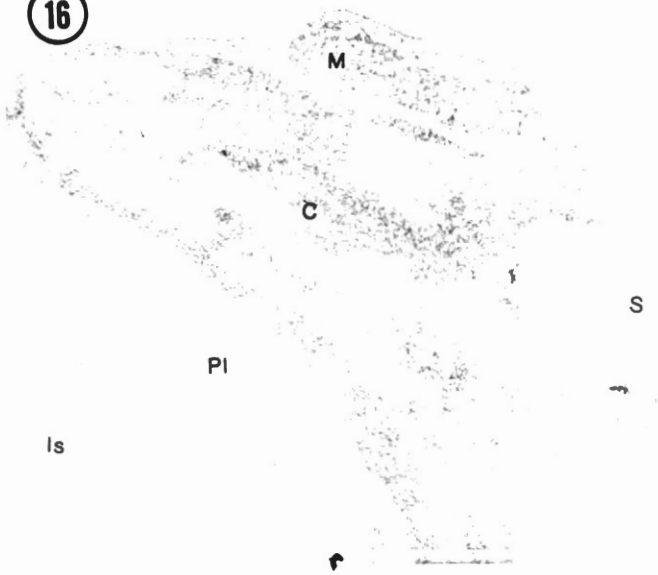

9
PP
10
E
Cc
Is

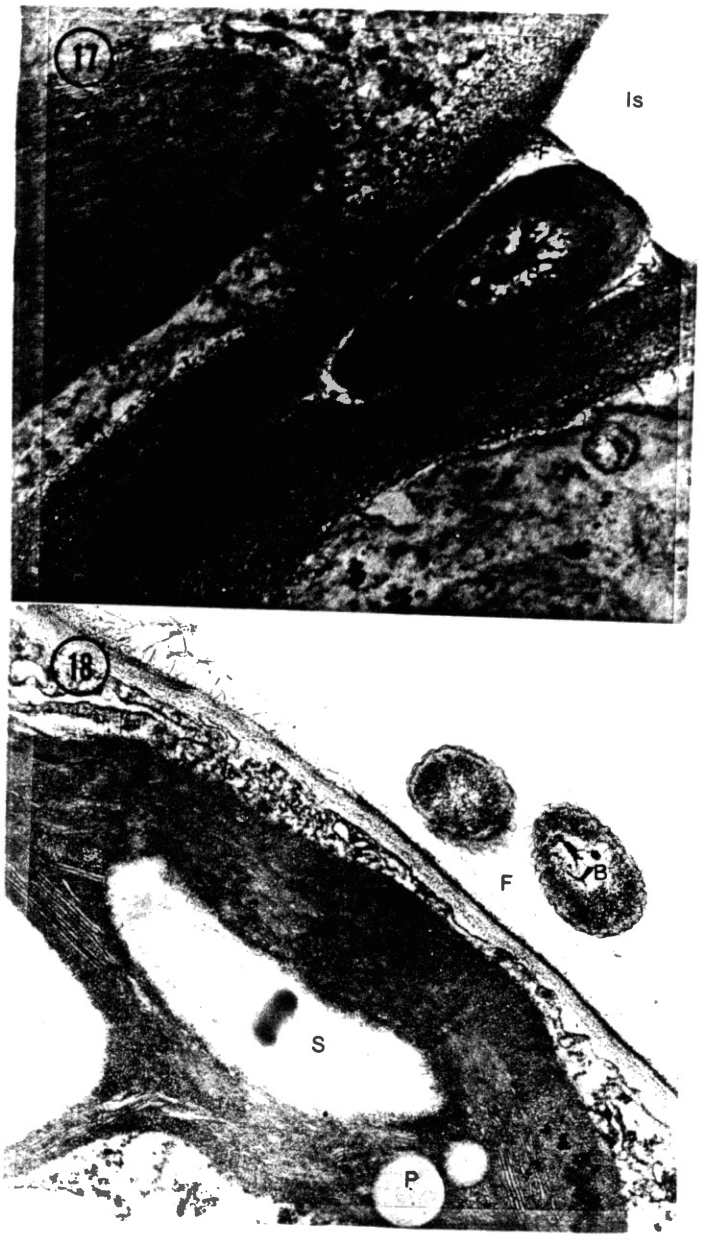

12
Cw
Pl
B
12
C
S
Er
Is
P

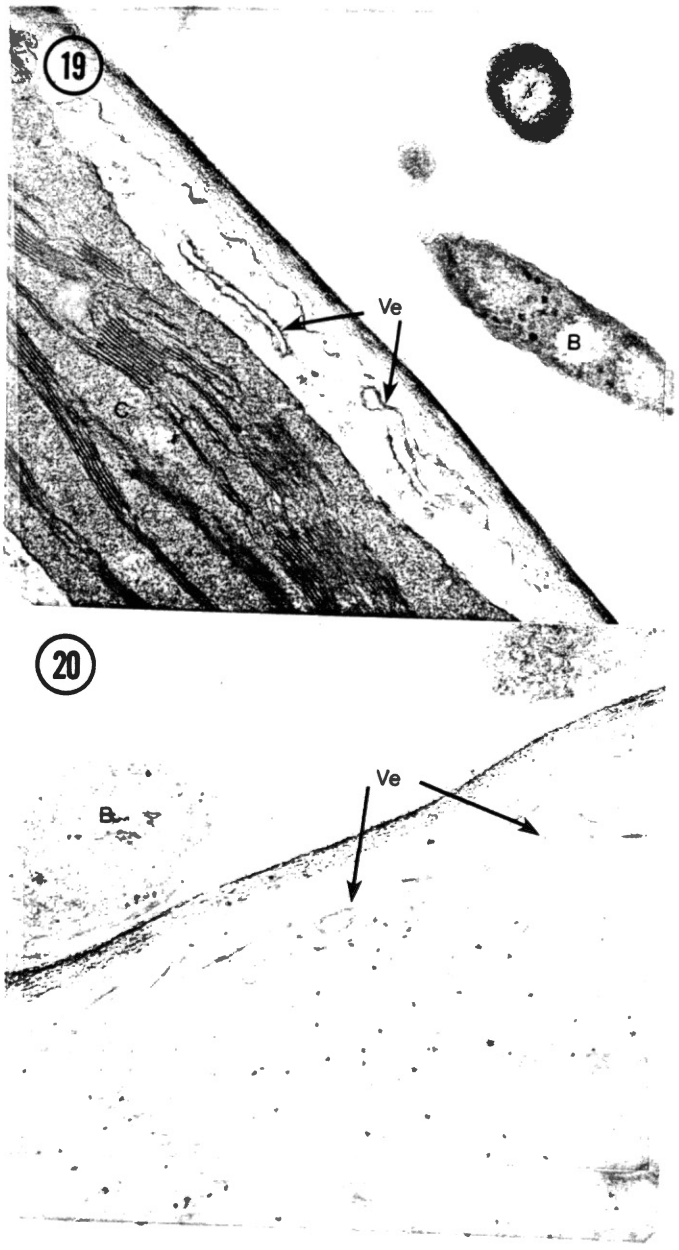

13
F
B
Cw
14
Mb
C
Is
F

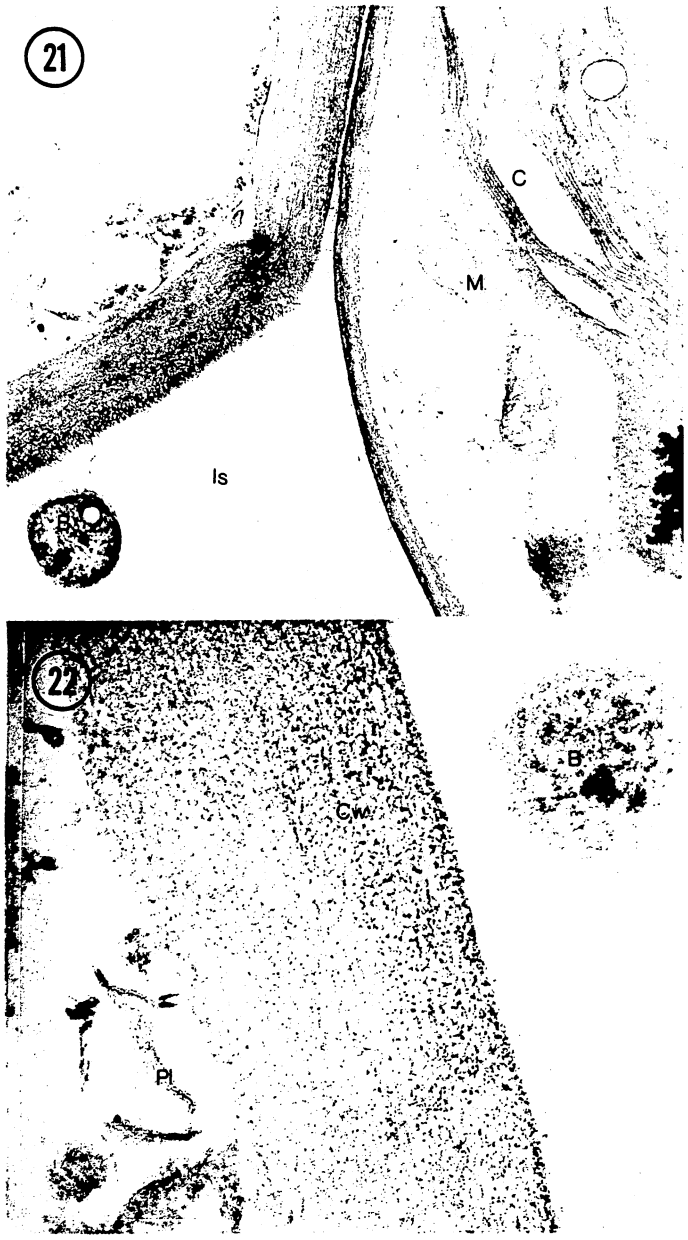


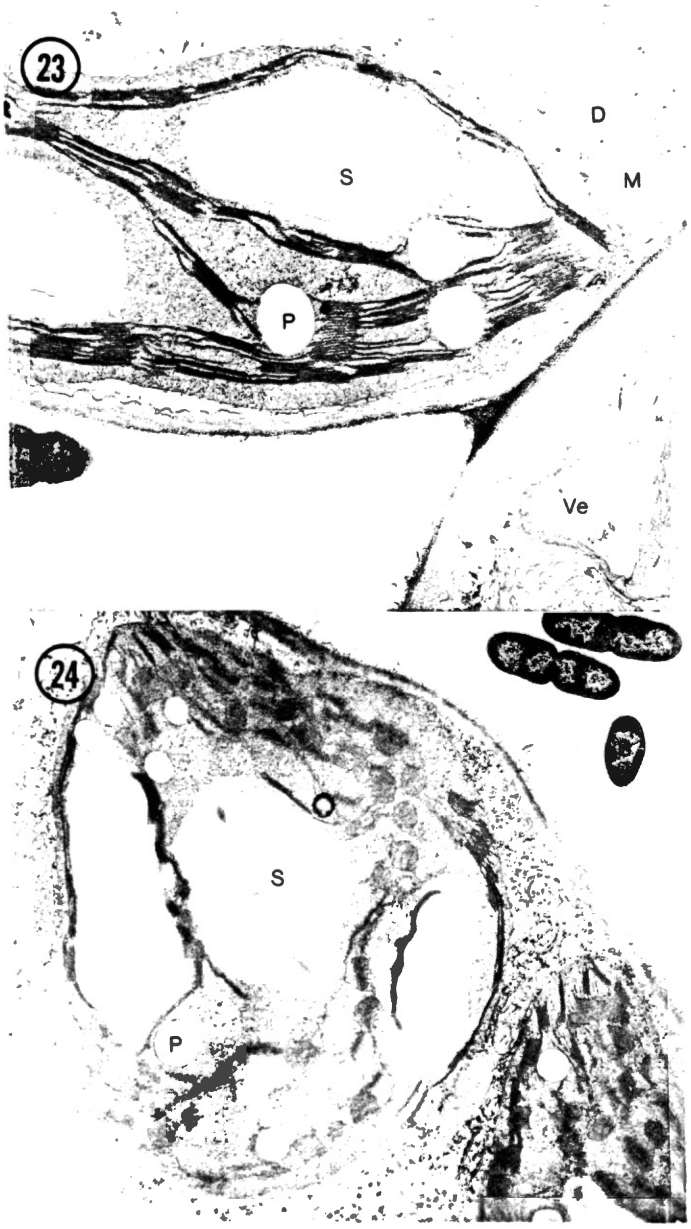
16

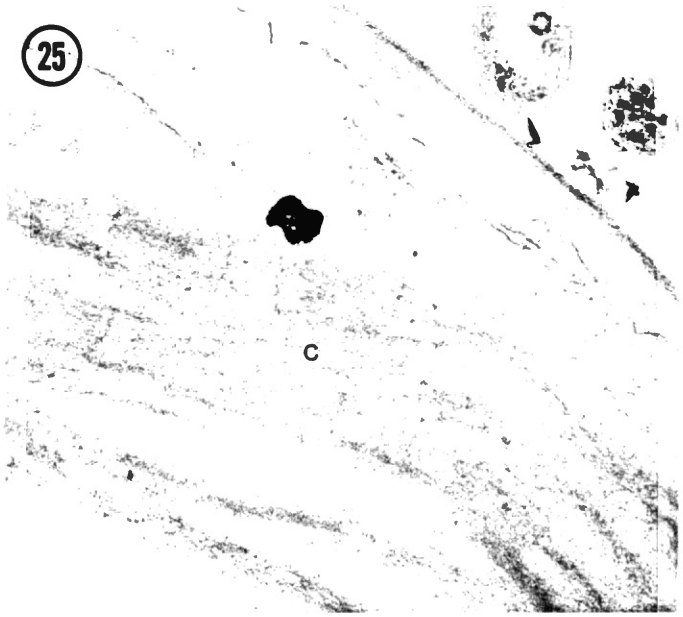


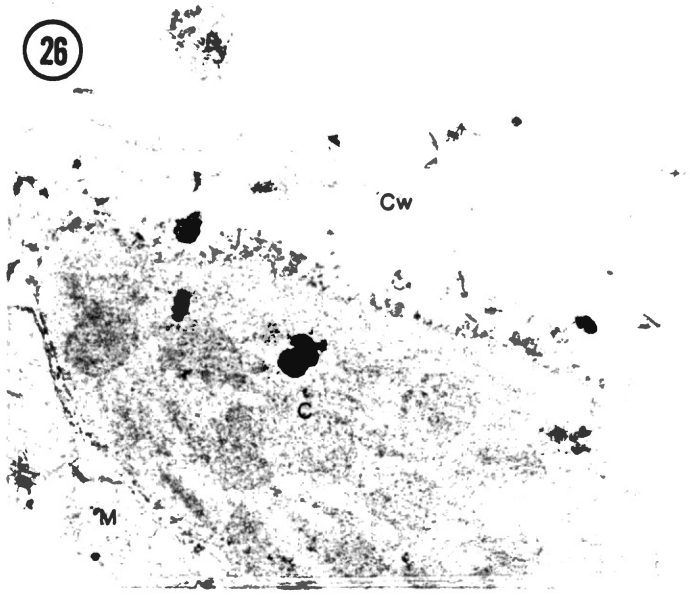

17
Is
18
F
B
S
P

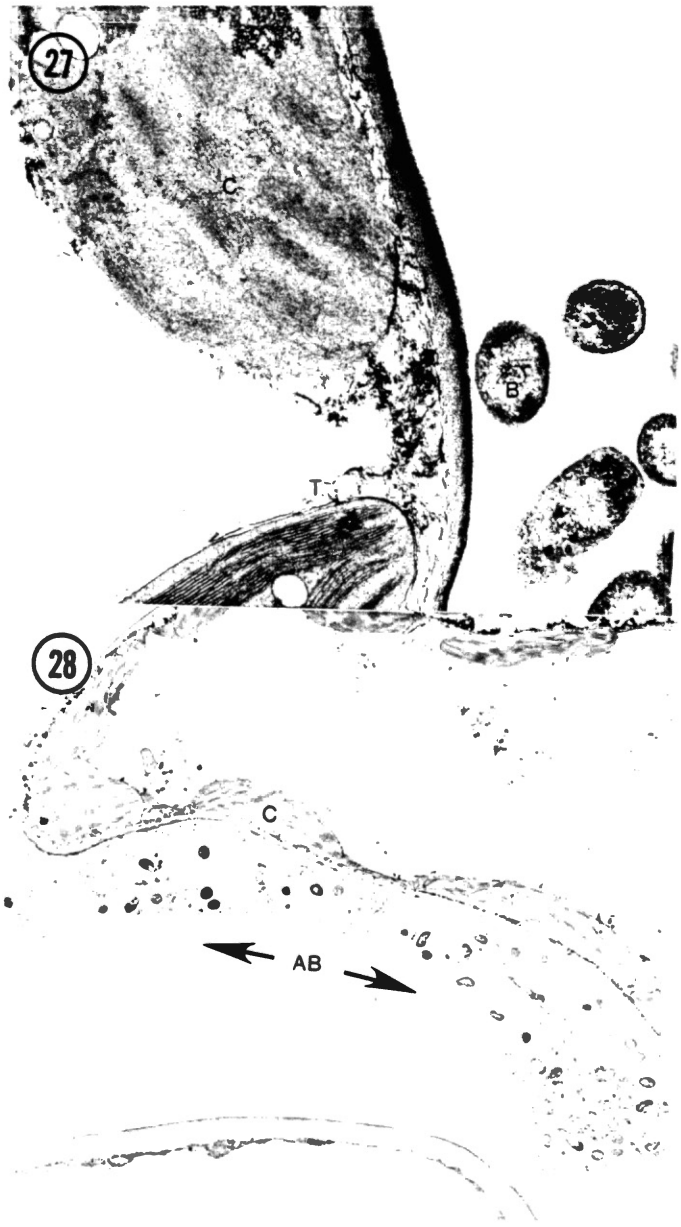

19
Ve
B
C
20
Ve
B

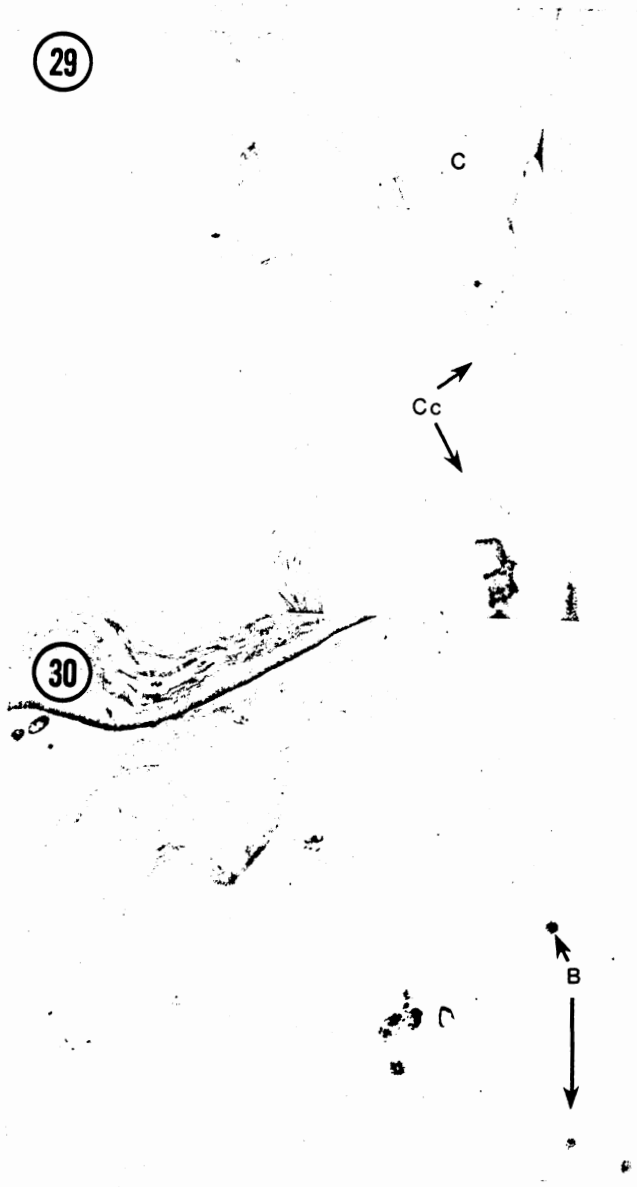

21
C
M
Is
B
22
B
Cw
Pl


23
D
S
M
P
Ve
24
S
P


25
C


26
Cw
C
M


27
C
B
T
28
C
AB

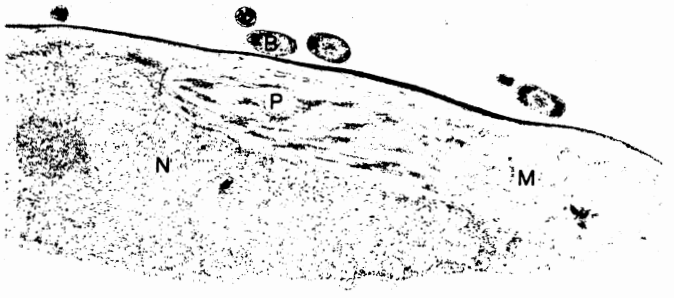

29
C
Cc
30
B


31
C
C
B
32
C
P
B
Ve


33
B
CW
C
34
M
CW
C
B

35

36



VITA

Paul Wilm

Candidate for the degree of

Master of Science

Thesis: CALCIUM NUTRITION OF A BLIGHT-SUSCEPTIBLE COTTON AND ITS EFFECTS ON INTERACTIONS WITH XANTHOMONAS CAMPESTRIS PV. MALVACEARUM.

Major field: Botany

Biographical:

Personal Data: Born in Chicago, Illinois, August 25, 1964, the son of M.F. Wilm and J.F. Wilm.

Educational: Graduated from Niles West High School, Skokie, Illinois. June 1982; attended Blackburn College, Carlinville, Illinois; received the Bachelor of Arts degree from Blackburn College in 1986; completed requirements for the Master of Science degree at Oklahoma State University in December, 1988.

Professional Experience: Teaching assistant, Department of Botany, College of Arts and Science, Oklahoma State University, 1986-1988.