EFFECT OF CALCIUM ON PEANUT (*Arachis hypogaea* L.) POD AND SEED DEVELOPMENT UNDER FIELD CONDITIONS

By

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To ParBrahmn, my parents, sister, advisor and beloved teachers
Ashok Kumar Vishwakarma, Dali Varghese, Sanjay Jambhulkar and Tapas Chaudhuri

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EFFECT OF CALCIUM ON PEANUT (*Arachis hypogaea* L.) POD AND SEED DEVELOPMENT UNDER FIELD CONDITIONS

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The effect of gypsum supplementation on peanut seed and pod development was studied for two varieties the field. Pod length was not affected by gypsum treatment. However, fewer two-segmented pods (P = 0.006), fewer pods with two seeds (P = 0.006), more immature or aborted distal seeds (P = 0.002), and more asynchronized fruit (P = 0.01) were observed in plots without gypsum applications. Non-treated gypsum plots at 100 and 130 DAP had the highest amount of aborted seeds (8%) and asynchronized fruits. The effect of gypsum onseed and fruit development was greater for C-99R than for Georgia Green.

Additionally, subsamples were analyzed for their mineral composition (Ca, Mg, K, P, S, Fe, Zn, Mn, Cu, Ni, and Na). All nutrients, except Cu and Mn, had their highest concentrations in immature pods and seeds and these levels decreased as fruits matured. Gypsum application increased Ca and S concentrations of both pods and seeds, decreased Mg concentrations only in seeds, and decreased Na and P concentrations of both pods and seeds. Calcium concentrations were two times higher in pods compared to seeds. Georgia Green accumulated more Ca in pods and seeds than did C-99R. A linear increase in Ca concentration was observed in pods and seeds at the same physiological stages when sampled over time.

Peanut seeds and pods also were analyzed for CDPK expression during their development in gypsum-treated and non-treated soils. In seeds, CDPK transcript and protein expression profiles were biphasic. High CDPK levels were observed in very immature seed stages and these levels dropped in immature stages, rose again to high levels in mature stages and then dropped significantly in very mature stages. In pods, CDPK transcript and protein levels were consistently high until the very mature stage when levels were significantly diminished. Seeds at all developmental stages showed 2- to 3-foldlower CDPK transcript and protein levels under gypsum treatment. Histolocalization data showed decoration of immunoreactive CDPK primarily in the outer most cell layers of the pericarp and around vascular bundles, as well as in the single vascular trace that supplies nutrients to the developing ovule.

CHAPTER 1
INTRODUCTION

Cultivated Peanut

Peanut or groundnut (*Arachis hypogaea* L.) is one of the most widely cultivated oil seed crops. Its seed is used directly for food or as a source of oil. It is produced primarily in India and China, followed by the US. Sixty-five percent of the world's peanut production is consumed by China, the US and India. In 2008-09, compared to a global total production of 34.3 million metric tons (Mmt), the US produced 2.34 Mmt of peanuts (USDA-FAS, 2010). Due to its low cost and good flavor, Americans eat approximately 1.7 billion lbs. of peanuts per year. The seed has high nutrient content containing essential vitamins, folic acid, "good" fats and high protein levels.

Peanut Reproduction and Calcium

Peanut reproduces via geocarpy, which is rare in the plant kingdom. However, geocarpy is a characteristic of all *Arachis* species. The underground developing fruits of peanut differ in a number of ways from those that develop aerially. First, peanut fruits are not photosynthetically active. Additionally, developing peanut fruits uptake nutrients directly from the soil and this process affects seed maturation. It is well established that calcium is critical for proper peanut seed development (Cox *et al.*, 1982; Gascho and Davis, 1994), and that maximum calcium levels in the seed are reached approximately 60 days after the peg enters the soil (Mizuno, 1959). However, when an adequate amount of calcium is unavailable to developing seed tissues, it results in pods containing no seed or severely underdeveloped seed. To avoid deficiencies, calcium is supplied to the soil as gypsum or limestone. Previous research has focused on the effect of calcium early in the season to: 1) identify specific calcium requirements for various peanut cultivars (Gascho *et al.*, 1994; Cox *et al.*, 1982; Walker *et al.*, 1976), 2) determine factors

affecting calcium uptake and the mechanisms of uptake (Kvein *et al.*, 1988; Boote *et al.*, 1982; Skelton and Shear, 1971), 3) define fruit filling characteristics (Colwell and Brady, 1945), 4) or at the end of the season to determine the effect on final yield (Sorensen and Butts, 2008). However, the seed developmental stage at which calcium is most crucial for normal maturation is unknown. Thus, the first objective of the current research was to address this question using two runner varieties, C-99R and Georgia Green, under field conditions in low calcium soils with and without calcium supplementation.

Macronutrient and micronutrient accumulation or composition in the developing peanut fruit and seed is affected by Ca (Pickett, 1950; Pattee *et al.*, 1974). This effect has been extensively studied in solution culture (Zharare *et al*., 2009a and b; Zharare *et al*., 1998; Zharare *et al.*, 1993; Pal and Laloraya, 1967). However, the effect of calcium sources, such as gypsum, on nutrient composition and nutrient changes within pods and seeds are not well understood. Therefore, a second objective of the above study was to determine the effect of gypsum supplementation on the concentration of calcium and other minerals in the developing peanut fruit.

The physiological basis of calcium uptake by the peanut fruit has been reported (Skelton and Shear, 1971; Wiersum, 1951). However, the molecular mechanisms involved in this process are not understood. One approach to understand the molecular basis of calcium uptake is to study a class of calcium sensors known as Calcium Dependent Protein Kinases (CDPKs). These proteins are ubiquitous in plants; there are at least 34 genes encoding CDPKs in the *Arabidopsis* genome (Cheng *et al.*, 2002; Harmon *et al.*, 2001). There is evidence that CDPKs are important for normal seed development. Frattini *et al.* (1999) showed that two rice CDPK isoforms, OsCDPK2 and OsCDPK11, were differentially expressed during seed development. Low

expression of the OsCDPK2 protein was observed during early developmental stages increasing through maturity, while OsCDPK11 showed down regulation during later stages of development. In a later study, over expression of *OsCDPK2* in transgenic rice blocked seed development at a very early stage (Morello *et al.*, 2000). In *Arabidopsis*, knockouts of the CDPK, CPK28, resulted in embryo lethality (Harper *et al.*, 2004). Likewise a CDPK was identified in sandalwood embryogenic cultures that accumulated to high levels during somatic and zygotic embryogenesis, endosperm development and seed germination, but was undetectable in mature organs such as shoots and flowers (Anil *et al.*, 2000). Dasgupta (1994) characterized a 53 kDa peanut CDPK (GnCDPK) from mature seed. However, little is known regarding a role for CDPKs in peanut fruit development. Because peanut's subterranean seed development relies primarily upon the sensing and response of the young, underground developing fruit to calcium levels in the soil, the third objective of this research was to examine changes in CDPK expression during peanut fruit development in low calcium soils and the same soils supplemented with calcium.

CHAPTER 2
LITERATURE REVIEW

Peanut Geocarpy

Angiosperms during reproduction usually flower and fruit aerially. However, in peanut (*Arachis hypogaea* L.), flowers are produced aerially, but fruits form underground. This phenomenon is called geocarpy and it is an atypical trait in the plant kingdom. Lev-Yadun (2000) reported 20 active geocarpic species in Israel. In addition, Barker (2005) reported 50 species including leguminosae members exhibiting an active, passive, amphi or geophytic geocarpy in African and Madagascan flora. However, geocarpy is a characteristic of all *Arachis* species.

The Peanut Flower

The papillionaceous, sessile and often orange-colored peanut flowers are borne on a spike inflorescence present on the primary and secondary braches of the plant (Pattee and Stalker, 1995; Smith, 1950). In the gynoecium, the ovary is unilocular, sessile and inferior. The ovules are anatropus, crassinucellate and bitegmic (Periasamy and Sampoornam, 1984).

Pollination and Peg Initiation

The flower opens after sunrise. Self pollination occurs in the enclosed keel. The flower usually withers within 24 hrs after pollination. Fertilization and syngamy occur 10 to 18 hrs following pollination (Smith, 1956). After syngamy, the peanut fruit is initiated as a stalk like structure that carries the fertilized ovules within 1 mm of the tip. Botanically, this stalk is widely known as gynophore, colloquially it is known as a peg (Smith, 1950). Within the peg, the intercalary meristem behind the fertilized ovules divides resulting in elongation. Anatomically and morphologically, the peg is a stem-like structure, however due to its positively geotropic

nature it behaves like a root (Moctezuma, 2003). The peg continues to elongate until it reaches the soil.

Fruit Initiation

Once the peg enters the soil, the intercalary meristem ceases its activity (Periasamy and Sampoornam, 1984). Thompson *et al.* (1992) showed that seven days after entry into the soil phytochrome is present in the developing ovules and embryos, but not in the peg tissues. The cells derived from the innermost layers of the peg increase around the proximal ovule first followed by the distal ovule, forming an inner zone. This marks fruit initiation. Then cells surrounding the inner zone divide rapidly on the dorsal side near the proximal ovule. Due to this unequal growth of the inner zone tissue, the fruit assumes a horizontal position and runs parallel to the soil. The inner zone has spongy parenchymatous tissue contributing to the major portion of the fruit wall (Periasamy and Sampoornam, 1984). The epidermis of the outer zone is replaced by the periderm due to an increase in cell number. As the fruit enlarges in the soil, the vascular bundles become interlinked by lateral connections through fibrous plates. Based on physiological growth and biochemical changes, fruit development has been classified into various stages (Young *et al.*, 2004; Boote, 1982; Pattee *et al.*, 1974). The current classification system of peanut fruit development (Figure 2-1) described below has been adapted from Young *et al.* (2004) and Paik-Ro *et al.* (2002).

Fruit Development Classification

Stage 1: Very Immature

Pod. The pod is very watery, soft and spongy. This appearance is due to the presence of parenchymatous tissue. The wall consists of an inner zone, outer zone, and 10-13 vascular bundles joined by lateral connections. At this stage, the pod acts as a storage organ for the developing seeds and has the highest levels of sugar and starch compared to later stages.

Seed. The seed is very small and flat with a white seed coat. Anatomically, the seed has an outer and inner surface epidermis having rectangular (20 - 40 µm) and irregular cells, respectively. The epidermal cells consist of a dense cytoplasmic network surrounding the organelles including starch grains and protein bodies. The provascular bundles range from 3 - 8 µm in diameter and are placed equidistant. The mid region parenchyma cells consist of numerous vacuoles, protein bodies and starch granules. Lipid bodies can also be seen at this stage. Stomata cannot be distinguished with electron microscopy at this stage.

Stage 2: Immature

Pod. The pod is watery and soft. However, it begins to show signs of dehydration.

Seed. The seed is round. Its seed coat is pink at the tip of the embryo axis, but the remainder is white. Sugar levels are high in the seed coat. Cells of the outer epidermis are now 30 - 50 µm and the cells of the outer epidermis are angular. At this stage, stomata can be easily seen on the inner surface of the epidermis. At this stage of development, starch and sugar levels are approximately equal (Pattee *et al.*, 1974); however the lipid content is low. The seed weight is approximately 300 mg.

Stage 3: Mature

Pod. The parenchymatous tissue begins to disintegrate; the inner pericarp dries and cracks giving a white papery appearance. Sugar and starch content decreases, however the content of hemicelluloses increases.

Seed. The seed coat begins to dry out and turn a light pink. The epidermal cells of the outer surface increase in size to 60 - 80 µm. The inner surface epidermal cells possess angular shaped cells with distinct stomata. Lipid bodies start accumulating in the seed. Starch and sugar content gradually decreases in the seed coat and increases in the seed. The seed weight is approximately 600 mg.

Stage 4: Very Mature

Pod. The parenchymatous tissue is gone. The outer and inner cell layers contain hemicellulose deposits. The 10 - 13 vascular bundles are interconnected through the sclerenchymatous layers. In cross section, the vascular bundles are in a Y-shaped groove of the sclerid layer. This Y-shaped grove is responsible for the reticulation of the outer surface of the pod (Halliburton *et al.*, 1975). Brown black splotches are on the inner pericarp as a result of complete loss of parenchymatous tissue.

Seed. The seed coat is completely dry and dark pink. The rounder outer surface epidermal cells are 70 - 100 µm in size and the inner surface epidermis possesses angular shaped cells. Vascular bundles can be seen in both cotyledons (Young and Schadel, 1990). A major portion of the cotyledons consist of parenchyma cells. Lipid bodies reach their maximal levels. Starch and sugar levels in the seed coat are the lowest since development initiated and are the highest in the cotyledons. The seed weight is approximately 600 mg.

Calcium Movement and Location in Peanut

In peanut roots, calcium moves upward via the xylem, but a non-significant amount of calcium moves back from the leaves downward to the developing reproductive organs (Mizuno, 1959; Wiersum, 1951; Bledsoe *et al.*, 1949). Mass flow also supplies low amounts of calcium through the upward movement of water from the gynophores to the plant tops. Skelton and Shear (1971) showed the presence of radio-labeled calcium in fruit exposed to the air and very little still in solution. They concluded that there was water movement by transpiration which transported calcium from root to gynophore, but not enough to satisfy the requirements of normal seed development. This result shows that the developing pod absorbs calcium directly from the soil solution in the fruiting zone. Consequently, calcium within the soil fruiting zone is required in relatively high concentrations. The first 20 days following entry of the peg in the soil

is critical for development because 92% of the total calcium is taken up during that period (Pattee and Stalker, 1995). Electron micrograph analysis of peanut fruits revealed that calcium was mainly concentrated in the exocarp and mesocarp of the pod and in the inner and outer surface of the seed coat. The lignified pod endocarp and the seed had the lowest calcium concentration (Smal *et al.* 1989).

Because calcium cannot be mobilized from older tissues and redistributed via the phloem, it creates pressure on the developing tissues to use the immediately available calcium supply from the xylem. Since the calcium supply in the xylem is transpiration dependent, and transpiration is low in developing fruits, young leaves and other developing tissues, calcium deficiency can result. In peanut, calcium deficiency results in an increased number of pods that contain no seeds or seeds that are severely underdeveloped. The deficiency can also result in a darkened plumule or black heart. It also can adversely affect pod production, seed viability and germination rates (Pattee and Stalker, 1995).

Factors Affecting Calcium Availability, Concentration and Distribution in Peanut

Sumner *et al.* (1988) found that larger seeded pods with less surface area to weight ratio require a higher soil calcium concentration since they are less efficient in diffusion compared to smaller seeded pods. In addition, other factors including pod maturity period, weight, thickness and volume also contribute to differences in calcium requirements for different cultivars (Kvien *et al.*, 1988).

Abiotic factors also influence the availability, concentration and distribution of calcium in peanut fruits. Murata *et al.* (2008) showed that low pH can adversely affect normal pod setting irrespective of calcium levels in solution culture. They showed that a pH of 3.5 at three different calcium levels, 500 µM, 1000 µM and 2000 µM, produced only 58 % normal pods

compared to 94 % normal pods produced in all three calcium levels with a pH of 5.0 and 6.5. The seed calcium concentration was highly reduced at pH 3.5, as well.

Oxygen supply is also an essential component for normal fruit development. In a solution culture experiment, Zharare *et al.* (1998) showed that failure to provide the proper aeration (oxygen) resulted in poor seed formation at the proximal end and no seed formation at the distal end.

Drought has a severe effect on calcium uptake by developing fruits. Boote *et al.* (1982) showed poor calcium uptake during a drought period. It resulted in severe calcium deficiency symptoms with reduced seed and pod calcium concentrations compared to a well-watered treatment. Cultivar differences may also affect moisture requirements. Wright. (1989) showed that the yield of a Virginia peanut was unaffected by low moisture, while a reduction in yield was obtained for a Spanish variety under the same conditions.

Gypsum particle size and its date of application can affect the availability of calcium in the soil. Walker *et al.* (1981) found that application of coarse gypsum at planting in a sandy loam soil resulted in higher calcium availability than fine particle gypsum applied at early flowering. However, yield was unaffected by these factors.

Soil texture also affects leaching ability of soil calcium. Leaching ability of calcium in a gypsum amended sandy loam soil was higher than in a sandy clay loam (Alva and Gascho, 1991). The gypsum amendment also increased the leaching ability of K and Mg in the sandy loam. The authors postulated that the high leaching ability of K and Mg in the gypsum amended sandy loam maintained the appropriate cationic balance to produce high quality peanuts.

Interactions of Calcium (Gypsum) and Other Ions in Peanut

Pal and Laloraya (1967) showed that adding NaCl to a peanut solution culture significantly reduced pod set. Peg tips turned brown and showed necrosis. Pods that did develop were small

and had necrotic lesions. Sodium was inhibiting the active uptake of calcium by the developing pods. Addition of gypsum to the saline-stressed plants alleviated the calcium deficiency and increased fruit yield.

Gypsum also affects potassium concentrations. Sullivan *et al*. (1974) showed that when gypsum was applied in combination with potassium, the potassium did not affect seed quality or yield; however, a potassium only application reduced the total yield. A soil calcium to potassium ratio of 3:1 resulted in the best yields because it increased the chances for calcium uptake. A high concentration of potassium and a low concentration of calcium also resulted in poor peanut seed germination. Magnesium is also inversely correlated with calcium. The addition of K_2SO_4 or $MgSO_4$ in the cultivation of Virginia Bunch increased pod breakdown, external damage to the seeds, and reduced seed calcium concentrations (Hallock and Garren, 1968).

Addition of gypsum also influences phosphorus uptake. Zhu and Alva (1994) showed a 54% decrease in phosphorus transport in a gypsum-amended sandy loam soil. It was postulated that the calcium precipitated the phosphorus and thereby decreased its transport in the soil.

Boron, like calcium, is phloem immobile and can decrease peanut yields under deficient conditions. Keeratikasikorn *et al.* (1991) showed that calcium and boron probably act in concert. Deficiencies of both ions resulted in a reduction of seed size, shelling percentage, number of pods per plant, and number of seeds per pod. Application of both ions increased the yield and seed quality.

Calcium as a Secondary Messenger

Calcium, apart from being a nutrient as described above, is also a central key ion that serves as a secondary messenger in a diverse array of plant signal transduction pathways. A stimulus specific increase in the $[calcium]_{cyt}$ is known as a calcium signature. There are numerous calcium signatures. The basic unit is a single spike. Other types include double

(biphasic) and multiple responses (oscillations). In theory, the signaling information can be encoded in a spike's magnitude, duration, frequency or sub-cellular location. Proteins are the receptors of these calcium signatures, known as calcium binding proteins. These proteins include the following (Harmon *et al.*, 2001):

1. **C**alcium **D**ependent **P**rotein **K**inases (CDPKs)
2. **C**alcium Dependent Protein Kinase **R**elated **K**inases (CRKs)
3. **Ca**l**m**odulin **D**ependent **P**rotein **K**inases (CaMKs)
4. **Ca**lcium and **Ca**l**m**odulin **A**ctivated **K**inases (CCaMKs)
5. **Sn**f1-**R**elated **K**inases (SnRKs)
6. **C**alcineurin **B**- **L**ike Calcium Binding Proteins (CBLs)
7. **C**aM-**B**inding Protein **K**inase (CBKs)

Based on the mechanism of activation, these calcium sensors are either "sensor responders" or "sensor relays" (Klimecka and Muszyńska, 2007; Harmon *et al.*, 2001). CDPKs are calcium responders, while CRKs, CCaMKs, and SnRK3s are sensor relays. CDPKs are found in plants and protists including the malarial parasite *Plasmodium falciparum* (Harper *et al.*, 2004), but not in mammals, fungi or insects (Harmon *et al.*, 2001). CDPK genes are thought to have evolved from the combination of protein kinase and calmodulin genes through recombination of ancient introns (Zhang and Choi, 2001).

Functions of CDPKs

CDPKs have been associated with a wide array of functions in signal transduction. An in depth discussion of all the functions is beyond the scope of this literature review. However, in broad terms, they are involved in stress and hormone responses, and reproductive and vegetative growth (Klimecka and Muszyńska, 2007; Ludwig *et al.*, 2004; White and Broadley, 2003). Such a wide range of diverse functions probably contributes to their phylogenetic grouping into

several subfamilies due to differences in structure, site specific localization (Klimecka and Muszyńska, 2007), and requirement for different promoters for tissue specific expression (Harper *et al.*, 2004).

Responses to Stress Signals

In rice, out of the 31 identified CDPKs, 11 have been reported to play an active role in stress signaling of 10-day old seedlings. Transcript levels were either up regulated or down regulated when exposed to different stress stimuli. CDPK sequence analysis revealed the presence of *cis*-acting elements upstream of the promoter region that were correlated to stress responses (DeFalco *et al.*, 2010; Ye *et al.*, 2009). In tobacco, the NtCDPK1 and NtCPK4 genes were transcriptionally up regulated after 1-2 hrs of osmotic stress.

In *Arabidopsis,* the zinc finger domain AtDi19, a drought responsive element, was phosphorylated by AtCPK4 and AtCPK11. These zinc finger domains are thought to have an important role in stress tolerance to salinity and drought. AtCPK23 played a positive role in stomatal opening and the acquisition of potassium ions when plants were under salinity stress (Ma and Wu, 2007). Salt stress also affects the subcellular localization of CDPKs. The ice plant McCPK1 was associated with actin filaments during osmotic stress and was translocated from the plasma membrane to the trans cytoplasmic ER and then to the nucleus where it phosphorylated MsCSP1, a pseudo response regulator of salt stress (Patharkar and Cushman, 2000).

CDPKs also play a role in elicitor and wound induced responses. The tobacco NtCDPK1 gene was up regulated by fungal chitin elicitor. Similar fungal elicitor responses also have been reported for NtCDPK2 (Romeis *et al.*, 2001), and in *in vitro* analysis of maize ZmCPK10 (Murillo *et al.*, 2001).When tomato leaves were wounded, the LeCDPK1 transcript displayed a transient increase at the wound site. The transient increase was correlated with an increase in the

amount and activity of cytosol soluble CDPKs suggesting a role in plant defense. A similar response was observed in maize when ZmCPK11 transcripts showed an increase during injury (Klimecka and Muszyńska, 2007).

Responses During Reproductive Development

CDPK levels change during fruit ripening (Leclercq *et al.*, 2005; Duan *et al.*, 2003) and seed development (Frattini *et al.*, 1999). The role of CDPKs in fruit development has been studied in apple (Duan *et al.*, 2003), tomato (Anguenot *et al.*, 2006), sandalwood (Anil *et al.*, 2003) and grape berry (Shen *et al.*, 2004). The role of CDPKs in seed development has been reported only in rice (Frattini *et al.*, 1999).

CDPK involvement in fruit development mainly has been related to sucrose metabolism (Anguenot *et al.*, 2006; Duan *et al.*, 2003). In tomato, a 55 kDa membrane associated and soluble CDPK was identified in developing tomato fruit. The membrane associated CDPK activity was highest in young fruits and gradually decreased as the fruit matured. This membrane CDPK phosphorylated sucrose synthase which was responsible for partitioning of sucrose synthase during fruit development.

Duan *et al.* (2003) obtained phosphatidylserine (PS) activated and membrane associated membrane CDPK and calcium-independent mitogen-activated protein kinase-like (MAPK-like) proteins from developing apple fruit. CDPK expression was highest in young fruit and middle fruit stages and decreased with maturity.

Anil *et al.* (2000) characterized two CDPKs of 55 to 60 kDa in sandalwood (swCDPK). swCDPK levels were high in mature fruits, but insignificant in shoots and flowers. The 55 kD CDPK was strongly associated with oil bodies (Anil *et al.* 2003). The high swCDPK activity and amount during oil body maturation suggests a regulatory role of CDPK in oil body biogenesis.

Kawasaki *et al.* (1993) reported that the *spk* gene encoding CDPK was located upstream of the gene encoding the starch branching enzyme, *sbe1,* in developing rice. The expression of *spk* was found to be similar to the expression of *sbe1*. It was exclusively expressed in developing seeds. It was postulated that *spk* might be involved in the partitioning of starch during seed maturation.

Frattini *et al.* (1999) showed that two rice CDPK isoforms, OsCDPK2 and OsCDPK11, were present at different levels during seed development. OsCDPK2 protein was low in early seed development and increased in later stages, while the converse was true for OsCDPK11. In a later study, over expression of OsCDPK2 in transgenic rice resulted in a drastic reduction of seed production (Morello *et al.*, 2000). Only 7% of the transgenic flowers produced seed. The conclusion was that the abnormally high expression of OsCDPK2 blocked early seed development. The mechanism for this inhibition of seed development is not known.

A)

B)

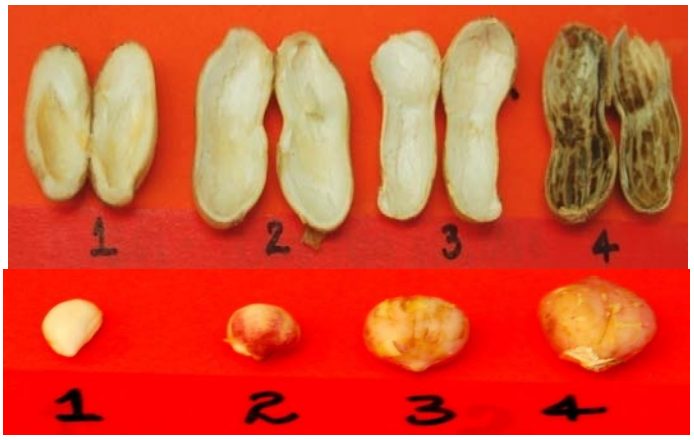

Figure 2-1. Stages of peanut fruit development. (A) Pod stages; (B) Seed stages. 1: Very Immature; 2: Immature; 3: Mature; 4: Very Mature (Pattee *et al*., 1974).

CHAPTER 3
THE EFFECT OF CALCIUM ON DEVELOPING PEANUT (*Arachis hypogaea* L.) SEEDS AND PODS IN THE FIELD

Introduction

Peanut (*Arachis hypogaea* L.) is one of the most widely cultivated oil seed crops of the 21st century. Peanut seed is a major source of oil and protein and it is used directly for food consumption. Although the anatomy and morphology of the developing peanut fruit has been described (Gregory *et al.* 1951), the physiological and molecular mechanisms governing its development are not well understood. However, it is certain that calcium is the most critical nutrient in the soil for peanut production, particularly seed development (Cox *et al.*, 1982; Gascho and Davis, 1994). Once the peanut peg has penetrated the soil, root-absorbed calcium is no longer translocated to the developing fruit. The peanut pod must absorb calcium directly from the soil (Skeleton and Shear, 1971). Consequently, soil calcium levels in the fruiting zone (0 -10 cm) must be in the range of 1200 to 1600 lbs/acre in order to produce high quality seed. A calcium deficiency, whether due to insufficient levels in the soil or to drought conditions which inhibit calcium uptake by the pod, results in seed abortion and numerous empty pods (Smith, 1956).

To amend a deficiency, calcium is applied to the soil as gypsum or limestone. Previous research has examined the effect of calcium on peanut production either very early in the growing season or as it relates to yield at the end of growing season (e.g. Sorenson and Butts, 2008). Other studies have examined the calcium requirements of various cultivars (Cox *et al.* 1982; Walker *et al.* 1976), factors affecting calcium uptake and the mechanisms controlling uptake (Kvein *et al.*, 1988; Boote *et al.*, 1982; Skelton and Shear, 1971), and the influence of calcium on fruit filling characteristics (Colwell and Brady, 1945). Although it is known that peanut can absorb calcium for as long as 60 days after the peg enters the soil (Mizuno, 1959), the

time during development at which calcium is most crucially required for normal maturation is unknown. In an attempt to answer this question, two runner varieties, C-99R (large-seeded) and Georgia Green (small-seeded), were studied under field conditions with low calcium soils supplemented with gypsum.

Materials and Methods

Two peanut runner cultivars, C-99R (large-seeded) and Georgia Green (small-seeded), were grown at the University of Florida's Plant Science Research and Education Unit (Citra, FL) on Candler fine sand (Buster, 1979) in 2007, 2008 and 2009. This soil is composed of 97% sand-sized particles and 2% silt (Gregory *et al.*, 2006). Soil samples were analyzed (Waters Agricultural Laboratories, Camilla, GA) 4 wks before planting for P (51, 43 and 62 lbs/acre), K (40,19 and 66 lbs/acre), Mg (35,14 and 63 lbs/acre), Ca (333, 201 and 1011 lbs/acre), B (1.1, 0.2 and 0.4 lbs/acre), Mn (4, 2 and 5 lbs/acre), and pH (5.5, 5.4 and 7.85) in 2007, 2008 and 2009, respectively.

Every year, each cultivar was given two external gypsum (68.5% Ca) applications of 1500 lbs/acre each at 30 and 60 days after planting (DAP). The plots were arranged in a split plot design with two calcium treatments (low calcium soil and two external gypsum applications to that low calcium soil) as the main plot and cultivars as the subplot with six replications. Each plot was comprised of two rows with an area of 4.5 m x 2 m.

In order to make terms simpler, a pod with seeds will be referred to as a fruit, unless otherwise stated. Developing fruits were sampled and analyzed for six parameters from a randomly measured 1 m section per replication at 70, 100 and 130 DAP. Parameters were measured on each fruit sampled for all sampling dates except 70 DAP in 2007. Pod length was measured in cm by a calibrated digital Vernier caliper (Fisher Scientific, Pittsburgh, PA). Seed and pod developmental stages were classified as described by Pattee *et al.* (1974) as 1, 2, 3 or 4

with 1 being very immature and 4 being very mature. Pod segments were noted based on the number of segments observed for each fruit. In two-segmented fruit, the seed closest to the peg was classified as a proximal seed and the seed nearest the apical end was classified as a distal seed. The number of seeds per pod were also scored. Fruit development was classified into two categories and was based on the synchronization of seed and pod stages. Seeds and pods at the same stage of development were classified as synchronized fruit, whereas aborted seeds, shriveled or immature seeds in a mature pod i.e. seeds which did not synchronize with the pod developmental stages were termed as asynchronized fruit.

Data Analysis

For pod length, number of pod segments, number of seeds per pod, and fruit development, the very immature stage 1 fruits were not included in the analyses. An overall Analysis of Variance (ANOVA) was performed by PROC GLIMMIX (SAS *v.* 9.2, 2008). Year and replication were random effects while gypsum treatments, cultivars, and stages of development were fixed effects. For pod length, the ANOVA was performed on the average pod length over all stages of pod development. For the number of segments, the average frequency percent at the three sampling dates was calculated for each plot in all three years and an ANOVA was performed. For the number of seeds per pod, the average frequency percent of one seed per one segmented pod, one seed per two-segmented pod and two seeds per two-segmented pod was calculated for each plot and sampling date for all three years and an ANOVA was performed. For seed developmental stages, the frequency percent of each seed stage at the proximal and distal ends was calculated for each plot in all three years and sampling dates. An ANOVA was performed for the sampling date, cultivar and treatment. For fruit development, the frequency percent of proximal and distal seed stages was calculated for each pod stage and ANOVA was performed on the frequency percentage for differences in fruit development due to gypsum

treatment and cultivars. The probability values were from t-tests of the mean differences based on planned comparisons ($P \leq 0.05$).

Results

Pod Length

Pods increased in length for both cultivars throughout development regardless of gypsum treatment (Table 3-1). However, cultivar differences were observed (Table 3-2). As expected, C-99R had longer pods than Georgia Green, since C-99R is a larger seeded cultivar compared to Georgia Green.

Number of Pod Segments

A higher percentage of one-segmented pods (13.7 %) was obtained in control plots (non-treated) than when gypsum was applied (10.3 %; $P = 0.006$) (Table 3-3). Cultivars also differed in the number of pod segments. Georgia Green had a higher percentage of two-segmented pods (90.6 %) than C-99R (85.5 %; $P < 0.0001$) (Table 3-4).

Number of Seeds per Pod

For two-segmented pods, there was a higher percentage of two seeds (84.2 % versus 78.1%, $P= 0.006$, Table 3-3) when gypsum was applied. Cultivars also differed in the number of seeds per pod. Georgia Green had a higher percentage of two-segmented pods filled with two seeds (85.0 %) than C-99R (77.3 %) (Table 3-4). Since low soil calcium can result in one seed in two-segmented pods, the total number of fruits with one seed in a two-segmented pod was also analyzed. In this case, there was no difference observed due to gypsum treatment ($P = 0.8$, Table 3-3) or cultivar ($P = 0.38$, Table 3-4). However, the control plots produced more one-segmented pods containing one seed (20.7 %) than those plots receiving gypsum (15.2%) (Table 3-3).

Proximal Seed

The percentage of proximal seeds was not affected by gypsum treatment, cultivar or sampling date (Table 3-5). However, proximal seed development was affected (seed stage < 0.0001) and there were significant interactions of cultivar*seed stage and seed stage* sampling date (Table 3-5). Georgia Green produced a higher percentage of very mature proximal seeds (18.66 %) compared to C-99R (14.38 %, P < 0.0001), but had a similar percentage of proximal seeds in all other seed stages (Table 3-7). In the seed stage* sampling date interaction (Table 3-8), more very immature and immature proximal seeds were produced at 70 DAP than at 100 DAP and 130 DAP. However, more mature proximal seeds were found at 100 DAP than at 70 or 130 DAP. No very mature proximal seeds were found at 70 DAP compared to 18.25 % at 100 DAP and 31.73 % at 130 DAP (P < 0.0001).

Distal Seed

Similar to proximal seeds, the number of distal seeds produced was not affected by the gypsum treatment, cultivar or sampling date, but seed stage and the interaction effects of gypsum treatment*seed stage (P = 0.0033), cultivar*seed stage (P < 0.0001) and seed stage*sampling date (P < 0.0001) influenced the percentage of distal seeds (Table 3-5). A seed position analysis revealed that it was significant for all three sampling dates (Table 3-6). Georgia Green produced more very mature distal seeds (17.68 %, P = 0.002) and fewer aborted seeds (4.48 %, P = 0.05) than C-99R (Table 3-7), however no differences were observed in other seed stages for these cultivars. When gypsum was applied, fewer aborted distal seeds (4.68 %, P = 0.005) and more very mature distal seeds (16.61 %, P = 0.022) were produced compared to the control. However, other stages were similar regardless of gypsum treatment (Table 3-9). In the sampling date*seed stage analysis, seed stages were inconsistent among sampling dates. For instance, more aborted seeds were observed in the distal section at 100 (6.9 %) and 130 DAP (6.76 %) than at 70 DAP

(4.33 %) (Table 3-10). At 70 DAP, more immature and very immature seeds were produced than were produced at 100 and 130 DAP. When sampled at 100 and 130 DAP, more mature and very mature seeds were produced (Table 3-10).

Asynchronized Fruit

Fruit development was based on the overall degree and synchronization of pod and seed physiological stage development. For example, if pods were staged as mature or very mature and seeds within them were staged as immature, aborted or shriveled, then fruits were characterized as asynchronized. If pods and seeds were at the same stage of development, then fruits were characterized as synchronized or fully developed. The overall production of asynchronized fruit was not affected either by gypsum application or cultivar, but fruit stages were significantly different ($P < 0.0001$, Table 3-11). When each fruit stage was analyzed separately, the frequency percentage of immature fruits was not affected by gypsum treatment ($P = 0.97$), cultivar ($P = 0.63$), or an interaction of cultivar*gypsum treatment ($P = 0.58$, Table 3-12), but differences were significant for mature and very mature fruit stages due to gypsum treatment ($P = 0.01$ and 0.02, respectively) and cultivar ($P = 0.02$, Table 3-12). In the non-treated condition, a higher percentage of asynchronized mature (24.27 %) and very mature (12.91 %) fruits were obtained than in the gypsum treated condition which had 16.57 % mature ($P = 0.01$) and 8.34 % ($P = 0.02$) very mature fruits (Table 3-13), respectively. Georgia Green had a lower percentage of asynchronized mature (17.71 %, $P = 0.02$) and very mature (8.60%, $P = 0.02$) fruits than C-99R (Table 3-14).

Discussion

The effect of calcium and its supplementation in field studies have mainly focused on yield at the end of the growing season. This study examined the effect of the addition of calcium via gypsum application to low calcium soils during the development of peanut seeds and pods

throughout the growing season. Six parameters were measured at three sampling dates and at harvest for two cultivars that differed in seed size.

Pod length was not affected by gypsum treatment. However, C-99R had longer pods than Georgia Green. This result was expected since C-99R is a larger-seeded variety that normally has greater pod length, breath and width than Georgia Green. It is possible that pod length is controlled more by genetic factors, like other pod characteristics including surface area and volume (Kvein *et al.* 1988), than by the external environment, particularly soil calcium levels.

However, pods were affected by gypsum application in the number of segments that formed. Higher percentages of two-segmented pods were formed when gypsum was applied. Additionally, these two-segmented pods were more likely to contain two seeds when gypsum was applied. These effects were more pronounced for the smaller-seeded Georgia Green than the larger-seeded C-99R. These results support the observations made by Colwell and Brady (1945) where they obtained more two-segmented pods and more two-seeded pods when gypsum was applied to Spanish and Runner cultivars.

Botanically, the two ovules in a unilocular ovary form the proximal and distal segments. These segments can be either marked by the depth and form of constrictions depending on the shapes and orientation of the seeds (Smith, 1950). Since, the distal ovule is farther from the stigma during fertilization, it is not uncommon to have that ovule abort in the leguminosae due to being unfertilized (Teixeira *et al.*, 2006). In peanut, the abortion of mostly unfertilized ovules is more common in the distal segment (Periasamy and Sampoornam, 1984) and results in a one-segmented pod. Calcium's role in promoting fertilization is well-known (Ge *et al.*, 2007). Hence it is possible that calcium may exert an early effect on peanut reproduction by increasing the successful fertilization of the distal ovule, thereby increasing the number of two-segmented

pods carrying two seeds. Additionally, distal ovules can abort even if properly fertilized due to the competition of nutrients (Teixeira *et al.*, 2006). During peanut fruit development, the proximal ovule begins to develop first and it is closer to the peg. Thus nutrient loading is more likely to favor the proximal ovule than the distal ovule.

Colwell and Brady (1945) were unable to determine when peanut ovule abortions took place. Periasamy and Sampoornam (1984) showed that either little or no parenchymatous tissues were present surrounding unfertilized peanut ovules. Hence, it is likely that unfertilized ovules are aborted before or soon after the gynophore enters the soil. We observed that when soil did not receive gypsum supplementation, distal seed abortion was higher (~ 7 % versus ~ 5 %) (Table 3-9). Similarly, more aborted distal seeds were observed at 100 DAP and 130 DAP (~ 7 %) than at 70 DAP (~ 4 %) (Table 3-10). The indeterminate nature of peanut could play a role in calcium availability to pods set later in the season. Boote *et al*. (1982) showed that peanut has exponential growth in fruit load from 49 - 89 DAP and later it slows. Mizuno (1959) and Kvein *et al.* (1988) showed that the peak time for calcium absorption by the developing pod is up to 60 days after the gynophore enters the soil. Hence the initial fruit load receives all maternal assimilates and enough calcium to progress toward maturity. However, after 89 DAP, when fruit set decreases and peanut has reached its maximum fruit load capacity, the newly set seeds, particularly the distal seeds, may receive an insufficient amount of calcium supply thus slowing seed maturation and leading to a higher rate of abortion as was observed at 100 and 130 DAP in the control plots. From the present research, it is clear that the majority of ovule abortions due to nutrient deficiency manifest themselves sometime between 70 and 100 DAP at a time well after pod segmentation is evident.

Georgia Green had a lower percentage of aborted seeds than C-99R (Table 3-7). Georgia Green also had higher calcium concentrations in both pods and seeds in the non-gypsum treatment than C-99R (see Chapter 4). C-99R is a larger-seeded cultivar with greater length, breadth, width and hull thickness than Georgia Green. However the surface area to weight ratio is less than Georgia Green. Sumner *et al.* (1988) showed that a larger seeded pod is less efficient in diffusion than a smaller seeded pod due to less surface area to pod ratio. Hence it is possible that C-99R would not have been able to receive enough calcium by diffusion compared to Georgia Green and thus had a higher rate of seed abortion.

The analysis of fruit development showed that immature fruits appeared normal under lower calcium conditions with seed and pod developmental stages synchronized. However, a higher percentage of mature asynchronized fruits i.e. mature pods with immature, aborted or shriveled seeds, occurred in the non-gypsum treated condition. Hence, the analysis revealed that pod maturity was unaffected while seed maturity was delayed in low calcium soils.
Therefore, it can be hypothesized that under low soil calcium conditions two phenomena occur that affect peanut reproduction. First, distal ovules abort at a higher frequency most likely due to being unfertilized resulting in more one-segmented pods. Second, seed and pod development becomes asynchronized with seed maturity lagging behind pod maturity detectable by the middle (100 DAP) of the growing season.

Table 3-1. Pod length at three stages of pod development for peanuts grown near Citra, FL in 2007, 2008 and 2009. Each value represents the mean of two cultivars, three sampling dates and two calcium levels. Means followed by a different letter in the pod length column are different based on Tukey's mean separation ($P \leq 0.05$).

Pod stage	Pod Length (cm)
2	2.40a
3	2.45b
4	2.57c

Table 3-2. Pod length of two peanut cultivars grown near Citra, FL in 2007, 2008 and 2009. Each value represents the mean of three pod stages, three sampling dates and two gypsum treatments. Means followed by a different letter are different based on Tukey's mean separation ($P \leq 0.05$).

Cultivar	Pod Length (cm)
C-99R	2.57a
Georgia Green	2.37b

Table 3-3. Frequency percentage for number of pod segments and number of seeds/pod for gypsum treatments for peanuts grown near Citra, FL in 2007, 2008 and 2009 and their P-values. Each value represents the mean of two cultivars and three sampling dates.

Gypsum Treatment	No. of pod segments		No. of seeds / pod		
	1	2	1 seed /1 seg pod	1 seed/2 seg pod	2 seed/2 seg pod
Non-Treated	13.7	86.3	20.7	1.2	78.1
Treated	10.3	89.7	15.2	0.5	84.2
P-values	0.006	0.006	0.0006	0.38	0.006

Table 3-4. Frequency percentage for number of pod segments and number of seed/pod for two peanut cultivars grown near Citra, FL in 2007, 2008 and 2009 and their P-values. Each value represents the mean of two calcium levels and three sampling dates.

Cultivars	No. of pod segments		No. of seeds / pod		
	1	2	1 seed /1 seg pod	1 seed/2 seg pod	2 seed/2 seg pod
C-99R	14.5	85.5	21.9	0.8	77.3
Georgia Green	9.4	90.6	14	0.91	85
P-values	< 0.0001	< 0.0001	< 0.001	0.8	< 0.0001

Table 3-5. ANOVA probability values determined for proximal and distal seeds with gypsum treatment, two cultivars and sampling date for peanuts grown near Citra, FL in 2007, 2008 and 2009.

Effect	Proximal	Distal
Gypsum Treatment	0.45	0.47
Cultivar	0.44	0.46
Sampling Date (sdate)	0.86	0.9
Seed Stage	< 0.0001	< 0.0001
Gypsum Trt*Seed Stage	0.1	0.0033
Cultivar*Seed Stage	0.0003	< 0.0001
Seed Stage*sdate	< 0.0001	< 0.0001

Table 3-6. ANOVA probability values determined in the seed stages for position effect with gypsum treatment for two peanut cultivars grown near Citra, FL in 2007, 2008 and 2009.

Effect	70	100	130
Gypsum Treatment	0.21	0.0048	0.06
Cultivar	0.001	0.2962	0.001
Position	< 0.0001	< 0.0001	< 0.0001
Cultivar* Gypsum Trt	0.77	0.12	0.23
Gypsum Trt*position	0.91	0.12	0.34
Genotype*position	0.007	0.25	0.12

Table 3-7. Frequency percentage of proximal and distal seed developmental stages for peanuts grown near Citra, FL in 2007, 2008 and 2009. Each value represents the average of two cultivars, gypsum treatments and three sampling dates. The probability values result from t-tests of the mean differences based on planned comparisons.

Seed Stage	Proximal			Distal		
	C-99R	Georgia Green	P-value	C-99R	Georgia Green	P-value
Aborted	1.49	0.82	0.44	7.51	4.48	0.05
Very Immature	6.89	5.72	0.21	6	5.8	1.00
Immature	8.81	8.46	0.7	8.1	7.78	1.00
Mature	18.25	17.71	0.53	15.43	15.68	1.00
Very Mature	14.38	18.66	<0.0001	12.94	17.68	0.002

Table 3-8. Frequency percentage of proximal seed developmental stages determined at 70,100 and 130 days after planting (DAP) for peanuts grown near Citra, FL in 2007, 2008 and 2009. Each value represents an average of two gypsum treatments and two cultivars. The probability values result from t-tests of the mean differences based on planned comparisons.

Seed Stage	DAP			Differences (P-value)		
	70	100	130	70-100	100-130	70-130
Aborted	0.48	1.87	1.11	0.22	0.44	0.58
Very Immature	14.3	3.13	1.48	< 0.0001	0.15	< 0.0001
Immature	18.56	4.82	2.53	< 0.0001	0.0.4	< 0.0001
Mature	16.51	22.58	14.86	< 0.0001	<0.0001	0.97
Very Mature	-	18.25	31.73	-	<0.0001	-

Table 3-9. Frequency percentage of distal seed developmental stages for gypsum treatments for peanuts grown near Citra, FL in 2007, 2008 and 2009. Each value represents the mean of two cultivars and three sampling dates. The probability values result from t-tests of the mean differences based on planned comparisons.

	Treatment		
Seed Stage	Non-Treated	Gypsum Treated	P-value
Aborted	7.31	4.68	0.005
Very Immature	6.12	5.68	0.66
Immature	7.56	8.31	0.45
Mature	14.82	16.28	0.11
Very Mature	14.19	16.61	0.022

Table 3-10. Frequency percentage of distal seed developmental stages determined at 70,100 and 130 days after planting (DAP) for peanuts grown near Citra, FL in 2007, 2008 and 2009. Each value represents an average of two gypsum treatments and two cultivars. The probability values result from t-tests of the mean differences based on planned comparisons.

	DAP			Differences (P-value)		
Seed Stage	70	100	130	70-100	100-130	70-130
Aborted	4.33	6.90	6.76	0.040	0.89	0.05
Very Immature	13.33	2.78	1.59	< 0.0001	0.34	< 0.0001
Immature	17.49	4.04	2.29	< 0.0001	0.13	< 0.0001
Mature	15.13	19.88	11.65	< 0.0001	< 0.0001	0.18
Very Mature	-	17.23	29.53	-	< 0.0001	-

Table 3-11. ANOVA probability values determined for developing peanut fruit for gypsum treatment, cultivar, developing fruit stage and the interaction of fruit stage by gypsum treatment, cultivar and cultivar by gypsum treatment for peanuts grown near Citra, FL in 2007, 2008 and 2009.

Effect	Asynchronized
Gypsum Treatment	0.17
Cultivar	0.38
Fruit Stage	< 0.0001
Cultivar*Gypsum Trt	0.27
Gypsum Trt*Fruit Stage	0.46
Cultivar*Fruit Stage	0.29

Table 3-12. ANOVA probability values determined for asynchronized peanut fruit at various developmental stages for gypsum treatment, cultivar and the interaction effect of gypsum treatment by cultivar for peanuts grown near Citra, FL in 2007, 2008 and 2009.

Effect	Fruit Stages		
	Immature	Mature	Very Mature
Gypsum Treatment	0.97	0.01	0.02
Cultivar	0.63	0.02	0.02
Cultivar*Gypsum Trt	0.58	0.47	0.33

Table 3-13. Frequency percentage of asynchronized peanut fruit at different developmental stages determined for gypsum treatment for peanuts grown near Citra, FL in 2007, 2008 and 2009. Each value represents an average of two cultivars and three different sampling dates. The probability values result from t-tests of the mean differences based on planned comparisons.

Gypsum Treatment	Fruit Stages	
	Mature	Very Mature
Non-Treated	24.27a	12.91a
Treated	16.57b	8.34b

Table 3-14. Frequency percentage of asynchronized fruit at different developmental stages determined for two peanut cultivars grown near Citra, FL in 2007, 2008 and 2009. Each value represents an average of gypsum treatments and three different sampling dates. The probability values result from t-tests of the mean differences based on planned comparisons.

Effect	Fruit Stages	
Cultivars	Mature	Very Mature
C-99R	23.12a	12.6a
Georgia Green	17.71b	8.6b

CHAPTER 4
THE EFFECT OF GYPSUM APPLICATIONS ON THE ACCUMULATION OF MINERALS IN PEANUT (*Arachis hypogaea* L.) PODS AND SEEDS

Introduction

Peanut seed is highly nutritious because it contains essential vitamins, folic acid, "good" fats and high protein levels. In the US, it is grown on sandy loam soils in seven states in the Southeast (Alabama, Florida, Georgia, Mississippi, South Carolina, North Carolina, and Virginia), and in Texas, Oklahoma and New Mexico in the Southwest. These soils are often deficient in calcium which is essential for proper seed development. To alleviate calcium deficiency, gypsum ($CaSO_4$) is applied to the soil. The effect of gypsum application on peanut has been widely studied for its effect on yields at the end of the growing season (Sorensen and Butts, 2008). The effect of calcium on the accumulation of other nutrients in peanut has been extensively studied in solution culture (Zharare *et al*., 2009a and b; Zharare *et al*., 1998; Zharare *et al.*, 1993; Pal and Laloraya, 1967). However, little is known about the accumulation of other nutrients in peanut pods and seeds following gypsum application to low calcium soils. Therefore, the objective of the present study was to investigate the effect of gypsum when applied to low calcium soils on the composition of minerals in the developing peanut fruit. To the best of our knowledge, this is the first report describing the mineral content of developing peanut fruits under the influence of gypsum.

Materials and Methods

Two peanut runner cultivars, C-99R (large-seeded) and Georgia Green (small-seeded), were grown at the University of Florida's Plant Science Research and Education Unit near Citra, FL on Candler fine sand (Buster, 1979) in 2007, 2008 and 2009. This soil is composed of 97% sand-sized particles and 2% silt (Gregory *et al.*, 2006) and are low in calcium. Soil samples were examined (Waters Agricultural Laboratories, Camilla, GA) 4 wks before planting for P (51, 43

and 62 lbs/acre), K (40, 19 and 66 lbs/acre), Mg (35, 14 and 63 lbs/acre), B (1.1, 0.2 and 0.4 lbs/acre), Mn (4, 2 and 5 lbs/acre), and pH (5.5, 5.4 and 7.85) in 2007, 2008 and 2009, respectively.

Every year of the study, each cultivar was given two external gypsum (68.5 % Ca) applications of 1500 lbs/acre each at 30 and 60 days after planting (DAP). The calcium levels in non-treated plots were 333, 201 and 1011 lbs/A in 2007, 2008 and 2009, respectively. The plots were arranged in a split plot design with two gypsum treatments (no treatment and external gypsum application) as the main plot and cultivars as the subplot with six replications. Each plot was comprised of two rows with an area of 4.5 m x 2 m. The underground developing fruits were sampled from random one meter sections at 70, 100 and 130 DAP.

Macronutrient and Micronutrient Analyses

Samples were washed with distilled water and then surface sterilized with 70 % alcohol. Seed and pod developmental stages were classified as described by Pattee *et al.* (1974) as 1, 2, 3 or 4 with 1 being Very Immature (VIm), 2 being Immature (Im), 3 being Mature (Ma) and 4 being Very Mature (VMa). Pods and seeds of all stages were separated and dried at 70°C until a constant dry weight was achieved.

In 2007, Ma and VMa stages for all three sampling dates were analyzed only for Ca by Waters Agriculture Laboratories (Camilla, GA) by inductively coupled argon plasma emission spectrophotometery/vacuum. Fungal contamination in younger peanut fruits precluded their use.

In 2008 and 2009, dried samples were ground to a fine powder using a stainless steel coffee grinder. The powdered samples were sent to Dr. Michael Grusak, Baylor College of Medicine (Houston, TX) for mineral analysis. A minimum of two sub-samples (~ 0.25 g/ DW) of each ground sample were digested and processed for elemental analysis. Specifically, sub-samples were weighed and placed in 100 mL borosilicate glass tubes for pre-digestion overnight

with 3 mL ultra-pure nitric acid. The following day, tubes were placed in a digestion block (Magnum Series; Martin Machine, Ivesdale, IL, USA) and maintained at 125 °C for a minimum of four hrs (with refluxing). If reddish nitric smoke was still emanating from certain samples after four hrs, this step of the protocol was lengthened until the smoke dissipated. Then tubes were removed from the block and cooled for 5 min before adding 2 mL of hydrogen peroxide, and then they were put back on the block at 125 °C for one hr. This hydrogen peroxide procedure was repeated two more times. Finally, the digestion block temperature was raised to 200 °C and samples were maintained at this temperature until they were dry. Once cooled (after removal from the block), digestates were resuspended in 2 % ultra-pure nitric acid overnight, then vortexed and transferred to plastic storage tubes until analysis for Ca, Mg, K, P, S, Fe, Zn, Mn, Cu, Ni, and Na concentrations. Elemental analysis was performed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (CIROS ICP Model FCE12; Spectro, Kleve, Germany) with the instrument calibrated daily with certified standards. Tomato leaf standards (SRM 1573A; National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along with the peanut samples to ensure accuracy of the instrument calibration.

The micronutrient and macronutrient data were analyzed using PROC GLIMMIX of SAS v 9.2 (SAS, 2008). Calcium levels, cultivars and developmental stages were the fixed effects while year and replication were considered as random effects. Analysis of Variance (ANOVA) was performed for all minerals using $P \leq 0.05$ and the means were separated by Tukey's HSD.

Results

Gypsum Application Differences

Gypsum application affected the concentration of Ca, S, and P in both pods (Table 4-1) and seeds (Table 4-4), and the Mg concentration only in the seed (Table 4-4). Of the

micronutrients tested, only Na in pods (Table 4-2) and Zn in seeds (Table 4-5) were significantly affected by gypsum treatment.

Ca concentration increased in pods and seeds due to the gypsum application when averaged across (a) cultivars, developmental stages and sampling dates (Figures 4-1 and 4-2); (b) developmental stages and cultivars for three different sampling dates (Table 4-8); and (c) sampling dates, cultivars, and calcium treatments for different developmental stages (Figures 4-3 and 4-4). Likewise, S showed an increase due to gypsum application in case (a) and (b), but not (c) as mentioned for Ca (Figures 4-1 and 4-2; Table 4-8). Except for Ca, other minerals did not show any significant differences in their concentrations at different sampling dates.

A reduction in P and Na concentration was observed in pods (Figure 4-1) and in P concentration in seeds (Figure 4-2). Mg concentration was reduced in the seed ($P = 0.006$), but not in the pod. Zn concentration in the pod was not affected by gypsum application, although it was reduced in the seed ($P = 0.005$) when treated with gypsum. Other macronutrients and micronutrients did not differ significantly in concentration due to gypsum application. There was no significant calcium level interaction with cultivars, developmental stages or sampling dates for any mineral analyzed.

Cultivar Differences

Cultivar differences were observed for Ca (Tables 4-1 and 4-4), P (Table 4-4) and Zn (Table 4-5). Georgia Green had a higher calcium concentration in both pods (Table 4-3) and seeds (Table 4-6) compared to C-99R, while P was higher in C-99R seeds and the Zn concentration was higher in Georgia Green seeds (Table 4-6). Other minerals did not show significant cultivar differences for pod or seed composition.

Macronutrients and Micronutrients During Fruit Development

Generally, the concentration of all macronutrients (Figure 4-5) and micronutrients (Figure 4-6) showed a decline during the maturation of both pods and seeds with the exception of Mn whose concentration increased in both pods and seeds (Figure 4-6). When the concentration of nutrients was compared between pods and seeds, the differences were unaffected by cultivar, gypsum treatment or stage of development. The concentrations of Cu, K and Ni were found to be almost equal in both pods and seeds. Concentrations of Ca, Fe, Na, and Mn were 1.5 to 2 times higher in pods than seeds. However, the reverse trend was observed for S, P, Mg and Zn, where concentrations were 1.5 to 2 times higher in seeds than pods.

Discussion

Sorensen and Butts (2008) reported no differences in Ca and S concentrations in leaves, pegs and immature pods for three different gypsum rates (0, 504, 1008 lbs/A) at 80 DAP. In the present study, gypsum application increased Ca and S concentration in both the pods and seeds (Table 4-8) starting at the initial sampling of 70 DAP. Differences in these two results can be attributed to drought and the leaching ability of gypsum. Sorensen and Butts (2008) mentioned that at the time of sampling, precipitation was very low, thus lowering gypsum's ability to leach into the rooting and fruiting zone. However, at the time of harvest, precipitation was relatively high, thus allowing gypsum to leach into the soil, and as a result Ca concentration in mature seeds increased with an increase in the gypsum application rate. During the sampling times in 2007, 2008 and 2009 of the present study, there was sufficient precipitation throughout the growing season (Table 3-7), allowing gypsum to leach into the fruiting zone and become available to the developing fruit, therefore showing significant differences even at 70 DAP.

In the current study, a linear increase in Ca concentration was observed (Figures 4-3 and 4-4) for pods and seeds at the same physiological stages sampled over time. However, this trend

was not observed for S or other nutrients that were affected by gypsum application. It can be hypothesized that the increase in calcium in the soil solution due to gypsum applications combined with increased transpiration rates as the plants grew over time, led to these linear increases in Ca concentration.

There was a small decrease in the P of both pods and seeds (Figures 4-1 and 4-2) sampled from the gypsum amended soils relative to the control. Gypsum amendment to a Pineda sand soil type resulted in a 54 % decrease in P transport in the soil (Zhu and Alva, 1994) probably due to Ca-P precipitation. It is possible that gypsum applications in the present study also precipitated the P, thus reducing its ability to leach into the soil and its availability to the developing tissues in the soil solution for absorption, ultimately resulting in reduced uptake by the peanut plant.

Gypsum application significantly decreased the concentration of Na ions in pods compared to the non-treated control (Figure 4-1). Several studies have shown an interaction between Ca and Na ions in relation to fruit development. For example, high sodium or salinity restricts Ca uptake, availability, and transport to the growing regions of plants and reduces fruit yields (Cramer 2004; Kaya *et al.*, 2002; Pal and Laloraya 1967). Gypsum application ameliorates the effect of high saline conditions (Khosla *et al.*, 1979) and increases fruit yields (Kaya *et al.*, 2002; Mizrahi, 1982). Pal and Laloraya (1967) showed that the addition of NaCl to a peanut pod culture solution did not allow the pegs to develop into pods. They suggested that the Na ions may have played a role in inhibiting the uptake of Ca ions by the growing pegs. Kaya *et al.* (2002) obtained higher strawberry yields when supplementary Ca was given to salt stressed plants, thus alleviating Ca deficiency symptoms.

We observed a higher Ca concentration in the pods (Table 4-3) and seeds (Table 4-6) of the smaller seeded Georgia Green compared to the larger seeded C-99R. These values for each

cultivar agree with previously reported values (Tillman *et al*., 2010; Sorenson and Butts, 2008). C-99R has a thicker, denser hull (20.3%) compared to hulls of Georgia Green (18.5%, $P \leq 0.0001$) with a smaller surface area:weight ratio. Sumner *et al.* (1988) found that larger seeded pods with a smaller surface area:weight ratio require a higher soil calcium concentration since they are less efficient in diffusion compared to smaller seeded pods. Consequently, it is likely that C-99R may have absorbed less Ca than Georgia Green, resulting in a lower Ca concentration.

Similar to studies examining changes in mineral composition of developing Persimmon fruit (Clark and Smith, 1990) and primrose seeds (Zerche and Ewald, 2005), in the present study the concentration of all nutrients, except Mn, was highest in immature peanut pod and seed stages and declined with maturity. The Persimmon study also showed that 80 % of Ca uptake was completed before the seed began to mature. Pattee *et al.* (1974) described the pod as the initial metabolic reserve for developing seeds. The young developing pod takes up Ca via passive diffusion from the soil solution for subsequent seed development, and later the pod becomes metabolically inert due to lignifications of the pod wall. Ultra structural analysis of peanut cotyledons (Young *et al*., 2004) showed active synthesis of starch granules during early stages of seed development. This requires the activation of plastidial ADP glucose pyrophosphorylase. Sukhija *et al.* (1987) reported that 10-15 days after pod formation (stage 2 of fruit development) was the crucial time period for oil body formation. P, S, and Zn are required for the formation of oil bodies. Thus, the high concentration of nutrients in immature peanut pods and seeds is required for their high metabolic activity.

Table 4-1. ANOVA probability values for macronutrients determined for gypsum treatment, cultivar and pod developmental stage for peanuts grown near Citra, FL in 2007, 2008 and 2009. Probability values for Ca were calculated for 2007, 2008 and 2009 while those for S, Mg, K and P were calculated for 2008 and 2009.

Effect	Mineral P Values				
	Ca	S	Mg	K	P
Gypsum Treatment	<0.0001	<0.0001	0.36	0.45	0.01
Cultivar	<0.0001	0.6	0.02	0.48	0.48
Stage	<0.0001	<0.0001	<0.0001	0.0007	<0.001

Table 4-2. ANOVA probability values for micronutrients determined for gypsum treatment, cultivar and pod developmental stage for peanuts grown near Citra, FL in 2008 and 2009.

Effect	Mineral P Values					
	Cu	Mn	Ni	Fe	Na	Zn
Gypsum Treatment	0.33	0.28	0.33	0.05	0.03	0.72
Cultivar	0.08	0.15	0.23	0.34	0.4	0.86
Stage	0.33	0.33	0.26	0.002	0.006	<0.0001

Table 4-3. Mineral analysis of peanut pods for Ca, P and Zn for two peanut cultivars. Each value for Ca represents the mean of at least three replicates taken in 2007, 2008 and 2009, while each value for P and Zn represents the mean of at least three replicates taken in 2008 and 2009. Means followed by the same letter within each element are not different at $P \leq 0.05$.

Effect	Mineral Composition		
Cultivar	Ca†	P†	Zn‡
C-99R	1.75a	1.52a	19.73a
Georgia Green	2.15b	1.46a	19.58a

†Concentration in mg/g
‡ Concentration in µg/g

Table 4-4. ANOVA probability values of macronutrients determined for gypsum treatment, cultivar and seed developmental stage for peanuts grown near Citra, FL in 2007, 2008 and 2009. Probability values for Ca were calculated for 2007, 2008 and 2009 while those for S, Mg, K and P were calculated for 2008 and 2009.

Effect	Mineral P Values				
	Ca	S	Mg	K	P
Gypsum Treatment	<0.0001	<0.0001	0.006	0.29	0.045
Cultivar	0.0022	0.78	0.15	0.62	0.03
Stage	<0.0001	0.0002	0.001	<0.0001	0.004

Table 4-5. ANOVA probability values for micronutrients determined for gypsum treatment, cultivar and seed developmental stages for peanuts grown near Citra, FL in 2008 and 2009.

Effect	Mineral P Values					
	Cu	Mn	Ni	Fe	Na	Zn
Gypsum Treatment	0.17	0.9	0.88	0.27	0.12	0.005
Cultivar	0.24	0.08	0.67	0.22	0.29	0.02
Stage	0.04	<0.0001	0.0002	<0.0001	0.001	<0.0001

Table 4-6. Mineral analysis of peanut seed for Ca, P and Zn for two peanut cultivars. Each value for Ca represents the mean of at least 3 replicates taken in 2007, 2008 and 2009, while each value for P and Zn represents the mean of at least 3 replicates taken in 2008 and 2009. Means followed by different letters within each element are different at $P \leq 0.05$.

Effect	Mineral Composition		
Cultivar	Ca†	P†	Zn‡
C-99R	0.83a	3.93a	38.13a
Georgia Green	1.07b	3.81b	39.16b

†Concentration in mg/g

‡ Concentration in µg/g

Table 4-7. Monthly average rainfall data for the 2007, 2008 and 2009 peanut growing seasons at Citra, FL. Data obtained from the Florida Automated Weather Network (FAWN).

Period	2m Rain total (inches)
May-07	0.16
Jun-07	4.36
Jul-07	6.5
Aug-07	3
Sep-07	5.69
Oct-07	6.85
May-08	0.6
Jun-08	7.04
Jul-08	4.05
Aug-08	4.57
Sep-08	1.74
Oct-08	1.73
May-09	9.01
Jun-09	6.48
Jul-09	4.31
Aug-09	4.21
Sep-09	4.59
Oct-09	1.52

Table 4-8. Ca and S content of pods and seeds at 70,100 and 130 DAP for peanuts grown near Citra, FL in 2007, 2008 and 2009 with and without gypsum treatment. Each value for Ca represents the mean for two peanut cultivars and all developmental stages in 2007, 2008 and 2009, while each value for S represents the mean for two peanut cultivars and all developmental stages in 2008 and 2009. Means followed by different letters in each DAP are significantly different at $P \leq 0.05$.

Gypsum Treatment	Days After Planting		
	70	100	130
		Pod-Ca*	
Non-Treated	1.2a	1.64a	1.72a
Treated	2.06b	2.55b	2.68b
		Seed-Ca*	
Non-Treated	0.55a	0.87a	0.77a
Treated	1.07b	1.14b	1.16b
		Pod-S*	
Non-Treated	1.15a	1.08a	NA
Treated	2.17b	2.08b	
		Seed-S*	
Non-Treated	2.11a	1.97a	NA
Treated	2.62b	2.64b	

*Concentration in mg/g

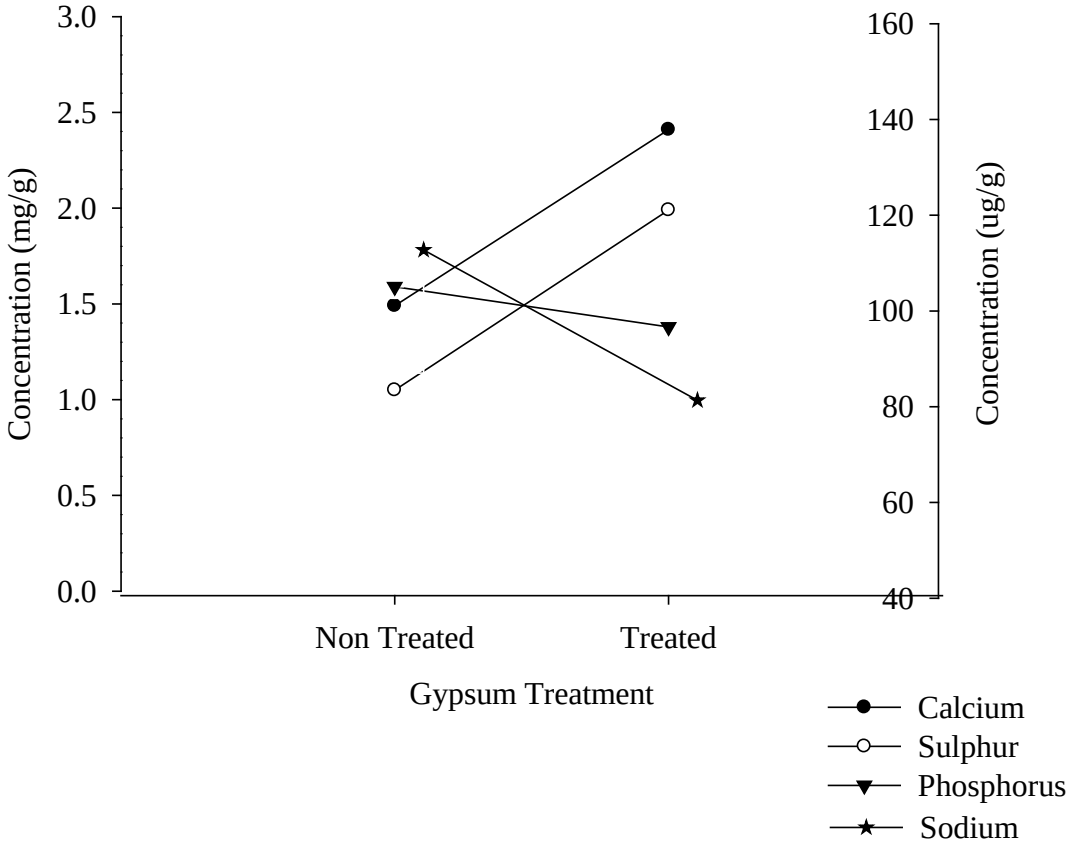

Figure 4-1. Effect of gypsum treatment on pod nutrient composition. Each value represents the mean of at least three replicates. Values for calcium represent means of at least three replicates for the years 2007, 2008 and 2009. Values for the other minerals represent the mean of three replicates for the years 2008 and 2009. The Y-axis on the right estimates the Na concentration and the Y-axis on the left estimates Ca, P and S concentration. Gypsum application increased the Ca ($P < 0.0001$) and S ($P < 0.0001$) concentrations, and decreased the P ($P = 0.01$) and Na ($P = 0.03$) concentrations.

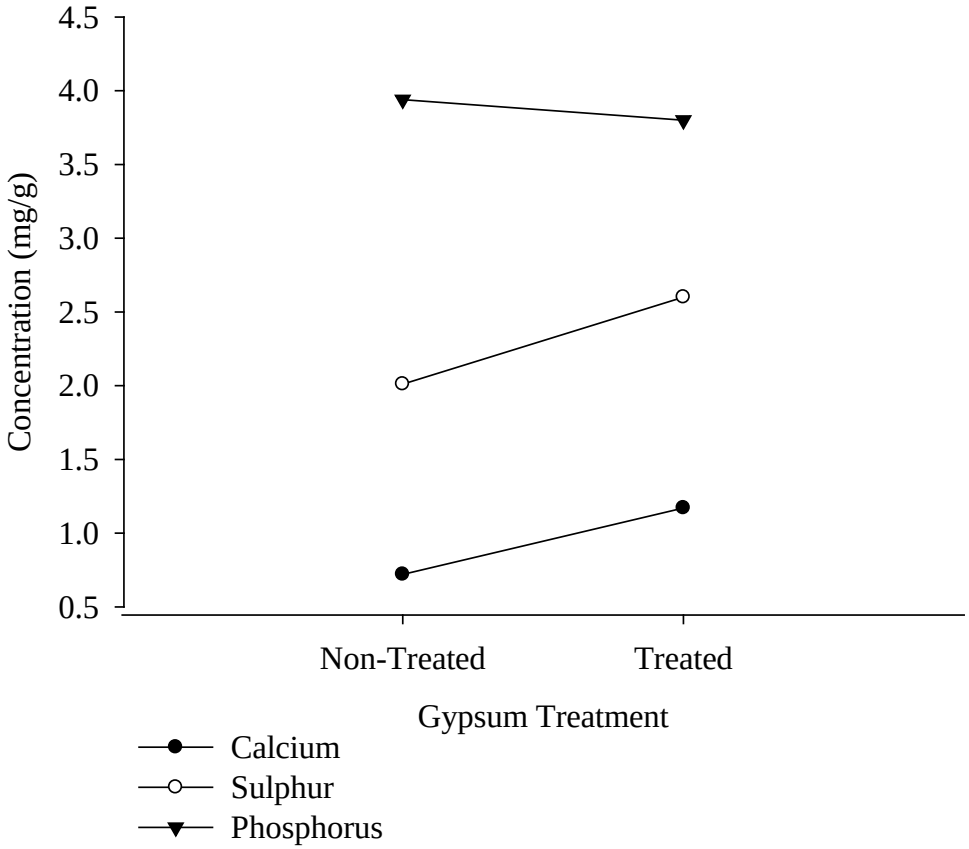

Figure 4-2. Effect of gypsum application on seed nutrient composition. Each value represents the mean of at least three replicates. Values for calcium represent the mean of at least three replicates for the years 2007, 2008 and 2009. Values for the other minerals represent means of three replicates for the years 2008 and 2009. Gypsum application increased the Ca ($P < 0.0001$) and S ($P < 0.0001$) concentrations, and decreased the P ($P = 0.04$) concentrations.

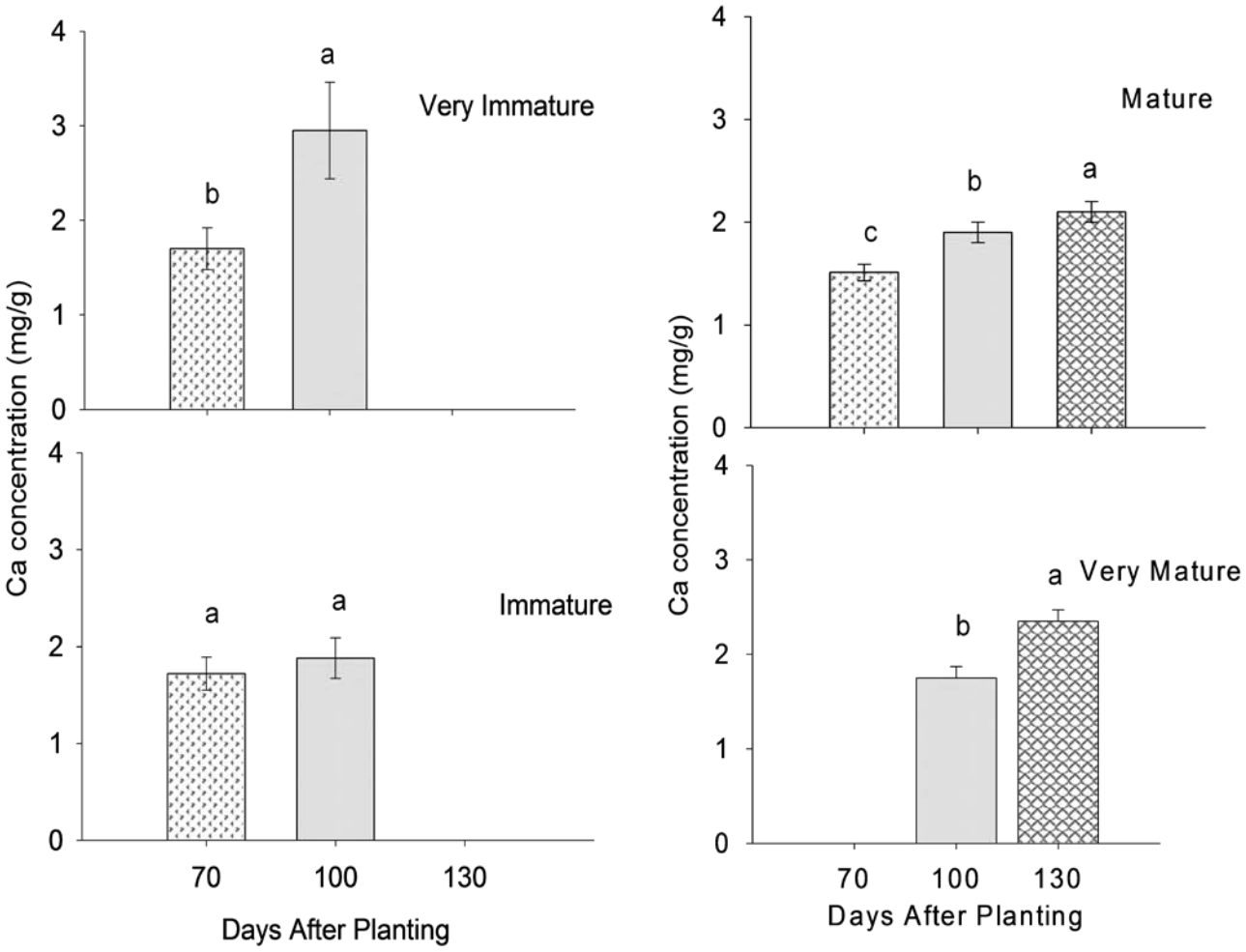

Figure 4-3. Ca concentration of peanut pods for four developmental stages sampled at 70,100 and 130 DAP. Each value represents the mean of at least three replicates averaged over gypsum treatment and two cultivars during 2007, 2008 and 2009. Standard errors are represented as vertical bars. Mean value data points followed by the same letter in the same graph are similar based on Tukey-Kramer's mean separation ($P \leq 0.05$).

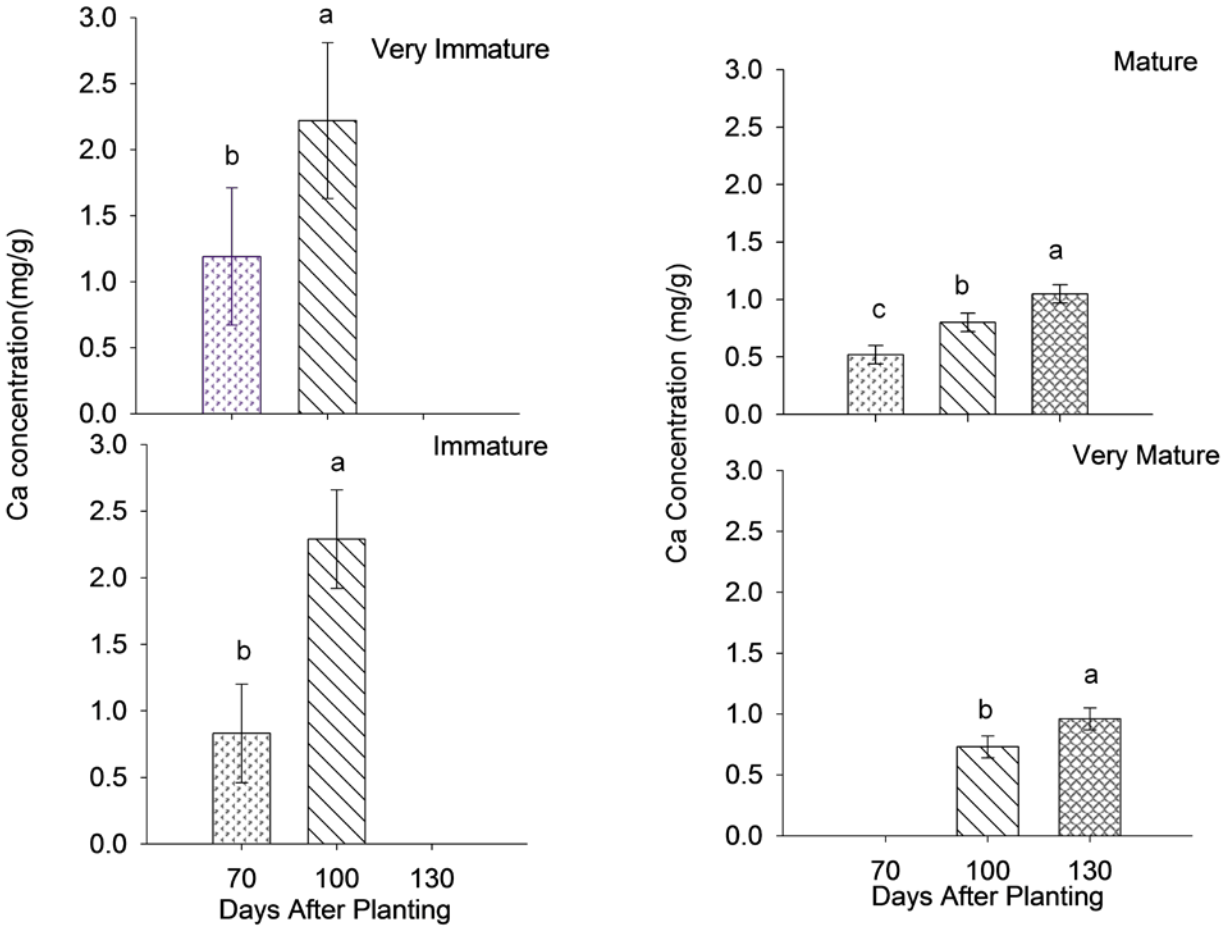

Figure 4-4. Ca concentration of peanut seeds at for four developmental stages sampled at 70,100 and 130 DAP. Each value represents the mean for at least three replicates averaged over gypsum treatment and two cultivars during 2007, 2008 and 2009. Standard errors are represented as vertical bars. Different letters within the same graph are different based on Tukey-Kramer's mean separation ($P \leq 0.05$).

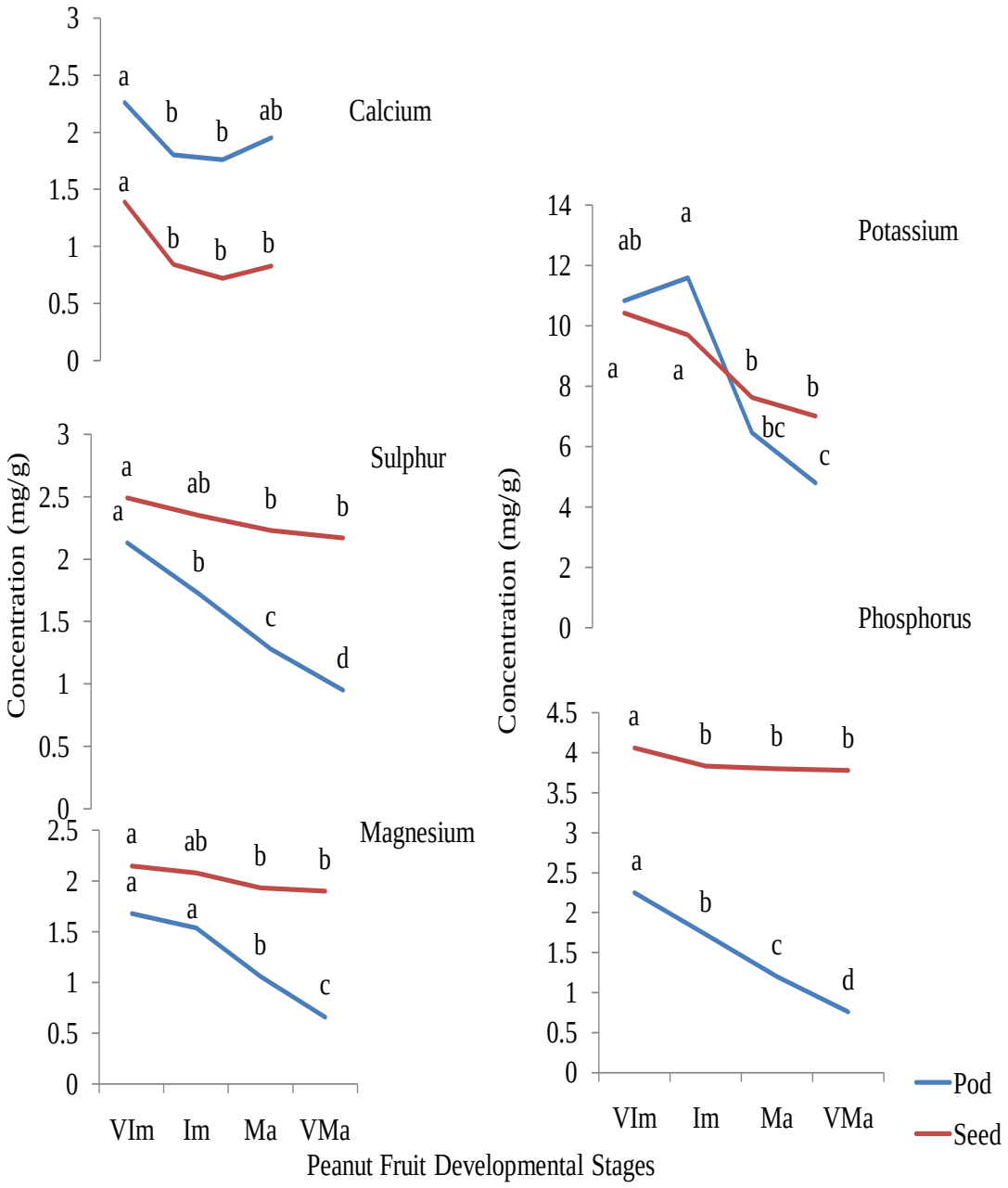

Figure 4-5. Concentration of macronutrients in four stages of peanut pod and seed development. Except for calcium, each value represents the mean of at least three replicates for the years 2008 and 2009 with standard errors shown by vertical bars. Values for calcium represent the mean of at least three replicates for the years 2007, 2008 and 2009. Mean value data points with the same letter in the same tissue within each graph are not different based on Tukey-Kramer's mean separation ($P \leq 0.05$). VIm: Very Immature; Im: Immature; Ma: Mature; VMa: Very Mature.

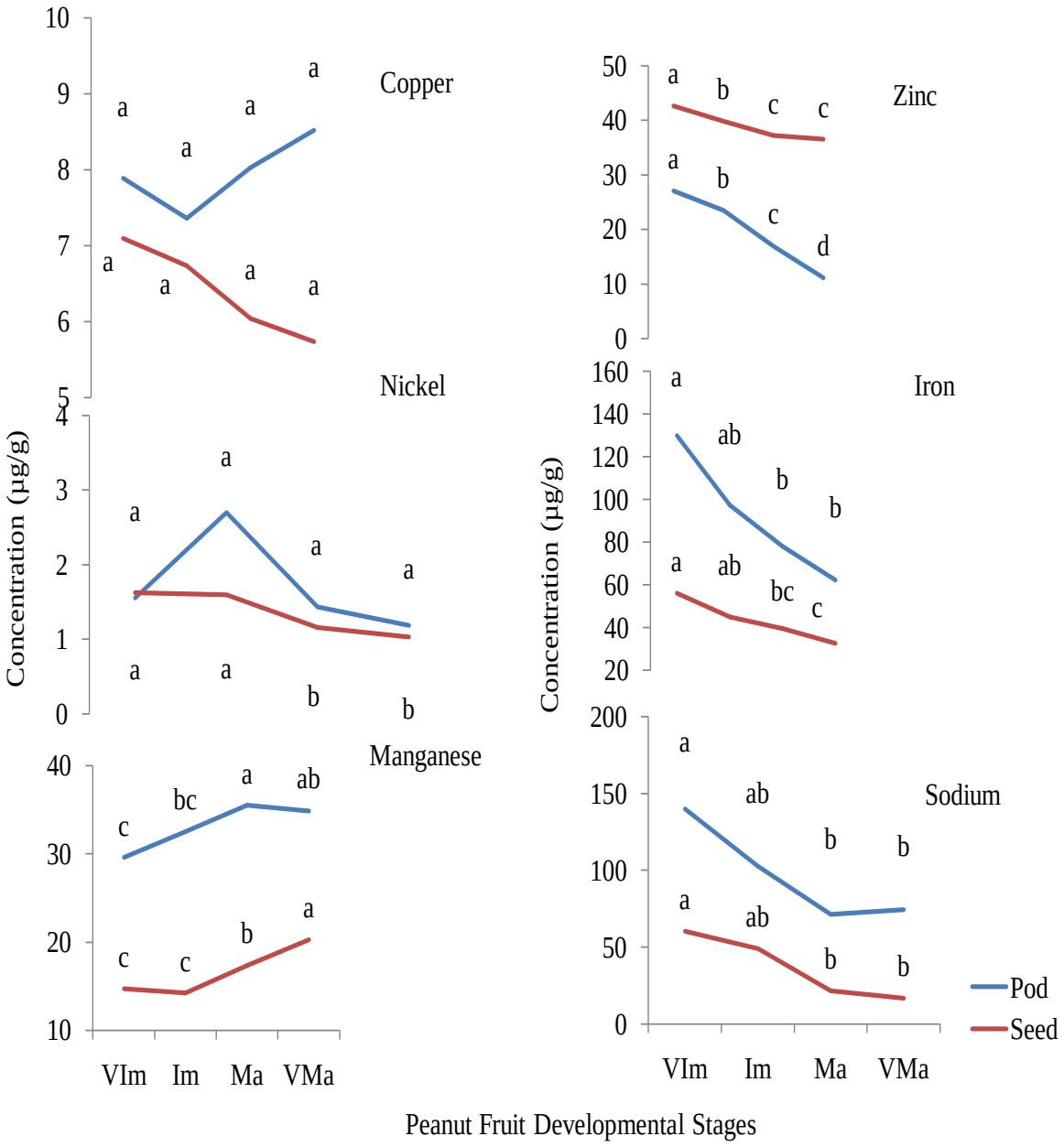

Figure 4-6. Concentration of micronutrients in four stages of peanut pod and seed development. Each value represents the mean of at least three replicates for the years 2008 and 2009 with standard errors shown by vertical bars. Mean value data points followed by the same letter in the same tissue within each graph are not different based on Tukey-Kramer's mean separation ($P \leq 0.05$). VIm: Very Immature; Im: Immature; Ma: Mature; VMa: Very Mature.

CHAPTER 5
THE EFFECT OF CALCIUM ON EXPRESSION AND LOCALIZATION OF CALCIUM DEPENDENT PROTEIN KINASES IN PEANUT FRUIT

Introduction

Arachis hypogaea L., cultivated peanut, is a relatively large seeded legume that differs from most other plant species in its geocarpic reproductive growth, which is marked by underground development of fruit following pollination and fertilization of air-borne flowers. Consequently, the young, developing fruit is photosynthetically inactive and relies on a maternal assimilate supply. Calcium cannot be repartitioned from older to actively growing tissue via the phloem route. Therefore, younger and actively growing fruit must fulfill its requirement utilizing either immediately available xylem-mobile calcium or that passively absorbed via epidermal layers of pod, thus posing additional burden on the growing fruit developing underground and lacking significant transpirational pull. Although low soil calcium concentrations do not inhibit the developmental progression of pods, they do increase seed abortion rates resulting in “pops” or empty pods (Smith, 1956).

The physiological basis of calcium’s effect on peanut fruit development has been reported (Skelton and Shear, 1971; Wiersum, 1951). However, the molecular mechanisms involved in these processes are not understood. One approach to understanding the molecular basis of calcium signaling in peanut is to study a class of calcium sensors known as Calcium Dependent Protein Kinases (CDPKs). CDPKs are unique to vascular and nonvascular plants, green algae and protists, and possess a calcium-regulated calmodulin-like regulatory domain located at the C-terminal end of the enzyme (Chandran *et al*., 2006). CDPKs recognize changes in intracellular calcium concentrations as a result of calcium directly binding to their C-terminal domain which allows for conformational change and the activation of an N-terminal kinase domain (Klimecka and Muszyńska, 2007). Along with CDPK-related kinases (CRKs), phosphoenolpyruvate

carboxylase kinases (PPCKs), PEP carboxylase kinase-related kinases (PEPRKs), calmodulin-dependent protein kinases (CaMKs), calcium and calmodulin-dependent protein kinases (CCaMKs), and Sucrose non-fermenting 1 related kinases (SnRK1, 2 and 3), CDPKs form the highly diverse and widespread Ser/Thr protein kinase superfamily, and represent 4% of the predicted 25,500 genes in *Arabidopsis* (Hrabak *et al*. 2003).

There is evidence that CDPKs are important for normal seed development. Frattini *et al.* (1999) showed that two rice CDPK isoforms, OsCDPK2 and OsCDPK11, were differentially expressed during seed development. Low expression of the OsCDPK2 protein was observed during early developmental stages increasing through maturity, while OsCDPK11 showed down regulation during later stages of development. In a later study, over expression of *OsCDPK2* in transgenic rice blocked seed development at a very early stage (Morello *et al.*, 2000). In *Arabidopsis*, knockouts of CPK28 resulted in embryo lethality (Harper *et al.*, 2004). Likewise, a CDPK was identified in sandalwood embryogenic cultures that accumulated to high levels during somatic and zygotic embryogenesis, endosperm development, and seed germination, and was undetectable in mature organs such as shoots and flowers (Anil *et al.*, 2000). Dasgupta (1994) characterized a 53 kD peanut CDPK (GnCDPK) from mature seed. However, little is known regarding a role for CDPKs in peanut fruit development. Because peanut's subterranean seed development relies primarily upon the sensing and response of the young, underground developing fruit to calcium levels in the soil, changes in CDPK expression were examined during peanut fruit development in low calcium soils and in the same soils supplemented with calcium via gypsum application.

Materials and Methods

Peanut Fruit Samples

In 2008, soil samples (Candler fine sand, Citra, FL) from experimental plots were analyzed (Waters Agriculture Laboratories, Camilla, GA) four wks before planting and contained Ca at 221 lbs/acre, P at 43 lbs/acre, K at 19 lbs/acre, Mg at 14 lbs/acre, B at 0.2 lbs/acre and Mn at 2 lbs/acre with a pH of 5.4. Peanut fruit of cv. Georgia Green at all four developmental stages, Very Immature (VIm), Immature (Im), Mature (Ma) and Very Mature (VMa), was collected at 100 DAP from untreated (Ca at 221 lbs/acre) and gypsum treated (2500 lbs/acre) soils, as described in previous chapters. Pod and seed samples were immediately frozen in liquid nitrogen upon collection and stored at -80 °C until further use.

Total Protein Isolation

Total protein was isolated using modified protocols from Koppelman *et al.* (2001) and Anil *et al.* (2000) as follows. Frozen seeds and pods were separated and ground to a fine powder in liquid nitrogen. To 100 mg of this ground powder, 1 ml of protein extraction buffer was added which contained 20 mM Tris-HCl (pH 7.2), 2.5 mM EDTA, 0.1 % β-mercaptoethanol and 10 µl of plant protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was stirred at room temperature for 10 min, and then centrifuged at 5000 rpm (4 °C) for 30 min. The clear supernatant was removed and further centrifuged at 10,000 rpm (4 °C) for 60 min to remove traces of oil bodies. Protein extracts were stored at -20 °C until further use. Protein concentration was determined using the Dc Protein Assay kit with BSA as the standard following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed by a modified method of Lammeli (1970) using the Mini-Tran system (Invitrogen Corporation, Carlsbad, CA).

Protein extracts were mixed with 2× sample buffer and 1 mM DTT and denatured at 70 °C for 10 min followed by centrifugation for 15 sec. Total protein was resolved on precasted 4 – 12 % NuPAGE gels (Invitrogen Corporation, Carlsbad, CA) in running buffer (5.23 g/L 3-(N-morpholino) propanesulfonic acid (MOPS), 3.03 g/L Tris-Base, 0.5 g/L SDS and 0.15 g/L EDTA) at 120 V for 90 min. Prior to transfer and Western blotting, an identical gel was stained and checked for equal sample loading by staining in 0.1 % Commassie Brilliant Blue R-250 (CBB) for 3 to 5 hrs and then destaining (40 % methanol, 10 % glacial acetic acid and 50 % D/W) for 1 hr followed by dehydrating the CBB-stained gels on a gel dryer (Model 583, BioRad-Laboratories, Hercules, CA). SeaBlue Plus2 pre-stained molecular weight markers (191-14 kD) were used as the standard marker (Invitrogen Corporation, Carlsbad, CA).

CDPK Antibody

A polyclonal soybean CDPK antibody was kindly provided by Dr. Alice Harmon (Dept. of Biology, University of Florida). The antibody was raised in rabbits against the calmodulin-like domain of soybean CDPKα and affinity purified using the recombinant calmodulin-like domain from soybean CDPKα (Bachmann *et al.*,1996). This antibody recognizes many isoforms including soybean CDPK alpha, gamma, beta and *Arabidopsis* CPK1 and CPK4 (Harmon, personal communication).

Western Blot

Proteins were transferred onto nitrocellulose membranes (GE Water and Process Technologies, Trevose, PA) using a transfer buffer of 25 mM Tris-Base, 192 mM Glycine and 20 % methanol overnight at 15 mA. Then the membranes were stained with Ponceau S (0.5 % in 3 % tricarboxylic acid) to verify the transfer efficiency. Membranes were destained with 1x TBS buffer for 15 min.

The Westernbreeze chemiluminescent immunodetection kit (Invitrogen Corporation, Carlsbad, CA) was used for Western blot analysis. The polyclonal soybean CDPK antibody was diluted to 1:6000 with blocking solution. The Western blot was performed as described by the manufacturer. The nitrocellulose membrane was incubated with blocking solution for 30 min, followed by a wash with distilled water for 5 min. The membrane was incubated for 1 hr with primary CDPK antibody on a glass plate covered with a piece of parafilm ironed on the surface to make it adhere. The blot was washed 3 times for 5 min each. The alkaline phosphatase conjugated secondary antibody was incubated for 30 min with the blot in a similar manner as the CDPK antibody incubation, followed by 3 antibody washes each for 5 min and 2 washes of distilled water each for 2 min. The chemiluminescent substrate with enhancer was added to the membrane and the signal was developed for 5 min. Any excessive substrate was soaked with blotting paper and the blot was sandwiched between two transparency sheets. The sandwich was then exposed to X-ray film (Kodak BioMax, Rochester, NY). Seeds protein blots were exposed for 7 min, while pod protein blots were exposed for 15 min due to lower protein concentrations.

Total RNA Isolation

Total RNA was isolated using the Trizol method (Invitrogen Corporation, Carlsbad, CA). Frozen tissue (100 mg) was ground to a fine powder, resuspended in 1 ml Trizol reagent and incubated at room temperature for 10-15 min. Chloroform (200 µl) was added and the extracts were vortexed for 10 sec, followed by gentle shaking for 10 min. The two phases were separated by centrifugation at 13,000 rpm for 15 min at 4 °C. The upper aqueous phase was removed to a new tube, and RNA was precipitated by adding 500 µl isopropanol and incubated at room temperature for 10 min, followed by centrifugation at 13,000 rpm for 10 min at 4 °C. The RNA pellet was washed in 200 µl of 75 % ethanol (in DEPC-treated water), air dried for 10 min and

resuspended in 80 µl of DEPC-treated water. The concentration of total RNA was quantified using the Nano drop (Thermo Scientific, Wilmington, DE).

First Strand cDNA Synthesis

First strand cDNA synthesis was performed using the VILO cDNA synthesis kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. 5×VILO reaction mix, 10× reverse transcriptase, and DEPC-treated water were added to 2.5 µg total RNA in a 20 µl final volume. The reaction contents were gently mixed and incubated at 25 °C for 10 min followed by further incubation at 42 °C for 5 min. The reaction was terminated by incubating at 85 °C for 5 min.

Quantitative RT-PCR of CDPK

Real-time quantitative PCR was performed using the Brilliant II SYBR® Green qPCR mix on an *Mx3000P* platform (Stratagene, Agilent Technologies, Santa Clara, CA). The PCR reactions were prepared according to the manufacturer's instructions and contained 200 nM of both the forward and reverse gene-specific primers (Table 5-1) and 2 µL of the 5-fold diluted RT reaction in a final volume of 25 µL. The thermal cycling protocol entailed activation of SureStart *Taq* DNA polymerase at 95 °C for 15 min. The PCR amplification was carried out for 40 cycles with denaturation at 94 °C for 10 s, and primer annealing and extension at 56 °C and 72 °C for 30 s each, respectively. Optical data were acquired following the extension step, and the PCR reactions were subject to melting curve analysis beginning at 55 °C through 95 °C, at 0.2 °C s^{-1}. Elongation factor 1 alpha (*EF1a*) was used as the endogenous reference gene for normalizing the transcript profiles. The primers used for qRT-PCR analysis are summarized in Table 5-1. The real time PCR data were calibrated relative to the transcript levels in VIm stages, following the $2^{-\Delta\Delta Ct}$ method for relative quantification (Livak and Schmittgen, 2001). The data are presented as

an average ± S.D. of three independently made RT preparations used for the PCR run, each having three replicates.

Immunolocalization of CDPK in Peanut Fruits

CDPK was localized in VIm fruits by the SuperPicTure™ Polymer Detection Kit (Zymed, Invitrogen Immunodetection, Carlsbad, CA). Thin hand cut transverse sections of peanut pods were floated on PBS buffer, and incubated in 30 % H_2O_2: methanol (1:9 v/v) for 10 min, followed by washing twice in PBS for 2 min each. The sections were incubated in primary Ab (1:6000 dilution, 1 hr, 4 °C) in a moist chamber. The sections were washed in PBS, and covered in HRP polymer conjugate for 10 min. The color was developed after washing the sections again in PBS, and incubating in DAB chromogen for 5 min. The peroxidase catalyzes the substrate (H_2O_2) and converts the chromogen to a brown deposit to visualize the location of the antigen. The peroxidase reaction was stopped by flushing water. The sections were dehydrated in a series of alcohol and xylene, and mounted in Histomount™ (Invitrogen Corporation, Carlsbad, CA) prior to imaging.

Results

Transcript profiles of *CDPK* during development of peanut seeds and pods are represented in Figures 5-1A and 5-2A, respectively. In seed, quantitative RT-PCR data showed that *CDPK* expression is spatiotemporally regulated, and is also responsive to gypsum. Regardless of gypsum treatment, *CDPK* transcripts had a bimodal profile with the highest levels observed in VIm seed and in Ma seed with significantly lower levels in Im seed and the lowest levels in VMa seed (Figure 5-1A). Gypsum treatment resulted in down regulating the expression of *CDPK* with 2- to 3-fold lower transcript levels observed at each seed developmental stage. Unlike developing seed tissue, *CDPK* transcription was similar for VIm, Im and Ma pods, and was unaffected by gypsum application (Figure 5-2A).

The fidelity of the qPCR amplification product was confirmed by cloning and sequencing of the PCR amplification product, and by dissociation curve analysis. The cloned amplified product was 100 % identical to the *Arachis* CDPK (GenBank Acc. # DQ074454), and the melting curve analysis also showed a single sharp peak centered around 80.5 °C. Therefore, even though the deduced amino acid sequence for the qRT-PCR product spans the extremely conserved CDPK catalytic kinase domain (spanning the 213-248 aa residue region), it is highly unlikely that the qRT-PCR primers used in the present investigation yielded any nonspecific reaction products.

The CDPK protein expression patterns corroborated the qRT-PCR data (Figures 5-1B and 5-2B) excluding the likelihood of posttranscriptional regulation of CDPK expression during development of either the peanut seed or pod. In seeds and pods, a 53 kDa band was obtained, as previously reported by Dasgupta (1994), for the VIm, Im and Ma stages of development. VMa fruit stages consistently showed negligible (seed) or undetectable (pod) expression of CDPK protein.

Tissue specific expression of CDPK was further validated by immunolocalization in VIm fruits. The immunoreactive CDPK was primarily found in the outer most cell layers of the pericarp and around vascular bundles linked by lateral connections in the pod (Figure 5-3A), as well as in the single vascular trace that supplies nutrients to the developing ovule (Figure 5-3B).

Discussion

To the best of our knowledge, this is the first report describing developmentally regulated CDPK expression profiles in peanut fruits. Given that a) calcium is phloem-immobile, and b) consequently peanut's hypogeal reproductive development is entirely dependent upon sensing and response of the young, underground developing fruit to calcium levels in the soil; changes in

CDPK expression were examined during peanut fruit development under deficient and adequate calcium levels following gypsum treatment.

Developing peanut fruit is highly heterogeneous, consisting of the maternal seed coat and pod tissues and the filial embryonic and cotyledonary tissues. Their successive development and differentiation is a sequential and continuous process and must rely on successful integration of environmental and developmental cues, supported by adequate assimilate partitioning from the mother plant. Calcium is an important macronutrient passively absorbed through the soil, and required by developing peanut fruits both for nutritional needs, as well as signaling functions. Generally, CDPK expression was higher in young developmental stages, in both seeds and pods, and declined considerably in mature desiccated fruit, reflective of its high metabolic status and steep growth kinetics. Notably, young seeds showed a bimodal profile for CDPK transcript and protein abundance (Figure 5-1), in sharp contrast to pods (Figure 5-2). Dynamic reorganization of cytoskeleton elements is deemed essential for mitotic growth of young developing seeds, and may be dependent upon CDPK activity. In plant cells, G- and F-actins co-localize with CDPK (Putnam-Evans *et al*. 1989), and Ca-dependent reversible phosphorylation of G- and F-actins is necessary for actin-mediated interactions (Grabski *et al.*, 1998, Smertenko *et al*., 1998). While seed mitotic activity is restricted to early developmental stages followed by cell expansion and storage product accumulation later on, pods have to undergo continued cellular proliferation and rapid expansion in order to accommodate cotyledonary growth during the storage phase, thus explaining consistently high CDPK expression in pods throughout the early developmental stages.

In addition to a role in early mitotic growth, CDPKs also have a significant influence on storage product biosyntheses. Carbohydrates are the major storage metabolite during early

development of peanut seed, until lipid bodies take over later during maturation (Basha *et al*., 1976, Pattee *et al*., 1974). Hale (1978) showed that regardless of the calcium level in the soil fruiting zone, the highest concentration of sucrose on a dry weight basis was found in immature fruit. Sucrose is the primary photoassimilated metabolite transported through phloem to sink organs, where it is hydrolyzed by sucrose synthase or invertase (Chourey *et al*. 1998, Winter and Huber 2000). Sucrose synthase-mediated reversible conversion of sucrose in the presence of UDP to UDP-glucose and fructose is the committed step in storage starch biosynthesis (Chourey *et al*. 1998). CDPK-mediated phosphorylation has earlier been implicated in activation of sucrose synthase (SuSy)-dependent post-phloem assimilate unloading in developing filial tissue. Highly abundant *SuSy* transcript levels in developing seeds in rice (Shimada *et al*., 2009), tomato (Anguenot *et al*., 2006) and *Vicia faba* (Weber *et al*., 1996) are reflective of active sucrose turnover reactions. Sucrose synthase activity is regulated by reversible phosphorylation. Shimada *et al*. (2009) showed that SPK (CDPK)–mediated phosphorylation of serine residue at the RXXS site is essential for the degradation of sucrose by SuSy in endosperm. Furthermore, transgenic rice plants expressing antisense *SPK* showed normal vegetative growth and reproductive phase transition, however, failed to accumulate storage starch thus compromising the sink strength (Shimada *et al*., 2004). Transient upregulation of a sucrose-inducible CDPK, and transcript localization in sucrose accumulating cell layers was observed in stolon tips during tuber formation in potato (Raices *et al*., 2003). In tomato, Auguenot *et al*. (2006) reported the association of membrane associated CDPK enhancing the efficiency of SuSy for sucrose metabolism in young tomato fruits. Phosphorylation of sucrose synthase by CDPK, represents a key event during transition from mitotic to growth/expansion phase, and metabolic switch to sucrose synthase-mediated control of storage product accumulation. The elevated CDPK

expression (Figure 5-1) during transition to storage phase supports the regulatory role of CDPK during peanut seed development.

Higher CDPK expression in the Ma seed stages, as compared to the Im stages, may also be related to the biogenesis of oil bodies which accumulate during late stages of seed maturity (Pattee *et al*., 1974). Li *et al.* (2009) showed that two peanut seed specific oleosin gene transcripts, *AhOleo17.8* and *AhOleo18.5,* were highly abundant in the mature embryo. Oleosins are small molecular mass proteins located on the oil body surface that prevent the phospholipid layers from contacting and coalescing with each other. Anil *et al.* (2003) showed immunoreactive CDPK in the oil body-associated protein fraction of mature seeds of sandalwood, sunflower, safflower, sesame, cotton and peanut. This suggests a direct role of CDPKs with oil body biogenesis and oil formation.

Studies addressing the physiological and biochemical properties of the peanut pod are limited. Pattee *et al.* (1974) showed higher levels of total sugar and starch in the pod of VIm and Im stages declining rapidly as maturity was reached, in line with the metabolic role of sucrose supporting high growth rate and mitotic activity in young pods and for providing C moieties for cell wall biosynthesis during maturation. Also, an increase in the residue weight due to deposition of lignocellulosic material was observed as the pod was maturing. The authors postulated that the decline in sugar levels and the increase in lignocellulosic material were due to the conversion of sugars to lignocelluloses. The high *CDPK* transcript levels observed in the first three developmental stages indicates that the pod is metabolically active.

Unlike seeds, soil calcium levels did not affect the expression of *CDPK* in pod tissue (Figure 5-2). Phenotypic analysis revealed that pods developed (length) normally with or without gypsum amendement (see Chapter 3, Table 3-1). Also, low soil calcium has more

deleterious effects on seed filling than pod development (Colwell and Brady, 1945). At maturity, calcium is required for the structural integrity of the pod, while for seed it is required primarily as a nutrient and signaling molecule. Hence the data reported here on pod CDPK expression supports observations made by Pattee *et al.* (1974) that at young stages, the pod is a nutritional reservoir for the developing seed and at maturity it is a protective barrier.

Gypsum amendment may adversely affect mobility, transport and bioavailability of soil phosphorus by precipitating it out of the soil solution (Zhu and Alva, 1994). Also, CDPK expression has been shown to be elevated when phosphorus availability is low, for example in the moss *Funaria hygrometrica* (Mitra and Johri, 2000) and in *Arabidopsis* roots (Wu *et al*., 2003). Despite the significantly lower total phosphorus content in both peanut pods and seeds produced under gypsum amended soil conditions (Figures 4-3 and 4-4, Chapter 4), no such regulatory role of phosphorus availability on CDPK expression was discernible during peanut pod and seed development (Figures 5-1 and 5-2).

The addition of gypsum to the soil also provides sulphur to the developing peanut fruit. As with phosphorus, sulphur starvation was shown to increase the transcript levels of CDPK in the moss *Funaria hygrometrica* (Mitra and Johri, 2000). In the present study, seeds from the non-gypsum amended soil had high CDPK transcript levels and protein expression compared to those from gypsum amended soil, however the sulphur concentration in both tissues did not increase when gypsum was supplied (Table 4-8, Chapter 4). Therefore, it decreases the possibility that enhanced CDPK expression under the non-gypsum condition was a result of sulphur starvation. It may, therefore, be argued that CDPK expression in peanut fruit is primarily dependent on the direct availability of soil calcium, rather than other macronutrients.

Table 5-1. Primers for qRT-PCR of CDPK and EF1α at four stages of peanut fruit developmental for cv. Georgia Green produced under gypsum treated or gypsum non-treated soils in Citra, FL during 2008.

Type	Accession No	Forward	Reverse
CDPK	DQ074454	5′-TCA AAC GAG AAC CTT GGC CGA GTA-3′	5′-GCT CAA GCA CCT GTT TGG CAG TTA-3′
EF1α	EZ748096	5′-AGTTTGCTGAGCTCCAGACCAAGA-3′	5′-TCCCTCACAGCAAACCTTCCAAGT-3′

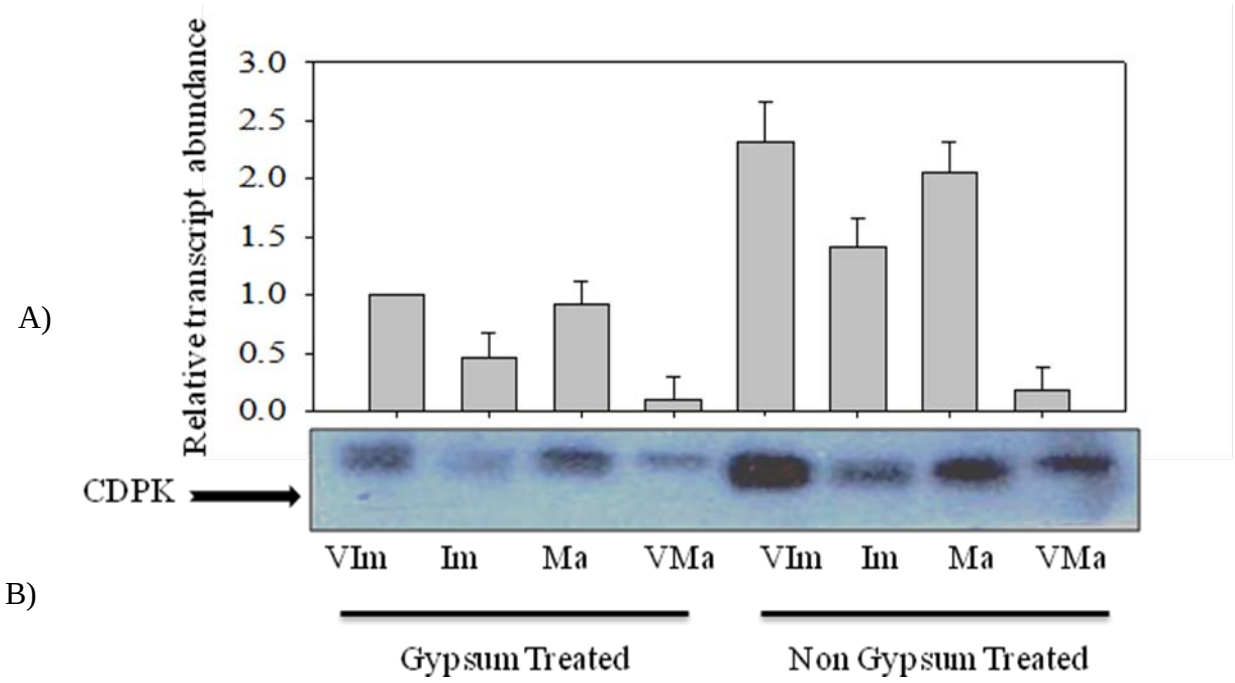

Figure 5-1. CDPK expression in four seed developmental stages of cv. Georgia Green at 100 DAP produced under gypsum treated or gypsum non-treated soils in Citra, FL during 2008. A) Quantitative RT-PCR with the Very Immature (VIm) seed under gypsum treatment used to calibrate expression levels. Elongation factor 1 alpha (EF1α) was used as the endogenous reference gene for normalizing transcript profiles. The data represent avg +/- SD for three independently made RT reactions, each having three technical replicates. (B) Western blot analysis with soybean CDPK antibody (1:6000). Developmental stages are described below the western blot image. VIm- Very Immature seed; Im- Immature seed; Ma- Mature seed; VMa- Very Mature seed.

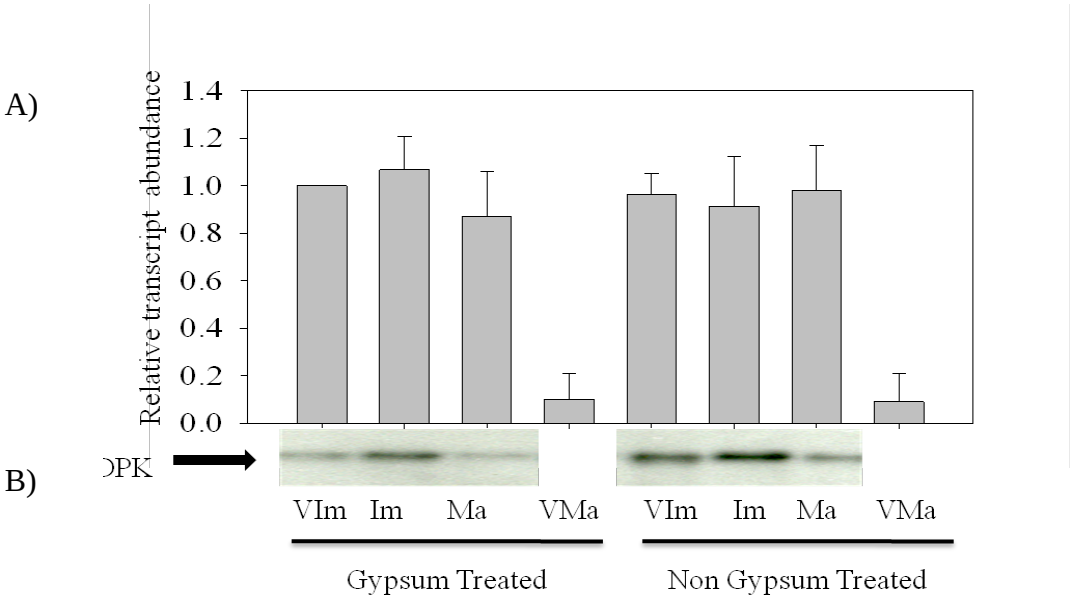

Figure 5-2. CDPK expression in four pod developmental stages of cv. Georgia Green at 100 DAP produced under gypsum treated or gypsum non-treated soils in Citra, FL during 2008. A) Quantitative RT-PCR with the Very Immature (VIm) pod under gypsum treatment used to calibrate expression levels. Elongation factor 1 alpha (EF1α) was used as the endogenous reference gene for normalizing transcript profiles.The data represent avg +/- SD for three independently made RT reactions, each having three technical replicates. (B) Western blot analysis with soybean CDPK antibody (1:6000). Developmental stages are described below the Western blot image. VIm- Very Immature seed; Im- Immature seed; Ma- Mature seed; VMa- Very Mature seed.

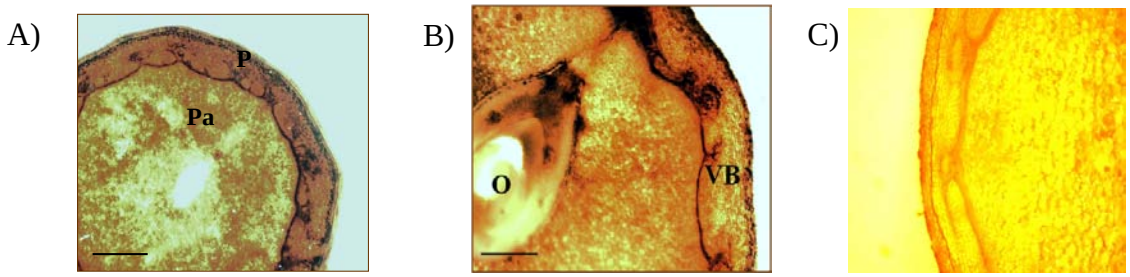

Figure 5-3. CDPK immunolocalization in very immature fruit of cv. Georgia Green. (A) Transverse section of fruit; (B) Transverse section of fruit with developing seed evident. Dark stained regions indicate the presence of CDPK. Magnification bar is 1mm. C) Transverse section of fruit as the CDPK negative control using secondary antibody. Pa: Parechymatous Tissue; P: Pericarp; O: Developing Ovule; VB: Vascular Bundles.

CHAPTER 6
CONCLUSION AND FUTURE RESEARCH

It is clear that calcium plays a major role in peanut fruit development, particularly as it relates to distal seed production and proper fruit maturation with the effects being significant by mid-season. The present study used destructive sampling to investigate the effect of calcium on the development of peanut pods and seed. Future research should focus on real-time tracking of calcium under different calcium regimes to more accurately assess its role in peanut fruit development. Likewise, controlled environment experiments should be developed to eliminate the variability associated with field environmental factors such as rainfall, light, temperature and pests.

Apart of fromits role as a nutrient, calcium also acts as a secondary messenger. Calcium dependent protein kinases (CDPK) are a class of calcium sensors that play a vital role in sensing different calcium levels as a response to environmental stimuli and developmental processes. In peanut fruit, CDPK expression appears to be under transcriptional control. Pod analysis showed that CDPK expression was not affected by gypsum treatment or by development except at the most mature stage. In contrast, seeds had lower CDPK transcript and protein levels under gypsum treatment, suggesting that seed CDPKs, unlike pod CDPKs, can sense differences in soil calcium levels. Additionally, seeds showed a bimodal expression pattern over maturation indicating that seed CDPKs are also regulated by developmental cues. In the future, CDPK expression should be examined in other peanut tissues such as roots, leaves, stems and flowers. Additionally, since peanut is an allotetraploid with a relatively large genome, there are probably numerous peanut CDPK genes. This study was limited in that it could not distinguish members of the peanut CDPK gene family. Future work should focus on the cloning of peanut CDPK genes to characterize them and to better understand their contributions to peanut development.

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BIOGRAPHICAL SKETCH

Bhuvan P. Pathak was born in Ahmedabad, India. She graduated from Sardar Patel University with a B.S. in botany (Gold Medal) in 2002 and M.S. in biotechnology in 2004. During her master's degree at Sardar Patel University, she joined Bhabha Atomic Research Centre for an internship where she worked on the molecular characterization of Indian mustard. In May 2006, she joined Charotar Institute of Technology, Changa, India as a Lecturer in Biotechnology where she taught plant biology to undergraduate pharmacy students. In 2007, she joined the University of Florida for her master's degree in agronomy. She is the eldest daughter of Pareshchandra Pathak and Jayshree Pathak followed by a younger sister Dhruva Pathak and a wife to Samarth Bhatt, Postdoctoral Associate in Human Genetics at the Institute of Anthropology and Human Genetics, University of Jena, Jena, Germany.