THE RELATIONSHIP OF CALCIUM TO THE ETIOLOGY OF PEANUT POD ROT

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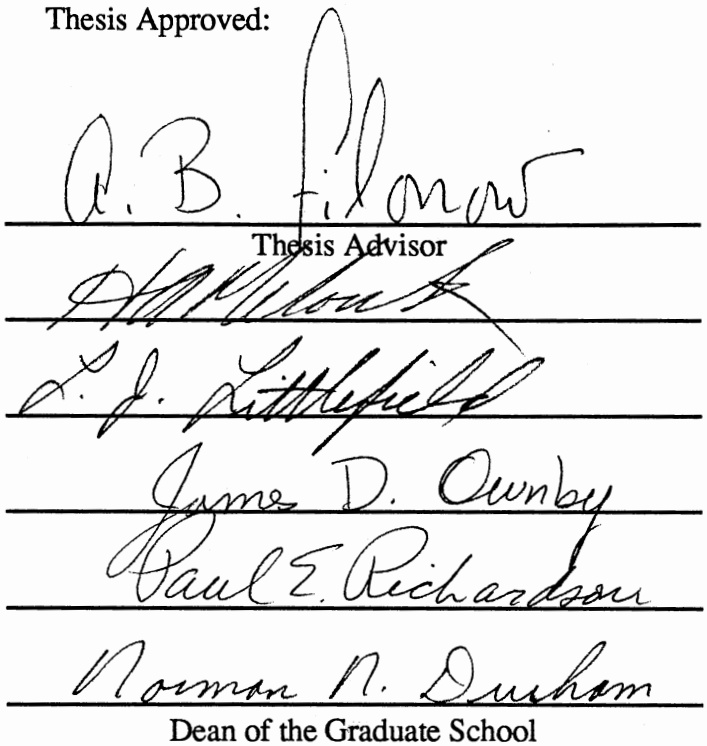

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PREFACE

The influence of calcium within the fruiting zone on the initiation of peanut pod rot was evaluated in laboratory, greenhouse and microplot studies. Peanut (Arachis hypogaea L.) plants were raised under gnotobiotic conditions to determine if a calcium deficiency alone can be considered a primary cause of the disease. The relative distribution of K, Mg, and Ca within the pericarp of fruit from the experiments was determined. Greenhouse and microplot studies were conducted to evaluate the effect of calcium sulfate in reducing pod rot in soil containing increasing populations of Pythium myriotylum. The relationship of calcium rates applied at early bloom to the calcium content of the pericarp and the level of disease was determined.

I wish to dedicate this entire thesis to the memory of my grandparents, Silvio and Arcangela Clemente. I would also like to acknowledge the support of my parents, Elmo and Teresa Clemente.

My sincere thanks to my major advisor, Dr. A. B. Filonow, for his unconditional support throughout my course of study at Oklahoma State University. I am also grateful to my other committee members, Dr. H. A. Melouk, Dr. L. J. Littlefield, Dr. J. D. Ownby and Dr. P.E. Richardson for their advisement on this project.

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Pod rot of peanut (Arachis hypogaea L.) is a very destructive disease often causing severe economic losses in Oklahoma annually. Symptoms include dark brown to black lesions on the pericarp surfaces of the fruits, which can lead to a total decay of tissue. The above ground vegetative portions of the plant usually remain unaffected and the disease can often go unnoticed until harvest. An essential part of any practical management program is a full understanding of the etiology of the disease trying to be controlled. The etiology of peanut pod rot remains obscure. During the past 25 years conflicting reports have appeared in the literature concerning the effect of a nutrient imbalance within the fruiting zone on disease incitement.

Many biotic pathogens have been implicated in playing a role in the final expression of disease symptomology [37]. The principal pathogens implicated in disease incitement have been reported to be the fungi Pythium myriotylum Drech [13], Fusarium solani Mart [13] and Rhizoctonia solani Kuhn [18]. Since these fungi may produce disease symptoms alone or in combination some researchers have referred to the disease as a disease complex [9,13]. These reports leave little doubt that biotic plant pathogens play a role in the etiology of the disease. However, peanuts treated in the field with soil calcium amendments, such as gypsum, lime or dolomite, have been reported to have less fruit decay, whereas potassium sulfate or magnesium sulfate tended to increase fruit decay [8,9,23,24]. These reports suggest an abiotic aspect in the etiology of peanut pod rot.

The unique fruiting habit of peanut makes the plant's developing fruits very

susceptible to localized calcium deficiency disorders. Calcium translocation within plant systems is primarily via xylem vessels, with limited mobility through phloem tissue [31]. Once the developing pegs go subterranean, they cease to uptake root absorbed xylem sap. Thus, the calcium supply from the root system is halted, and the developing fruit must take up its needed calcium requirement directly from the local geocarposphere soil solution [4,6,7,44]. Since the fruits are relatively ineffecient absorbers of calcium, ample supplies of calcium must be maintained within the upper 10 cm of the fruiting zone during the early stages of development [37].

Gypsum ($CaSO_4 \cdot H_2O$) has long been used as a calcium soil amendment by peanut growers to raise the level of fruiting zone calcium, and its use has been associated with increased quality and yields of fruit [1,41,48]. Garren [17] was the first to report that an increase in fruiting zone calcium resulted in a consistent reduction in pod rot. In a later study conducted by Hallock and Garren [24], the data were inconsistent over a three year period, but generally, fruits with less than 0.20% calcium content of the pericarp appeared more susceptible to pod rotting pathogens.

To evaluate the relationship between gypsum and pod rot, Walker and Csinos [49] performed a series of experiments from 1977-1979, in which gypsum was applied as a top dressing during early bloom at increasing rates up to 1,680 Kg/ha. Their data indicated an inverse relationship between gypsum levels applied and incidence of peanut pod rot. In another study Csinos and Gaines [8] suggested that increased magnesium and potassium concentration in the fruiting zone aggravated the disease by their competitive effect on calcium uptake, inciting a localized calcium deficiency in the pericarp. Gypsum added to the soil increased calcium in the fruiting zone, reducing the disease. They suggested that the disease was similiar to known calcium deficiency disorders, such as the blossom-end rot of fruits[10], bitter pit of apple [40], and black heart of celery [43], with the exception that the subterranean fruiting habit of peanut

makes it very susceptible to colonization by fungi and other microorganisms. Thus, fungal pathogens may take advantage of a decrease in cell wall integrity due to a localized calcium deficiency, resulting in greater invasion of fruit tissue.

Evidence from Oklahoma indicates no relationship between gypsum levels and peanut pod rot. Filonow et al.[12] found no significant decrease in pod rot with gypsum rates of 1,120 kg/ha - 3,360 kg/ha even though gypsum application significantly increased pericarp and kernel calcium content of the fruit. Moore and Wills [35] in a series of greenhouse studies found no relationship between calcium levels applied and susceptibility of fruits to both P. myriotylum and R. solani. The researchers however, failed to determine the calcium content of the fruits and its relationship to the susceptibility to these pathogens.

Thus, there is some uncertainty to the influence of calcium on the etiology of peanut pod rot. Therefore, the purpose of this thesis was to investigate the relationship of calcium to the etiology of peanut pod rot by determining the following:

1) if a calcium deficiency in gnotobiotically grown peanut pods can incite pod rot symptoms. The content and relative distribution of Ca, Mg, and K within pericarp tissue of the pods will be determined.
2) if the failure of gypsum to reduce pod rot in soil infested with P. myriotylum may be related to the inoculum density of the fungus.

CHAPTER II

LITERATURE REVIEW

Relationship of Biotic Factors to Incidence of Peanut Pod Rot

Pod rot is a very destructive disease of peanut which can vary in symptomology from slight lesion development on the pericarp surface to a complete decay of the fruit. The above ground vegetative parts usually remain symptom free and the disease can often go unnoticed until harvest. In Oklahoma an estimated 40% of the peanut fields sampled in 1985 had pod rot ; within these fields disease incidence ranged from 5.0-36.7% [Filonow, unpublished]. Several soilborne fungal pathogens have been reported to induce pod rot, including Pythium myriotylum Drech. [13], Rhizoctonia solani Kuhn [18] , Fusarium solani Mart [37] and Sclerotium rolsii Sacc.. In addition, plant parasitic nematodes [15,37] and soilborne mites [42] have also been implicated in playing a role in the final expression of disease symptomology. Although many of these organisms have been reported to cause disease symptoms by themselves, there have been reports to suggest combinations of these pathogens as cofactors in the etiology of the disease [14,18].

P. myriotylum is a fungus of the class oomycetes. It grows best in warm, moist soil. It is a major pathogen of peanut and is capable of causing pre- and post-emergence damping-off in addition to causing pod rot [37]. It attacks both peg and developing fruit throughout the growing season and is often isolated from rotted pods. Porter et al [39] reported about 80% isolation of P. myriotylum from rotted fruit in a field with a history of pod rot. In the peanut production areas of Oklahoma, P. myriotylum is frequently found in soils and may be one of the limiting factors influencing yields.

R. solani (anastomosis group 4) has also been reported to cause pod rot [37]. Garren [18] demonstrated that R. solani produced symptoms on peanut fruits indistinguishable from those induced by P. myriotylum , although symptom development occurred at a much slower rate with a R. solani infection. He suggested that competition between P. myriotylum and R. solani for infection sites may occur and and that P. myriotylum was favored under certain conditions. Isolations of two or more potential pathogens from the same rotted fruit is not uncommon, suggesting a complex etiology in the field.

R. solani and P. myriotylum are considered the most important biotic factors in the etiology [12,17,37,39]. In fields where both fungi are present isolation of one fungus preferentially over the other was influenced by the sequence of colonization on the fruit, presence of antagonistic organisms in the soil and other environmental conditions [16]. The influence of antagonistic organisms on the survival of P. myriotylum in soil was demonstrated by Garren [19] who observed a increase in P. myriotylum survival in soils in which the fungus was not indigenous. He hypothesized that soils native to P. myriotylum may contain antagonistic microbes that decreased P. myriotylum's saprophytic ability to persist. Pythium has been regarded as a latent endophyte of peanut fruit and Pythium spp. are commonly associated with apparently healthy fruit.

Frank [14] showed that inoculation of plants with F. solani, followed by a subsequent inoculation with P. myriotylum resulted in a significant increase in fruit decay compared to P. myriotylum alone. He suggested that Fusarium spp. predisposed the fruit to greater infection by P. myriotylum. Also, an interaction between the mycophagus mite, Caloglyphus micheali and P. myriotylum has been reported[42]. The mite was attracted to cultures of P. myriotylum preferentially over five other fungi isolated from rotted fruit. Oospores extracted from fecal pellets of the mite remained viable after passing through the alimentary canal, suggesting the mite may influence the spread of pod rot.

S. rolsii [37], and Sclerotina sp. [37] also can cause pod rot. However, these fungi produce visible sclerotia within the infested fruit differentiating their infection from that caused by P. myriotylum or R. solani.

Relationship of Calcium to the Incidence of Peanut Pod Rot.

Garren [17] was the first to report that increased levels of available calcium within the fruiting zone of peanut resulted in a consistent reduction of pod rot. There are several reports in the literature on the influence of calcium on this disease which confirm the suppressive influence of gypsum on disease [8,9,20,49]. A lack of sufficient calcium within the fruiting zone can lead to decreased calcium content within the pericarp tissue [9], resulting in a decrease in cell wall integrity of the tissue layers composing the pericarp and rendering the fruit more susceptible to a potential pathogen. This hypothesis is supported by the work of Bateman [2,3]. He demonstrated that increased levels of calcium increased resistance of been hypocotyl tissue to infection by R. solani. This increase in resistance was associated with the increased formation of calcium pectate moieties that were not utilized by the enzyme, polygalacturonase, and therefore decreased the ability of R. solani to ingress hypocotyl tissue.

A second hypothesis suggests that the activity of the pathogen can be influenced by calcium. A direct effect of the cation on the reproduction of Pythium sp. was reported by Yang and Mitchell [50], who found that calcium ions were essential for the in vitro formation of the oogonial wall . Hallock and Garren suggested [24] that the ionic balance in the soil may act on the life cycle of the fungus. Higher levels of calcium may stimulate sexual reproductive stages and would cause a subsequent decrease in energy available for the pathogenetic activities. A more indirect effect of calcium on the activity of Pythium within soil was reported by Kao and Ko [27], who suggested that high calcium levels and high microbial activity are two factors required for the induction of

soils suppressive to P. splendens in Hawaii. Soils conducive to P. splendens activity were low in calcium and microbial activity. Suppressiveness could be induced in the conducive soils by the addition of calcium and microflora indigenous to the suppressive soil.

An increase in the incidence of pod rot due to the application of potassium or magnesium salts [8,23] to soil also suggests a nutritional component involved in the etiology of pod rot. Csinos et al. [8] reported that calcium content within the pericarp tissue was significantly correlated negatively with incidence of peanut pod rot and suggested that increased levels of potassium or magnesium in the geocarposphere may have a competitive effect on the uptake of calcium into the developing fruit. They further hypothesized that the initiation of disease was of a similar mechanism reported for the blossom-end rot of fruits and other known calcium deficiency disorders. In an earlier study, Hallock and Garren [24] reported a similar finding and showed that pericarps with <0.20% calcium were more susceptible to pod rot than those with a greater calcium content.

Contradictory to these reports Moore and Wills [35] found no correlation between rates of calcium used and susceptibility to either P. myriotylum or R. solani incited pod rot. These workers suggested that under certain environmental conditions calcium may aid in the reduction of pod rot, but that calcium deficiency alone did not seem to be the primary factor involved in the susceptibility of the fruit to disease. Studies in Oklahoma over several years indicated no relationship between rates of gypsum applied to soil in greenhouse, microplot and field studies and the severity of peanut pod rot [12, Filonow unpublished]. Nor was there any linear correlation between calcium content in pericarps and pod rot severity.

Control of pod rot has proven difficult in some states, including Oklahoma. An overall pod rot management program, incorporating genetic resistance, cultural practices and timely fungicide sprays directed at appropriate pathogens would be the best

approach. However, an efficient management program will not be obtained until there is a better understanding of its etiology.

Calcium Translocation in Plants and Deficiency Symptoms

Calcium is a relatively abundant soil element and yet physiological plant disorders due to calcium deficiencies still occur [43]. The primary reason is due to the translocation of the element within plant systems. Many parameters are involved in the transmobility of the element and localized deficiencies are not uncommon . A holistic approach is sometimes necessary to effectively diagnose a possible calcium related disorder. The possibility of a plant pathogen inducing calcium deficiency-like symptoms should always be considered. Calcium translocation within plants is limited primarily to xylem tissue, with limited cell to cell or phloem mobility [31]. Calcium and possibly boron are the only two mineral nutrients of plants with functions primarily outside the cytosol in the apoplast [31]. Within the apoplast the majority of calcium is bound to carboxyl groups of polygalacturonic acid residues composing the middle lamella [25,32].

There have been over thirty physiological disorders associated with local deficiencies of calcium [43]. Symptoms on fruits range from cracking, water soaked areas to the formation of pits and cavities [45]. Under severe calcium stress meristematic and leaf tip necrosis may occur. These macroscopic symptoms are the result of microscopic alterations in plasmalemma permeability and cell wall integrity [45] of the affected areas. Symptoms are relatively unique as compared to other mineral deficiencies, but very similar symptoms can be induced by plant pathogens. For example, the soft rot bacteria can reduce tissue to a watery mass by the degradation of middle lamella areas through enzymatic activity [45]. The increase in electrolyte leakage triggered by the toxin victorin, produced by the fungus Helminthosporium victoriae, is thought to involve the sequestering of calcium ions from membranes, thus inducing a

localized deficiency.

Fruits with symptoms related to calcium deficiency have lower calcium concentrations than leaves of that same plant [45]. Why sufficient calcium concentrations can be found in the leaves while deficient amounts exist in the fruits can be explained by the transmobility of the element. Leaf tissues are supplied with calcium via the roots through xylem vessels. Once in the leaves calcium remains there and very rarely will be remobilized to another site within the plant [31,45]. Within developing fruits however, the supply of calcium via xylem slows down as the fruit begins to enlarge due to a decrease in transpiration and subsequent increase in phloem constituents flowing into the enlarging fruit [45]. Therefore, as fruits develop the actual concentration of calcium will decrease, which can cause a localized calcium disorder even under relatively high levels of soil calcium .

Environmental parameters can influence disorders associated with calcium deficiencies [43]. Both low and high soil moisture have been shown to aggravate blossom-end rot of tomato [46]. High light intensity tends to increase the incidence of calcium disorders in lettuce and oranges [43].

The ionic balance within the soil solution has a well documented influence on calcium disorders [43,46]. Nitrogen in the form of ammonium ions has an adverse effect on calcium by two primary mechanisms. First, with increasing applications of ammonium salts, the pH of the soil solution will drop causing calcium to be readily leached and secondly, there is a direct competition between the ions for uptake into the plant [43]. Potassium and magnesium also have been shown to aggravate calcium disorders [43]. The ratio of these elements to calcium has been shown to increase in blossom-end rot of fruits [10].

A calcium deficiency disorder usually can not be solely corrected by the addition of a calcium soil amendment. Other parameters such as concentrations of other cations in the soil solution, soil pH, moisture, humidity and the requirements of the particular crop

being grown, must all be considered before incorporating any corrective cultural practice. Since many calcium deficiency disorders can be very localized, analysis of entire fruits may be deceiving.

Translocation of Calcium in Peanut and Influence of Calcium on Yield and Quality: The unique fruiting habit of peanut makes the developing fruit of the plant very susceptible to calcium deficiency. Once the elongating peg penetrates the soil surface, the developing fruit undergoes a rapid decrease in transpiration rate, causing a decrease in calcium influx [45]. Under acidic soils in the U.S., calcium is the most limiting nutritional factor influencing yields [37]. The peanut essentially has two modes of uptake for required calcium nutrition, via the root system for vegetative parts and direct uptake through pericarp tissue in the developing fruit. Unlike other developing fruits, which usually serve in a total sink action , the peanut fruit also has some root like action. Calcium labeling studies have demonstrated this phenomenon [6,7]. Beringer and Taha [4] demonstrated the ability of calcium to be directly absorbed from the local geocarposphere into the developing fruits, and that this auxiliary mode of calcium uptake was necessary for proper fruit development. Skelton and Shear [44] demonstrated that calcium supplied to the rooting zone would not translocate at sufficient rates for proper fruit development. Conversely, Chahal and Virmani [7] found that 88.3% of radioactive calcium absorbed through the fruits remained in the fruit and only a trace could be detected in the vegetative parts.

Cultivars of peanut differ in their ability to take up calcium through the developing fruit . Symptoms of a deficiency in fruits include development of 'pops' (fruits with shriveled or aborted seeds), healthy appearing kernels with depression in center of cotyledons and/or blackness of embryo [37]. Cultivar differences for calcium requirements in developing peanut fruit have been shown [28, 47,48,49]. Small seeded Spanish type cultivars were less susceptible to calcium related disorders than Bunch type

cultivars which are prone to calcium disorders. Boote et al. [5] have implicated the surface to volume ratio of the fruit as a factor influencing fruit calcium concentration. They argued that to reach equal fruit calcium concentration of the small seeded variety, the large seeded variety must transport more calcium through a larger unit area of fruit surface during the calcium uptake period from initial pegging (R2 reproductive stage) to full seed, (R6 stage). Mizuno [34] found that 8% of calcium concentration of the final fruit was obtained between 0 and 20 days after penetration of peg into soil, whereas between 20 and 30 days after peg penetration, 69% of the final calcium concentration was reached. The final calcium concentration was reached by 80 days after peg penetration. At 30 days after peg penetration 91% of the calcium in the fruit was found in the pericarp and this slowly decreased to 44% at harvest. This translocation of calcium from the pericarp into the developing kernels was demonstrated to be more efficient in the smaller seeded varieties and was hypothesized to play a role in susceptibility to calcium related disorders [4].

Other factors that influence calcium concentration in peanut fruits include pericarp thickness and inherent cation exchange capacity and soil temperature, which can influence the respiration of the fruit, thus indirectly influencing calcium uptake [29].

CHAPTER III

MATERIALS AND METHODS

Inducement of a Calcium Deficiency in Peanut Fruit Grown in Gnotobiotic Conditions

All experiments were done in a plastic isolator purchased from Standard Safety Inc. (Pallatine, IL; Figure 1). The 4 mil thick plastic isolator (1.54 X 0.6 X 0.8 m) was fitted with rubber sleeve gloves, a cylindrical (30.0 cm in diameter X 30.0 cm) stainless steel entry port and inlet and outlet filters. Positive air pressure from an electric blower passed through the inlet filter into the isolator, keeping it inflated. The inlet filter and outlet filters consisted of stainless steel supports wrapped with several layers of fiberglass and enclosed in plastic sleeves. Filters were autoclaved for 2 hours prior to fitting them to the isolator. Two weeks prior to use the entire isolator was fumigated with 250 ml of 4% (v/v) peracetic acid in water [21] applied with a compressed air sprayer.

All necessary supplies were aseptically brought into the isolator by a process outlined by Hale [21,22], using a 4% peracetic acid solution as the sterilizing agent. A 30.0 cm dia. X 60.0 cm stainless steel sterilizing cylinder (Figure 2), with holes in the cylinder, was wrapped with 3 layers of cotton and a top layer of cheesecloth. A fine mesh screen was fastened with two large pipe clamps to secure the filtering material around the cylinder. Supplies were placed inside the cylinder, and the open end of the cylinder was covered with a mylar film and sealed with mylar tape. The entire cylinder was placed in an autoclave for sterilization. Liquid materials were sterilized for 1.5 hours and all soils were sterilized for 5.5 hours on three successive days with the last

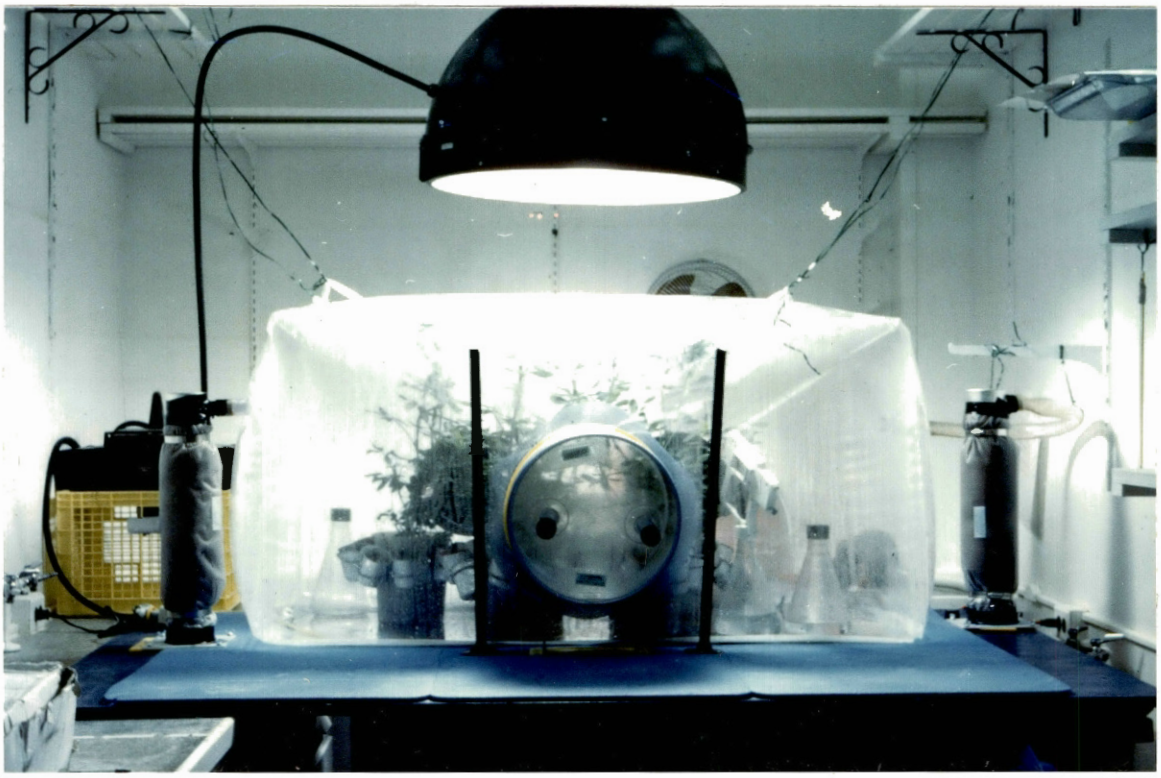


Figure 1: The Isolator Used for the Two Gnotobiotic Experiments

Figure 2: Sterilization Cylinder Used to AsepticallyTransfer Supplies Into the Isolator.*

*All supplies were placed within the cylinder and the open end was subsequently wrapped with a mylar film. The entire cylinder was then autoclaved. The cylinder was then fastened to the entry port via a plastic sleeve, which was filled with peracetic acid.

2.0 hours within the cylinder. After autoclaving, the sterilization cylinder was fitted to the entry port of the isolator via a 30 cm dia X 86 cm plastic sleeve. The sleeve was then filled with 30 ml of peracetic acid solution through the sprayer. Two days after spraying, the inside door of the entry port was opened and large stainless steel tongs were used to rupture the mylar film and bring in materials.

The cultivar Early Bunch was used in all gnotobiotic experiments. Seed with coats intact were surfaced disinfested under UV light for three days. Embryo rescues were then preformed on the seeds. The embryos were placed on an agar medium containing the macronutrients of Linsmaier/ Skoog [30] and the micronutrients of Murashige/Skoog [36] with no growth regulators. Embryos were allowed to grow aseptically for 18-20 days on the medium within magenta boxes placed in a growth chamber at 25^{o} C and with a 16/8 hour light/dark regime. Plantlets were brought into the sterilized isolator by spraying each magenta box with peracetic acid and placing them directly inside the entry port. The entry port would then be filled with peracetic acid. After 2 hours the inside door of the entry port was opened and the magenta boxes were brought in. The lids of the magenta boxes were partially removed after 2 days in the isolator, after which the lids were removed for 5 hours on 3 successive days to acclimate the plantlets to the rapid decrease in humidity from the boxes to the interior of the isolator. If this was not done in a timely manner severe necrosis occurred on the young tissue.

Four plants were grown within the isolator for each experiment. Each plantlet was transplanted into a glass cylinder (3.7 cm X 12.5 cm) which was placed centrally into a 2,000 cc polypropylene beaker. Both the cylinder and the beaker contained deionized-water-washed sand/ vermiculite mix (1:1 v/v). The cylinder served as a rooting zone for the developing plant elevating the crown of the plant about 7.5 cm above the beaker. This allowed the pegs to be easily placed into fruiting zones. At the early pegging stage, four 100 cc polypropylene beakers per plant, filled with acid-washed sand/vermiculite mix, served as the fruiting zones for the developing pods.

These were arranged around the glass cylinder and were inside the 2,000 cc beaker. Fruiting zone beakers were color coded and wrapped in aluminum foil to prevent light from penetrating the fruiting zone (Figure 3). The beakers received one of the following treatments, 0 Kg/ha, 280 Kg/ha, 560 Kg/ha or a 1,120 Kg/ha calcium equivalent of gypsum added as anhydrous calcium sulfate in a full strength Hoagland's solution [26] minus the calcium nitrate component.

At transplanting, the sand/vermiculite mix in the rooting zone was saturated with half-strength Hoagland's solution. Thereafter, rooting zones were watered daily with 75-100 ml of distilled water and every other day with half-strength Hoagland's solution. On the days that the nutrient solution was added to the rooting zone 25-30 ml of distilled water were added to the glass cylinder portion of the rooting zone to prevent splashing of the nutrient solution into the fruiting zone beakers. Fruiting zones received 10-15 ml of distilled water every five days. Lighting was supplied by a metal halide lamp which produced light intensities of approximately 500 uE/m^2/sec at plant height within the isolator. The light regime was 16/8 hour light/dark cycle. Day time temperatures were approximately 32^o C and night temperatures approximately 25^o C.

Throughout the experiments sterility checks were conducted once every two weeks by taking 40 random swabs within the isolator, plating the swabs on potato dextrose agar and nutrient agar, and incubating the plates for 10 days at 30^o C. At the completion of the experiment, portions of stem, root and soil samples and additional swabs from the isolator surfaces were plated on six different media in order to detect a broader spectrum of microorganisms. Agar media included potato dextrose, nutrient, Maconkey's, Sabaroud's, brain heart infusion, and tryptic soy broth. Plates were incubated at 30^o C for 10 days.

At harvest fruits were visibly inspected for pod rot like symptoms. Dry weights of the pods were determined. Two pods from each replication were randomly selected. Portions of the pericarps along with soil samples from the fruiting zones were sent to an

Figure 3: Experimental Design of the Gnotobiotic Experiment*

*The 100 cc beakers were filled with a acid-washed sand/vermiculite (1:1 v/v) mix. Each plant had all calcium treatments within the same rooting zone.

analytical laboratory (Servi-Tech Inc. Dodge City, KA) for atomic absorption analysis for total calcium, magnesium and potassium content. The remaining portions of the pericarps from the two pods were saved for scanning electron microscopy x-ray microanalysis.

Calcium, Potassium and Magnesium Content
and Distribution in the Pericarp

The content and relative distribution of calcium, potassium and magnesium within the various layers of the pericarps were determined. Scanning electron microscopy (SEM) was done using a JOEL 35U SEM equipped with a Tracer Northern 1000 x-ray microanalyser.

SEM x-ray microanalysis was preformed on 10 air-dried subsamples of the pericarps. The subsamples were mounted on two carbon stubs with a carbon based paint and subsequently carbon coated. Among these 10 subsamples from each replication, 5 random readings were taken from the exocarp, sclerenchyma band and the remaining portions of the mesocarp tissue (avoiding any vascular bundles and the entire endocarp area). Therefore, a total of 20 readings per tissue type per treatment were taken for the experiment.

The regions of interest for the elements were 1.18-1.3 kiloelectron volts (KeV), 3.34-3.54 KeV and 3.55-3.7 KeV for magnesium, potassium and calcium, respectively. Net counts in each elemental spectrum were determined and a ratio of Ca/Mg+K counts was calculated.

Calcium Sulfate Effectiveness in Suppressing Pod Rot
Related to Populations of Pythium myriotylum: Greenhouse

The cultivar Early Bunch was chosen for greenhouse studies, as it has been shown to be very susceptible to pod rot , but responsive to soil calcium inputs and their

ameliorating effect on pod decay [8]. Pregerminated seeds were planted into 3.7 cm diameter PVC pipes filled with a sand/vermiculite mix (1:1 v/v). The PVC pipes were in the center of 22.5 cm diameter X 30 cm plastic pots filled with sand/vermiculite (1:1 v/v). The pipes served as the rooting zones for the plants and seeds were planted about 8.7 cm above the pot surface. Four plastic 250 ml cups were placed around the PVC pipe which served as the fruiting zone for the plants (Figure 4). As the pegs elongated they were trained into the fruiting zones and care was taken not to allow any pegs to develop inside the PVC pipe, which could impede translocation due to the excess pressure as the pod swells.

Treatments consisted of three levels of fungal inoculum and three levels of soil calcium within the fruiting zones. Treatments were added at the early pegging stage of the peanut development. All fruiting zones of a given plant received the same treatment. Inoculum densities of P. myriotylum were 0 propagules per gram of soil (p/g), 10 p/g and 50 p/g. Cultures of P. myriotylum were grown in aluminum oven pans (7.5 X 30 X 45 cm) lined with autoclave bags and filled to 3.7 cm in depth with a 5% (w/w) cornmeal/sand mix amended with a 30 ppm cholesterol solution [33]. The pans were wrapped in aluminum foil and put inside autoclave bags. Pans were autoclaved for one hour on two successive days. The medium was aseptically inoculated with plugs from a 2-3 day old culture of P. myriotylum. Pans were allowed to incubate at 24-26^{o} C for 21 days prior to use. At this point a thick mycilial mat completely covered the surface and was interwoven throughout the culture. The cornmeal/sand cultures were then diluted to the required inoculum densities by hand mixing with a steam pasteurized sand/vermiculite mix (1:1 v/v). Inoculum densities were estimated by spreading 1:20 and 1:200 dilutions on 5 plates of a Pythium selective medium (PSM) [11] and incubating them at 24-26^{o} C for 18-24 hours prior to reading the plates.

Calcium treatments consisted of the addition of 0 Kg/ha, 560 Kg/ha or 1,120 Kg/ha calcium equivalent of gypsum to the different inoculum densities being tested. Calcium

Figure 4: Geenhouse Experimental Design.*

*Fruiting zone cups were arranged around the interior of the rooting zone pots. Pegs were trained into the cups as they elongated. All cups within the same rooting zone received the same treatment.

was added as anhydrous calcium sulfate in a full strength Hoagland's solution minus the calcium nitrate component.

Rooting zones were watered once a day. Twice per week the water contained full strength Hoagland's solution. Fruiting zones were watered approximately every third day with 40-50 ml of de-ionized water. Malathion, Pentac, Kelthane and O-mite were used in rotation to control insects.

The experimental design was of a 3 X 3 factorial completely randomized with 4 replications. At maturity the plants were harvested, pods were washed and rated for pod rot severity according to the following: 1= a healthy pod, 2= 1-24% of pericarp surface decayed, 3= 25-49% of pericarp surface decayed, 4= 50-74% of pericarp surface decayed, and 5 greater than 75% of the pericarp surface decayed. For each replication, pods that fell in severity class 3, 4 and 5 were summed, and divided by the total number of pods in that replication. This fraction was termed the pod rot index, and served to evaluate disease response to treatments.

Portions of the pericarp samples from each replication were surfaced sterilized with a 15% bleach solution for 30 seconds and double rinsed in water. Five samples were placed on each of two plates of PSM and incubated at 37^{o} C for 16 hours. Dilutions of soil samples from each replication were plated on PSM to estimate populations of P. myriotylum in the fruiting zone, and samples from both soil and pericarps were sent to an analytical laboratory (Servi-Tech, Dodge City, KA) for calcium content determination by atomic absorption spectrophometry.

Calcium Sulfate Effectiveness in Suppressing Pod Rot Related to Populations of Pythium myriotylum in Soil: Microplots. The cultivars Spanco and Florunner were used in the microplot studies. These cultivars were chosen because they are extensively grown in Oklahoma, and Spanco was considered less susceptible than Florunner to disease. Microplots were located on the Plant Pathology Farm, Oklahoma State

University and were situated on bottom land (MP 1) or on a terrace (MP 2). MP 2 microplots were built of wooden planks (2.4 X 2.4 X 0.3 m) filled with sandy loam soil (72% sand, 12% silt and 16% clay: pH 6.6). MP 1 microplots were built of railroad ties (2.4 X 2.4 m) filled with a sandy loam soil (pH 7.6). Three weeks prior to planting the microplots were rototilled and fumigated with 0.45 Kg of methylbromide per box. Plots were allowed to aerate until planting. Each microplot had one row of each cultivar, with about 15 plants per row. Two terrace and one bottom land microplot experiments were conducted. MP 2 microplots were planted June 6, 1987 and May 21,1988. MP 1 microplots were planted May 26, 1988.

Treatments consisted of 3 inoculum densities of P. myriotylum and 3 concentrations of calcium. Inoculum of P. myriotylum was grown as described for greenhouse experiments. Cornmeal/sand cultures were mixed with steam pasteurized sand/topsoil (1:1 v/v) to give about 85 p/g, and this was diluted with soil from the microplots. Infestation was done by digging a 10 X 7.5 cm deep trench along each side of a row of peanut plants at flowering and filling the trenches with soils infested with 0, 10, and 50 propagules of P. myriotylum per gram of soil. Microplots were infested with P. myriotylum on July 27,1987 and July 17, 1988 (MP 2) and August 3, 1988 (MP 1). In the noninfested microplots trenches were dug and refilled with microplot soil. Both the rows within the same microplot recieved the same treatment. Microplots were irrigated (5 cm water) after infestation was completed. Calcium treatments were added 1-2 days after infestation as agricultural gypsum at rates of 0, 1,120 and 2,240 Kg/ha in a band over each row.

Microplots were irrigated once every 6-8 days if no precipitation occurred. Plots were hand tilled on a regular basis to control weeds and sprayed with malathion and liquid sevin to control thrips and the corn leaf borer, respectively. In 1988, an application of a 1% ferrous ammonium sulfate solution was applied to microplots in both locations to correct what appeared to be the onset of iron chlorosis.

Both cultivars were harvested October 17 in 1987. In 1988 Spanco was harvested October 11 and October 16 for the MP 2 and MP 1 microplots, respectively. Florunner was harvested October 23 and November 1 for the MP 2 and MP 1 microplots, respectively. Soil samples were collected along each row on three sampling dates after infestation. Two samples from the pegging zone of each row were randomly taken and composited, and populations of Pythium spp. in the soils were determined. Ten grams of soil were added to 90 ml of 0.1% water agar in flasks, and shaken for 30 minutes. Aliquots of 0.5 ml from two dilutions were then spread on five PSM plates. Plates were incubated at 24-26° C for 18-24 hours. The mean populations (p/g) of the two dilutions were averaged for an overall approximation of population.

At harvest pods were separated by cultivar and treatment replication, washed under running water, and rated for pod rot as described for the greenhouse experiments. A pod rot index was calculated as described above. Pods were randomly selected from disease severity classes 3, 4, and 5 and pericarp pieces were surfaced sterilized in 15% bleach solution for 30 seconds followed by a double rinse in water. Ten pieces from each cultivar replication were plated on two PSM plates and incubated at 37° C for 16 hours. Pericarp samples from each cultivar were randomly selected from the entire production for that replication, placed in coded coin envelopes, and sent to an analytical laboratory (Servi-Tech, Dodge City, KA) for atomic absorption analysis to determine calcium content.

The experimental design consisted of a 3 X 3 factorial arranged in a randomized complete block with 4 replications. All data were subjected to statistical analysis by analysis of variance and significant treatment effects were separated by orthogonal contrasts. Linear and parabolic trends were checked by orthogonal polynomials.

CHAPTER IV

RESULTS AND DISCUSSION

Inducement of a Calcium Deficiency in Peanut Fruit Grown in Gnotobiotic Conditions

Early gnotobiotic experiments were hampered by unforeseen problems associated with growing plants under such controlled environmental conditions. A first attempt was aborted due to low light intensities (a minimum of 350 uE/m^2/sec is required for the initiation of flowering [37]) and an unsatisfactory soil mix for the rooting zone. A mixture of perlite/soil/sand 1:1:1 v/v evidently produced toxic substances after the sterilization process, since the root systems of the plants were severely stunted with no apparent microbial activity detected. Microbial contamination was another problem that led to the abortion of two other experiments. Two experiments were carried out to completion, and they were designated gnotobiotic experiment one (G 1) and gnotobiotic experiment two (G 2).

The G 1 experiment was maintained essentially axenically. The sterility checks during the course of the experiment showed no detectable microbial growth. At the completion of the experiment, plant soil and additional swabs plated on six different media resulted in two colonies on tryptic soy broth agar and three colonies on brain heart infusion agar. At harvest a total of 2 out of 20 pods at the 0 Kg/ha calcium treatment and 1 out of 24 pods at the 280 Kg/ha were visible discolored. Discolored areas had a shiny appearance, ranging in surface area from a portion at the distal tip to approximately 75% of the pod. The discolored areas lacked the characteristic shredding and defined lesion areas associated with pod rot under field conditions. The induction of 'pops' (pods with

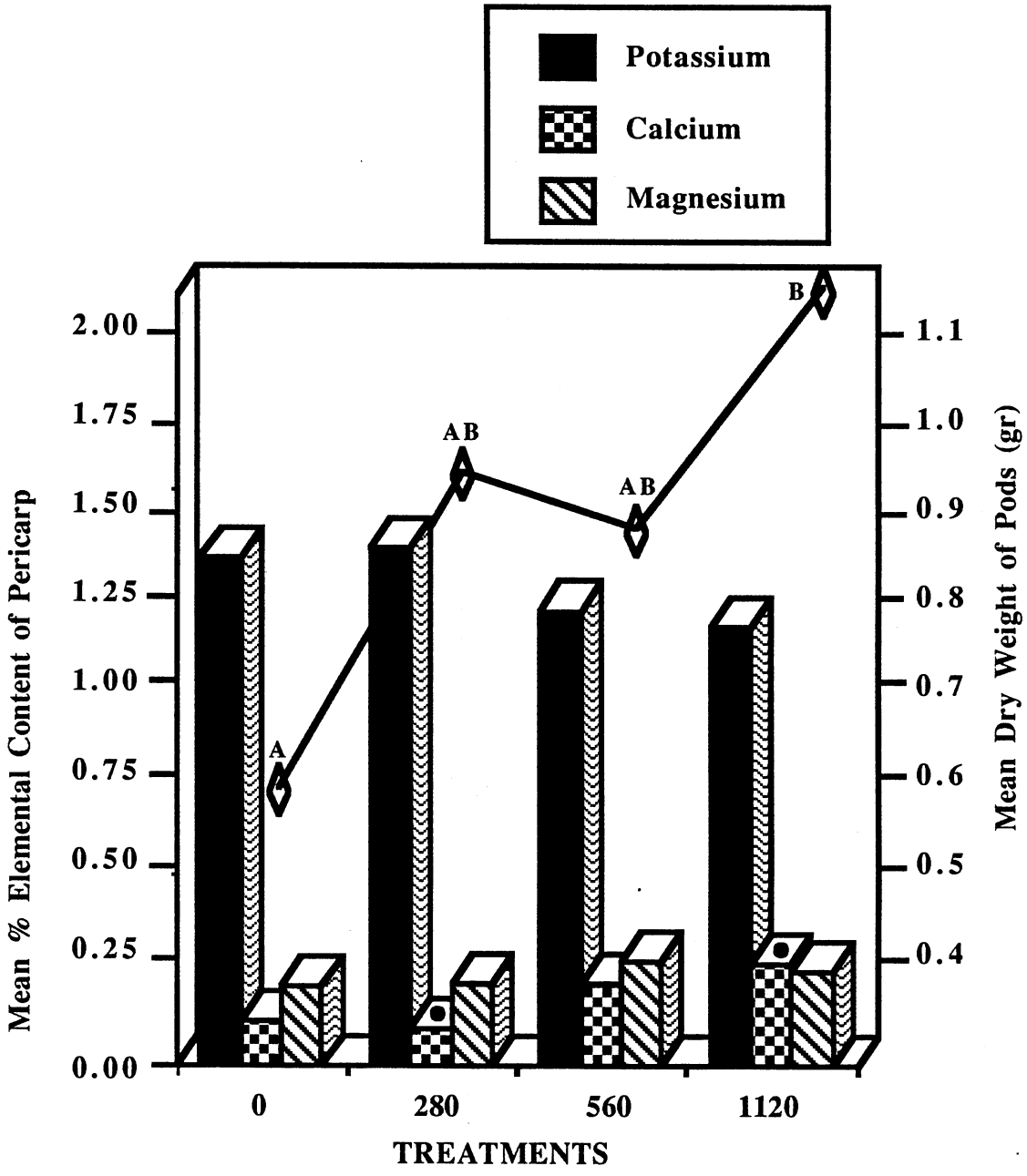
aborted or poorly filled seed) occurred at all levels of calcium in the fruiting zones; However, 'pops' occurred at a much higher frequency under the lower calcium levels. The increase in the occurence of 'pops', at the lower calcium treatments, was reflected in the significant increase in dry weight of the pods within the higher calcium treatments (Figure 5). All kernels were aborted in discolored pods. These observations demonstrated the influence of calcium stress on the developing peanut pod. Severe calcium stress caused an autolysis of pericarp tissue, while less severe calcium stress can caused 'pops' formation. Therefore, if peanut pod rot is induced by a similar mechanism as that reported for the blossom end rot of fruits, it would be likely that the majority of decayed pods would contain aborted or shrivelled kernels. In Oklahoma, this is rarely observed. The majority of mature pods that are discolored and/or decayed possess full kernels.

The mean percent Ca, Mg, K contents are shown in Figure 5. Potassium was found in greatest concentrations in the pericarps, and generally decreased with addition of greater calcium sulfate to soil. Calcium content in the pericarps ranged from .09% to .39% for the 0 to 1,120 Kg/ha treatments, respectively. Percent calcium in pericarps generally increased with higher soil calcium levels, but not all the increases were significant (P= .05). Calcium content in pericarps was positively correlated (r=0.67, P=.05) with added soil calcium. Magnesium content of the pericarps was also positively correlated (r=0.79, P=.05) with calcium content of pericarps. Under field conditions magnesium in the pericarps has been shown to be inversely correlated with pericarp calcium [9]. A plausible explanation for the latter atypical observation could be that a separation of the rooting and fruiting zones would allow Mg influx into the pod to be unaffected by the calcium treatments applied to the fruiting zones.

In a preliminary study, the SEM x-ray analysis of elements in peanut pericarps, indicated relative levels of Ca, K, and Mg comparable to the actual content as analyzed through atomic absorption. Further studies using SEM x-ray analysis followed only the

Figure 5: Mean Elemental Content of Pericarps and Dry Weight of Pods: Gnotobiotics 1.*

*The treatments are in calcium equivalent of gypsum, as anhydrous calcium sulfate in a full strength Hoagland's solution, in kilograms per hectare (Kg/ha) applied at early bloom. Both dry weights and elemental content (by atomic absorption) means were differentiated by Duncan's Multiple Range Test at P= .05. Mean dry weights with the same letter are not statistically different The only significant difference among the elemental contents occurred within the calcium content between the 280 Kg/ha and 1120 Kg/ha as indicated by the● . Neither the Mg nor the K levels fluctuated significantly.


Potassium
Calcium
Magnesium
Mean % Elemental Content of Pericarp
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Mean Dry Weight of Pods (gr)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
A
AB
AB
B
0
280
560
1120
TREATMENTS

ratio of Ca/Mg+K rather than levels of individual elements. This was done to reduce the inherent problem of fluctuations in beam current that occurred over time which influences spectrum height. For example, if the beam spot could be returned the following day to the identical area of a previous analysis, the peaks of elements may fluctuate in height, but all at equal levels. Therefore, by using this ratio the day to day beam current fluctuations that affect the individual peaks were eliminated.

Pericarp samples of G 1 exhibited a consistently greater Ca/Mg+K ratio within the sclerenchyma band compared to either the exocarp or the remaining portions of the mesocarp tissue (Figure 6). This was expected, considering that cells composing the sclerenchyma band have much thicker walls compared to those comprising the parenchymatous cells of the exocarp or the mesocarp, not considering the vascular bundles (Figure 7) , and calcium primarily functions outside the cytosol in the apoplast of cells [31]. However, for a particular tissue type no increase in the Ca/Mg+K ratio corresponding to the increased soil calcium was observed using this technique. On the other hand, mean Ca/Mg+K ratio, according to atomic absorption analysis, significantly increased with increasing fruiting zone calcium.

The SEM x-ray data were subjected to statistical analysis as a split plot design, with the calcium treatments serving as the main plot treatments and the tissue type serving as the subplot treatments. When this was done the tissue type variable was the only significant term within the AOV (P=.007). The equality of the variances for the ratios obtained for all tissue types across the treatments was checked. Data obtained from in each treatment were fit to a model incorporating the plant (block effect) +tissue type +plant*tissue type interaction term. Comparison of the mean squares of the interaction term indicated that variances among the treatments were not equal. Therefore, the level of significance in the tissue type variable within the split plot design model was questionable. Furthermore, application of AOV to the sclerenchyma band alone, showed that the treatment variable did not have a significant effect on the ratio, thus, no

Figure 6: Mean Ratio of Ca / Mg+K Distribution Across Pericarp Layers: Gnotobiotics 1.*

*The bars represent the actual mean ratio for each treatment according to atomic absorption analysis. Bars with the same letter are not statistically different at P= .05 according to Duncan's Multiple Range Test. Each point on the three lines represents the mean of 20 readings by the x-ray microanalysis technique. Treatments are in kilograms per hectare calcium equivalent of gypsum applied at early bloom.

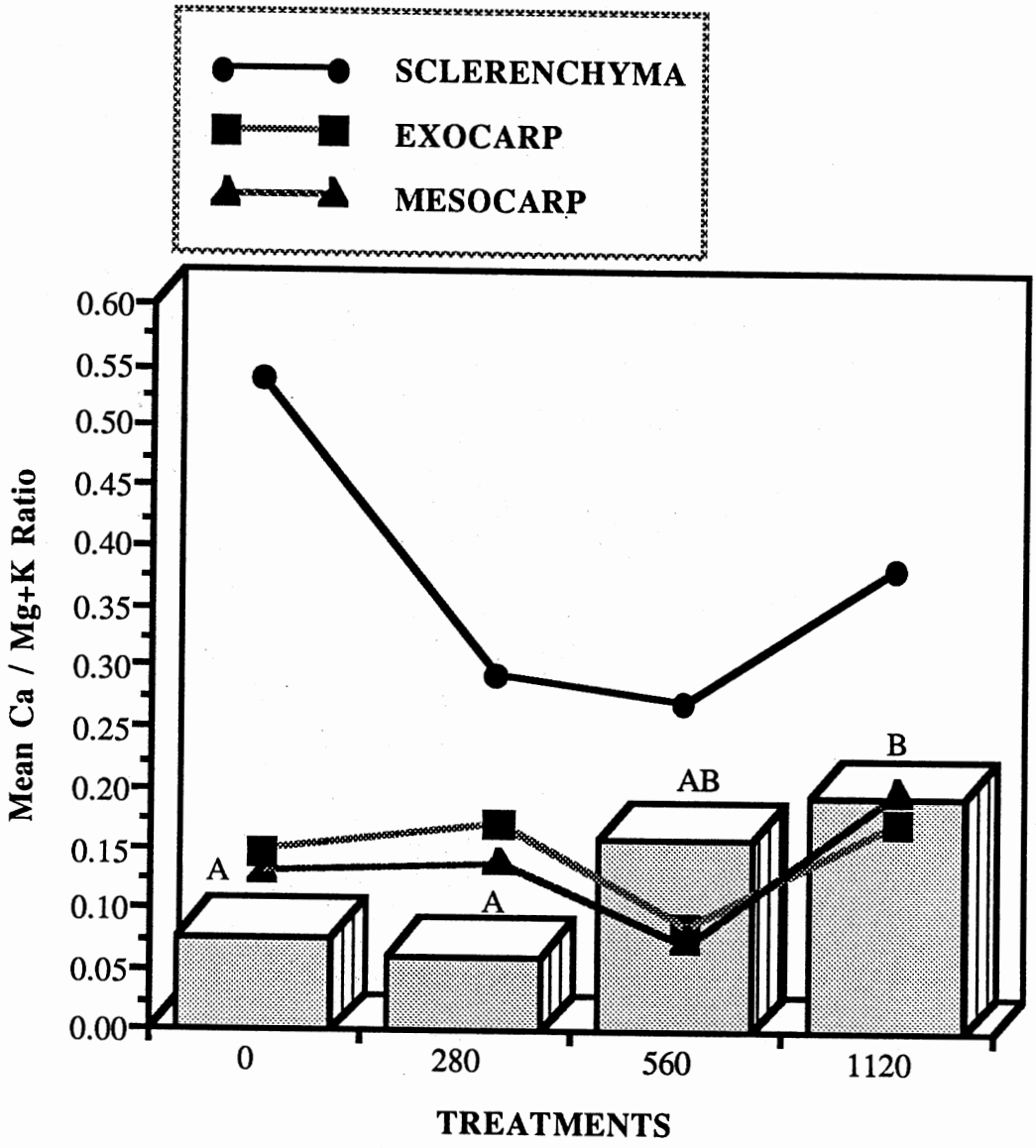

SCLERENCHYMA
EXOCARP
MESOCARP
Mean Ca / Mg+K Ratio
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A
A
AB
B
0
280
560
1120
TREATMENTS

Figure 7 (A and B): Scanning Electron Micrographs of Peanut Pericarps*

7A ——▶ Immature peanut pericarp. The arrows point to the respective areas (A--Exocarp, B--Mesocarp, C--Endocarp, D--Vascular Bundle, E--Sclerenchyma band).

7B ——▶ Mature peanut pericarp. As the pericarp matures the sclerenchyma band extends up to the base of the exocarp. Vascular bundles break down and the endocarp compacts up against the sclerenchyma band [38].

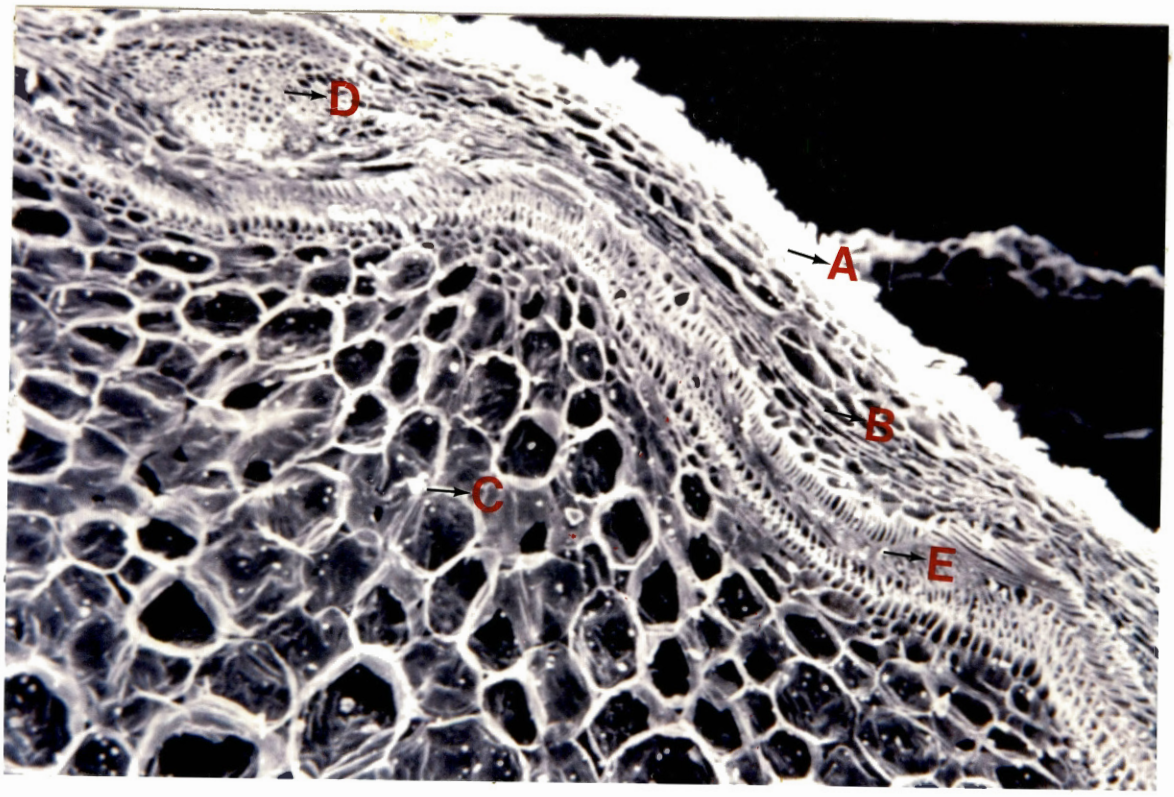


Figure 7A

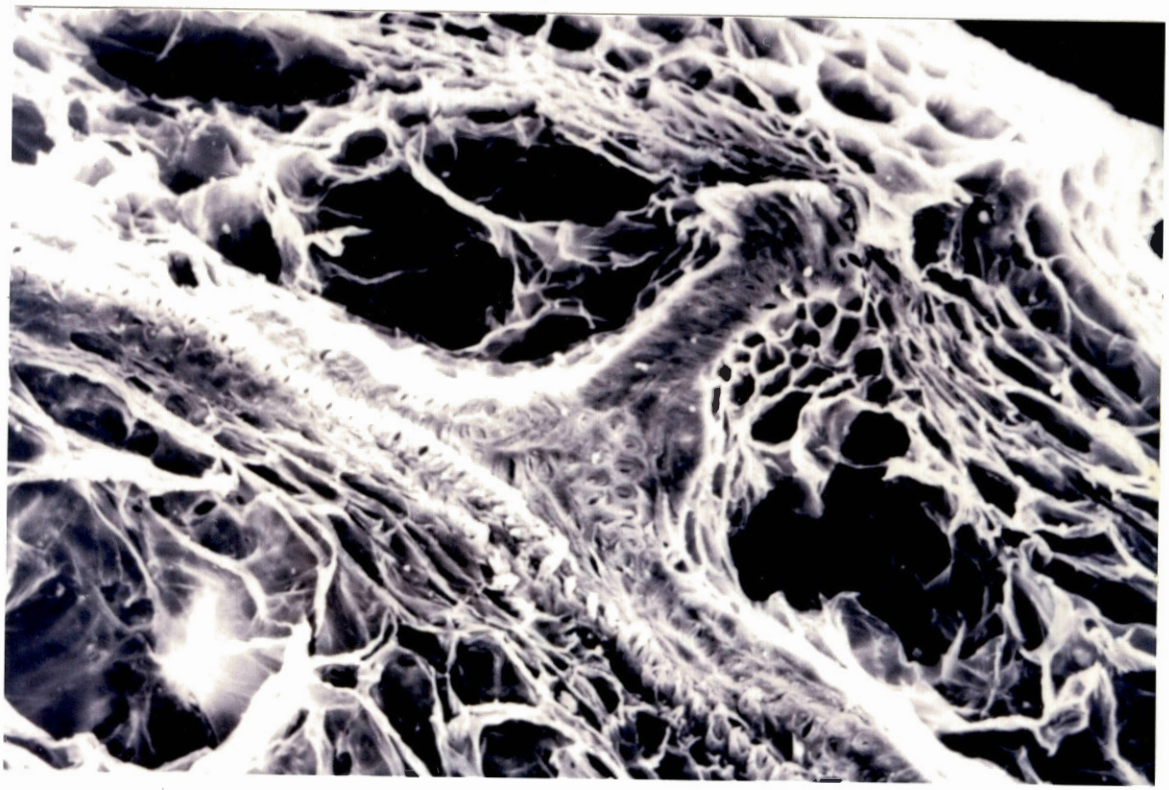


Figure 7B

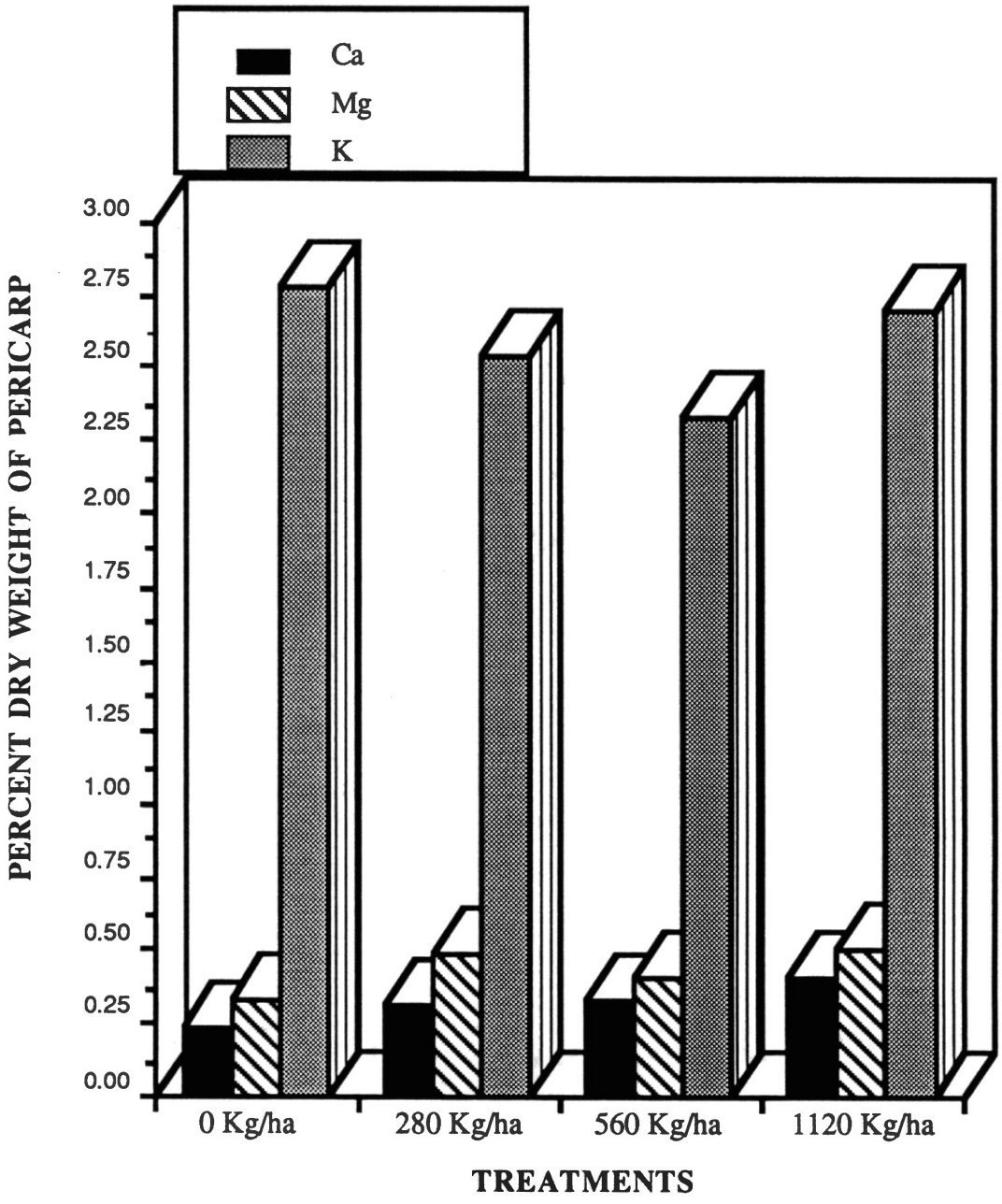
really significant fluctuation occurred within the sclerenchyma band, although there was quite a dramatic drop in the means.

It should be noted that there are some inherent problems associated with SEM x-ray analysis [51]. The number of x-rays that actually reach the detector, during the accumulation period, is influenced primarily by three factors: (1) the topography of the surface being scanned, (2) the density of the surface being scanned, and (3) the beam current at the time of scanning. For these reasons, statistical analysis of data obtained by this technique is difficult to interpret with confidence, and care should be taken in interpretation of data. These problems are not encountered in transmission electron microscopy microanalysis, where data are quantifiable and appropriate standards can be incorporated. This tool would be very effective for attempting to understand the elemental distribution within peanut pericarp. However, preparation of pericarp samples for this type of analysis may be problematic.

The G 2 experiment had bacterial contamination detected early in the experiment. However, no fungi were detected throughout the experiment. Early in the experiment circumstances forced the use of reverse osmosis rather than de-ionized water throughout the majority of the experiment. Mean concentrations of Ca, Mg,and K in pericarps as determined by atomic absorption analysis (Figure 8) were higher than those for experiment G 1. Most likely, this was due to the higher concentrations of these elements in the reverse osmosis water. The order of elemental concentrations, however, was the same as in the G 1, with K>Mg>Ca. Calcium and magnesium contents in the pericarps increased with added calcium applied to the fruiting zones; however, none of these increases were significant ($P<.05$). There was a positive linear correlation ($r=.92$, $P<.05$) between Mg and Ca in the pericarps. Potassium content in the pericarps fluctuated considerably and no trend related to added soil calcium was seen. Percent content of Ca in the vermiculite/sand from the fruiting zones was not significantly correlated ($r=.13$) with added calcium sulfate. No visible discoloration in pods was

Figure 8: Mean Elemental Content of Pericarps Gnotobiotics 2*

*The treatments are in calcium equivalent of gypsum, as anhydrous calcium sulfate in a full strength Hoagland's solution, in kilograms per hectare (Kg/ha) applied at early bloom. No significant differences occurred ($P<.05$) among the elemental contents across the calcium treatments.


Ca
Mg
K
PERCENT DRY WEIGHT OF PERICARP
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0 Kg/ha
280 Kg/ha
560 Kg/ha
1120 Kg/ha
TREATMENTS

observed in this experiment; however, 'pops' occurred. There was no relationship between the frequency of 'pops' and the added calcium sulfate.

SEM x-ray microanalysis of pericarps was not done due to the untimely equipment failure at Oklahoma State University. An effort was made to use the SEM x-ray analysis instrument at the University of Oklahoma; however, this instrument also required repair and was not available.

Calcium Sulfate Effectiveness in Suppressing Pod Rot Related to Populations of Pythium myriotylum in Soil: Greenhouse

Several experiments were conducted, but some of these were failures. Maintenance of the proper density levels of P. myriotylum in the fruiting zones to cause significant pod rot created some problems. In two experiments, very low densities of P. myriotylum in the sand/vermiculite disappeared prior to harvest and no incitement of pod rot incurred. In another experiment densities of 60 and 350 p/g persisted in cups, but zoospores in water leaking from the cups into the root zones severely stunted or killed mature plants. P. myriotylum was isolated at a frequency of 100% from the roots of these plants. In addition, spider mites were a continued nuisance in the greenhouse during spring and summer months and most likely affected yield. Several insecticides were used to minimize this problem with variable success.

Two experiments were completed and data taken. These were designated Greenhouse Experiment 1 (GH 1) and Greenhouse Experiment 2 (GH 2). In both experiments, no visible symptoms of pod rot were observed in noninfested controls (Figures 9-11), whereas at 10 p/g soil and 50 p/g soil severe pod decay generally occurred, although some pods escaped infection. Analysis of variance showed significant pod rot effects due to inoculum densities at $P < .01$ for GH 1 and $P = .002$ for GH 2. Pythium was not recovered from pods of the controls, but was isolated from 80-100% of the pericarps pieces from infested pots plated on PSM.

Figure 9 (A, B, C): Photograph of Pods from Greenhouse Experiment 2*

*9 A ⟶ The 0 p/g P. myriotylum inoculum density level, all pods are lesion free across all levels of calcium .

9 B ⟶ The 10 p/g P. myriotylum inoculum desitiy level, pods with visible lesion formation across all levels of calcium.

9 C ⟶ The 50 p/g P. myriotylum inoculum level, pods with visible lesion formation across all levels of calcium

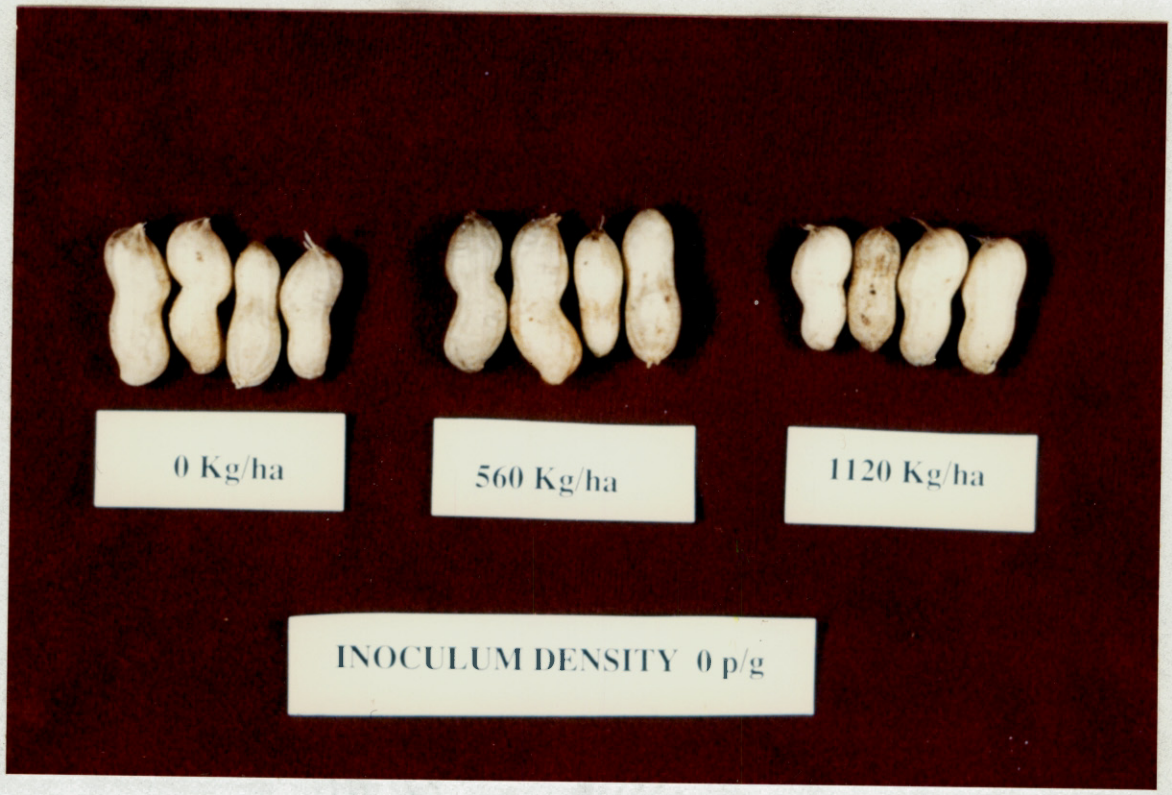


Figure 9 A

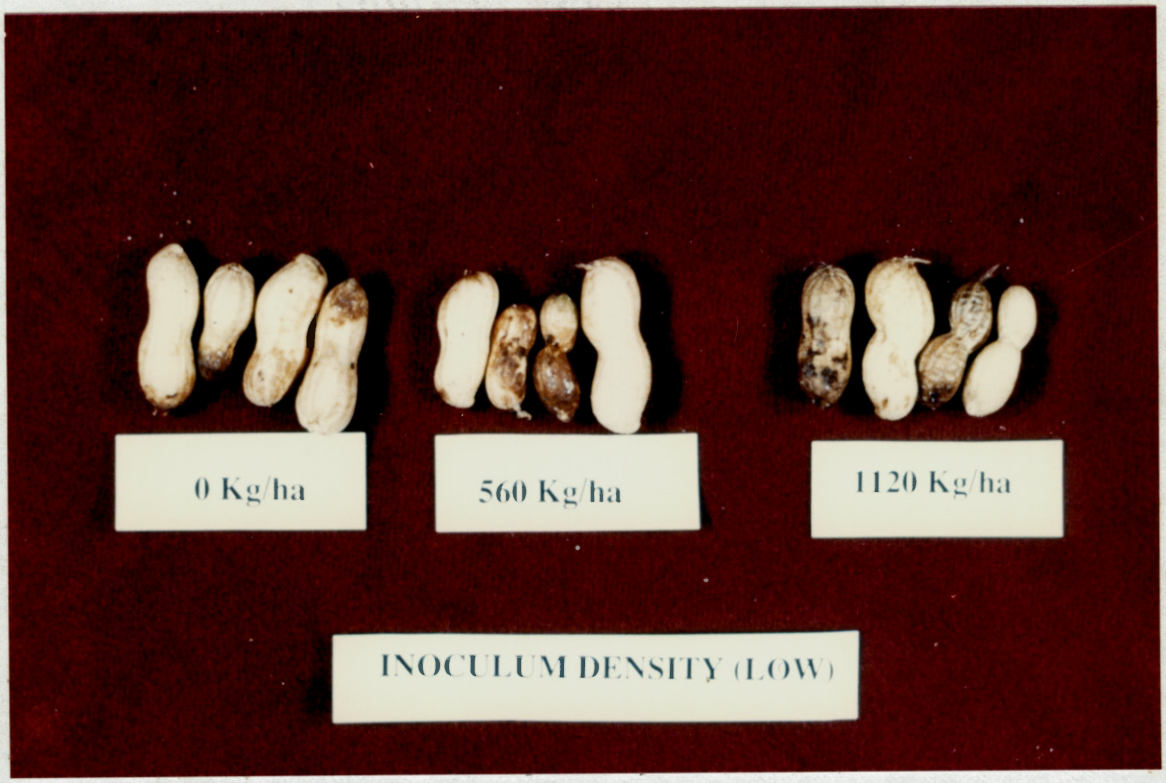


Figure 9 B

Figure 9C

Inoculum densities had no influence on the response of pod rot to applications of calcium sulfate. In both experiments and for all calcium sulfate treatments, pod rot was not significantly ($P<.05$) greater at 50 p/g then at 10 p/g. Furthermore, there was no significant ($P<.05$) linear correlation between percent calcium content in pericarps and pod rot for GH 1 ($r= -.003$) or GH 2 ($r= -.290$). In both experiments, calcium content was usually the lowest in pericarps of pods grown in vermiculite/sand infested with 50 p/g of P. myriotylum (Figures 10 and 11).

At harvest, no propagules of Pythium spp. were detected in noninfested vermiculite/sand (Figures 12 and 13), whereas at the 10 and 50 p/g infestation densities, propagules of Pythium were consistently detected. Populations of Pythium in some cases were higher than at infestation. In experiment GH 1 (Figure 12) there were no differences ($P=.05$) between populations at any of the infestation densities, except in the high calcium treatment where the population in the 50 p/g level was significantly ($P=.05$) greater than the control. In experiment GH 2 (Figure 13) populations in all treatments infested at the 50 p/g were significantly different from the noninfested controls, and in some cases different ($P<.05$) than populations in the treatments infested at 10 p/g. Pythium was isolated from a few pods taken from fruiting zones without detectable Pythium propagules. Conversely, Pythium propagules were recovered from fruiting zones with no recovery from pods. The calcium sulfate applied or the final calcium in the fruiting zones had no significant ($P=.05$) effects on populations of Pythium in the fruiting zones.

At harvest, atomic absorption results indicated no significant differences in fruiting zone calcium concentrations among all treatments for both experiments. At the 0 Kg/ha calcium sulfate rate, fruiting zone calcium concentrations were 250-350 ppm for GH 1 and 291-349 ppm for GH 2. At the 1,120 Kg/ha calcium sulfate rate, fruiting zone calcium concentrations were 272-391 ppm for GH 1 and 321-467 ppm for GH 2. These results indicated that extensive leaching of calcium might have occurred during the

Figure 10: Greenhouse Experiment One (GH1): Mean Pod Rot Rating Index versus Mean % Calcium Content of Pericarp.*

*Treatments are arranged by calcium levels (0 kilogram/hectare (Kg/ha), 560 Kg/ha and 1120 Kg/ha for the low Ca, Med Ca and High Ca respectively), in all combinations of inoculum densities of P. myriotylum (0,10 and 50 are in propagules per gram). The bars represent the mean pod rot rate (obtained for each replication by dividing the number of pods with greater than 25% of pericarp surface decayedby the total number of pods in the replication). Pod rot rate means were statistically differentiated by orthogonal contrasts. Bars with the same letter (A,B) are not statistically different at P= .05. Mean percent calcium content (on a dry weight basis) of the treatments are represented by the center point of the solid triangles. No significant differences occurred among all treatments at P= .05 according to analysis by orthogonal contrasts

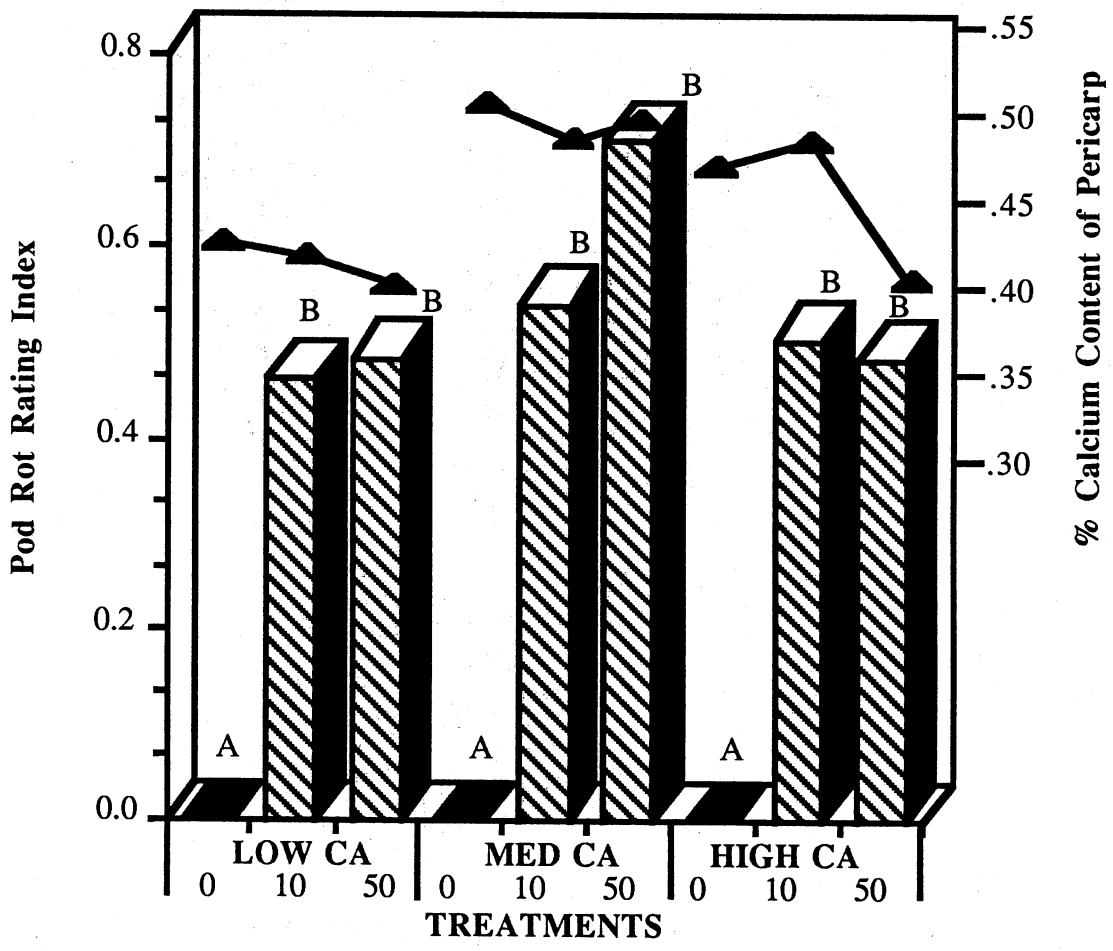

Pod Rot Rating Index
0.8
0.6
0.4
0.2
0.0
.55
.50
.45
.40
.35
.30
% Calcium Content of Pericarp
A
B
B
A
B
B
A
B
B
LOW CA
MED CA
HIGH CA
0
10
50
0
10
50
0
10
50
TREATMENTS

Figure 11: Greenhouse Experiment Two (GH 2): Mean Pod Rot Rating Index versus Mean % Calcium Content of Pericarp.*

*Treatments are arranged by calcium levels (0 kilogram/hectare (Kg/ha), 560 Kg/ha and 1120 Kg/ha for the low Ca, Med Ca and High Ca respectively), in all combinations of inoculum densities of P. myriotylum (0,10 and 50 are in propagules per gram). The bars represent the mean pod rot rating (obtained for each replication by dividing the number of pods with greater than 25% of pericarp surface decayedby the total number of pods in the replication). Pod rot rating means were statistically differentiated by orthogonal contrasts. Bars with the same letter (A,B) are not statistically different at P= .05. Mean percent calcium content (on a dry weight basis) of the treatments are represented by the center point of the solid triangles. No significant differences occurred among all treatments at P= .05 according to analysis by orthogonal contrasts.

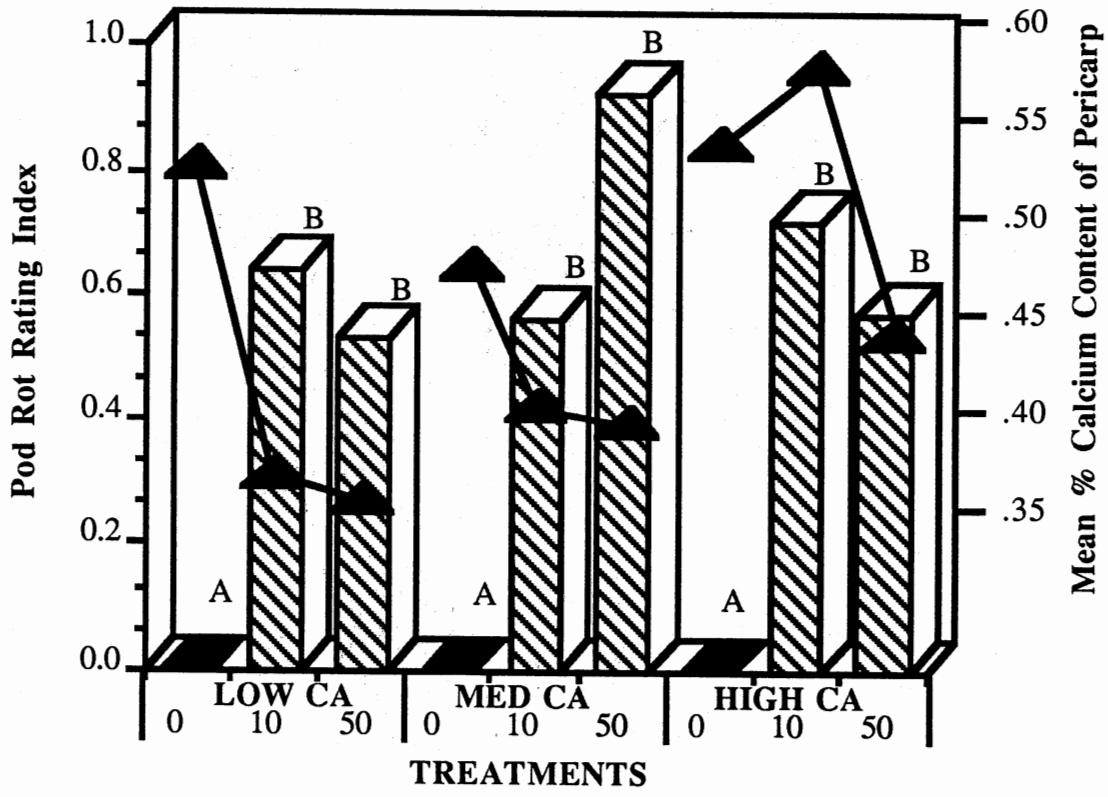

Pod Rot Rating Index
1.0
0.8
0.6
0.4
0.2
0.0
.60
.55
.50
.45
.40
.35
Mean % Calcium Content of Pericarp
A
B
B
A
B
B
A
B
B
LOW CA
MED CA
HIGH CA
0
10
50
0
10
50
0
10
50
TREATMENTS

Figure12: Greenhouse Experiment One (GH 1): Survival of Pythium myriotylum Within the Fruiting Zone at Harvest.*

*Treatments are arranged by calcium levels (0 kilogram/hectare, Kg/ha, 560 Kg/ha and 1120 Kg/ha for the low Ca, Med Ca and High Ca respectively), in all combinations of inoculum densities of P. myriotylum (0, 10 and 50 propagules per gram fruiting zone mix). The bars represent the mean propagules per gram dry weight of fruiting mix. Mean propagules per gram dry weight for each treatment was determined through a dilution plating technique incorporating a Pythium Selective Medium. The means were statistically differentiated by orthogonal contrasts. Bars with the same letter (AB) are not statistically different at P=.05.

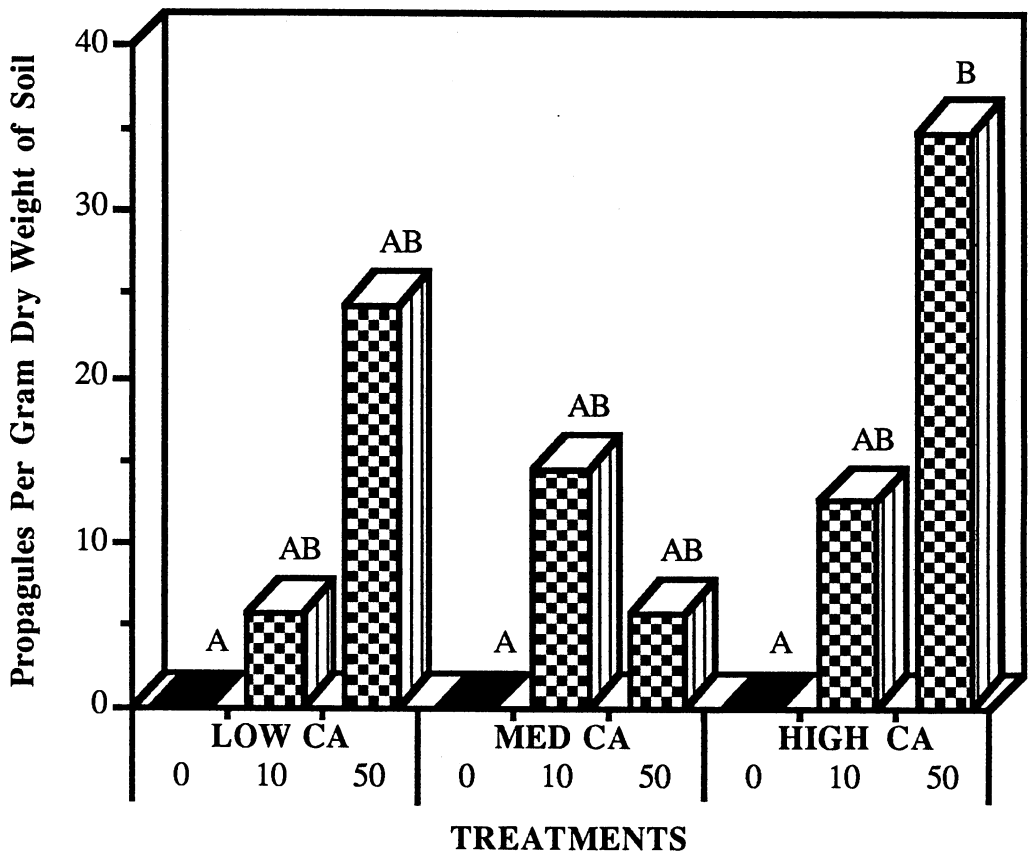
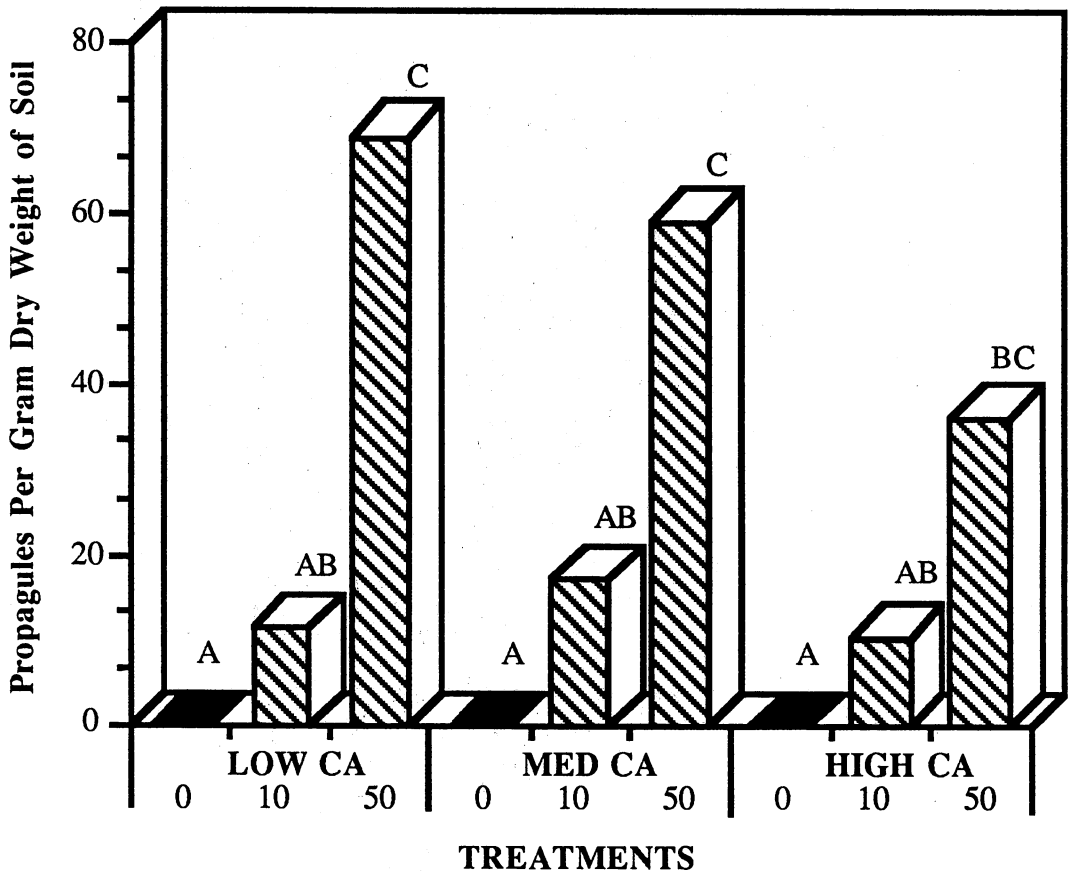

Propagules Per Gram Dry Weight of Soil
40
30
20
10
0
A
AB
AB
A
AB
AB
A
AB
B
LOW CA
0 10 50
MED CA
0 10 50
HIGH CA
0 10 50
TREATMENTS

Figure 13: Greenhouse Experiment Two (GH 2): Survival of Pythium myriotylum Within the Fruiting Zone at Harvest.

*Treatments are arranged by calcium levels (0 kilogram/hectare, Kg/ha, 560 Kg/ha and 1120 Kg/ha for the low Ca, Med Ca and High Ca respectively), in all combinations of inoculum densities of P. myriotylum (0, 10 and 50 propagules per gram fruiting zone mix) The bars represent the mean propagules per gram dry weight of fruiting zone mix. Means were determined through a dilution plating technique incorporating a Pythium Selective Medium. The means were statistically differentiated by orthogonal contrasts. Bars with the same letter (A,B,C) are not statistically different at P= .05.


Propagules Per Gram Dry Weight of Soil
80
60
40
20
0
A
AB
C
A
AB
C
A
AB
BC
LOW CA
MED CA
HIGH CA
0
10
50
0
10
50
0
10
50
TREATMENTS

experiments. The failure of the vermiculite/sand to maintain the calcium sulfate levels applied was reflected in the calcium content of pericarp samples. In both experiments, no significant difference occurred among all treatments for calcium content of pericarps. Although fluctuations did occur in both experiments, with a greater degree of fluctuation in GH 2, no relationship between calcium in the pericarps and either calcium sulfate applied or final concentrations of calcium in the fruiting zone was observed. Although calcium was apparently leached out of the fruiting zone, percent calcium content of pericarps were greater than the .20% level suggested by Hallock and Garren [24] as rendering pods less susceptible to disease.

These greenhouse experiments clearly demonstrated the ability of P. myriotylum to incite pod rot. Koch's Postulates were fulfilled. The experiments demonstrated that pod rot is not necessarily a disease complex. The disease can be incited by one organism; however, as demonstrated by others [14,16] organisms may interact as cofactors in the incitement of disease. The greenhouse results support the work of Moore and Wills [35], where they demonstrated that the calcium levels applied had no effect on the susceptibility of pods to infection by P. myriotylum. This sugests that calcium is not a primary factor in disease incitement, but may play a role as a secondary influence on the etiology by some unknown mechanism(s).

Calcium Sulfate Effectiveness in Suppressing Pod Rot Related to Populations of Pythium myriotylum in Soil: Microplots. In 1987 microplot experiments were affected severely by chlorosis and ultimately necrosis of the foliar portions of the plants. This problem was believed to be abiotic in nature, but no conclusive evidence was ascertained. Although similar symptoms occurred in 1988, the extent was not so severe as in the prior year. The MP 2 experiment in 1988 had little if any symptomology as in 1987, while the MP 1 experiment, not used in 1987, developed some symptoms. For this reason only results obtained from 1988 will be dealt with in depth and limited

reference to the 1987 data will be made.

Discolored pods were found in noninfested microplots, possibly due to the presence of Sclerotina minor in some of the microplots. Nevertheless, more pod rot was consistently found in infested microplots than in noninfested ones. Spanco peanut from infested plots had greater pod rot severity than controls in the MP 1 experiment (Figure 14) or in the MP 2 experiment (Figure 15). In both experiments the majority of the sum of squares associated with the effect of inoculum density on pod rot was accountable by a linear trend. In the MP 1 experiment a significant (P=.04) block effect occurred.

Pod rot severity was also greater on Florunner in infested microplots of MP 1 experiment (Figure 16) and MP 2 (Figure 17). Although a linear trend accounted for the sum of squares associated with the effect of inoculum densities on pod rot, a parabolic trend also was suggested for the significant inoculum density term in both experiments, which indicated the variability in disease within the cultivar. Florunner consistently had higher severity than Spanco in all experiments over both years, which is consistent with what has been reported in the literature for the Spainish market type [39].

Application of calcium sulfate did not significantly decrease pod rot severity on Spanco peanut in the MP 1, although the MP 2 experiment exhibited a significant decrease in pod rot severity under the 10 p/g inoculum level. Calcium content of pericarp of Spanco in the MP 2 experiment was significantly (P=.035) affected by the interaction of added calcium and inoculum density. Application of calcium sulfate resulted in significant increases in calcium content of pericarps of Spanco peanut from both experiments. A similar result was observed in the 1987 experiment as well. Calcium accumulation in the pericarps tended to be linear. However, pod rot was not significantly linearly correlated with calcium content in pericarps of Spanco peanut from the MP 1 experiment (r= -.268) or MP 2 (r= -.286).

Added calcium sulfate did not significantly reduce pod rot of Florunner peanut in either experiment, although it did significantly raise the calcium content of pericarps.

Figure 14: Spanco MP 1 Experiment: Pod Rot Rating Index versus Mean % Calcium Content of Pericarp.*

*Treatments are arranged by inoculum densities (ID) (0 propagules per gram,p/g, 10 p/g and 50 p/g for the Low ID, Med ID and High ID respectively) in all combinations with gypsum application levels applied at early bloom (rates equivalent to 0 kilograms per hectare (Kg/ha),1120 Kg/ha and 2240 Kg/ha). The bars represent the mean pod rot rate (obtained for each replication by dividing the number of pods with greater than 25% pericarp surface decayed by the total number of pods in the replication). Pod rot rate means were statistically differentiated by orthogonal contrasts. Bars with same the letter (A,B,C,D) are not statistically different at P=.05. Mean percent calcium content of pericarp tissue, on a percent dry weight basis, are represented by the solid squares. Squares with the same letter (X,Y,Z) are not statistically different at P=.05 as analyzed by orthogonal contrast. The box contains P values associated with the linear and parabolic components of the variables within the AOV (calcium level, ID level and calcium level*ID level interaction term). A check indicates the variable was significant at P=.05 in the analysis of variance.

		P-value PRR		P-value %Ca in Pericarp
CALCIUM LINEAR		.062	✓	.000
CALCIUM PARABOLIC		.217	✓	.048
PM LINEAR	✓	.003		.295
PM PARABOLIC	✓	.009		.056
CA LINEAR* PM LINEAR		.218	✓	.001
CA PARABOLIC*PM LINEAR		.847	✓	.452
CA LINEAR*PM PARABOLIC		.694	✓	.534
CA PARABOLIC*PM PARABOLIC		.420	✓	.695

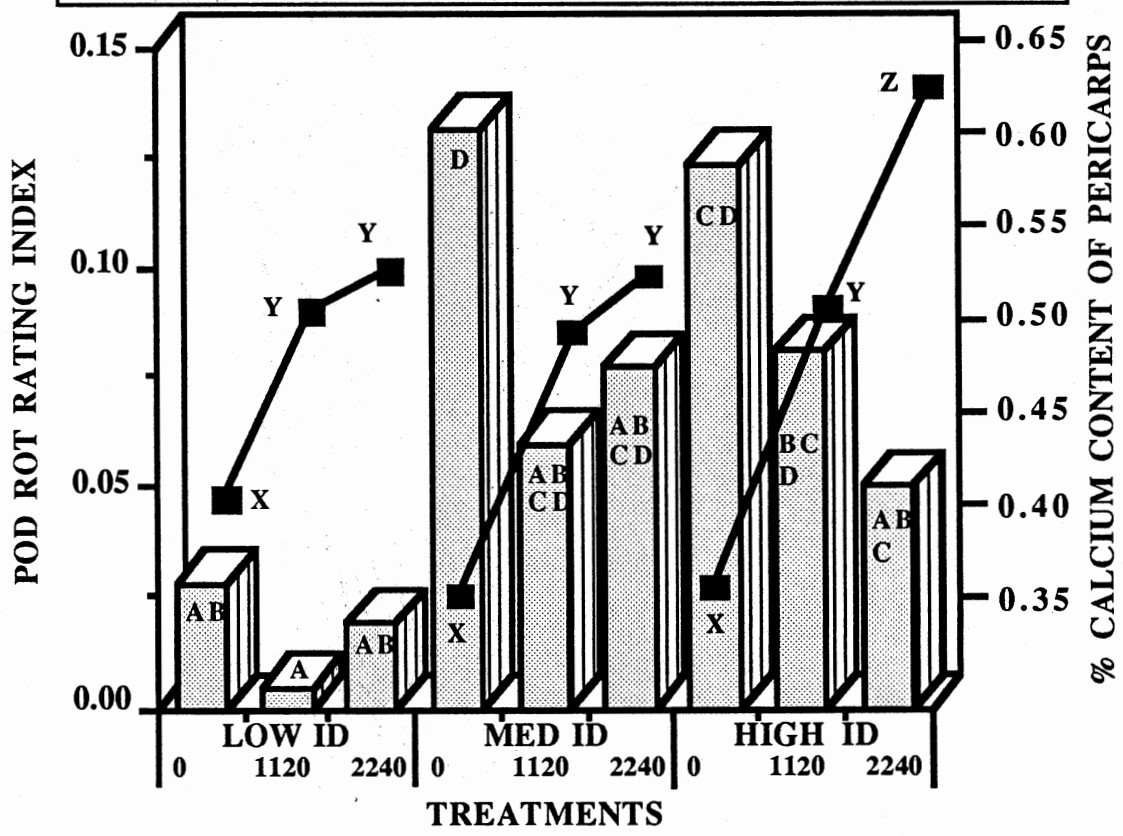


Figure 15: Spanco MP 2 Experiment: Pod Rot Rating Index versus Mean % Calcium Content of Pericarp.

*Treatments are arranged by inoculum densities (ID) (0 propagules per gram,p/g, 10 p/g and 50 p/g for the Low ID, Med ID and High ID respectively) in all combinations with gypsum application levels applied at early bloom (rates equivalent to 0 kilograms per hectare (Kg/ha), 1120 Kg/ha and 2240 Kg/ha). The bars represent the mean pod rot rate (obtained for each replication by dividing the number of pods with greater than 25% pericarp surface decayedby the total number of pods in the replication). Pod rot rate means were statistically differentiated by orthogonal contrasts. Bars with same letter (A,B,C,D) are not statistically different at P=.05. Mean percent calcium content of pericarp tissue, on a percent dry weight basis, are represented by the solid squares. Squares with the same letter (X,Y,Z) are not statistically different at P=.05 as analyzed by orthogonal contrast. The box contains P values associated with the linear and parabolic components of the variables within the AOV (calcium level, ID level and calcium level*ID level interaction term). A check indicates the variable was significant at P=.05 in the analysis of variance.

		P-value PRR	P-value %Ca in Pericarp
CALCIUM LINEAR		.025	✓ .000
CALCIUM PARABOLIC		.708	✓ .175
PM LINEAR	✓	.002	.883
PM PARABOLIC	✓	.029	.641
CA LINEAR* PM LINEAR		.995	.420
CA PARABOLIC*PM LINEAR		.045	.604
CA LINEAR*PM PARABOLIC		.080	.105
CA PARABOLIC*PM PARABOLIC		.157	.387

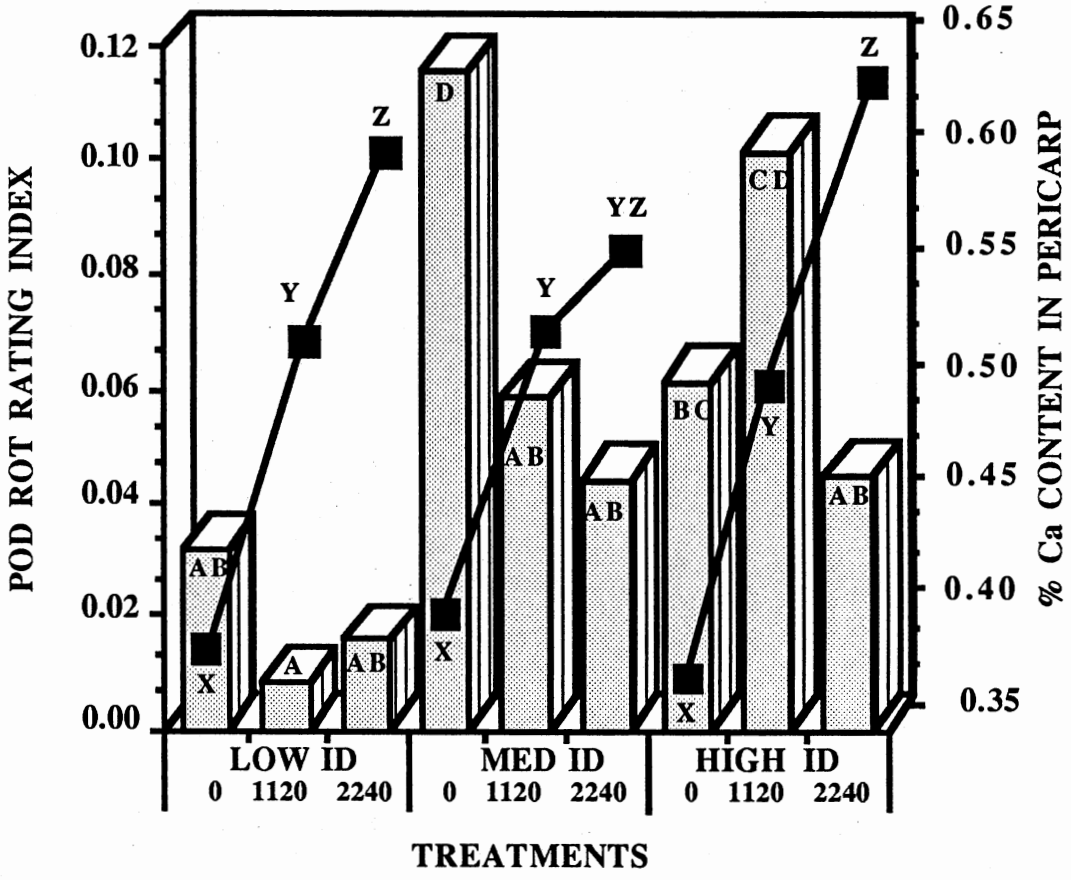


Figure 16: Florunner MP 1 Experiment: Pod Rot Rating Index versus Mean % Calcium Content of Pericarp.*

*Treatments are arranged by inoculum densities (ID) (0 propagules per gram, p/g, 10 p/g and 50 p/g for the Low ID, Med ID and High ID respectively) in all combinations with gypsum application levels applied at early bloom (rates equivalent to 0 kilograms per hectare (Kg/ha), 1120 Kg/ha and 2240 Kg/ha). The bars represent the mean pod rot rate (obtained for each replication by dividing the number of pods with greater than 25% pericarp surface decayed by the total number of fruits in the replication). Pod rot rate means were statistically differentiated by orthogonal contrasts. Bars with the same letter (A,B,C,D) are not statistically different at P=.05. Mean percent calcium content of pericarp tissue, on a percent dry weight basis, are represented by the solid squares. Squares with the same letter (X,Y,Z) are not statistically differentat P=.05 as analyzed by orthogonal contrast. The box contains P values associated with the linear and parabolic components of the variables within the AOV (calcium level, ID level and calcium level*ID level interaction term). A check indicates the variable was significant at P=.05 in the analysis of variance.

	P-value PRR	P-value %Ca in Pericarp
CALCIUM LINEAR	.031	✓ .000
CALCIUM PARABOLIC	.968	✓ .022
PM LINEAR	✓ .000	.163
PM PARABOLIC	✓ .009	.967
CA LINEAR* PM LINEAR	.992	.044
CA PARABOLIC*PM LINEAR	.412	.486
CA LINEAR*PM PARABOLIC	.664	.772
CA PARABOLIC*PM PARABOLIC	.020	.191

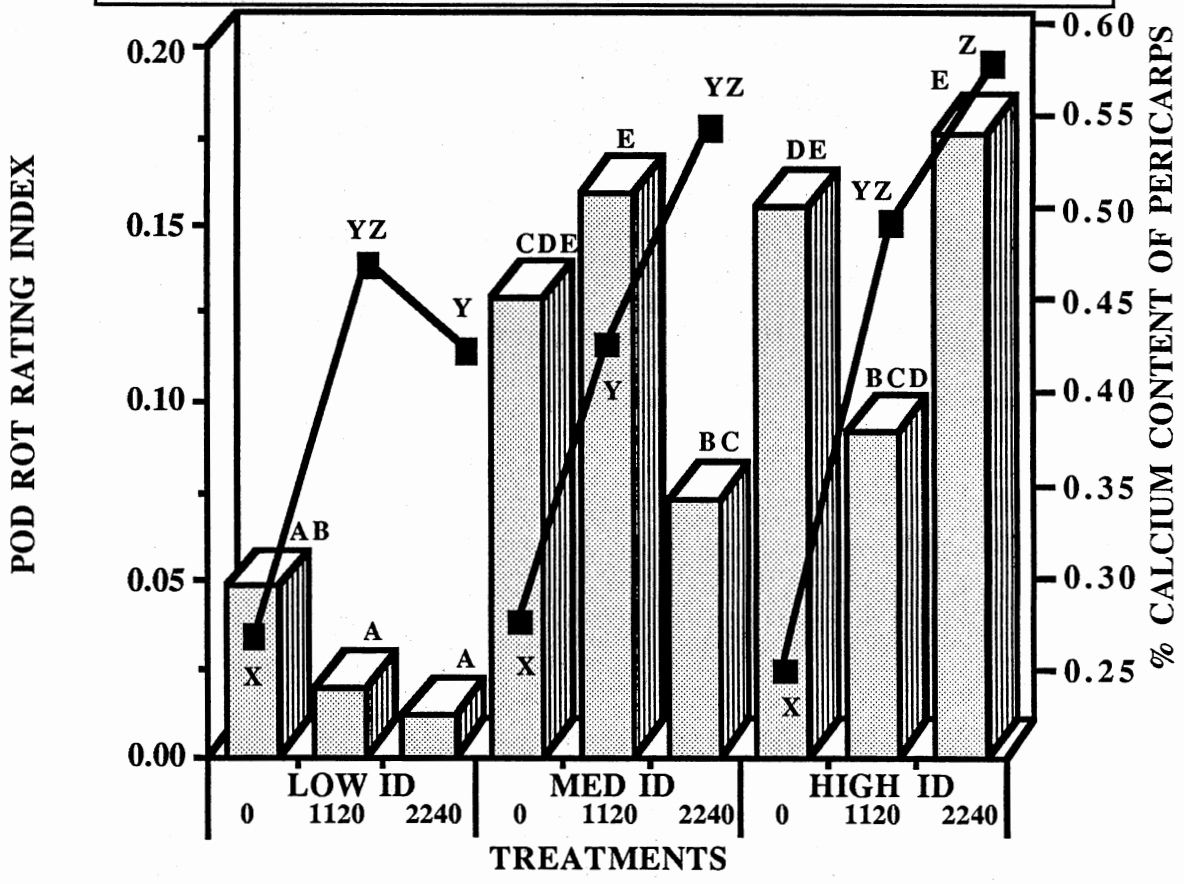
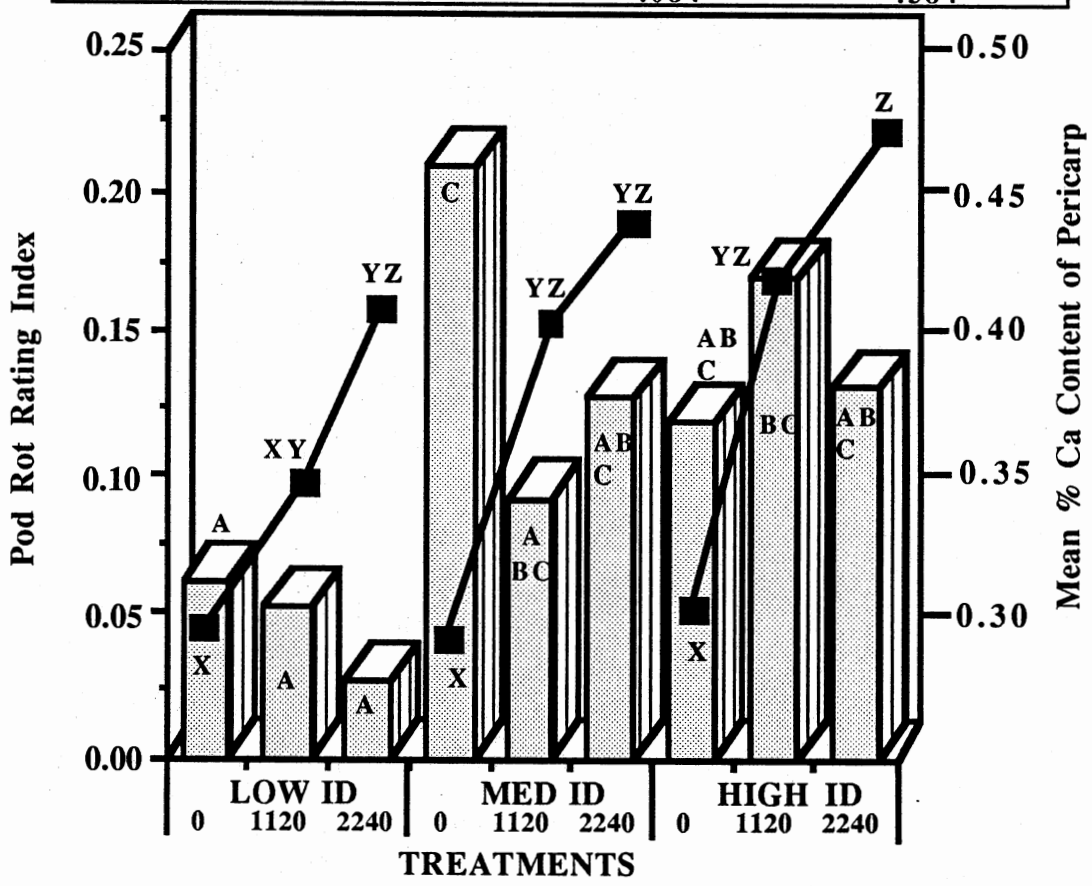


Figure 17: Florunner MP 2 Experiment: Pod Rot Rating Index versus Mean % Calcium Content of Pericarp.

Treatments are arranged by inoculum densities (ID) (0 propagules per gram,p/g, 10 p/g and 50 p/g for the Low ID, Med ID and High ID respectively) in all combinations with gypsum application levels applied at early bloom (rates equivalent to 0 kilograms per hectare (Kg/ha), 1120 Kg/ha and 2240 Kg/ha). The bars represent the mean pod rot rate (was obtained for each replication by dividing the number of pods with greater than 25% pericarp surface decay by the total number of pods in the replication). Pod rot rate means were statistically differentiated by orthogonal contrasts. Bars with same letter (A,B,C) are not statistically different at P=.05. Mean percent calcium content of pericarp tissue , on a percent dry weight basis, are represented by the solid squares. Squares with the same letter (X,Y,Z) are not statistically differentat P=.05 as analyzed by orthogonal contrast. The box contains P values associated with the linear and parabolic components of the variables within the AOV (calcium level, ID level and calcium level*ID level interaction term). A check indicates the variable was significant at P=.05 in the analysis of variance.

	P-value PRR	P-value % Ca
CALCIUM LINEAR	.289	✓ .000
CALCIUM PARABOLIC	.776	✓ .449
PM LINEAR	✓ .007	.105
PM PARABOLIC	✓ .089	.950
CA LINEAR* PM LINEAR	.564	.432
CA PARABOLIC*PM LINEAR	.590	.595
CA LINEAR*PM PARABOLIC	.311	.790
CA PARABOLIC*PM PARABOLIC	.084	.584



Calcium contents in Florunner pericarps were consistently lower than those of Spanco pericarps. A linear trend accounted for the majority of the sum of squares associated with the significant calcium treatment variable when calcium content of the pericarp was the dependent variable. However, for the MP 1 experiment, a parabolic trend also displayed a significant P value as a component of the main variable, calcium treatment. Pod rot of Florunner pods was not significantly, linearly correlated with calcium content of pericarps from the MP 1 experiment (r= -.192) or the MP 2 experiment (r= -.021).

In both experiments, the frequency of Pythium isolations from the pericarps of diseased Spanco (Table 1) or Florunner (Table 2) was, for the most part, significantly greater from infested than noninfested microplots. Pythium colonies recovered from pods were not positively identified to species, although recovery after incubation at 37° C for 16 hours suggested that they were mainly P. myriotylum. Pod rot in infested soils then, was attributed to P. myriotylum .

The microplot studies demonstrate the ability of P. myriotylum to incite greater levels of pod rot regardless of calcium sulfate levels applied or calcium concentration of the pericarp. This work concurs with the work of others in Oklahoma [12]. The significant increase in pericarp calcium content with increasing rates of gypsum, with Spanco maintaining higher calcium content compared to Florunner, was consistent with the literature [5,29]. Furthermore, Florunner maintained higher pod rot ratings than Spanco across all treatments.

TABLE 1

MEAN PERCENT RECOVERY OF PYTHIUM FROM DECAYED SPANCO PERICARP SAMPLES *

Treatment	Spanco MP 2	Spanco MP 1
0/0	0.0 A	0.0 A
0/1120	0.0 A	5.0 A
0/2240	5.8 A	3.8 A
M/0	71.3 B	57.5 B
M/1120	66.3 B	41.3 B
M/2240	25.0 A	35.0 B
H/0	70.0 B	46.6 B
H/1120	71.3 B	41.3 B
H/2240	78.6 B	40.0 B

* Numbers within the table are in percent recovery from pericarp samples. Means, within the same column, sharing the same letter are not statistically differant at P=.05 as analyzed by orthogonal contrast. Treatments are 0 p/g, 10 p/g and 50 p/g for the L, M and H ID of P. myriotylum respectively. The calcium levels are in kilograms per hectare applied at early bloom.

TABLE 2

MEAN PERCENT RECOVERY OF PYTHIUM FROM DECAYED FLORUNNER PERICARP SAMPLES*

Treatment	Florunner MP 2	Florunner MP 1
0/0	0.0 A	0.0 A
0/1120	17.5 AB	0.0 A
0/2240	8.8 A	0.0 A
M/0	53.8 CD	56.3 C
M/1120	23.8 ABC	36.3 BC
M/2240	67.5 D	30.0 BC
H/0	48.8 BCD	35.0 BC
H/1120	37.5 BCD	11.3 AB
H/2240	66.3 D	12.5 AB

* Numbers within the table are percent recovery from pericarp samples. Means, within the same column, sharing the same letter are not statistically differant at P=.05 as analyzed by orthogonal contrast. Treatments are 0 p/g, 10 p/g and 50 p/g for the L, M and H ID of P myriotylum respectively. The calcium levels are in kilograms per hectare applied at early bloom.

CHAPTER V

SUMMARY AND CONCLUSIONS

In relation to objective 1 of this study, results show that a calcium deficiency in pericarps of pods did not incite pod rot symptoms. Atomic absorption spectrophometry showed that a positive linear correlation existed between calcium and magnesium contents of pericarps, a finding that is atypical of field conditions.

SEM, x-ray microanalysis indicated that the sclerenchyma band had a consistently higher Ca/Mg+K ratio compared to the other tissues of the pericarp. X-ray microanalysis may be a valuable tool for determining the relationship of elemental content and distribution to the integrity of pericarp tissue.

In relation to objective 2 of the study, results showed that P. myriotylum caused pod rot in the greenhouse at inoculum densities of 10 p/g or higher. Calcium sulfate at rates of 1,120 and 2,240 Kg/ha did not significantly reduce pod rot severity in these studies across the inoculum density treatments of P. myriotylum tested. Survival of P. myriotylum from soil mix of fruiting zones did not appear to be related to the rate of calcium sulfate applied or final calcium concentrations in the fruiting zones.

More pod rot occurred on both cultivars in infested microplots compared to noninfested microplots. Calcium sulfate applications significantly increased percent calcium content of pericarp compared to nontreated microplots. No significant, linear relationship existed between percent calcium content of pericarps and pod rot severity. Pod rot of Spanco was reduced in infested microplots to levels not significantly different than those in noninfested microplots, but the reductions were not related to the calcium content of pericarps. Florunner had greater pod rot severity than Spanco for all

treatments, whereas Spanco maintained maintained a higher content of calcium in the pericarps compared to pericarps of Florunner. High temperature Pythium spp. were consistently isolated from lesion areas of pericarp tissue, with greater recovery from infested microplots over noninfested microplots, indicating P. myriotylum as the species recovered.

Calcium alone does not seem to be the primary determinate for peanut pod rot. Whether the element has a secondary influence to the etiology, either as increasing cell wall integrity or acting on the activity of potential pathogens in the geocarposphere has yet to be elucidated.

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APPENDIX A

POPULATIONS OF PYTHIUM SPP. IN MICROPLOTS TREATED WITH CALCIUM SULFATE

During the 1988 growing season, soils in both microplots experiments were monitored for Pythium sp. populations at three sampling periods after infestation. Soil samples were taken at 1 week post-infestation, early September, and at harvest. Two samples were collected from along each row within each microplot, and composited. A ten gram subsample was randomly selected and soil dilutions of 1:20 and 1:200 were used plated on Pythium Selective Medium [11]. Propagule counts in 5 plates per dilution factor were averaged and the combined average of the two dilution factors was used as the final population. Plates were incubated at 24-26° C for 18-24 hours prior to reading.

The results are shown in figures 18-21. In general, no trends could be observed across all calcium levels for both cultivars. Infestation density had no effect on populations over the sampling periods. Populations at all sampling periods had a great deal of variability. Cultivar did not have an influence on Pythium populations detected. The Pythium populations detected within the noninfested microplots evidently were inhibited by incubation at 37° C or not able to colonize pericarp tissue efficiently as reflected in Pythium recovery from pericarp pieces of pods (Tables 1 and 2).

Figure 18: Mean Pythium Populations During Three Sampling Periods Under Three Soil Calcium Levels: Florunner MP 1 Experiment.*

*Each point represents the mean p/g of four replications according to dilution plating on a Pythium Selective Medium (dilution factors of 1:20 and 1:200 were used with 5 plates per dilution factor). No significant relationship was observed between soil calcium treatment and the propagules detected.

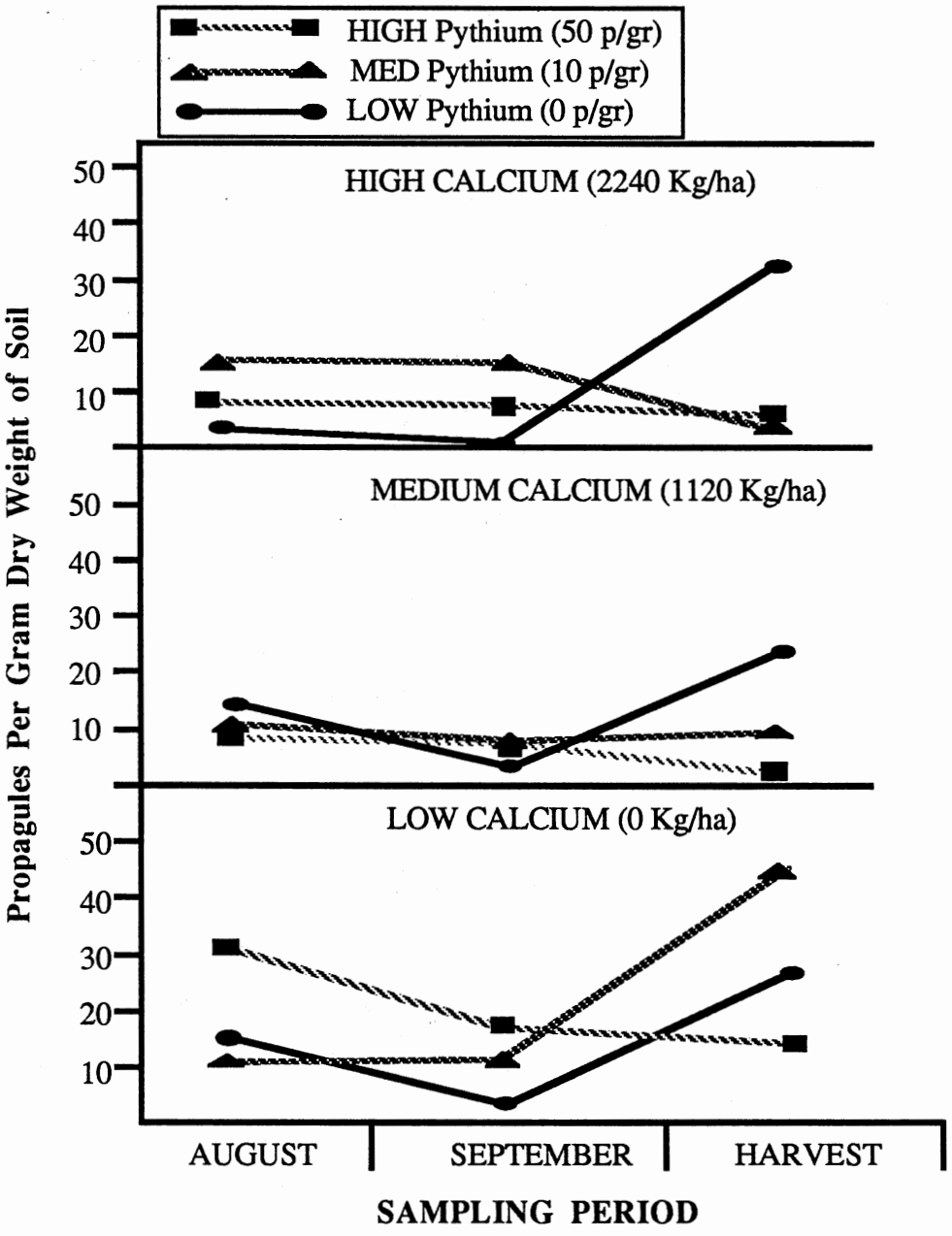

HIGH Pythium (50 p/gr)
MED Pythium (10 p/gr)
LOW Pythium (0 p/gr)
HIGH CALCIUM (2240 Kg/ha)
MEDIUM CALCIUM (1120 Kg/ha)
LOW CALCIUM (0 Kg/ha)
Propagules Per Gram Dry Weight of Soil
50
40
30
20
10
AUGUST
SEPTEMBER
HARVEST
SAMPLING PERIOD

Figure 19: Mean Pythium Populations During Three Sampling Periods Under Three Soil Calcium Levels: Florunner MP 2 Experiment.*

*Each point represents the mean p/g of four replications according to dilution plating on a Pythium Selective Medium (dilution factors of 1:20 and 1:200 were used with 5 plates per dilution factor). No significant relationship was observed between soil calcium treatment and the propagules detected.

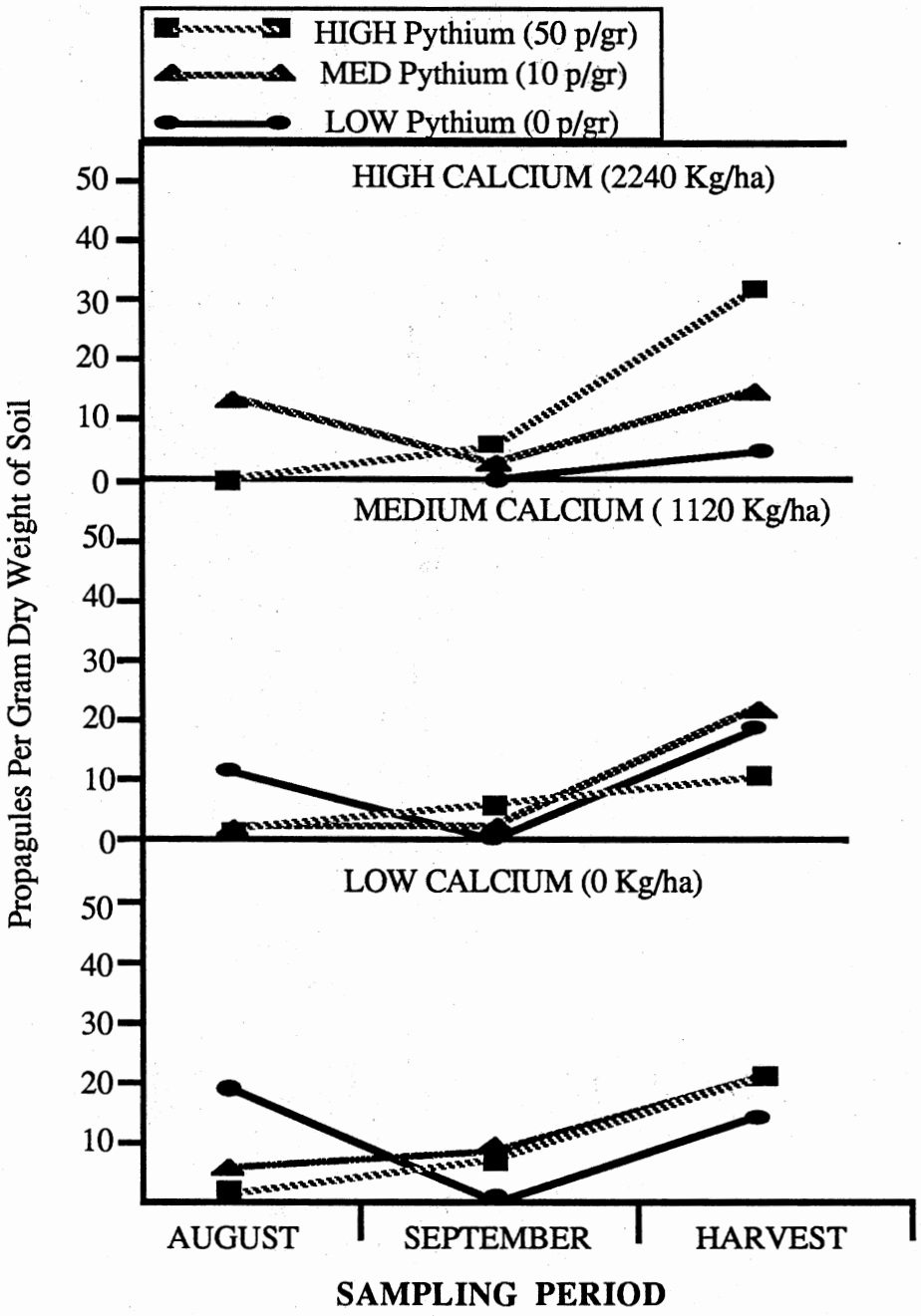

HIGH Pythium (50 p/gr)
MED Pythium (10 p/gr)
LOW Pythium (0 p/gr)
HIGH CALCIUM (2240 Kg/ha)
MEDIUM CALCIUM (1120 Kg/ha)
LOW CALCIUM (0 Kg/ha)
Propagules Per Gram Dry Weight of Soil
50
40
30
20
10
0
AUGUST
SEPTEMBER
HARVEST
SAMPLING PERIOD

Figure 20: Mean Pythium Populations During Three Sampling Periods Under Three Soil Calcium Levels: Spanco MP 1 Experiment.*

*Each point represents the mean p/g of four replications according to dilution plating on a Pythium Selective Medium (dilution factors of 1:20 and 1:200 were used with 5 plates per dilution factor). No significant relationship was observed between soil calcium treatment and the propagules detected.

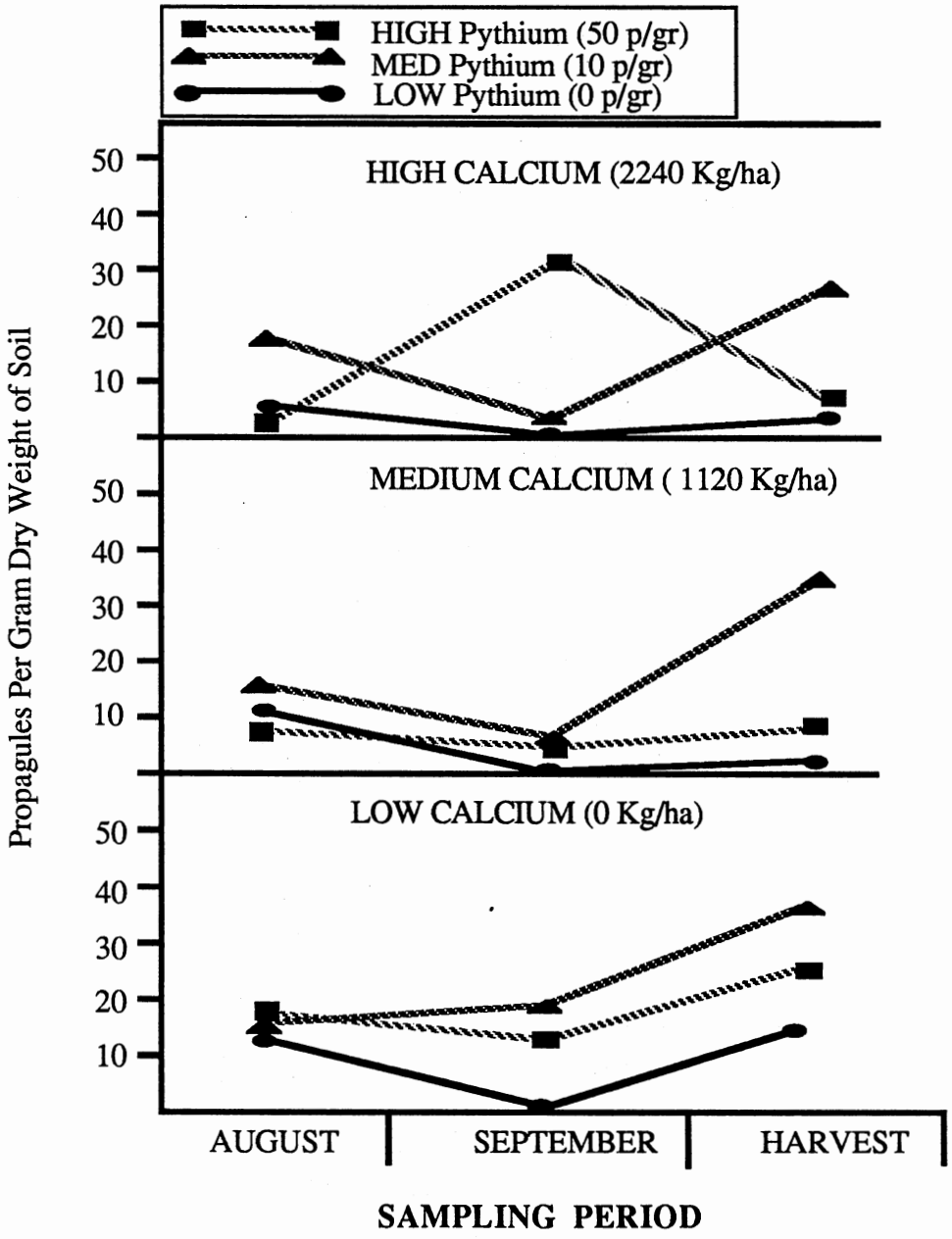
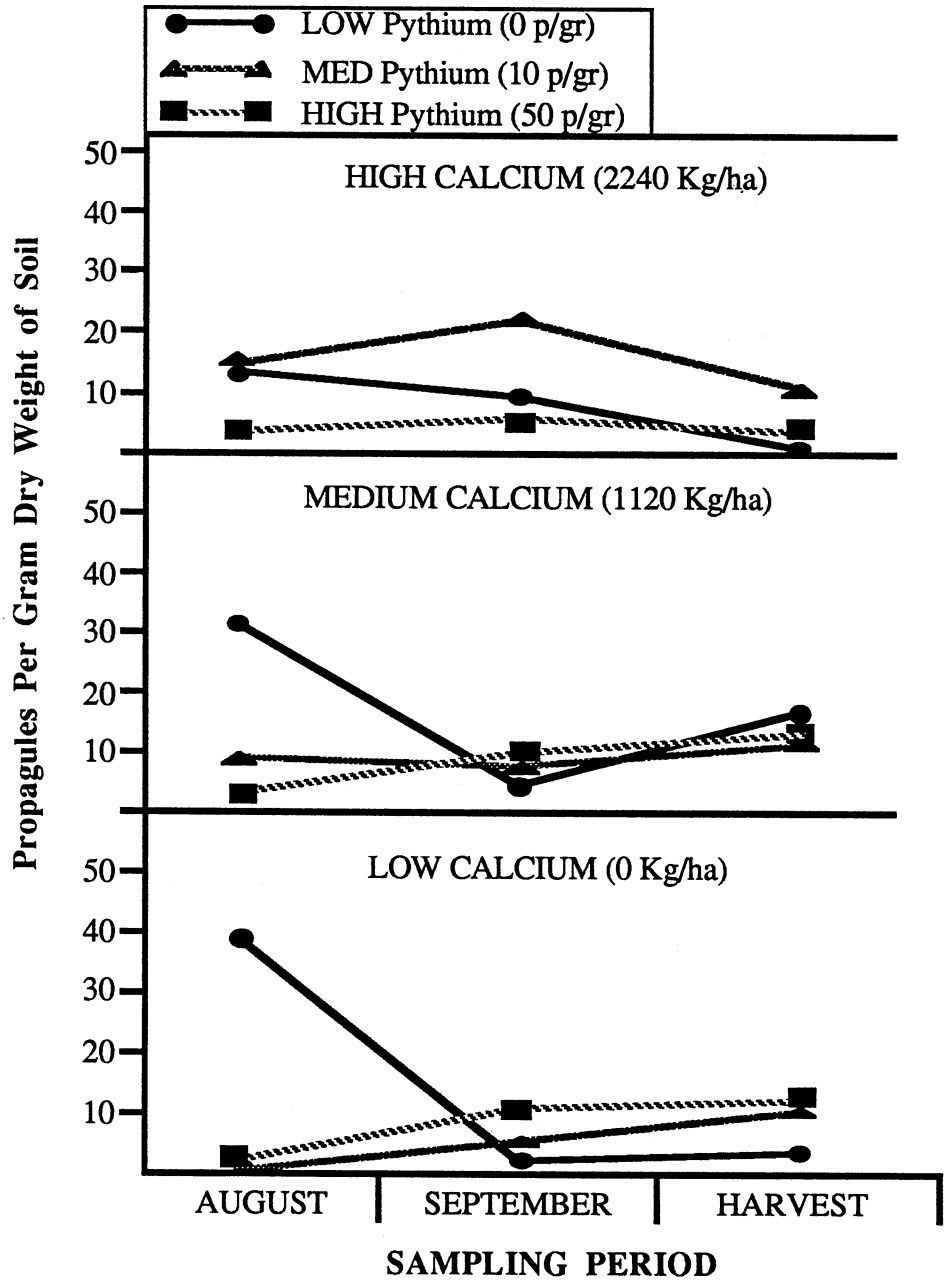

HIGH Pythium (50 p/gr)
MED Pythium (10 p/gr)
LOW Pythium (0 p/gr)
HIGH CALCIUM (2240 Kg/ha)
MEDIUM CALCIUM (1120 Kg/ha)
LOW CALCIUM (0 Kg/ha)
50
40
30
20
10
Propagules Per Gram Dry Weight of Soil
AUGUST
SEPTEMBER
HARVEST
SAMPLING PERIOD

Figure 21: Mean Pythium Populations During Three Sampling Periods Under Three Soil Calcium Levels: Spanco MP 2 Experiment.*

*Each point represents the mean p/g of four replications according to dilution plating on a Pythium Selective Medium (dilution factors of 1:20 and 1:200 were used with 5 plates per dilution factor). No significant relationship was observed between soil calcium treatment and the propagules detected.


LOW Pythium (0 p/gr)
MED Pythium (10 p/gr)
HIGH Pythium (50 p/gr)
HIGH CALCIUM (2240 Kg/ha)
MEDIUM CALCIUM (1120 Kg/ha)
LOW CALCIUM (0 Kg/ha)
Propagules Per Gram Dry Weight of Soil
50
40
30
20
10
AUGUST
SEPTEMBER
HARVEST
SAMPLING PERIOD

VITA

Thomas Elmo Clemente

Candidate for the Degree of

Master of Science

Thesis: THE RELATIONSHIP OF CALCIUM TO THE ETIOLOGY OF PEANUT POD ROT

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