CALCITE DISSOLVING BACTERIA: A NEW APPROACH TO SUSTAINABLE PEANUT PRODUCTION IN CALCIUM DEFICIENT SOIL

by

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ABSTRACT

Peanut (*Arachis hypogaea* L.) is an important commercial crop for the state of Georgia, which produces about 50% of the total U.S. peanut crop. However, compared to other commodity crops, peanut production has one of the highest costs due to the extensive usages of fungicides and fertilizers. One of those fertilizers is calcium, in the form of gypsum or lime. Insufficient calcium during flowering and fruiting may lead to significant reduction in yield and increased disease severity. To explore the function of soil microbes in calcium cycling, we conducted experiments to isolate calcite dissolving bacteria (CDB) from Georgia peanut-producing fields and characterized their potential to elevate soluble calcium in soil. CDB refers to a functional group of bacteria that can solubilize calcite, the mineral form of calcium, and release soluble calcium making it available to plants. A total of 65 CDB were isolated representing 15 unique strains belonging to 10 different genera from peanut fields at the Black Shank farm, Tifton, GA. A subset of the CDB, named as “Top mix” due to their high capacity to dissolve calcite, increased soluble calcium level in soil. CDB abundance in soil was negatively corelated with calcium level. We further conducted a survey of CDB in 15 peanut-producing fields belonging to three regions with distinct soil characters in southern Georgia. We found that three genera of "core" CDB, namely *Paenibacillus, Niallia* and *Rossellomorea*, were present in all regions. CDB belonging to *Staphylococcus, Mesobacillus, Pseudarthobacer,* and *Schouchella* were unique to a single field of the three regions surveyed. The core and unique CDB showed comparable capacity to dissolve calcite on plate. To explore the impact of CDB on peanut pod development, we developed an "In-Tube growth" (ITG) system that allows roots and pods on the same peanut plant to grow in differently controlled soil. We demonstrated that peanuts grown in ITG tubes were comparable to those in soil in terms of their normal development and responses to calcium deficiency. Future research is needed to determine the molecular mechanisms of CDB-mediated calcite solubilization. CDB may one day be used as a bio-fertilizer to replace or complement the current usage of mineral fertilizers in peanut production.

INDEX WORDS: Calcite Dissolving Bacteria, CDB, *Arachis hypogaea* L., Peanut, Calcium, bio-fertilizer

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DEDICATION

To My Friends and Family

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CHAPTER 1

INTRODUCTION

Peanut, originated from South America, is native to Peru, Bolivia, Argentina, and Brazil (Bertioli et al., 2011; Tillman & Stalker, 2010). Domesticated peanut (*Arachis hypogaea* L.) is an annual herbaceous, allotetraploid species growing 12 to 20 inches in height and has leaves that are opposite and pinnate with four leaflets with no terminal leaflet (Janila & Mula, 2015; Krapovickas, 2007; Putnam et al., 1991). Peanut leaves are nyctinastic meaning that they close at night (Augusto Joao, 2012; Ueda Minoru, 2000). After producing small yellow flowers on the stem above ground, peanuts develop their fruit below ground, and thus are classified as a geocarpic plant. Peanut plants produce gynophores (pegs) from pollinated flowers, that contain the fertilized ovules at the tip of the peg and enter the soil between 4 to 7 days after pollination (Bledsoe Roger W, 1949). The peg enters the soil 2-8 cm below soil surface, known as the pegging zone, and the embryo within the peg begins to uptake nutrients. The swelled embryo forms seeds within the protective pod (Bledsoe Roger W, 1949; Moss, Rao, Pattee, & Stalker, 1995; Rashid et al., 2016; Zamski & ZIV, 1976). As the pod continues to swell and expand it actively uptakes nutrients (calcium and other inorganic salts) and moisture (Bledsoe Roger W, 1949; Inanaga & Yoshihara, 1997; Pattee & Mohapatra, 1987; A. Singh, 2004b). The peanut fruit or pod typically contains one to four seeds. A large number of peanut cultivars can be grouped broadly into four market types: Runner, Virginia, Spanish and Valencia (Board, 2023; Tillman & Stalker, 2010).

Economic importance of peanut production in the U.S. and Georgia

The United States is one of the leading countries in peanut production. The state of Georgia contributes about 50% of all peanuts produced in the United States, bringing in over 850 million dollars in crop value alone (Report, 2021) However, commercial peanut production has the highest total percent yield loss of a commodity crop (25.7%), due to disease pressure; and the second highest cost for fungal and herbicidal control at 60 million dollars (Report, 2021). Peanuts are the 3rd highest ranked commodity crop in the state of Georgia, just after cotton and broilers (Report, 2021). Peanut production has remained constant and continues to increase in both value and land usage. The majority of peanuts, specifically runner type, are grown in the southeastern coastal plain of Georgia within 75 counties, utilizing 771,843 acres of land (Board, 2023; Report, 2021).

Importance of Calcium in Peanut Pod Formation

Calcium is critical for proper peanut pod and embryo development. Deficiency of soluble calcium (<400 ppm) in the soil surrounding the developing pods leads to abortion of the seed (Bledsoe Roger W, 1949; Kvien, Branch, Sumner, & Csinos, 1988). Even if the seed is not aborted, its quality is severely affected, diminishing its commercial value. Low quality seed is a persistent problem for commercial seed producers. Importantly, calcium is essential for pod development is directly absorbed by the pod rather than being transported from the roots (Bledsoe Roger W, 1949; Kvien et al., 1988; Skelton & Shear, 1971; Yang, 2014). Bledsoe et al. in 1949, demonstrated the separation of the pod absorption of calcium, and how calcium is transported through the root system (Bledsoe Roger W, 1949). The gynophore (peg) and pod actively absorbed 3.5 micrograms µg) of calcium compared to <0.25 µg transported by the rooting system (A. Singh, 2004a; A. L. Singh, Goswami, Nakar, Kalariya, & Chakraborty, 2014). 90% of nutrients are directly absorbed by the peanut pod itself with only 10% of nutrients being supplied by the root system (Bledsoe Roger W, 1949).

Calcium deficiency in soil also increases the susceptibility to soilborne pathogens. For example, disease occurrence and disease severity of the pod rot complex (*Pythium myriotylum, Rhizoctonia* sp. and *Fusarium* sp.) is significantly higher in peanuts grown in calcium deficient soils (Csinos & Gaines, 1986; Taylor & Locascio, 2004). Similarly, blossom-end rot of fruits occurs when calcium deficiency combined with drought leads to immobility of calcium (Taylor & Locascio, 2004). A major pathogen in peanut production, *Aspergillus flavus*, has increased presence and disease severity in calcium deficient soils (Chourasia & Sah, 2017; Pickova Darina, 2021; Reding, Harrison, & Kvien, 1993; Uppala, 2011; Yang, 2014). In peanut production, *Aspergillus flavus* costs farmers $126 million every year in the United States due to its ability to produce a carcinogenic toxin (aflatoxin) (Pickova Darina, 2021; Smith, 2021). The regulation on aflatoxin is enforced by agencies such as Food and Drug Administration (FDA) in the United States and European Food Safety Authority (EFSA) in EU. For example, in EU, the maximum allowable amount of Aflatoxin B1 in peanuts and processed products is 2 parts per billion (ppb). (Dohlman, 2001; Njoroge, 2018).

Physiological features such as the pod shape and hull thickness, play an important role in the peanut pods ability to absorb necessary calcium (Kvien et al., 1988). Using eight different peanut genotypes in the same soil condition, Kvien et al. in 1993, demonstrated that thin, light hulls allowed high calcium concentrations within the seed, while thick, dense hulls have lower calcium concentrations in the seed. These results indicate that calcium uptake by the pod is based on the surface to volume ration of the hull and the length of time for a pod to reach maturity (Kvien et al., 1988). High calcium concentrations in the seed correlate with improved seed quality (Cox, Sullivan, & Martin, 1976; Kvien et al., 1988). In combination with pod characteristics, environmental stresses (e.g., drought) can also lead to decreases in calcium uptake and, consequently, seed quality (Cox et al., 1976; Kvien et al., 1988; Yang, 2014). Calcium is considered as an immobile nutrient; without sufficient water the pod and root are unable to uptake calcium (Adams & Hartzog, 1980; Sikora, 2020; Wright et al., 2016). Timing is also critical for calcium supply. Sufficient calcium is needed particularly during the initial pegging and full seed; once the seed has reached maturity minimal calcium is absorbed (Adams & Hartzog, 1980).

To mediate for a shortage of soluble calcium in the field, gypsum ($CaSO_4$) is often applied at a rate of 0.5 tons/acre or 0.1 kg/m^2 during the peanut's pegging time (Wright et al., 2016). Application of gypsum can increase soluble calcium to the optimal level >600 ppm (Deonne, 2021; Travis Faske, 2021). Alternatively, lime ($CaCO_3$) can be applied at the beginning of season due to its low solubility (Wright et al., 2016). Importantly, applying lime also alters the pH of the soil. Peanuts grow optimally between a pH of 6.2-6.5 and lime should be added if soil pH is below 5.8; low pH leads to zinc or aluminum toxicity and other issues with plant nutrient availability associated with highly acidic soils (Adams & Hartzog, 1980; Wright et al., 2016).

Calcite Dissolving Bacteria

Calcite dissolving bacteria (CDB) can solubilize calcite ($CaCO_3$), an insoluble or mineral form of calcium, by secreting citric acid, oxalic acid and sanazine pigment (Jacobson & Wu, 2009; Peper, Brenneman, & Yang, 2022; Subrahmanyam, Vaghela, Bhatt, & Archana, 2009; Sulu-Gambari, 2012; Tamilselvi, Thiyagarajan, & Uthandi, 2016). Similarly, Phosphate Solubilizing Microbes (PSMs) have been characterized to contain a secondary metabolite that produces acid as a means of competitive advantage, modifying the environment or improving nutrient acquisition (Gyaneshwar, Kumar, & Parekh, 1998; Kalayu, 2019; Taurian et al., 2010). CDB were previously identified in calcareous soils, farmland, animal waste and limestone quarries located in the middle east (Rana Gopinath, 2015; Subrahmanyam et al., 2009; Tamilselvi et al., 2016). CDB were proposed as an alternative approach to reclaim calcareous sodic soils, and to remove calcite deposits from pipes in commercial operation and other unwanted calcite formation (Eroglu Seckin, 2012; Rana Gopinath, 2015).

Currently, the primary method to isolate CDB is by using a selective medium supplemented with 1% calcite (Peper et al., 2022; Tamilselvi et al., 2016). A clear zone or “halo” forms around the bacterial colony as a result of the suspended and insoluble calcite being dissolved (Peper et al., 2022; Tamilselvi et al., 2016). Using this method, a highly diversified group of CDB were isolated, belong to three taxonomy groups: the *Bacillus*, *Brevibacterium* and *Pseudomonas* genera (Rana Gopinath, 2015; Sulu-Gambari, 2012; Tamilselvi et al., 2016).

Microbes play key roles in plant nutrient uptake and soil nutrient cycling. Plant Growth-Promoting Rhizobacteria (PGPR) have been well characterized and used as a model for studying beneficial microbe-plant interactions (Ashrafuzzaman et al., 2009; Bhattacharyya & Jha, 2012; Gupta Govind, 2015; Kenawy et al., 2019; Riaz et al., 2021; Sukul et al., 2021). Nitrogen fixing bacteria and their formation of nodules on the peanut root is a prime example of the impact microbes can have on nutrient uptake and cycling (Ibáñez, Wall, & Fabra). Since the identification of nitrogen fixing bacteria, further research has identified a broad range of beneficial microbes that support plant health through various modes of action (Basu, Bhadoria, & Mahapatra, 2008; Ibáñez et al., 2017; Taurian et al., 2010). The mode of action varies between each PGPR, through secretion of acids, hormones, secondary metabolites, and manipulation of soil nutrients (Ashrafuzzaman et al., 2009; Bhattacharyya & Jha, 2012; Rana Gopinath; Riaz et al., 2021). For example, Phosphate solubilizing Microbes (PSM) can solubilize phosphorus though the use of organic acids, siderophores and chelating agents, providing mobile phosphorus for the plant to uptake (Anand, Kumari, & Mallick, 2016; Kalayu, 2019; Taurian et al., 2010). Similar to CDB identification, PSMs are isolated using media containing recalcitrant P sources and observation of a clear zone phenotype (Kalayu, 2019; Taurian et al., 2010). PSMs are from a highly diverse phylogeny like CDB however, the primary PSMs are *Firmicutes, Actinobacteria* and *Proteobacteria*, which have not been identified as CDB (Jeong, Moon, Shin, & Nam, 2013; Pei-Xiang, 2012; Zaidi, Khan, Ahemad, & Oves, 2009).

Prior to our study, it was unclear whether CDB play a role in soil calcium cycle in peanut-producing fields. Given the importance of calcium to peanuts and the agricultural practice to supplement gypsum/lime, we hypothesize that CDB will facilitate the solubilization of mineral calcium to enhance peanut growth. We carried out research on three topics:

1) Develop an experimental system to study the pod-specific response to low calcium.
2) Isolate and characterize CDB from a research farm in Georgia.
3) Explore the diversity and distribution of CDB in peanut-producing farms in Georgia.

Potential Social and Economic Impact

Calcium must be readily available during flowering time for uptake by the embryo and pod to properly develop high-quality peanut seeds (Kvien et al., 1988; Wright et al., 2016). CDB may potentially serve as a bio-fertilizer to increase soluble calcium within the pegging zone, which is expected to reduce the need for gypsum or lime application. The average cost per acre to apply 0.5 tons of gypsum is 52 dollars per acre (Report, 2021). Application of CDB through irrigation as a soil drench could reduce the need to apply a large amount of gypsum. The application of CDB coinciding with application of lime is expected to increase the amount of available calcium released, reducing the need for excessive lime applications. Also, CDB can be used in calcium deficient soils and be used to reduce or reclaim highly calcareous soils (Peper et al., 2022; Tamilselvi et al., 2016). Lastly, CDB are ideal for use in organic peanut production, a growing market in the southeast, with Georgia opening its first certified organic shelling plant.

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CHAPTER 2

A NOTE ON THE DEVELOPMENT OF AN IN-TUBE GROWTH SYSTEM TO STUDY POD-SPECIFIC RESPONSES OF PEANUT (*ARACHIS HYPOGAEA* L.).

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Abstract

As a geocarpic plant, the fruits of peanuts mature below ground. Developing pods share a similar nutritional and microbial environment as roots, albeit they have distinct physiology and responses to environmental stimuli. Separating pods and roots into different environments to study a pod-specific or a root specific response to stimuli is still technically challenging. Research was conducted to develop an easy and affordable system to support the growth of individual pods in a controlled environment that is separate from roots, named the In-Tube Growth (ITG) system. The data indicate that the growth of pods of various varieties in ITG is comparable to those in open soil. The ITG system was used to investigate the defects caused by calcium deficiency in the geocarposphere, demonstrating it as a useful system for studying pod development and their responses to biotic or abiotic stresses.

Introduction

Peanut (*Arachis hypogaea* L.) is the only economically important geocarpic plant species, producing flowering structures above ground but forming fruits below ground. After pollination, a gynophore (peg) is produced which contains the fertilized ovules at the tip (Smith, 1954). The peg is positively geotropic, and between 4 and 7 days after pollination it enters the soil to a depth of 2 to 8 cm below the surface, otherwise known as the pegging zone. Here the embryo within the peg begins to swell (Bledsoe, Comar, & Harris, 1949; Moss, Rao, Pattee, & Stalker, 1995; Rashid et al., 2016; Zamski & Ziv, 1976; Ziv, 1981), and then forms the peanut pod and the seeds (Smith, 1954; Xi, 1991). As the pod swells, it actively uptakes nutrients (calcium and other inorganic salts) and moisture from the surrounding soil, while photosynthates are translocated from mesophyll cells to developing pods (Bledsoe et al., 1949; Inanaga & Yoshihara, 1997; Pattee & Mohapatra, 1987; Singh, 2004).

Although peanut pods and roots are both developed in soil, they have distinct interactions with soil microbiome and different needs for nutrition. Comparison of rhizosphere, geocarposphere and bulk soil microbiota revealed differentiated bacterial and fungal populations associated with each organ (Kloepper & Bowen, 1991; Subrahmanyam & Rao, 1977). The preferential association of some fungi and bacteria with early developmental stages of pods indicates that these microorganisms may be adapted for colonizing the geocarposphere (Kloepper & Bowen, 1991; Rashid et al., 2016; Subrahmanyam & Rao, 1977). Such pod-associated microbiota could influence the level of infection by fungal pathogen *Aspergillus flavus* and the production of aflatoxin (Chourasia & Sah, 2017; Li et al., 2020). Selected geocarposphere bacteria were also proposed as a biological approach for controlling *Aspergillus* infection and aflatoxin biosynthesis in peanut (Dong et al., 2020; Lyu, Yang, Wu, Zhang, & Li, 2020; Mickler, Bowen, & Kloepper, 1995; Yan et al., 2010).

As an essential nutrient for pod development, calcium is directly absorbed by developing pods from the geocarposphere rather than being transported from roots (Bledsoe et al., 1949; Brady, 1948; Skelton & Shear, 1971; Wiersum, 1951; S. Yang et al., 2020). Soluble calcium is critical for proper pod and embryo development. Deficiency of soluble calcium (<400 ppm) in the soil surrounding the developing pods leads to abortion of seed development (Dey, Pal, Bhatt, & Chauhan, 2004; Kissel & Sonon, 2008; R. Yang, 2014). If the seed is not aborted, its quality is severely affected, diminishing its commercial value. Calcium-deficient soil promotes stress within the peanut plant and increases the severity of diseases caused by soil borne pathogens. For example, pod rot is caused by a group of fungal pathogens and is more severe in peanuts grown in calcium-deficient soil (Damicone, 2014). To mediate a lack of soluble calcium in the field, supplemental sources are used such as gypsum ($CaSO_4$) or lime ($CaCO_3$). Gypsum is highly soluble and therefore applied at pegging time, whereas lime is incorporated into the soil prior to planting to allow time for the calcium to become available. Gypsum has little effect on soil pH, whereas lime is used primarily to increase pH. A few studies investigating the global transcriptional response to calcium deficiency revealed that auxin and gibberellin (GA) pathway genes were differentially expressed in pods grown in calcium-deficient or calcium-sufficient soil, as well as genes involved in Ca^{2+} signal transduction, nutrition absorption and microRNA function (Chen et al., 2019; S. Yang et al., 2017; S. Yang et al., 2020). However, in these studies, both roots and pods were exposed to calcium deficiency, so it is unclear which responses observed in pods are secondary results of calcium deficiency sensed via root (Chen et al., 2019; S. Yang et al., 2017; S. Yang et al., 2020). The object of this study is to develop an experimental system that allows researchers to study pod development and microbial conditions in a controlled environment independent of the root environment.

Materials and Methods

Plant source and growth conditions

Peanut varieties used in this study were Numex High Oleic 01 (Reg. No. CV-123, PI 670460) and Georgia-06G (CV- GA 011557). Seeds were germinated in soil and grown in a growth chamber (Model CMP3244 Winnipeg, Canada) at 26 ˚C day/ 23 ˚C night, with 12 hr day/ 12 hr night and with a 62% humidity. Plants were grown to full maturity within 1.3-gallon tulip pan circular pots containing Professional Growers Mix potting soil (Sungro Horticulture, Agawam MA, USA 01001). Plants were watered to soil saturation twice a week. Plants were fertilized with an all-purpose 24-8-6 water soluble fertilizer (Miracle-Gro, Marysville OH, USA 43040) every other week based on the manufacturer application rate.

The In-Tube Growth (ITG) System

The ITG system was developed to support individual pod growth in a controlled environment (Figure 2.1). A 50 ml Falcon conical tube (Thermo Fisher Scientific Waltham MA, USA 02451) was modified to hold soil and the developing pod. The lid with five 6-mm holes served as a gas exchange system. The perforated lid was then covered in three alternating layers of 1-inch 3M Micropore tape (3M, Saint Paul MN, USA 5122). On the interior side of the cap was a 0.5 mm gridded mesh. The tube was sealed around the cap-tube junction with parafilm. The gas exchange system allowed proper moisture and humidity control, while simultaneously providing the exchange of gases to prevent an anaerobic environment. The standard 50 mL tube had one 0.5mm hole drilled at its bottom, covered with Parafilm (Bemis Company, Neenah WI, USA 54956), and can be filled with various growth media depending on the purpose of a study (Figure 1A). The ITG system was encased in a layer of aluminum foil. After filling the tube with soil to a volume of 35 ml, 50 ml of sterile DI water was added and passed through the soil. The humidity of soil was maintained around 20% for the duration of the experiment; measured using a standard soil humidity meter. No additional water was added to the tubes throughout the experiment.

Application of the ITG system

When a peg elongated to about 2.5 cm, it was inserted into a tube with soil (Figure 2.1B). Only aerial pegs were used for the system; pegs that contacted the soil surface were no longer used for the ITG system, preventing the initiation of nutrient uptake by a peg. Multiple ITG tubes could be assembled on a single plant (Figure 2.1C). A peanut pod was then grown for an average of 90 days in a tube (Figure 2.1C). The pegs used as open soil control were labeled with a tag at the same time when ITG tubes were applied onto experimental pegs.

Comparison of ITG system and pot grown peanuts (open soil)

Nine Georgia-06G plants were grown in Professional Growers Mix potting soil (Sungro Horticulture, Agawam MA, USA 01001) to the flowering stage for tube attaching. Each tube contained 35 g of potting soil; and adjusted to 20% humidity. On each individual plant, 5-10 pegs as open soil control were labeled using a plant tag. At the same time, 4-6 tubes were assembled on the same plants. To reduce the noise coming from physiological variation of individual plants, pods grown in open soil or tubes from about 10 plants were pooled. Tubes were attached randomly based on peg availability. The experiment was conducted three times. The same experiment was conducted for the Valencia type peanut Numex high oleic 01.

Low and high calcium growth medium

Low calcium growth medium used in ITG system contained 3 parts of sand and 1 part of perlite (Miracle Gro, Marysville, OH, 4304). Murashige & Skoog Medium Basal Medium without calcium, sucrose, and agar (PT018, Himedia, Mumbai, India) was added to supplement nutrition.

High calcium growth medium was comprised of the same artificial soil and amended with 5 g of gypsum (Pennington, Madison, GA, USA 30650) per 300 g of sand.

Low and high calcium pod response

Nine pots of Georgia-06G plants were grown to flowering stage for the attaching tubes. Plants were grown in potting mix. A total of 50 tubes filled with 35 g of either low or high calcium artificial soil were assembled on these 9 plants. On average, each plant carried about 5 low calcium and 5 high calcium tubes.

Data Collection and Analysis

Tubes were harvested at the end of the growing season which was 90-120 days after applying them. All tubes were removed by cutting the pegs from a plant. The peanuts were then removed from the tubes and rinsed under DI water to remove attached soil. Extra water was removed using a paper towel. Pod fresh weight and size were measured immediately after harvest. Pods were then shelled, and the fresh weight of seeds was also determined. Student t-test was used to test a null hypothesis.

Results and Discussion

The assembly of the ITG system was illustrated in Figure 2.1 (Figure 2.1A). Tubes were applied on Georgia-06G plants to examine the growth of pods in ITG system (Figs. 1.1B-1.1D). The development of Georgia-06G pods and seeds in the ITG system was comparable to those from the same plants grown in open soil (Figs. 1.2A and 1.2B). In some cases, deformed pods were found in tubes, probably due to the restriction in space (Figure 2.2C). The deformed pods were included in the analysis of pod weight and seed weight. A decrease in the number of deformed pods were observed when perlite was added into the soil mix, probably due to a reduction in the compaction. Pods grown in open soil had an abortion rate of 19%, while the abortion rate was slightly lower in tubes, at 13%. The abortion rate was calculated by dividing the number of aborted pods by the total number of pegs assembled in tubes. When compared to pods that were tagged at the same time and grown in open soil, pods development in tubes were slightly delayed. No significant differences in fresh pod and seed weight were observed (Figs. 1.2D and 1.2E). The average dry weight of pods and seeds developed in tubes surpassed those developed in open soil (Figs. 1.2F and 1.2G), probably due to the limited exposure to pathogens in the enclosed environment. The increased peanut pod performance in tubes could also be due to differences in environmental and nutritional conditions in open soil and a tube. The growth of a Valencia type variety, Numex High Oleic 01, was also tested in the ITG system. The development of pod and seeds were comparable to those in open soil. The average pod weight was 1.06 g and 1.27 g in open soil and in ITG, respectively (Figure 2.2H). Taken together, the data demonstrated that the ITG system could support normal pod growth for multiple varieties. The problem of deformed pods could be severe when assaying varieties with large pods, such as Georgia-11J. Adding perlite into the ITG system or using a large tube may alleviate the issue.

Results showed that within a 120-day growth period, the ITG system supported comparable pod development as those in open soil. Supplementation of nutrition and water may be required for varieties with late maturity.

Developing pods directly absorb calcium from the geocarposphere, which is independent of the calcium level in the rhizosphere. The ITG system was used to test the pod-specific response to calcium deficiency. Plants were grown in a potting mix (calcium concentration at 12,000 ppm). Artificial soil mixtures with either a low (42 ppm) or a high (1046 ppm) calcium concentration were added into tubes (Figure 2.3A). On average five tubes with low and five tubes with high calcium levels were assembled on one plant (Figure 2.3A). As expected, low calcium treatment caused reduced seed weight (Figure 2.3B and Table 2.1). Low calcium treatment led to a high rate of aborted pegs (50% in the low calcium condition vs 32% in the high calcium condition) and a low fresh pod weight (Table 2.1). Pods grown in a low calcium condition produced less than half of seeds from high calcium-treated pods (Table 2.1). These seeds from low calcium condition also showed reduced dry weight (Table 2.1). Thus, the ITG system can be used to test a pod-specific response to nutritional calcium and perhaps other conditions.

Conclusions

Research results showed that the ITG system could support pod growth in a controlled soil environment separated from the root. This system should be suitable to study pod-specific responses to a range of factors associated with the geocarposphere. It also has utility for investigation of soil nutritional conditions, specifically calcium levels. The ITG system may also be applied to studying the interactions between pods and microbes associated with the geocarposphere. For example, soilborne pathogens or plant growth-promoting rhizobacteria can be added into either open soil or tubes to test their specific impact on pod development.

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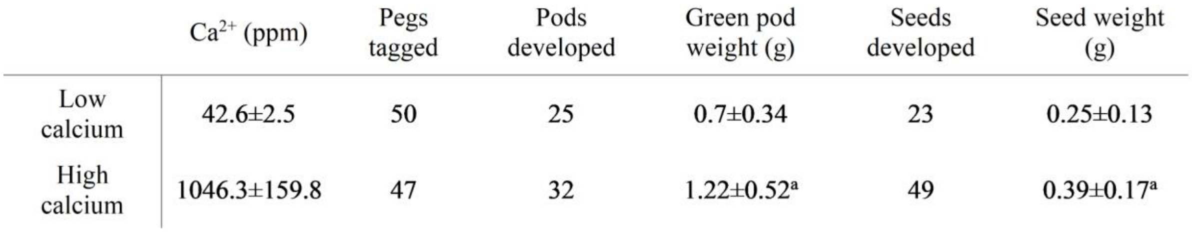
Tables

Table 2.1: Comparison of pod and seed developed in low and high calcium conditions.



	Ca^{2+} (ppm)	Pegs tagged	Pods developed	Green pod weight (g)	Seeds developed	Seed weight (g)
Low calcium	42.6±2.5	50	25	0.7±0.34	23	0.25±0.13
High calcium	1046.3±159.8	47	32	1.22±0.52[a]	49	0.39±0.17[a]

a: *p*-value < 0.05 using Student T-test

Figures

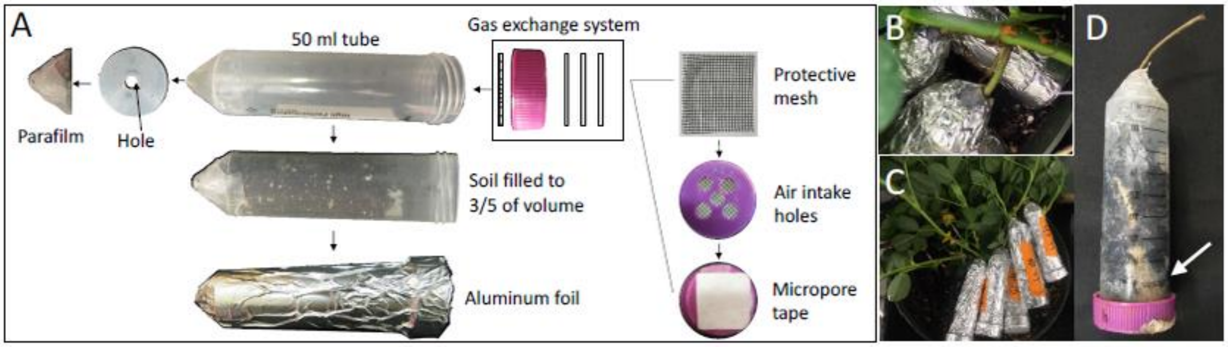


Figure 2.1 Assembly of the ITG system

A) Components of the ITG system. The lid contains five 6 mm holes, three layers of 3M micropore tape and a mesh. Soil was filled up to 35 ml in a 50 ml tube. The bottom of the tube has a single hole for insertion of the peanut peg. The whole system is wrapped in aluminum foil. B) Close-up view of a peg inserted in a tube. C) A representative image showing multiple tubes hosting individual pods attached on a peanut plant. D) A pod growing in a tube. Arrow points to a developed pod.

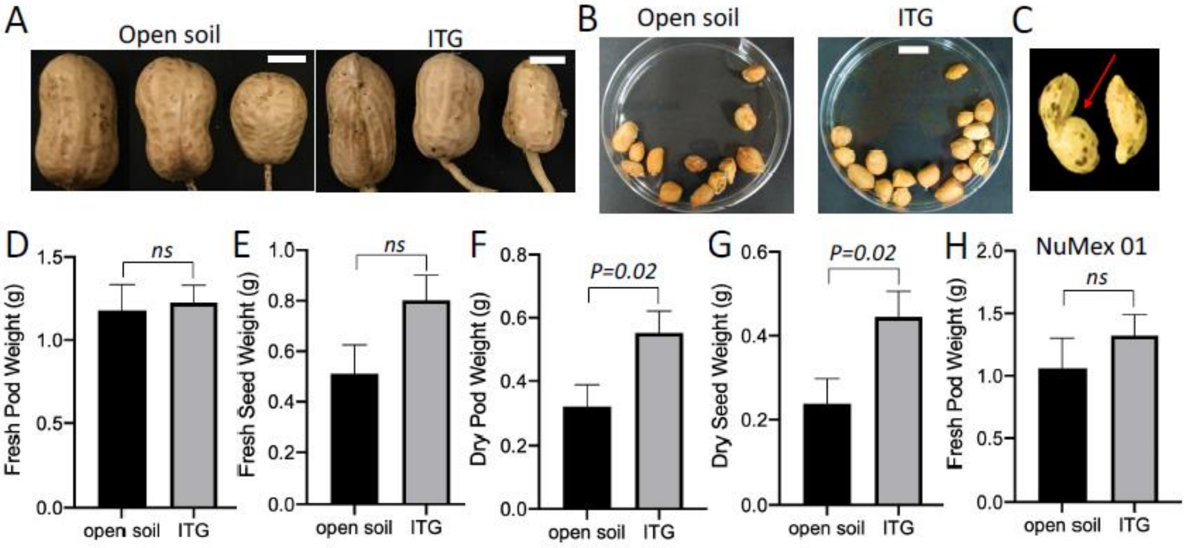


Figure 2.2 Comparisons of pod development in ITG and open soil

A) Representative images of Georgia-06G pods grown in the ITG system and open soil. Bar: 0.5 cm. B) Representative images of seeds developed in the ITG system and open soil. Bar: 1 cm. C) Representative image of deformed pods developed in the ITG system. D)-H): Measurement of fresh pod weight (D), seed weight (E), dry pod weight (F) and dry seed weight (G) from Ga-06G pods grown in ITG and open soil. n=42 for open soil samples, n=45 for ITG samples. H): Measurement of fresh pod weight of NuMex High oleic 01 in ITG and open soil. n=18 for open soil samples; n=40 for ITG samples. Error bars in C-G indicate SEM. ns: not significant based on a student two-tailed t-test. ITG tubes were opened 90 days after attachment.

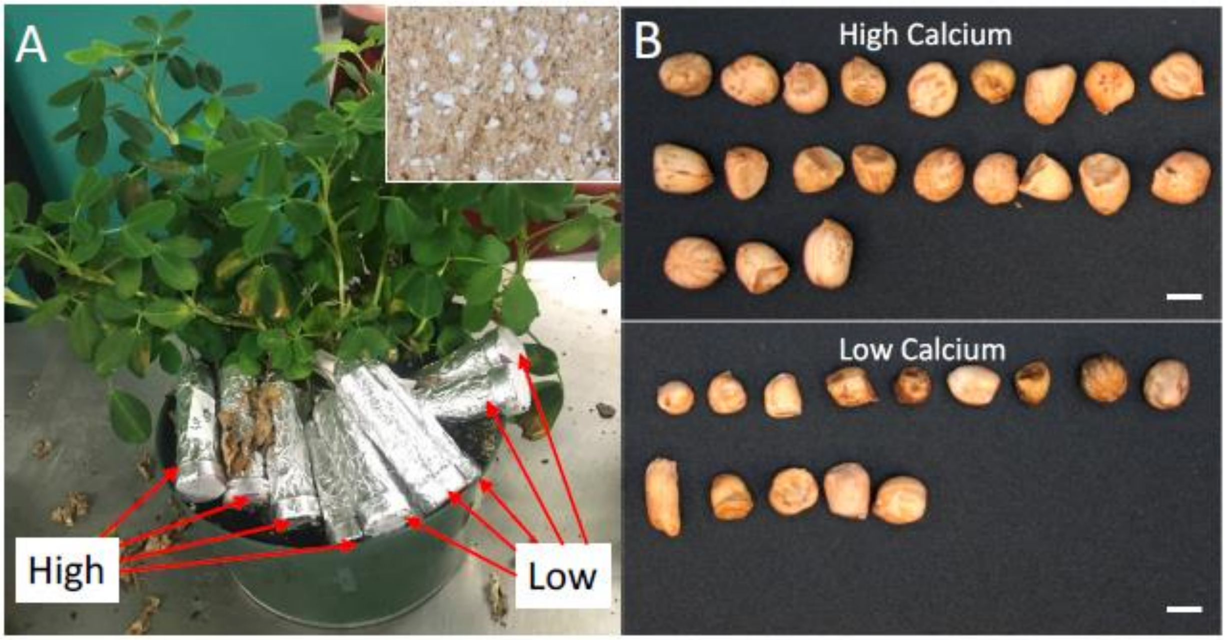


Figure 2.3 Experimental setting to compare pod growth in a low or high calcium conditions. A) An image showing tubes with low or high calcium artificial soil were assembled on a peanut plant. The insert at the up-right corner shows the artificial soil composed of sand and perlite. B) Seeds developed in tubes with a low or high calcium medium. Scale bar=5 mm A) An image showing tubes with low or high calcium artificial soil were assembled on a peanut plant. The insert at the up-right corner shows the artificial soil composed of sand and perlite. B) Seeds developed in tubes with a low or high calcium medium. Scale bar=5 mm

CHAPTER 3

CALCITE DISSOLVING BACTERIA FROM PEANUT (*ARACHIS HYPOGAEA* L.) PEGGING ZONE INFLUENCES SOIL CALCIUM LEVEL.

Reproduced, by permission, Peper, A. Brenneman, T. and Yang, L., (2022) “Calcite dissolving bacteria from peanut (Arachis hypogaea L.) pegging zone influences soil calcium level.*”*, *Frontiers in Microbiomes* 1:1019134. doi: https://doi.org/10.3389/frmbi.2022.1019134

Abstract

Microbial communities play critical roles in mobilizing soil nutrition and, consequentially, shaping plant growth and stress responses. Soluble calcium in the pegging zone is essential for peanut yield. Calcium starvation may lead to seed abortion and increased incidence of disease, such as pod rot. Currently, gypsum or lime are often used to supplement calcium in the pegging zone. Calcite Dissolving Bacteria (CDB) can dissolve poorly soluble calcite into Ca^{2+} thus making it available to plants. Here, we report the isolation and characterization of CDB from a peanut field in Tifton, Georgia. We identified 65 CDB isolates, representing 15 unique strains belonging to 10 different genera. When applied to field soil, most of these CDB survived only several days. However, a synthetic community of CDB strains increased soluble calcium levels when applied to field soil. We also found that CDB abundancy was negatively associated with a soluble calcium level in soil. In summary, we conclude that CDB has the capacity to influence calcium availability in soil, and the abundance of CDB in a bacterial community dynamically responds to soil calcium levels.

Introduction

The relationship between plants, microbes and nutrition is complex. Microbes can play a vital role in plant growth and stress responses (Schlaeppi and Bulgarelli, 2015; Egamberdieva and Ahmad, 2018; Saleem et al., 2019; Qu et al., 2020). For example, Plant Growth Promoting Rhizobacteria (PGPRs) are a group of rhizobacteria found in the plant rhizosphere that can promote plant growth through multiple modes of action. (Ashrafuzzaman et al., 2009; Bhattacharyya and Jha, 2012; Gupta et al., 2015). The mode of action varies greatly between each PGPR, from secretion of plant hormones, secondary. metabolites, volatile organic compounds, to manipulation of the abiotic environment (Vessey, 2003; Ahmed and Holmström, 2014; Kenawy et al., 2019; Riaz et al., 2021; Sukul et al., 2021). PGPRs can be developed into biofertilizers and biocontrol to support plant health and reduce the impacts of stress. For example, phosphorus solubilizing microbes (PSMs) provide mobile forms of phosphorus by solubilizing non-soluble forms of phosphorus using organic acids, siderophores, and chelating agents (Gyaneshwar et al., 1998; Vessey, 2003; Backer et al., 2018; Kalayu, 2019). Calcium is an essential element for plant growth. It is a limiting nutrition for peanut seed development in many peanut producing areas (Pegues et al.; Cox et al., 1976; Chen et al., 2022). As a geocarpic plant, peanut plants flower above ground, forming a peg carrying the developing embryo at its tip, and completes fruit development below ground. A developing peanut pod directly absorbs about 90% of all calcium supporting its growth from the soil, with only 10% of calcium coming from the root system (Bledsoe et al., 1949; Skelton and Shear, 1971). Soluble calcium in the geocarposphere is critical to proper pod development. Calcium starvation in the peanut pegging zone (5- 7 cm below ground) during seed development may lead to aborted embryo development, compromised seed quality and eventually yield loss (Brady, 1948; Bledsoe et al., 1949; Harris, 1949; Cox et al., 1976; Yang, 2014; Chen et al., 2022). Increased incidence of soil borne fungal pathogens were also associated with calcium deficiency (Zhang et al., 2021). To ensure proper seed development and resistance to pathogens, calcium supplements in the form of gypsum ($CaSO_4$) or lime ($CaCO_3$) are applied yearly (Singh, 2004; Kissel and Sonon, 2008; Yang et al., 2020). For example, gypsum is suggested to be applied at a rate of 560 kg/ha (500 lbs per acre) every year at the time of flowering and is often mandatory for seed peanuts due to its positive effects on germination (Kissel and Sonon, 2008; Eroglu et al., 2012; Pegues et al., 2017).

Soil microbes play key roles in the calcium cycle to create soluble calcium for plants to uptake. Calcinogenic bacteria can convert CO_2 into insoluble $CaCO_3$ (calcite) in presence of soluble calcium (Lüttge and Conrad, 2004). On the other hand, calcite dissolving bacteria (CDB) dissolute poorly soluble calcite to generate Ca^{2+} (Jacobson and Wu, 2009; Sulu-Gambari, 2012; Tamilselvi et al., 2016). CDB have previously been identified in calcareous soils, farmland, animal waste and limestone quarries (Subrahmanyam et al., 2009; Rana et al., 2015; Tamilselvi et al., 2016). Several mechanisms are proposed for CDB to dissolve calcite, including secreting citric acid, oxalic acid and sanazine pigment (Subrahmanyam et al., 2009; Rana et al., 2015). CDB can be selected from media containing 1% of calcite on which they form a clear zone or "halo" around the colonies (Subrahmanyam et al., 2009; Tamilselvi et al., 2016). Several commercial operations use CDB to remove calcite deposits from pipes and other unwanted calcite formations (Eroglu et al., 2012). Calcite dissolution by bacteria was also proposed as an alternative approach to reclaim calcareous sodic soils (Tamilselvi et al., 2016). In this study, we investigated the potential role of CDB in addressing calcium deficiency in peanut field. We identified 15 CDB unique strains from a peanut growing field in Georgia, U.S. A synthetic community containing selected CDB strains increased soluble calcium level in natural soil. A negative correlation between soil calcium level and CDB abundance was observed in laboratory setting and fields. Taken together, CDB may be a key component in regulating the dynamics of calcium level in soil.

Materials and Methods

Isolation of CDB from soil

One gram of soil collected from the peanut pegging zone in field was mixed with 9 ml of sterile water and 20 µl of cycloheximide, for fungal inhibition. After 2 minutes' vigorous shaking, 100 µl of the soil suspension was added to a 96-well plate for serial dilution, using a factor of 1:10. The suspension was diluted to the concentration for selecting single colonies, between 1:1,000 and 1:10,000 factor dilution. 100 µl of the final dilution was spread and plated on CDB differentiating media. Plates were incubated at 28 °C for 6 days. Colonies that indicate the clear zone phenotype were selected and re-isolated to obtain single CDB strains. CDB strains were then stored at -80 °C. Soil used for isolation and subsequent experiments was obtained from the UGA Blackshank Farms, Tifton GA. Five individual fields with various field histories and crop rotations were sampled from. Sample site histories are as below. Site 1: vegetable; site 2: peanut; site 3: peanut/cotton rotation; site 4: onion, and site 5: cotton. All sample sites had similar soil texture and soil type (loamy-Sand). The CDB differentiating media used in grams per liter was Glucose 5 g; Yeast extract 1 g; Peptone 1 g; K_2HPO_4 0.4 g; $MgSO_4$ 0.01 g; NaCl 5 g; $(NH_4)_2SO_4$ 0.05 g; $CaCO_3$ 5 g and Agar 7.5 g (Sulu-Gambari, 2012). The standard media used was Lysogeny broth (LB) in grams per liter Tryptone 10 g; Yeast Extract 5 g; NaCl 5 g and Agar 7.5 g.

16S RNA sequencing

High-Quality genomic DNA was extracted using Qaigen's DNEASY Microbial Extraction Kit (Qiagen, Hilden, Germany). The 16s rRNA V4-V5 region of the CDB was amplified using universal primers (515F: GTGCCAGCMGCCGCGGTAA and 926R: GGACTACHVGGGTWTCTAAT) and Sanger sequenced by Eurofins Genomics (Eurofin

Genomics, Louisville KY, USA 40299). The sequencing results were blasted against NCBI nucleotide collection to identify the closest hits.

Calcite solubilization index

An overnight culture of CDB was grown in Luria Broth (LB), pelleted, and resuspended to an OD_{600} = 0.001 at 1 ml. On a six-well culture plate (VWR, Randor PA, USA 19008), a single 10 µl droplet of CDB was placed in the center of each well containing 2 ml of CDB differentiating media. Six technical repeats were included for each CDB. The plates were incubated at 28 °C. Images were taken on day 7 to measure the diameter of the colony, and the clear zone was measured through ImageJ software (NIH, Bethesda MD, USA 20814). The solubility index was calculated for each CDB using the formula Solubility index = $\frac{Clear\ Zone+Colony\ Size}{Colony\ Size}$(Tamilselvi et al., 2016).

Application of CDB into soil

An overnight culture of CDB grown in LB was pelleted and resuspended in 1 ml of sterile water. The suspension was then diluted to either an OD_{600} = 0.01 or 0.1 in 30 ml. The suspension was then added to 300 g of field soil with either low or high calcium level. Low soil calcium level was obtained through washing of the soil to remove Ca^{2+}; low calcium ranged from 50 to 150 ppm. High calcium field soil was obtained by adding gypsum (Pennington Seed, Madison GA, USA 30650) into low calcium soil, increasing soil Calcium level to 1000 ppm. Gypsum was supplemented to achieve high calcium level. Soils with CDB were incubated 28 °C for 7 days and then air dried for 3 days. Control samples were treated with 30 ml of sterile water. The samples were analyzed by the Agricultural and Environmental Services Laboratories at University of Georgia for soil pH and compositions, including calcium, potassium, manganese, phosphorus, magnesium, and zinc.

CDB competition assay

Two colonies of CDB suspensions (10 ul at OD_{600} = 0.01) were spotted next to each other on a CDB differentiating media plate. The size of targeted colonies was measured at four days after plating. The colony size from a pair of the same CDB was used as control, and its colony size was set as "1". All members in the "TOP" mix were tested in a pairwise combination. Six repeats were performed for each combination.

Germination test

The peanut variety used in this study was Georgia-06G. Seedlings were grown in a greenhouse (UGArden Student Community Farm, Athens GA, USA 30605) at 26 °C day/23 °C night, with 12 hr day/12 hr night and with 62% humidity. 30 seeds per CDB strain were soaked in a suspension of CDB diluted to OD_{600} = 0.1 at 50 ml. Seeds were soaked for 10 minutes and then air dried for 5 minutes. 10 seeds were planted in each tulip bulb pot filled with Growers Mix potting soil, and 3 pots were used for each CDB strain. Control seeds were soaked in sterile water. A randomized block design was used to arrange the pots. Seeds were grown in the greenhouse for 16 days. The percent of germination was calculated as germinated seeds/total seeds. Seedlings with two open cotyledons were considered as germinated. Data was collected 16 days after planting.

Development of rifampicin resistant CDB strains through natural selection

A dense 5 ml overnight culture of selected CDB was grown in 5 ml of LB. The culture was pelleted and resuspended in 500 µl of sterile water. 100 µl of CDB suspension was spread onto a Rifampicin 60 plate (RF_{60}) (60 mg/ml) (Gold biotechnology, Olivette MO, USA 63132). Plates were incubated at 28 °C for 2 days. 25 surviving colonies were selected for each CDB and streaked onto a CDB differentiating media supplemented with Rifampicin. Mutants retaining both calcite dissolving ability and Rifampicin resistance mutants were selected for further study.

Survivability of CDB in soil

An overnight culture of a Rifampicin-resistant CDB strain was grown in 5 ml of LB with 5 µl of Rifampicin. The culture was pelleted and re-suspended in 1 ml of sterile water. The suspension was then diluted to an $OD_{600} = 0.1$ in 15 ml of sterile water. The diluted suspension was added to a 150 g of low or high calcium peanut soil. The suspension soil mixture was incubated at 28 °C for 8 days. One gram of soil was collected every two days and the CDB were counted on RF_{60} plates after serial dilution. The ratio of CDB was determined as the proportion of CDB colonies in total culturable colonies on a LB plate.

Soil test

Soil was oven dried (40 °C), ground, and sieved through a 2 mm screen. Soil was weighed (~5.0 g), adding 20 mL Mehlich I (0.025N H_2SO_4 + 0.05N HCl) extracting solution. Samples were immediately placed on shaker for 5 minutes at high speed (250 oscillations per minute). Samples were then filtered using Whatman #1 paper and the extracts are analyzed for P, K, Ca, Mg, Zn, Mn, by ICP-OES (Spectro Arcos FHS16). Soil pH and the lime buffer capacity were determined using an automated LabFit AS-3000 pH Analyzer equipped with direct titration capabilities. Soil pH was determined using a 1:1 soil: 0.01 M $CaCl_2$ suspension. The 0.01 M $CaCl_2$ readings were then converted to soil-water pH readings by adding a conversion factor of 0.6. The Lime Buffer Capacity was determined on samples with pH readings of less than 8.1 by direct titration using 0.023 M $Ca(OH)_2$.

Statistics

Student t-test was used to compare the treated samples with controls in (Figures 2.2A, C, D, E). Student t-test was also used to compare the CDB survivability in soils with low or high concentration of calcium. One-way ANOVA with Tukey multiple comparisons was used to compare differences in treatments in (Figures 2.4A, B). Pearson correlation coefficient (r) test was used to calculate the correlation in (Figure 3.5C).

Results

To identify potential CDB from a peanut producing field, we sampled the pegging zone soil from a field in continuous peanut culture at the UGA Blackshank farm (Tifton, GA) (Figure 3.1A). Soil samples were subject to a serial dilution in water. Suspensions were plated on a CDB differentiating media to visualize calcite dissolving ability of a colony. Colonies that created a clear zone were selected as potential CDB. We isolated 65 CDB strains and confirmed their ability to dissolve calcite in single colony streaks (Figure 3.1B). These CDB strains showed distinctly different colony morphologies (Figure 3.1C and Table 3.1), indicating that they belonged to different phylogenetic groups. We sequenced the 16s rRNA V4-V5 regions from 65 CDB colonies and identified 15 unique CDB strains. The CDB strains isolated from peanut field belongs to multiple genera including *Bacillus, Paenibacillus, Buttiauxella, Lelliottia, Cellulomonas, Enterobacter, and Staphylococcus* (Table 3.1). Multiple strains from *Bacillus, Paenibacillus, Buttiauxella* and *Lelliottia* genera were isolated. We measured the solubilization index of the 15 CDB strains on CDB differentiating plate. The solubilization index ranged from 2.1 to 5.89 (Table 3.1). We did not observe a correlation between solubilization index and bacteria genera.

Application of CDB alters soil calcium level

To test if adding CDB in soil can increase soluble calcium level, we collected field soil samples from the same location where CDB were isolated. The average calcium concentration was around 200 ppm. Soils were supplemented with individual CDB strains at $OD_{600} = 0.1$ in laboratory and incubated in 28 °C incubator. Increased calcium level was observed in many cases after adding individual CDB (Figure 3.2A). However, extensive variations were observed between repeats, despite our efforts to standardize the protocol. Only one CDB (95A) significantly increased calcium level. We reasoned that individual CDB had limited capacity to influence soil calcium level, and the heterogeneity of natural soil increased the variation between repeats. To improve the consistency of CDB application, we generated three synthetic communities of CDB. The "TOP" mixture contained 7 CDB strains that showed the highest solubilization index on plate (Figure 3.2B); the "ALL" mixture contained all 15 CDB strains; and the "*Bacillus*" mixture contains seven *Bacillus* and *Paenibacillus* CDB strains (Figure 3.2B). We chose *Bacillus/Paenibacillus*, because they can generate subtilis spore, a bacterial survival sturcture, which may increase survivability of CDB in soil. When applied at $OD_{600} = 0.01$, none of these mixtures changed the level of calcium in soil. At a high concentration ($OD_{600} = 0.1$), the "TOP" mixture significantly increased calcium level (Figure 3.2C). Interestingly, soil pH was not changed by the "TOP" mixture as well as the levels of Potassium, Manganese, phosphorus, magnesium, and zinc (Figure 3.2D). To explore the potential interaction between members of the "TOP" mix, we performed a competition assay to compare CDB multiplication in a pairwise combination on calcite plates (Figure 3.2E). We observed a complex interaction pattern between "TOP" CDB members. Mainly, 93A inhibited the colony growth of 4 out of 6 "TOP" community members, and 188A multiplication was inhibited by 4 members (54A, 93A, 95A and 100A), indicating potential interactions between these CDBs in soil (Figure 3.2E). We also found that CDB strains included in the "TOP" mix were not pathogenic, after application to peanut seeds, although one strain (100A) slightly reduced germination rate (Figure 3.2F). These results suggested that supplementing CDB could increase soluble calcium level in natural soil.

Survival rate of CDB

We hypothesized that the variation of individual CDB's impact on calcium level was due to the short survival time of supplemented CDB. To monitor the survival rate of supplemented CDB in soil, we generated mutants of CDB that are resistant to Rifampicin (CDB-RIF) (Figure 3.3A). Importantly, the Rif-resistant CDB still has the capacity to dissolve calcite (Figure 3.3A). We added individual Rif-resistant CDB into soil and measured the amount of CDB that could be recovered on a Rifampicin plate. For the tested eight CDB strains, their abundance dropped to less than 30% after the first two days. 95A-Rif maintained a higher survival rate compared to other CDB at day 4, which was consistent with its impact on soil calcium level when added individually (Figure 3.2A, B). One week after application, added CDB were not detectable (Figure 3.3B). Thus, supplemented CDB have short survival time even in native soil.

Correlation between CDB abundance and soil calcium level

To test the relationship between CDB abundance and soil Ca^{2+} level, we monitored the relative abundance of CDB in low calcium field soil supplemented with different levels of gypsum. The relative abundance of CDB was determined by the ratio of CDB colonies to the total number of culturable colonies on a calcite plate. We found a reduction of CDB proportion when Ca^{2+} concentration increased from 0 to 350 ppm (Figure 3.4A). No further reduction was observed when Ca^{2+} level exceeded a threshold of 350 ppm, indicating that the increase of CDB level in low calcium soil might be an adaptation to calcium deficiency. We further narrowed the threshold concentration to 250 ppm (Figure 3.4B). It is noteworthy that the calcium concentration in untreated field soil was around 200 ppm, which is close to the threshold. To directly observe CDB's response to calcium level and test which CDB contributed to this overall reduction of CDB population in response to increased calcium level, we inoculated a high (1000 ppm) and low (100 ppm) calcium soil with individual Rifampicin-resistant CDB strains (Figure 3.4C). Two days after inoculation, we compared the abundance of inoculated CDB on a Rif_{60} plate. Two of the 15 tested strains showed increased survivability in high-calcium soil. 54A-RIF,

93A-RIF, 100A-RIF, 130.2A-RIF, 141A-RIF and 188A-RIF strains had decreased survivability in high calcium soil (Figure 3.4C). It is noteworthy that we were not able to recover 188A-RIF from high calcium soil two days after inoculation. These results indicate that individual CDB strains have distinct response to soil calcium level, and a few CDB strains may contribute to the observed overall negative correlation between CDB abundance and calcium level. To test if the negative correlation between CDB abundance and soil calcium level occurs in nature, we sampled soils from five fields in the Blackshank farm (Figure 3.5A). The field soils had similar structure with 85% sand and 15% silt and clay (Figure 3.5B). We observed that soils from different fields contained variable amounts of Ca^{2+} (Figure 3.5C). Field 3 and 4 had the highest calcium level and the lowest CDB ratio (Figure 3.5C). There was a negative relationship between relative CDB abundance and soil Ca^{2+} level in these samples (Pearson correlation coefficient ($r = -0.9352$)). The negative relationship was consistent with what we observed from soils supplemented with different amounts of gypsum in lab (Figure 3.4A). Taken together, our observations indicated that the soil CDB abundance decreased as a response to elevated soil calcium level.

Discussion

Calcium is a well-known limiting nutrient in peanut production and has been linked to disease severity from soil borne fungal pathogens i.e., *Aspergillus flavus* (*A. flavus*) (Fernandez et al., 1997; Yang, 2014; Yang et al., 2017). In this study, we isolated CDB strains from a peanut growing field in Georgia, USA, representing species from different genera. Previously, CDB were identified from limestone quarries and animal fecal matter (Tamilselvi et al., 2016). Other research has shown CDB to be an effective means to solubilize calcite and have used them to reverse the desertification process, restore statues, and remove calcium build-up from pipes

(Eroglu et al., 2012; Rana et al., 2015; Anbu et al., 2016; Tamilselvi et al., 2016; Qu et al., 2020). Interestingly, CDB isolated from these studies and our research belong to distinct phylogenic groups, indicating that different bacteria with calcite dissolving ability adapted to these environments. The identification of CDB in peanut fields provides the potential for a deeper look into the peanut microbiome as well as a start to further investigate the microbial impact on the soil calcium cycle. As a geocarpic plant, peanut fruits share the same environment as their root. Their roots and pods associate with distinct and overlapping microbiomes, presumably cooperating with different physiological needs of these two organs (Bledsoe et al., 1949; Harris, 1949; Kvien et al., 1988; Smal et al., 1989). The Pod-associated microbiota was shown to affect A. flavus infection and the production of aflatoxin (Chourasia and Sah, 2017; Zhang et al., 2021). Selected geocarposphere bacteria have potential as a biocontrol for soil borne fungal pathogens such as Aspergillus (Chourasia and Sah, 2017; Li et al., 2020; Zhang et al., 2021). Since peanut pods actively absorb a large amount of calcium to support embryo and shell development, it is intriguing to know whether CDB are differentially associated with pods and roots and whether they can promote pod development. We found that single CDB strains had limited impact on the calcium level in natural soil (Figure 3.2A). Only one strain, 95A, significantly increased calcium level (Figure 3.2A). However, a synthetic community composed of CDB with high solubilization index significantly increased soluble calcium level (Figure 3.2C). It is likely that a single CDB strain is not competitive in the soil microbial community, leading to a short survival time as indicated by our CDB survival study. The ability of "TOP" synthetic community to increase calcium level implies that the solubilization index on plate could be an indicator of calcite dissolving ability in soil. It is also possible that the "TOP" synthetic community increases the survivability of members in soil. To develop CDB as a biofertilizer, it is a future challenge to further increase the potency of CDB community in soil. One approach is to identify the core microbiome associated with the peanut rhizosphere and geocarposphere. Core microbiomes have been used to stabilize synthetic communities (Armanhi et al., 2018; Toju et al., 2018; Bano et al., 2021; Fazeli-Nasab et al., 2022). Further investigation using tagged bacteria will help to track CDB in a complex soil environment and understand the mechanism underlying their low survivability in high calcium soils. Alternatively, CDB may be applied via other methods such as a seed coating or even an in-furrow spray (Malusá et al., 2012; Herrmann and Lesueur, 2013; Ma, 2019; Agake et al., 2021). One of the greatest threats to peanut production is the increased presence of *A. flavus* and its potential to produce the mycotoxin aflatoxin. Studies showed that *A. flavus* infection on peanut and aflatoxin accumulation was negatively correlated with soil calcium level (Zhang et al., 2021). Thus, CDB has the potential as another tool in management of *A. flavus*.

Conclusion

Our results show that CDB are present in Georgia peanut fields and can be successfully isolated. Individual CDB strains showed limited capacity to influence soil soluble calcium level, probably due to short survival time. However, a logically designed synthetic community increased calcium level in natural soil. The relative abundance of CDB in soil was negatively correlated with calcium level. Our study suggested the potential of CDB to engineer soil calcium availability for peanut production.

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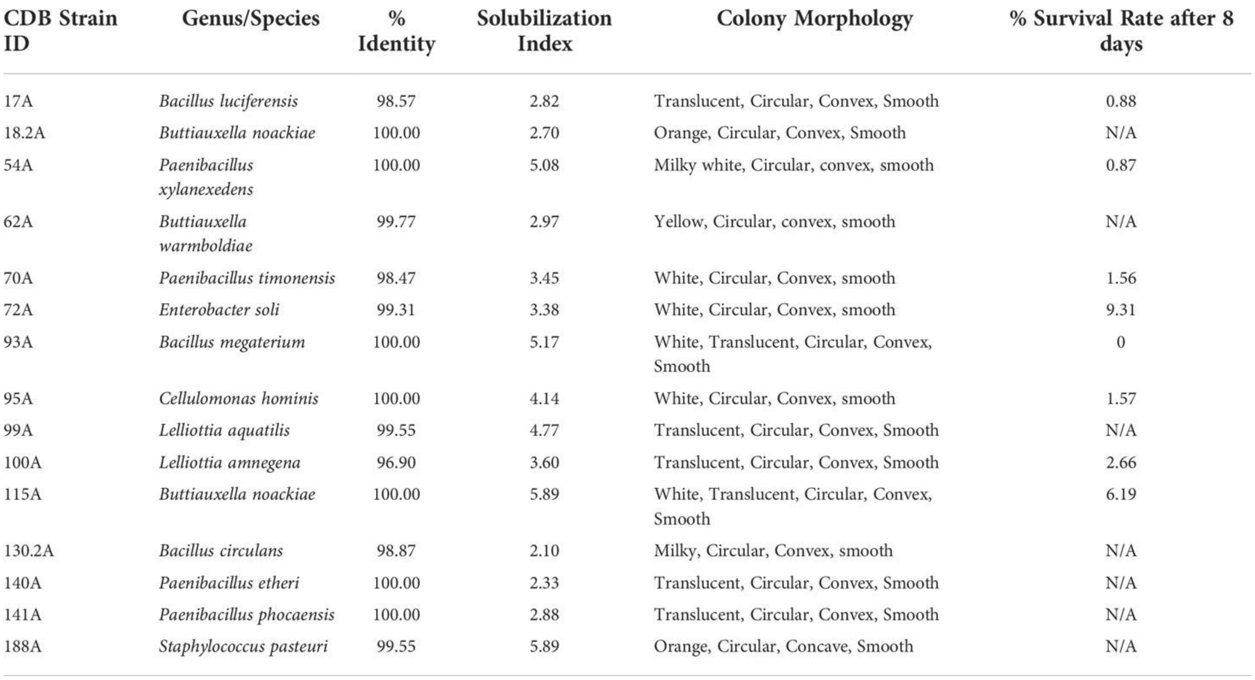
Tables

Table 3.1 Characterization and identification of CDB strains.



CDB Strain ID	Genus/Species	% Identity	Solubilization Index	Colony Morphology	% Survival Rate after 8 days
17A	*Bacillus luciferensis*	98.57	2.82	Translucent, Circular, Convex, Smooth	0.88
18.2A	*Buttiauxella noackiae*	100.00	2.70	Orange, Circular, Convex, Smooth	N/A
54A	*Paenibacillus xylanexedens*	100.00	5.08	Milky white, Circular, convex, smooth	0.87
62A	*Buttiauxella warmboldiae*	99.77	2.97	Yellow, Circular, convex, smooth	N/A
70A	*Paenibacillus timonensis*	98.47	3.45	White, Circular, Convex, smooth	1.56
72A	*Enterobacter soli*	99.31	3.38	White, Circular, Convex, smooth	9.31
93A	*Bacillus megaterium*	100.00	5.17	White, Translucent, Circular, Convex, Smooth	0
95A	*Cellulomonas hominis*	100.00	4.14	White, Circular, Convex, smooth	1.57
99A	*Lelliottia aquatilis*	99.55	4.77	Translucent, Circular, Convex, Smooth	N/A
100A	*Lelliottia amnegena*	96.90	3.60	Translucent, Circular, Convex, Smooth	2.66
115A	*Buttiauxella noackiae*	100.00	5.89	White, Translucent, Circular, Convex, Smooth	6.19
130.2A	*Bacillus circulans*	98.87	2.10	Milky, Circular, Convex, smooth	N/A
140A	*Paenibacillus etheri*	100.00	2.33	Translucent, Circular, Convex, Smooth	N/A
141A	*Paenibacillus phocaensis*	100.00	2.88	Translucent, Circular, Convex, Smooth	N/A
188A	*Staphylococcus pasteuri*	99.55	5.89	Orange, Circular, Concave, Smooth	N/A

Figures

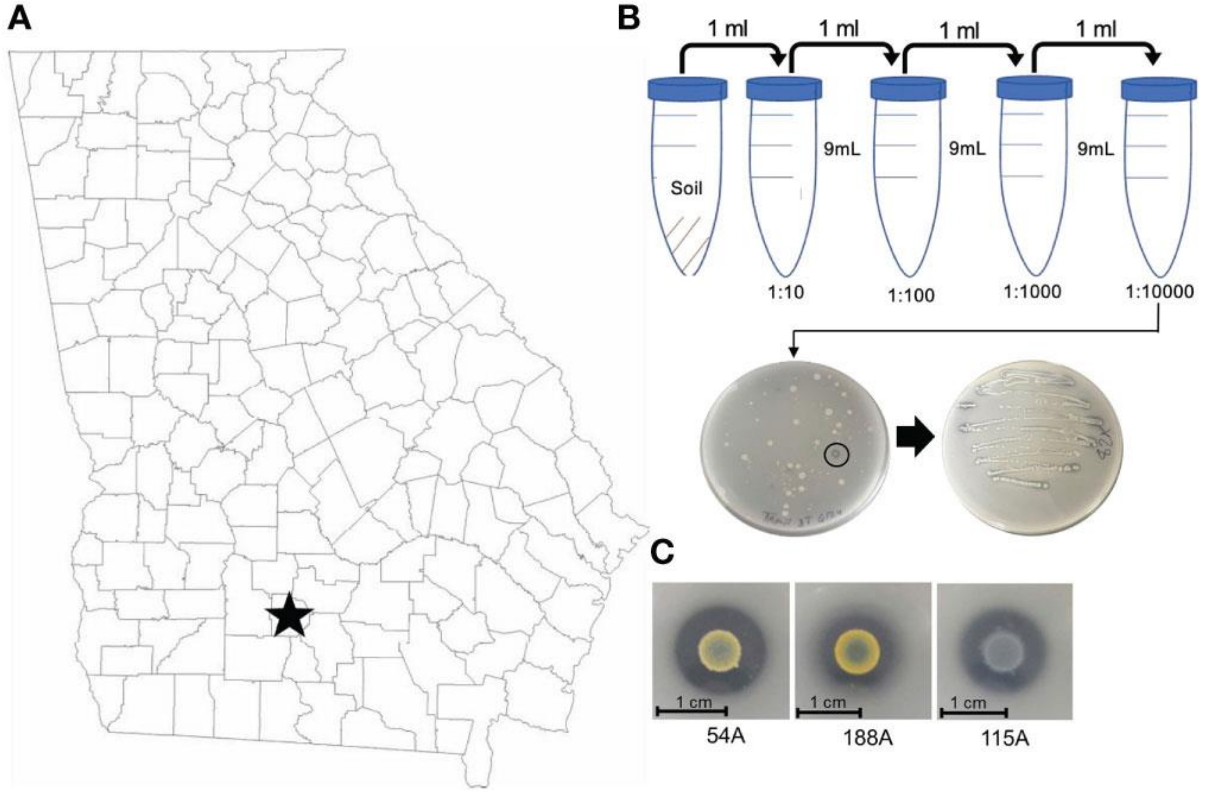
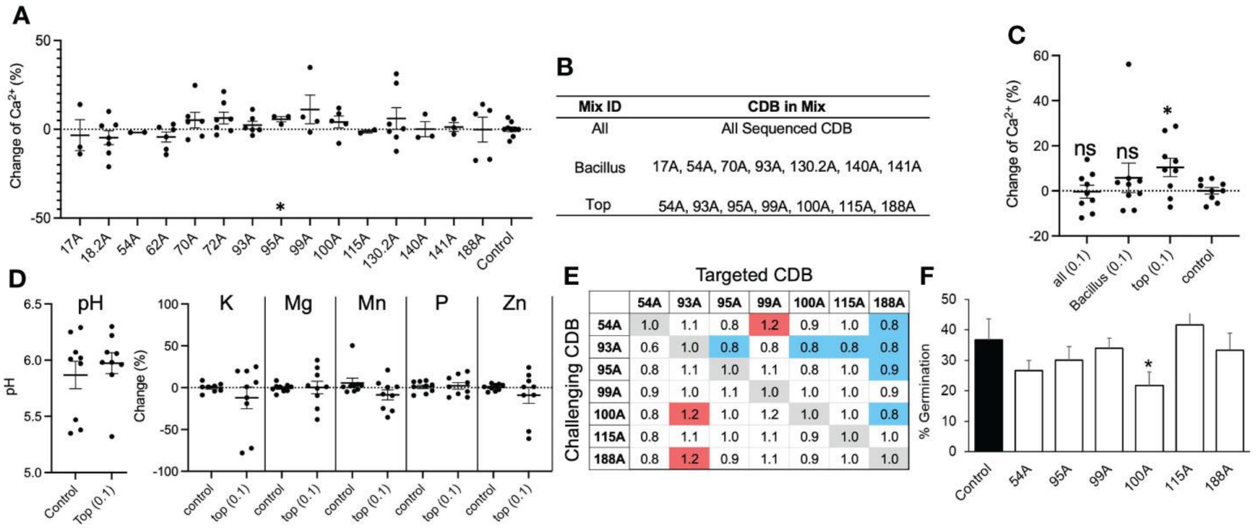


Figure 3.1: CDB isolation from peanut field in Tifton, Georgia, US. (A) Map of CDB survey region in Georgia, U.S. (B) CDB Isolation procedure from soil to single strains. Note the circled CDB colony with clear zone. (C) Representative CDB morphologies and images.



Mix ID	CDB in Mix
All	All Sequenced CDB
Bacillus	17A, 54A, 70A, 93A, 130.2A, 140A, 141A
Top	54A, 93A, 95A, 99A, 100A, 115A, 188A

	54A	93A	95A	99A	100A	115A	188A
54A	1.0	1.1	0.8	1.2	0.9	1.0	0.8
93A	0.6	1.0	0.8	0.8	0.8	0.8	0.8
95A	0.8	1.1	1.0	1.1	0.8	1.0	0.9
99A	0.9	1.0	1.1	1.0	1.0	1.0	0.9
100A	0.8	1.2	1.0	1.2	1.0	1.0	0.8
115A	0.8	1.1	1.0	1.1	0.9	1.0	1.0
188A	0.8	1.2	0.9	1.1	0.9	1.0	1.0

Figure 3.2 CDB alters soil calcium availability. (A) Percent change in soil calcium level after application of single strain CDB at OD_{600}=0.01. Water was used as a control. * above 95A indicate $p< 0.05$ when compared to control using student t-test. (B) Table listing components of each synthetic CDB community. (C) Soil calcium level after applying synthetic CDB communities. Each dot represents an independent test. Long bars and short bars represent means and standard errors, respectively. * indicates $p< 0.05$ when compared to control using student t-test. ns: not significant. (D) Soil pH and nutrient level after applying the “TOP” mix. (E) Pairwise interaction between members in the “TOP” CDB mix. The numbers indicate normalized colony size of targeted CDB. The targeted CDB colony size was normalized to “1” when the same bacterium was used as challenging CDB. Red and blue shade indicate significant promoting and inhibiting effect based on a comparison of colony size using student t-test. (F) Germination percent of Georgia-06G after seed soaking with single strain CDB suspension. indicates $p < 0.05$ when compared to control using student t-test.

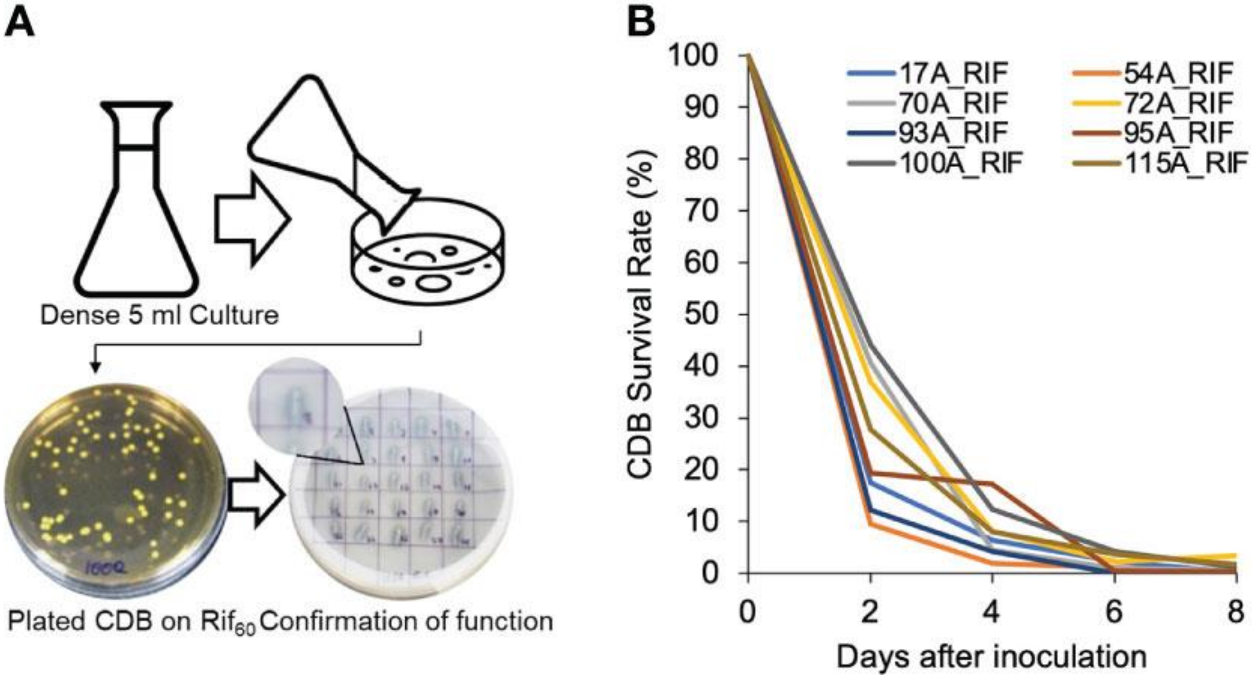


Figure 3.3 Supplemented CDB have short life in soil. (A) Procedure to create rifampicin resistant CDB mutants and confirmation of calcite dissolving phenotype. In the CDB differentiation plate, note the clear zone created by CDB strains recovered from Rif60 plates. (B) CDB-RIF survival rate in soil. CDB-RIF was added at a concentration of OD600=0.1. Survival rate was determined by comparing the colony forming unit of CDB recovered from a Rif60 at each day to that of Day 0. Measurements were taken every two days until day 8.

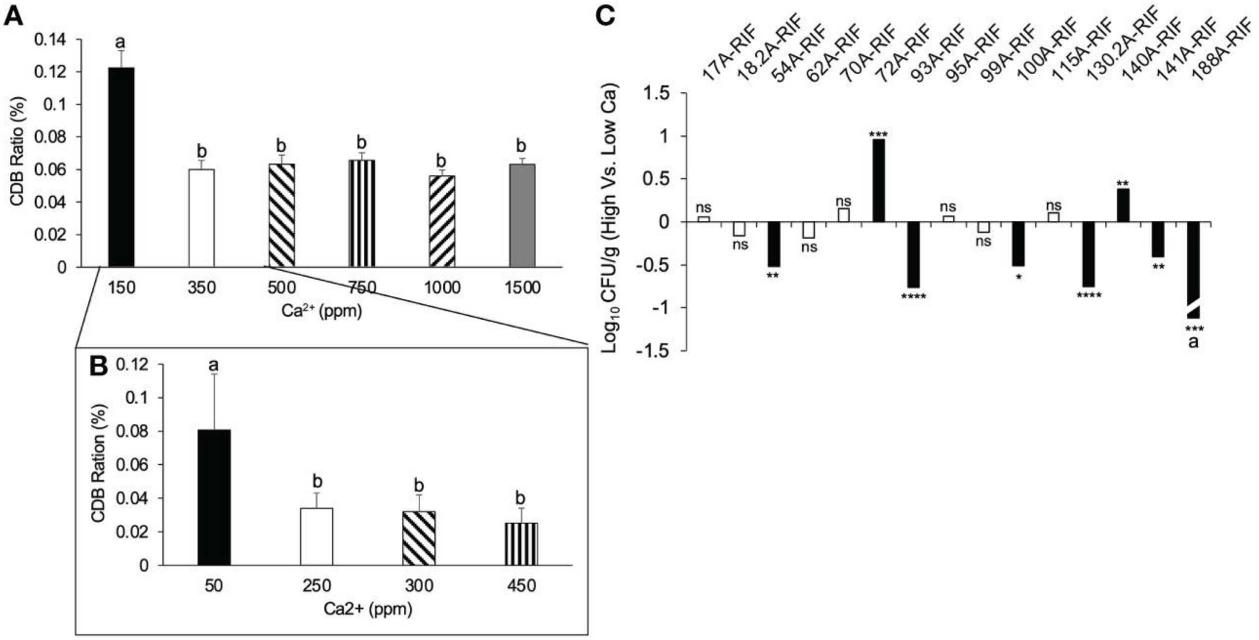


Figure 3.4 Correlation between CDB abundance and soil calcium level. (A) Relative CDB abundance in soils supplemented with different concentrations of calcium. error bars: standard error; different letters on top of each bar represent significant difference using One way ANOVA with a Tukey multiple comparison. Calcium was supplemented in the form of gypsum. (B) Relative CDB abundance responding to a fine scale of calcium concentration. CDB ratio was determined by dividing the number of CDB colonies to the number of total culturable colonies on CDB differentiation plate. (C) Individual CDB response to soil calcium level. *$p < 0.05$; **$p < 0.01$; ns: not significant. Student t-test was used to compare the abundance of CDB-RIF (Log(CFU/g)) from high and low calcium soil. a: 188A-RIF was only recovered from low calcium soil. ***$p < 0.001$; ****$p < 0.0001$.

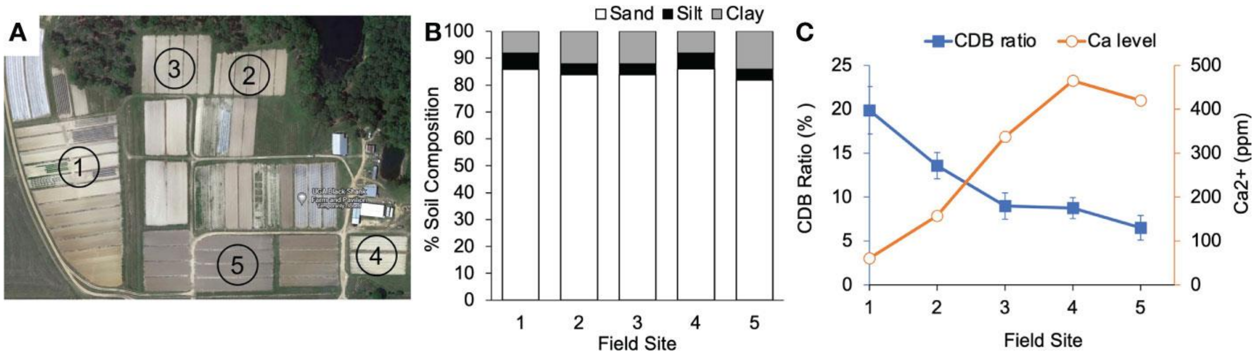


Figure 3.5 CDB population response to calcium in field soil. (A) Arial photo of sample sites in Blackshank Farm, Tifton, GA. Numbers in each field correspond to numbers on X axis in (B, C). (B) Soil texture analysis of the 5 field sample sites. (C) A negative correlation between calcium level in soil and relative abundance of CDB within field soils. CDB ratio was determined by dividing the number of CDB colonies to the number of total culturable colonies on CDB differentiation plate. 10-15 plates were used for each soil sample.

CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF CORE AND UNIQUE CALCITE DISSOLVING BACTERIA COMMUNITIES FROM PEANUT FIELDS

Reproduced, by permission, Peper, A., Newton, C., Lim, S., Zheng, W., Brenneman, T., & Yang, L. (2023). “Functional Characterization of Core and Unique Calcite Dissolving Bacteria communities from Peanut Fields”. Submitted to Phytopathology.

Abstract

Calcium deficiency is a leading cause of reduced peanut (*Arachis hypogaea* L.) seed quality and has been linked to increased disease susceptibility, specifically to soil borne fungal pathogens. Sufficient calcium at flowering time is critical to ensure proper pod development. Calcite Dissolving Bacteria (CDB) isolated from farming fields can dissolve calcite ($CaCO_3$) on plates and increase soluble calcium levels in soil. However, the phylogenetic diversity and geographic distribution of CDB is unclear. Here, we surveyed five peanut producing fields in each of three regions in southern Georgia, representing distinct soil compositions. We characterized the soil composition and identified 52 CDB strains. CDB abundance was not associated with any of the soil characteristics we evaluated. Three core genera, represented by 43 strains, were found in all three regions. *Paenibacillus* was the most common CDB found in all regions, making up 30 of the 52 identified strains. Six genera, represented by eight strains, are unique to one region. Members of the core and unique communities showed comparable solubilization index on plates. We conclude that a diversified phylogenetic population of CDB are present in Georgia peanut fields. Despite the diversity, as a population, they exhibit comparable function in modulating soil calcium level based on the solubility index.

Introduction

Calcium is a versatile and essential nutrient for plant growth and development as well as responses to environmental stresses (Adams 1980; Taylor 2004; Wright et al. 2016). Calcium deficiency in plants may lead to severe growth defects and enhanced disease susceptibility (Kvien 1988; Taylor 2004; Yang 2014). A large amount of calcium is required for peanut pod development (Chen et al. 2022; Cox 1976; Harris 1949; Pegues 2017). Pods directly absorb most calcium needed for their development, and only a fraction is transported via root uptake (Bledsoe R 1949; Kvien 1988). Calcium supplemented only to the roots were not able to rescue the growth defects in pods exposed to calcium deficient soil (Bledsoe R 1949; Skelton 1971). Insufficient calcium during pod expansion leads to abortion of embryos or a significant reduction in seed quality (Brady 1948; Chen et al. 2019a; Chen et al. 2022; Harris 1949; Yang 2014). In addition, calcium deficiency in soil has been linked to high incidence of soilborne fungal pathogens on pods, such as the pod rot complex (*Pythium myriotylum, Rhizoctonia* sp. and *Fusarium* sp.) (Csinos 1986; Taylor 2004; Zhang et al. 2021). Application of gypsum ($CaSO_4$) to peanut reduces disease severity, produces higher yields, and increases seed quality, similarly to the effects of calcium on blossom-end rot of fruits (Alva 1991; Zhang et al. 2021). Calcium deficiency and mineral imbalance increase plant susceptibility to blossom-end rot and pod rot (Taylor 2004). Transcriptomic studies reveal that a subset of miRNAs are induced by calcium deficiency. Targets of these miRNAs, including TCP3, AP2, EMB2750, and GRFs, are expressed in early embryo development and have been indicated in embryo development in other plants (Chen et al. 2019b; Chen et al. 2022; Yang et al. 2020). In addition, calcium deficiency limits MADS- box transcription factors, preventing swelling of gynophores and formation of a pod (Yang et al. 2017).

Microbial communities play key roles in soil nutrition cycling. They may be involved in decomposition, mineralization, nitrogen fixation, pH regulation and many other processes. Calcite Dissolving Bacteria (CDB) are a functional group of bacteria that can dissolute poorly soluble calcite (Jacobson 2009; Peper et al. 2022; Sulu-Gambari 2012; Tamilselvi et al. 2016). CDB have been isolated in calcareous soils, farmlands, animal waste and limestone quarries of the middle east, where the primary focus was the reclamation of low-quality farmlands (Rana et al. 2015; Subrahmanyam et al. 2009; Tamilselvi et al. 2016). We previously identified CDB from one experimental field in Georgia (Peper et al. 2022). These CDB are phylogenetically divergent with *Paenibacillus* as the most abundant CDB population (Peper et al. 2022). A synthetic community composed of CDB members showing high calcite solubilizing ability can increase calcium level in soil (Peper et al. 2022). We also observed that CDB abundance was negatively correlated with soil calcium level when it is below 150 ppm (Peper et al. 2022). Although CDB isolated from divergent geographic locations covers a wide range of genera (Peper et al. 2022; Rana et al. 2015; Tamilselvi et al. 2016), we have limited information on the occurrence, incidence, and composition of CDB community in farmlands with similar history. To obtain a comprehensive picture of CDB frequency, composition, and phylogeny in farmlands within a region, we surveyed 15 peanut producing fields in southern Georgia.

Here we report that the CDB communities are highly diversified even in a small geological region with similar soil composition. The abundance or phylogeny of CDB is not associated with tested soil nutrient levels. We identified core and unique CDB based on their distribution in regions with different soil types. The capacity of members in the core or unique communities to solubilize calcite on plate were not different. This work characterized CDB present in Southern Georgia and elucidates the impact of soil properties on CDB distribution.

Materials and Methods

Soil sampling and processing

We sampled soils from 15 in-production peanut fields distributed through three regions in South Georgia's coastal plain: the middle, southwest and upper. Tift, Mitchel, and Sumter counties were selected to represent these regions. Five in-production peanut field sites were selected from each region, based on soil type (Figure 4.1). Six soil cores were taken from the peanut pegging zone at a depth of 6 cm below the surface within each field. Sampling was conducted in a linear transect; samples were randomly collected to ensure coverage of the entire field. Each sample consisted of 0.5 kg of soil that was collected in individual plastic bags and transported in a cooler before being stored in a cold room at 4 ºC. Soil samples were processed within one week after sampling. Soil properties including nutrients (Ca, K, Mg Mn, P, Zn), pH, texture, and electrical conductivity were measured by Agricultural and Environmental Services Laboratories at University of Georgia (UGA) for each sample. To process soil samples, 20 ml of the Melich I extraction solution (0.025N H_2SO_4 + 0.05N HCl) were added to 5 g of 2 mm sieved soil (Melich 1953). Samples are placed on the shaker for 5 minutes (250 RPM) and then filtered through Whatman #1 filter paper before analysis by ICP- OES (Spectro Arcos FHS16) (Donohue 1983).

Isolation of CDB from field soils

CDB were isolated as previously described (Peper et al. 2022; Tamilselvi et al. 2016). Briefly, one gram of soil was suspended in 9 ml of sterile water by vigorous vortexing. The original suspension was 1:10,000 times diluted. To isolate CDB, a 100 µl of the 1:10,000 diluted suspension was plated on CDB differentiating media (Tamilselvi et al. 2016). In total, 10 biological replicates were conducted per sample site. The number of total and CDB colonies were counted. “CDB Ratio” was calculated by dividing the number of CDB colonies by the number of total colonies (Peper et al. 2022). Individual CDB colonies were picked from CDB differentiating plates and cultured for further characterization.

16S rRNA sequencing

CDB were overnight cultured in liquid Luria Broth (LB) and were collected for genomic DNA extraction using Wizard HMV DNA Extraction Kit (Promega, Madison WI, USA 53703). Sanger sequencing of the entire 16s rRNA region was conducted by Azenta (Azenta, Burlington MA, USA 01803) using proprietary Bifidobacterium optimized primers. The provided sequence data was trimmed and aligned using Genious software (Dotmatics, Bishop’s Stortford, United Kingdom). The aligned sequences were blasted against NCBI 16S ribosomal RNA sequence database to determine genus level identification (Silva, Braunschweig, Germany). The minimum threshold for a successful sequence was set at 95% identity.

Measurement of solubilization index

CDB were streaked onto CDB differentiating media and incubated at 28 °C. Upon visual confirmation of the CDB dissolving calcite on the CDB differentiating media, colonies were picked and washed in 1ml 10 mM $MgCl_2$, pelleted, and then resuspended at an $OD_{600} = 0.01$ in 1ml of 10 mM $MgCl_2$. Disks (7 mm in diameter) were made from 10 µm Whatman filter paper. Six discs were soaked for 5 minutes in the CDB suspension on a rocker at 28 °C. A single disk was placed in the center of a 6-well plate containing 1 ml of CDB differentiating media. The plates were then dried and incubated at 28 °C for 10 days. Photos of each well were taken on days 4, 7, and 10 for calculation of the solubility index. Images were collected on the ZEISS Axio Zoom. V16 microscope using PlanNeoFluar Z 1.x/0.25 FWD 56mm objective. Four overlapping images of an individual colony were collected at a zoom factor of 7 and stitched together using the ZEN 3.5 (ZEN pro) version 2.5.93.0001 software (Carl Zeiss AG, Jena, Germany). Colony images were further processed and measured using the ImageJ software (NIH, Bethesda MD, USA 20814). Images were converted to a binary format and the resultant image was measured for the area of the clear zone/halo and colony. The solubility index was calculated for each field CDB isolate using the formula Solubility index $=\frac{clear\ zone+Colony\ Size}{Colony\ Size}$ (Tamilselvi et al. 2016).

Determination of communities

Two CDB communities (unique and core) were determined by comparing solubility indexes. Unique CDB were identified as present in one region. CDB identified as core if their genera were identified in all 3 regions.

Phylogenetic mapping of CDB

16S rRNA sequences were aligned using the MAFFT multiple sequence alignment program (Katoh 2013). The phylogenetic relationship amongst the 16S rRNA sequences were screened for the appropriate evolutionary model using ModelFinder (Kalyaanamoorthy et al. 2017). The evolutionary model with the lowest BIC score was used to construct a Maximum Likelihood phylogenetic tree that was optimized after 167 iterations using IQ-TREE (Minh et al. 2020). The resultant phylogeny was visualized and annotated using iTOL (Letunic 2007).

PCA analysis for CDB and soil properties

We performed a principal components analysis (PCA) on our soil characteristics data matrix (Team 2023). Outliers were identified and removed by calculating the Minimum Covariance

Determinant estimator and utilizing this value for determining the Mahalanobis distance for the dataset (Team 2023). A chi-square test was conducted using a threshold of $P = 0.001$ for excluding outliers (Team 2023). Seven outliers were excluded from the PCAs. We then performed multiple principal components analyses on our soil characteristics data matrix (Team 2023).

CDB diversity analysis

We conducted multiple alpha diversity measurements based on the 16S rRNA sequencing data. A PERMANOVA was simultaneously conducted on the Bray-Curtis distance matrix to test if species composition differs by region (Oksanen et al.). The Simpson and Shannon index were both measured in addition to species richness (Oksanen et al.). A one-way ANOVA was conducted for the Simpson index, Shannon index, and species richness to test for any differences between regions in these alpha diversity measurements (Oksanen et al. ; Team 2010).

Results

Divergent soil characteristics in peanut producing fields.

To investigate the diversity and abundance of CDB population in fields, we sampled pegging zone soils from 15 peanut producing fields in southern Georgia (Figure 4.1). Each region has distinct soil characteristics. Region 1 has loamy sand soils; Region 2 has deep sand soils and Region 3 soil has a higher clay content. Soil tests confirmed the composition of each soil type (Table 4.1). We selected five fields in each region and six soil samples were collected on each field. In total, we collected 90 sample sites, and two replicates of each soil test were conducted. Soil tests of the 90 samples revealed a range of calcium level in each field from 62.65 to 785.15 ppm (supplementary Table 4.1). It is noteworthy that a high variation may exist among six samples collected from the same field. For example, in field 7, calcium level ranged from 62.65 to 203.9 ppm among the six samples, indicating heterogeneity of soil composition in each field (supplementary Table 4.1).

Divergent composition of CDB population in different fields

We selected three fields from each region to isolate CDB based on a wide range of soil calcium concentration, from 62.65 to 785.15 ppm. A total of 111 CDB colonies were isolated on CDB selection medium supplemented with 1% Calcite (Peper et al. 2022; Tamilselvi et al. 2016). Among them, 52 strains stably maintained calcite dissolving ability. The loss of calcite dissolving ability after culture may be due to different nutrition composition and microbial environment in soil and culture medium. The relative CDB abundance ranged from 29.26% in Field 1.3 (Region 1, field 3) to 6.85% in field 2.9 (Table 4.1).

Using the full length 16s rRNA sequences, we identified three genera of CDB that were present in all three regions, namely *Paenibacillus*, *Niallia* and *Rossellomorea*, which were named as "core" CDB (Figure 4.2). Among them, *Paenibacillus* is the dominant CDB, representing 30 out of the total 52 CDB isolates. On the other side, six genera of CDB are uniquely presented in only one region (*Pseudarthobacter, Shouchella, Mesobacillus, Staphylococcus, Priestia,* and *Neobacillus*), thus they were named as the "unique" CDB (Table 4.2).

Correlation between CDB abundance and soil features

The diversity of CDB phylogeny was not different by regions (Figures 3.3A-3.3C). Using Simpson's diversity index, we did not observe difference between regions based on genera dominance (Figure 4.3A). When considering species number and diversity, no difference between regions was observed using a Shannon diversity index and analyzing Genera Richness (Figures 3.3B-3.3C). Based on our analysis of the diversity, abundance, and presence of CDB in southern Georgia, we conclude that a core group of CDB widespread through all sampled regions and are not influenced by location.

We further investigated potential correlation between CDB abundance and a soil characteristic. Our field samples formed three distinct groups under this PCA which correlate to the three regions sampled providing support that our soil characteristics dataset is accurate (Figure 4.3D). Multiple PCAs were used to determine correlations between multiple factors. PCA comparing various soil nutrients and characteristics using all fields combined found no correlation between CDB abundance and soil characteristics (Figures 3.3D, 3.3E). PC1 explains 46.3% of the variation present and is largely determined by sand, Mg, clay, K and Mn; while PC2 explains 20.9% and is largely determined by P and Zn. At this resolution, we observed no pattern between CDB abundance, and the soil characteristics tested (Figure 4.3E). We further investigated the relationship between CDB abundance and soil characteristics in individual regions. Region 1's PC1 explains 52.9% of the variation present and is largely determined by Zn, K, Ca, Mg, Mn, P, and silt; while PC2 explains 27.9% and is largely determined by clay, sand, and pH (Figure 4.3F). Region 2's PC1 explains 33.1% of the variation present and is largely determined by Ca, pH, Mg, sand, silt, and K; while PC2 explains 23.2% and is largely determined by Mn, P, K, silt, and sand. (Figure 4.3F). Region 3's PC1 explains 47.6% of the variation present and is largely determined by sand, silt, Ca, clay, Mg, and Mn; while PC2 explains 21.4% and is largely determined by P, K, clay, and Zn (Figure 4.3F). Based on these results, CDB abundance exhibits no patterns with tested soil characteristics at a region-specific level. Taken together, the range of soil nutrients and texture observed in our survey have no impact on CDB abundance. We also investigated the correlation between calcium level and CDB abundance. Previously, we found that increasing calcium level in soil led to decrease in CDB abundance (Peper et al. 2022). However, no such correlation was observed when combining all soil data collected in this survey (Figure 4.3G). Our previous study demonstrated the inverse relationship between CDB abundance and calcium to occurred at soil calcium levels below 150 ppm (Peper et al. 2022). Only 4 sample sites from 2 regions in the current dataset were below 150 ppm of calcium, making it difficult to recapitulate the correlation. Combined this indicates a complex relationship between CDB abundance and soil calcium level in a natural condition (Figure 4.3G).

Calcite Dissolving Capacity of Core and Unique CDB

To test if core and unique CDB have different capacity to dissolve calcite, we measured the solubilization index for all 52 CDB strains (Figure 4.4, supplementary Table 4.2). The solubilization index, measured by the ratio of halo to colony, indicates a strain's capacity to dissolve calcite on plates (Figure 4.4A). We compared the solubilization index of core and unique CDBs at 4, 7, and 10 days after inoculating CDB plates. These days were chosen because halo associated with each CDB were evident on different days, and all 52 CDBs show halo after 10 day's culture. Among all 52 CDBs, a "unique" CDB belonging to *Staphylococcus* (F1.8.12) was the most robust CDB stain with a solubilization index of 2.29 (Figure 4.4B). We also noticed that strains in the same genera may show different solubilization index values, indicating they may represent different species (Figure 4.4B). Although we did not observe statistical difference in solubilization index between the core and unique CDBs as a group at 4, 7, and 10 days after inoculating CDB plates (Figure 4.4C), nine out of the top 10 CDBs with the highest solubilization index belong to the core CDB community, representing all three core CDB genera (Figure 4.4B, supplementary Table 4.2).

Discussion

Microbial communities in soil are critical for plant development, nutrition uptake and responses to biotic and abiotic stresses (Egamberdieva 2018; Qu et al. 2020; Saleem et al. 2019; Schlaeppi 2015). Most studies on soil microbiomes focus on the tripartite interactions among root, soil, and microbes. As a geocarpic plant, peanut plants (*Arachis hypogaea* L.) flower above ground and develop their fruits below ground. The geocarpic feature of peanut provides an interesting case to study the impact of microbiomes associated with geocarposphere, the area surrounding the peanut pod, on fruit development. Previous studies have indicated that fungi and bacterial populations may have distinct associations with peanut roots and pods (Ci et al. 2021; Kloepper 1991; Li et al. 2014). The association between nitrogen fixing bacteria and the peanut rhizosphere has been well characterized and is used as a model for Plant Growth Promoting Rhizobacteria (PGPRs) (Ashrafuzzaman et al. 2009; Bhattacharyya 2012; Gupta Govind et al. 2015; Kenawy et al. 2019; Riaz et al. 2021; Sukul et al. 2021). Characterizing microbes in the rhizosphere demonstrated that a core microbiome is necessary to prevent disease and maintain plant health (Fazeli-Nasab et al. 2022; Toju et al. 2018). Microbes including *Bacillus thuringiensis*, *Bacillus subtilis, Fluorescent Pseudomonas*, and *Trichoderma spp*. are associated with the geocarposphere providing protection from fungal pathogens (Anjaiah et al. 2006; Mickler et al. 1995). It is still unclear whether the microbiome associated with geocarposphere contributes to calcium availability or uptake during pod expansion. The capacity of CDBs to increase calcium solubilization makes them good candidates to be recruited by pods. Future research to explore the biodistribution of CDB in low and high calcium condition may shed light on their interaction with peanut plants.

Several mechanisms have been reported for microbe-mediated solubilization of calcite. Cyanobacterial isolate, strain BC008 can excavate carbonate minerals. It promotes spontaneous dissolution of calcite by up taking Ca^{2+} at the excavation front. Intracellular Ca^{2+} was transported and excreted at the distal borehole opening into the external medium. P-type Ca^{2+}-ATPases are key transporters required for the Ca^{2+} movement from excavation front to borehole opening (Garcia-Pichel 2010). Other studies indicated that bacteria solubilizing calcite can secrete citric acid, oxalic acid and sanazine pigment, contributing to a low pH environment (Rana et al. 2015). Siderophore and extracellular polysaccharides may also enhance the calcite solubilization (Rana et al. 2015). It would be interesting to explore what mechanisms are employed by CDB isolated from peanut fields to dissolve calcite.

Phosphate solubilizing microorganisms (PSMs) is a group of microbes that dissolves insoluble phosphorus compounds in the soil, making this essential nutrient more accessible to plants (Zaidi 2009). PSMs are usually isolated on media containing recalcitrant P sources, such as $Ca_3(PO_4)_2$, $FePO_4$, $CaHPO_4$ or $AlPO_4$. PSMs form halos on selective plate as CDB on calcite-supplemented media (Taurian 2010; Zaidi 2009). PSMs are from a high diversity of phylogeny, mainly from three phyla: Firmicutes, Actinobacteria and Proteobacteria (Koczorski 2022). Microbe-derived enzymes, such as acid phosphatase (encoded by olpA), alkaline phosphatase (phoD), phytase (appA), phosphonatase (phnX), and C-P lyase (phnJ), are able to release free orthophosphate from recalcitrant organic P forms (Liang 2020). In addition, a variety of organic acids (citric acid, gluconic acid, formic acid, malic acid, and oxalic acid) contribute to the microbial-induced solubilization of recalcitrant inorganic P (Liang 2020; Zaidi 2009). Due to overlapping capacity between CDB and PSM in producing acids, CDB may also dissolve recalcitrant P sources. However, we did not observe change of P level in CDB treated soil samples, despite an increase in soluble calcium (Peper et al. 2022). It could be due to low amount of recalcitrant P sources in the tested soil samples or unknown complex mechanisms beyond acid-mediated solubilization.

Peanut production has one of the highest uses of pesticides and fungicides compared to other commodity crops (Report 2021). The development of biofertilizer has become a critical endeavor to reduce the use of chemicals in peanut production. It has been well documented that application of single isolate bacterial bio-controls is less stable than those in a community. The development of a “core” group of CDB may set a foundation toward a community functioning in field. Identifying a diversity of CDB is critical in creating an effective and stable biocontrol that can be applied to native soils.

Conclusion

In summary, we surveyed CDB in three major peanut producing regions of southern Georgia and isolated a diversity of CDBs. From this survey, *Paenibacillus* was identified as the most dominant genus presented in all fields. A single *Staphylococcus* strain showed the highest rate of solubilization. Furthermore, we revealed that “core” and “unique” CDB populations showed comparable capacity to dissolve calcite on plates despite their distinct geographic distribution and phylogenetic diversity.

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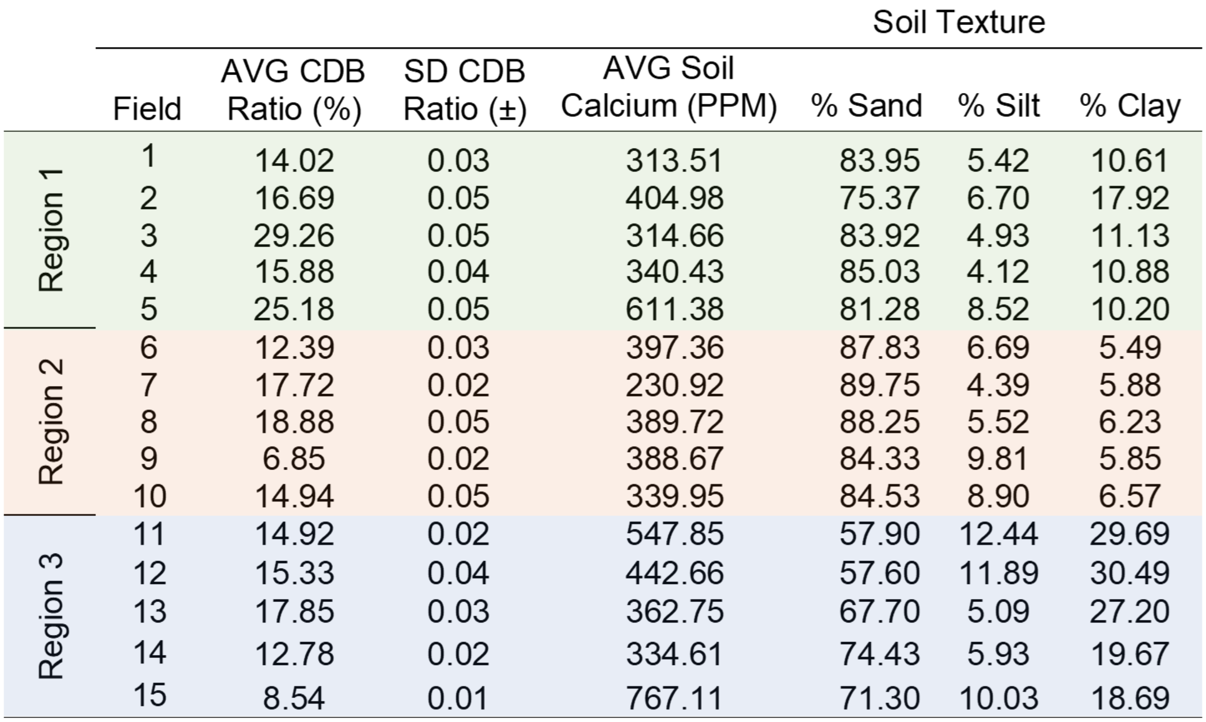
Tables

Table 4.1: CDB abundance and soil composition from survey fields.



					Soil Texture		
	Field	AVG CDB Ratio (%)	SD CDB Ratio (±)	AVG Soil Calcium (PPM)	% Sand	% Silt	% Clay
Region 1	1	14.02	0.03	313.51	83.95	5.42	10.61
	2	16.69	0.05	404.98	75.37	6.70	17.92
	3	29.26	0.05	314.66	83.92	4.93	11.13
	4	15.88	0.04	340.43	85.03	4.12	10.88
	5	25.18	0.05	611.38	81.28	8.52	10.20
Region 2	6	12.39	0.03	397.36	87.83	6.69	5.49
	7	17.72	0.02	230.92	89.75	4.39	5.88
	8	18.88	0.05	389.72	88.25	5.52	6.23
	9	6.85	0.02	388.67	84.33	9.81	5.85
	10	14.94	0.05	339.95	84.53	8.90	6.57
Region 3	11	14.92	0.02	547.85	57.90	12.44	29.69
	12	15.33	0.04	442.66	57.60	11.89	30.49
	13	17.85	0.03	362.75	67.70	5.09	27.20
	14	12.78	0.02	334.61	74.43	5.93	19.67
	15	8.54	0.01	767.11	71.30	10.03	18.69

Table 4.2: Distribution and phylogeny of CDBs from survey fields.

	Region 1: Tift			**Region 2: Mitchel**			**Region 3: Sumter**				
Genus	Field 1	Field 2	Field 3	Field 7	Field 8	Field 9	Field 12	Field 13	Field 14	Present in # of fields	Total Abundance
Paenibacillus	2	3	2	8	2	4	3	1	5	9	30
Niallia		1	4		1			1		4	7
Rossellomorea		1	3			1			2	4	6
Neobacillus							1	1		2	2
Priestia							1	1		2	2
Shouchella								1		1	1
Pseudarthobacter					1					1	1
Mesobacillus								1		1	1
Staphylococcus					1					1	1
Total Identified	2	5	9	8	5	5	5	6	7		52

Supplementary Table 4.1Field survey soil nutrient analysis replicates 1 and 2

Replicate 1

		ppm CaCO3/pH			Mehlich 1 mg/kg (ppm)					
Field ID	Site	LBC 1	LBCeq	pH 2	Ca	K	Mg	Mn	P	Zn
Field 1	1	188	502	5.95	398.1	85.3	45.52	7.84	34.24	1.63
Field 1	2	164	414	6.03	376.8	54.5	22	5.03	25.49	0.55
Field 1	3	134	304	4.97	130.8	40.3	13.44	6.49	48.01	0.84
Field 1	4	151	366	5.81	334.2	32.1	15.33	5.23	55.85	1.06
Field 1	5	147	351	6.14	437	34.2	19.89	6.35	56.87	1.39
Field 1	6	152	370	5.78	256.3	35.1	18.46	7.09	43.83	1.27
Field 2	1	177	461	5.8	294.1	68.2	43.09	4.13	6.24	1.67
Field 2	2	237	682	5.61	327.9	73.9	36.68	4.18	6.82	1.15
Field 2	3	219	616	6.7	516.4	78	55.93	4.44	6.96	0.88
Field 2	4	230	656	6.45	438.7	47.5	56.89	3.97	3.08	0.84
Field 2	5	241	696	6.79	508	47.9	80.23	3.37	2.95	0.64
Field 2	6	198	539	6.57	464	65.5	55.52	3.59	7.24	0.96
Field 3	1	138	318	5.75	267.5	55.2	31.95	13.06	31.01	0.97
Field 3	2	163	410	6.21	319.1	41.7	37.58	5.22	15.77	0.53
Field 3	3	147	351	7.05	381.7	45.8	42.64	4.94	30.14	0.62
Field 3	4	130	289	6.65	317.3	40.9	36.09	5.15	28.5	0.69
Field 3	5	156	384	6.13	337.6	51.4	38.3	5.18	17.71	0.7
Field 3	6	145	344	6.01	358.6	51.6	36.41	5	27.06	0.71
Field 4	1	128	282	5.9	421	22.2	18.92	8.86	10.18	1.24
Field 4	2	158	392	6.01	341.7	43.5	19.85	7.01	8.84	1.48
Field 4	3	128	282	6.07	280.9	36.1	19.97	5.03	8.93	1.04
Field 4	4	123	263	6.12	325.6	32.1	27.36	6.17	5.84	0.8
Field 4	5	137	315	5.69	357.8	43.4	18.78	6.04	4.95	0.89
Field 4	6	128	282	5.46	332.7	28.1	13.71	6.64	8.43	0.92
Field 5	1	232	663	6.67	781.9	88.3	90.68	16.25	90.87	3.29
Field 5	2	321	931	5.93	613.5	97.5	64.59	13.52	81.51	3.12
Field 5	3	287	832	5.61	509.9	91.9	56.17	14.08	72.99	2.87
Field 5	4	297	861	5.92	670.6	125.9	81.23	17.15	81.89	3.57
Field 5	5	275	798	6.26	805.6	110.2	92.19	18.34	93.8	4.23
Field 5	6	274	795	7.21	2805.3	72.3	176.01	15.68	102.21	3.36
Field 6	1	165	417	6.52	561	18	23.22	13.85	31.34	1.07
Field 6	2	169	432	6.78	478.6	16.1	36.32	11.86	39.74	1.17
Field 6	3	166	421	5.98	401.5	25.7	20.1	15.71	63.97	1.89
Field 6	4	194	524	5.84	364.2	22.9	16.74	14.14	54.8	2.3
Field 6	5	200	546	5.93	413.9	30.3	25.77	14.23	31.53	2.28
Field 6	6	140	326	5.83	260.3	14.8	8.7	11.3	23.36	0.98

Field 7	1	151	366	4.96	113.9	18.9	7.93	13.63	54.25	3.08
Field 7	2	138	318	4.77	82.8	12.9	5.83	14.11	59.21	2.12
Field 7	3	130	289	4.7	64.3	16.4	6.22	14.76	58.58	1.45
Field 7	4	199	542	5.17	214.4	31.4	17.21	24.77	98.27	3.24
Field 7	5	222	627	6.26	718.5	42.8	64.15	17.95	259.82	18.97
Field 7	6	167	425	5.24	183.1	18.7	11.1	12	61.13	2.7
Field 8	1	171	439	6.01	385.4	30.6	17.6	19.96	39.97	1.13
Field 8	2	162	406	6.2	387.5	28.3	17.05	20.19	35.9	1.14
Field 8	3	195	528	6.04	455	35.5	19.34	19.83	58.25	1.81
Field 8	4	165	417	6.11	319	31.8	20.2	16.09	32.71	0.87
Field 8	5	189	506	6.21	489.8	34.3	27.66	17.88	47.21	2.5
Field 8	6	135	307	5.94	257	22	14.55	11.78	47.2	1.26
Field 9	1	183	484	5.95	410.6	35	21	10.05	63.35	3.12
Field 9	2	127	278	6.35	339.3	18.1	14.2	8.23	50.41	1.8
Field 9	3	140	326	6.03	263.3	37.3	18.91	11.75	39.85	1.94
Field 9	4	174	450	6.27	436.1	43.4	24.29	17.64	73.75	3.8
Field 9	5	175	454	6.04	380	30.9	19.38	15.54	75.28	3.73
Field 9	6	172	443	6.33	487	31.7	26.22	16.93	70.41	3.45
Field 10	1	151	366	5.87	379.1	16.7	6.72	16.03	36.63	3.15
Field 10	2	154	377	5.89	376.2	13.7	7.19	12.18	45.07	3.41
Field 10	3	122	260	5.93	321.3	11.8	5.41	8.32	39.23	2.26
Field 10	4	165	417	5.69	275	18.5	8.27	11.77	34.03	1.91
Field 10	5	168	428	5.68	280	15.6	8.45	12.03	41.41	2.42
Field 10	6	174	450	6.28	417	18.5	7.54	10.94	21.8	1.36
Field 11	1	240	693	5.69	451.4	62.1	46.1	22.19	39.37	3.53
Field 11	2	301	873	5.87	502.6	39.5	50.96	23.89	17.09	2.4
Field 11	3	277	803	5.83	505.7	33.1	57.11	30.62	14.94	2.21
Field 11	4	349	1012	6.14	602.8	37.4	64.58	33.61	13.67	2.42
Field 11	5	299	867	6.03	586.8	52.6	64.53	26.46	12.78	2.39
Field 11	6	319	925	5.87	567.2	85	66.25	25.91	22.91	2.64
Field 12	1	257	745	6.32	453.7	82.4	121.92	16.66	39.91	3.43
Field 12	2	263	763	5.88	407.4	72.1	104	25.49	21.92	2.24
Field 12	3	306	887	6.03	443.1	107.9	118.72	38.43	17.58	2.25
Field 12	4	332	963	5.98	377.5	96.6	111.37	30.74	21.46	2.69
Field 12	5	261	757	6	369.5	107.1	109.12	28.56	22.89	2.75
Field 12	6	396	1148	6.13	546.3	185.8	142.87	53.2	26.16	2.92
Field 13	1	261	757	6.23	466.8	76.1	100.62	22.99	11.99	0.96
Field 13	2	261	757	5.77	368.4	87.6	75.54	22.94	7.92	0.92
Field 13	3	266	771	5.27	300.6	98.7	56.64	18.55	7.8	0.82
Field 13	4	238	685	5.55	342.7	67.2	57.88	17.22	12.73	1.24
Field 13	5	230	656	5.86	387.9	55	75.7	12.54	11.75	1.39

Field 13	6	262	760	5.59	329.6	60.6	63.09	12.66	9.99	1.16
Field 14	1	212	590	5.91	325	95.7	37.29	26.59	43.43	1.24
Field 14	2	210	583	5.8	306	86.3	41.45	23.16	36.06	1.55
Field 14	3	224	634	5.71	347.9	70.3	34.22	24.33	25.81	1.76
Field 14	4	238	685	5.76	348	73.9	39.29	28.49	20.67	1.73
Field 14	5	223	630	6.19	381.4	65.6	54.08	19.18	23.88	1.76
Field 14	6	239	689	5.53	266.7	52	32.04	19.75	26.08	1.81
Field 15	1	371	1076	6.43	953.5	106.7	263.62	17.47	35.87	3.99
Field 15	2	338	980	6.82	815.2	162.2	231.51	18.95	25.2	2.98
Field 15	3	294	853	6.57	603.7	163.2	146.15	32.8	47.89	4.49
Field 15	4	217	608	6.1	363.7	100.8	87.63	27.92	55.55	3.35
Field 15	5	280	812	6.68	685.7	137.8	172.17	22.9	46.22	3.16
Field 15	6	402	1166	6.58	841.6	123.2	197.79	20.47	44.01	5.2

Replicate 2

		ppm CaCO3/pH			Mehlich 1	mg/kg (ppm)				
Field ID	Site	LBC 1	LBCeq	pH 2	Ca	K	Mg	Mn	P	Zn
Field 1	1	192	517	5.96	353.9	71.76	48.13	5.83	27.7	1.4
Field 1	2	144	340	6.26	348.5	47.24	21.81	4.16	24.26	0.53
Field 1	3	135	307	4.94	125.6	33.59	13.43	4.52	44.86	0.84
Field 1	4	125	271	6.06	313.5	29.71	17.49	4.12	60.58	1.12
Field 1	5	155	381	6.45	446	28.77	23.31	4.89	53.76	1.29
Field 1	6	119	249	5.85	241.4	32.48	18.45	5.32	41.44	1.17
Field 2	1	180	473	5.75	256.7	58.61	43.05	3.04	5.17	1.49
Field 2	2	152	370	5.73	314.9	62.01	37.49	3.08	5.25	1.06
Field 2	3	211	586	6.77	499.7	65.47	57.99	4.13	7.21	0.91
Field 2	4	151	366	6.26	317.5	30.23	41.5	3	3.55	0.7
Field 2	5	246	715	6.77	504	44.89	81.63	3.07	2.6	0.64
Field 2	6	160	399	6.67	417.8	61.3	54.21	2.96	5.99	0.82
Field 3	1	143	337	5.67	243.2	53.37	31.15	4.64	24.45	0.74
Field 3	2	122	260	6.24	289	34.34	36.71	3.6	13.4	0.48
Field 3	3	153	373	7	355	41.86	44.49	4.33	32.96	0.72
Field 3	4	115	234	6.67	276.7	34.19	33.94	3.49	25.35	0.61
Field 3	5	152	370	6.1	306.2	40.08	36.2	3.49	18.14	0.74
Field 3	6	126	274	6.07	324	40.14	34.6	3.2	23.75	0.63
Field 4	1	131	293	5.91	402.6	19	26.63	7.5	9.37	1.24
Field 4	2	119	249	5.76	297	32.93	22.61	4.98	7.26	1
Field 4	3	134	304	6.17	292.5	30.13	21.16	3.92	8.44	0.89
Field 4	4	107	205	6.06	328.2	23.68	34.33	3.81	5.21	0.6
Field 4	5	142	333	5.81	409	33.5	24.64	5.56	4.31	0.74
Field 4	6	111	219	5.55	296.2	25.75	18.46	4.25	6.86	0.75
Field 5	1	241	696	6.75	750.3	74.83	88.82	12.1	79.38	3.01
Field 5	2	186	495	5.96	607.9	85.43	63.7	12.07	79.14	3.08

Field 5	3	278	806	5.6	516.8	81.62	58.05	10.41	73.79	2.76
Field 5	4	207	572	5.95	686.4	111.98	83.7	11.77	77.52	3.35
Field 5	5	286	829	6.18	764.7	99.65	86.3	13	85.11	3.89
Field 5	6	212	590	7.43	3317.8	61.17	165.56	13.2	109.23	3.47
Field 6	1	176	458	6.51	486	14.68	22.22	10.43	27.83	0.96
Field 6	2	142	333	6.92	490.3	13.95	42.91	9.72	37.32	1.64
Field 6	3	174	450	5.94	340.1	18.77	19.8	9.39	49.87	1.57
Field 6	4	144	340	5.9	348	18.23	17.53	9.63	49.34	2.1
Field 6	5	199	542	5.93	380.5	20.98	23.16	10.28	25.96	2.09
Field 6	6	122	260	5.94	243.9	9.93	8.01	8.23	20.16	0.89
Field 7	1	171	439	4.98	125.1	19.97	7.93	9.55	56.82	3.6
Field 7	2	116	238	4.84	74.7	11.7	5.59	8.66	54.27	2.18
Field 7	3	150	362	4.67	61	14.89	5.86	9.84	55.77	1.45
Field 7	4	138	318	5.23	193.4	26.55	17.06	13.6	72.06	2.88
Field 7	5	228	649	6.36	739.6	33.77	73.51	14.08	227.54	12.66
Field 7	6	140	326	5.3	200.2	17.7	12.17	8.96	66.87	3.16
Field 8	1	174	450	6.07	404.5	26.8	18.65	16.54	39.76	1.06
Field 8	2	136	311	6.31	421.7	26.63	18.21	17.63	37.66	1.16
Field 8	3	200	546	6.12	469.2	30.65	19.59	15.23	55.86	1.8
Field 8	4	130	289	6.23	326.4	27.8	20.96	12.28	30.64	0.82
Field 8	5	201	550	6.25	488.4	33.46	29.92	15.43	45.75	2.53
Field 8	6	123	263	6.06	272.7	23.23	16.01	9.73	49.08	1.36
Field 9	1	183	484	6.03	445.3	47.47	29.29	9.04	71.27	3.46
Field 9	2	111	219	6.48	357.4	16.71	17.51	7.03	47.49	1.96
Field 9	3	148	355	5.93	256.3	34.79	21.05	8.93	37.97	1.88
Field 9	4	132	296	6.25	433.4	44.31	24.67	13.52	69.14	3.63
Field 9	5	161	403	6.09	379.8	26.94	23.09	12.59	73.11	3.91
Field 9	6	149	359	6.38	475.5	33.31	28.49	13.59	71.27	3.6
Field 10	1	155	381	5.91	394.2	15.5	8.41	11.83	36.18	3.22
Field 10	2	121	256	5.98	351.8	11.27	7.6	9.55	38.08	3.12
Field 10	3	130	289	6	324.5	10.51	4.98	6.59	38.53	2.27
Field 10	4	119	249	5.87	296.8	14.99	7.85	8.74	36.13	1.93
Field 10	5	159	395	5.47	248.8	17.58	8.68	9.42	43.1	1.88
Field 10	6	132	296	6.31	414.7	18.09	9.82	8.83	19.95	1.34
Field 11	1	254	737	5.67	461.2	58.24	49.81	16.52	40.27	3.56
Field 11	2	203	557	5.98	479.8	33.5	54.58	17.61	15.34	2.21
Field 11	3	290	841	5.91	528	32.46	65.63	22.52	15.75	2.3
Field 11	4	217	608	6.28	646.8	35.26	70.07	24.36	14.13	2.49
Field 11	5	311	902	6.12	641.4	50.94	75.04	20.67	13.11	2.55
Field 11	6	225	638	6	600.5	76.57	73.83	20.93	21.01	2.8
Field 12	1	279	809	6.4	497.3	79.1	133.41	14.38	42.96	3.9
Field 12	2	190	509	6.02	411.6	66.29	106.92	18.48	23.65	2.59
Field 12	3	320	928	5.89	445.2	94.44	119.3	29.61	19.53	2.66
Field 12	4	201	550	6.04	371.9	91.81	107.97	19.82	19.95	2.56
Field 12	5	294	853	5.93	430.3	101.21	100.31	23.25	21.3	3.14

Field 12	6	255	740	6.17	558.1	122.61	146.71	32.93	21.59	2.71
Field 13	1	264	766	6.23	452.5	70.8	100.83	14.94	12.57	0.95
Field 13	2	187	498	5.86	360.6	84.8	76.66	15.27	7.15	0.88
Field 13	3	282	818	5.25	303.5	93.2	61.54	13.22	8.63	0.84
Field 13	4	186	495	5.64	312.3	60.26	58.11	10.45	10.39	1.1
Field 13	5	224	634	5.84	392.3	53.16	76.98	9.64	12.35	1.39
Field 13	6	173	447	5.69	335.8	57.26	65.54	8.84	11.17	1.1
Field 14	1	219	616	5.93	319.4	86.96	41.65	18.1	42.17	1.13
Field 14	2	167	425	5.9	295.6	80.6	45.19	18.08	40.81	1.67
Field 14	3	232	663	5.7	365.1	68.32	39.81	17.97	27.06	1.77
Field 14	4	196	531	5.97	370	73.08	47.66	20.66	21.19	1.75
Field 14	5	220	619	6.24	404.6	68.2	60.92	15.12	25.36	1.78
Field 14	6	160	399	5.69	285.6	53.85	39.28	13.46	27.53	1.77
Field 15	1	339	983	6.48	876.6	102.76	228.96	14.03	36.76	4.06
Field 15	2	253	734	7.05	929.8	151.39	250.22	16.03	31.14	3.46
Field 15	3	319	925	6.73	931.5	190.55	230.89	29.29	65.24	6.84
Field 15	4	159	395	5.94	246.3	99.17	62.94	17.04	38.79	2.2
Field 15	5	264	766	6.71	698.5	159.61	163.11	16.16	42.88	3.29
Field 15	6	258	748	6.91	1259.2	130.76	294.57	20.22	69.82	7.37

Supplementary Table 4.2 Characterization and identification of all 52 CDB isolates

Region	Field	Strain ID	Genus	Species	% ID	SI	SD SI
1	1	R1.1-14	Paenibacillus	sabinae	95.601	1.48	0.06
1	1	R1.1-17	Paenibacillus	sabinae	95.051	1.55	0.11
1	2	R1.2-1	Rossellomorea	marisflavi	100	1.91	0.21
1	2	R1.2-7	Niallia	circulans	99.698	2.08	0.15
1	2	R1.2-13	Paenibacillus	lautus	98.13	1.9	0.1
1	2	R1.2-14	Paenibacillus	mobilis	97.986	1.69	0.15
1	2	R1.2-15	Paenibacillus	lautus	98.516	1.65	0.05
1	3	R1.3-4	Paenibacillus	xylanilyticus	98.208	2.06	0.08
1	3	R1.3-5	Niallia	nealsonii	99.725	1.98	0.2
1	3	R1.3-7	Rossellomorea	marisflavi	99.99	1.95	0.16
1	3	R1.3-15	Niallia	nealsonii	99.725	1.81	0.03
1	3	R1.3-17	Niallia	nealsonii	99.269	1.72	0.05
1	3	R1.3-19	Niallia	nealsonii	99.368	2.11	0.18
1	3	R1.3-20	Rossellomorea	marisflavi	100	2.16	0.29
1	3	R1.3-27	Paenibacillus	illinoisensis	98.213	1.61	0.08
1	3	R1.3-33	Rossellomorea	marisflavi	98.806	1.81	0.06
2	7	R2.7-1	Paenibacillus	xylanexedens	98.891	1.83	0.15
2	7	R2.7-2	Paenibacillus	illinoisensis	97.92	1.39	0.18
2	7	R2.7-5	Paenibacillus	illinoisensis	99.267	2.21	0.26
2	7	R2.7-9	Paenibacillus	tuaregi	98.293	1.83	0.1
2	7	R2.7-10	Paenibacillus	lautus	99.664	1.5	0.11
2	7	R2.7-11	Paenibacillus	odorifer	99.086	1.69	0.17
2	7	R2.7-13	Paenibacillus	oralis	98.189	1.52	0.1
2	7	R2.7-15	Paenibacillus	tundrae	99.817	1.2	0.09

2	8	R2.8-7	Paenibacillus	mobilis	99.845	1.57	0.13
2	8	R2.8-12	Staphylococcus	hominis subsp.	97.828	2.29	0.14
2	8	R2.8-14	Pseudarthobacter	siccitolerans	98.224	1.44	0.07
2	8	R2.8-17	Paenibacillus	illinoisensis	98.265	1.76	0.07
2	8	R2.8-28	Niallia	nealsonii	98.665	1.69	0.11
2	9	R2.9-6	Paenibacillus	taichungensis	98.403	1.95	0.07
2	9	R2.9-8	Rossellomorea	marisflavi	100	1.56	0.09
2	9	R2.9-10	Paenibacillus	lautus	98.884	1.63	0.04
2	9	R2.9-11	Paenibacillus	lautus	99.181	1.64	0.03
2	9	R2.9-2	Paenibacillus	odorifer	96.908	1.41	0.03
3	12	R3.12-3	Neobacillus	cucumis	99.358	1.62	0.07
3	12	R3.12-4	Paenibacillus	xylanexedens	98.653	1.5	0.09
3	12	R3.12-5	Priestia	aryabhattai	99.627	1.56	0.1
3	12	R3.12-12	Paenibacillus	lautus	99.437	1.59	0.11
3	12	R3.12-13	Paenibacillus	illinoisensis	97.516	1.6	0.15
3	13	R3.13-1	Shouchella	gibsonii	99.722	1.41	0.05
3	13	R3.13-2	Niallia	nealsonii	99.633	1.64	0.15
3	13	R3.13-8	Neobacillus	cucumis	98.391	1.7	0.26
3	13	R3.13-9	Paenibacillus	lautus	98.873	1.83	0.06
3	13	R3.13-13	Mesobacillus	thioparans	99.624	1.88	0.06
3	13	R3.13-16	Priestia	megaterium	97.603	1.82	0.12
3	14	R3.14-5	Paenibacillus	mobilis	98.933	1.89	0.05
3	14	R3.14-6	Paenibacillus	mobilis	97.954	1.82	0.1
3	14	R3.14-11	Paenibacillus	illinoisensis	98.004	1.96	0.14
3	14	R3.14-13	Rossellomorea	marisflavi	99.698	2.02	0.12
3	14	R3.14-17	Paenibacillus	cellulositrophicus	96.151	2.09	0.13

3	14	R3.14-20	Paenibacillus	mobilis	98.881	1.67	0.07
3	14	R3.14-45	Rossellomorea	marisflavi	99.175	2.14	0.18

Figures

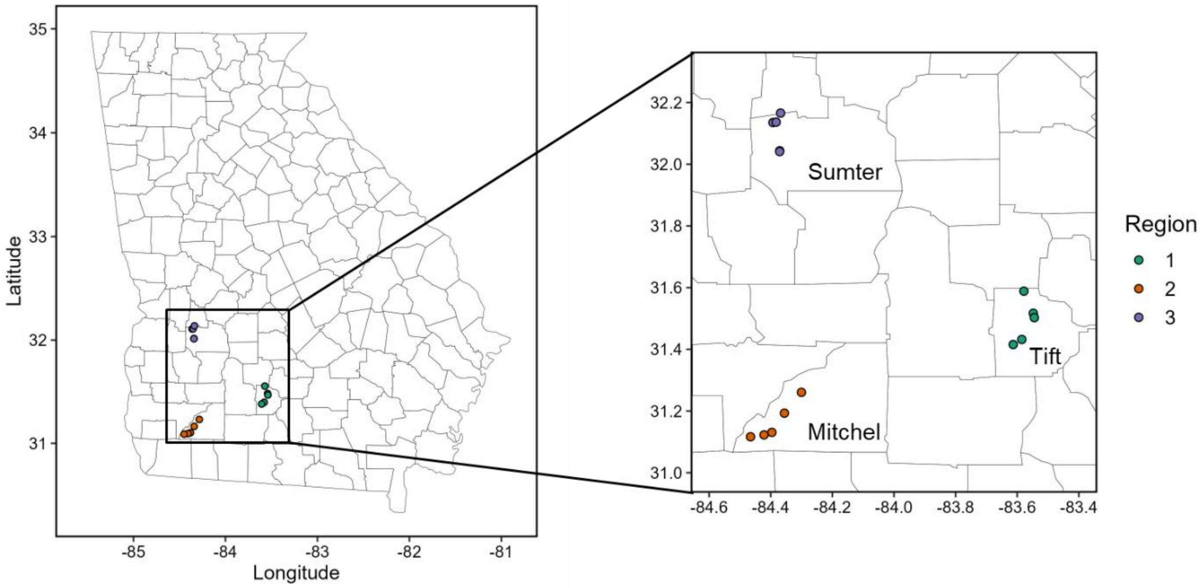


Figure 4.1: Field sites of CDB survey in southern Georgia. Map of CDB survey sites from Mitchel, Sumter, Tift counties in Georgia, U.S.

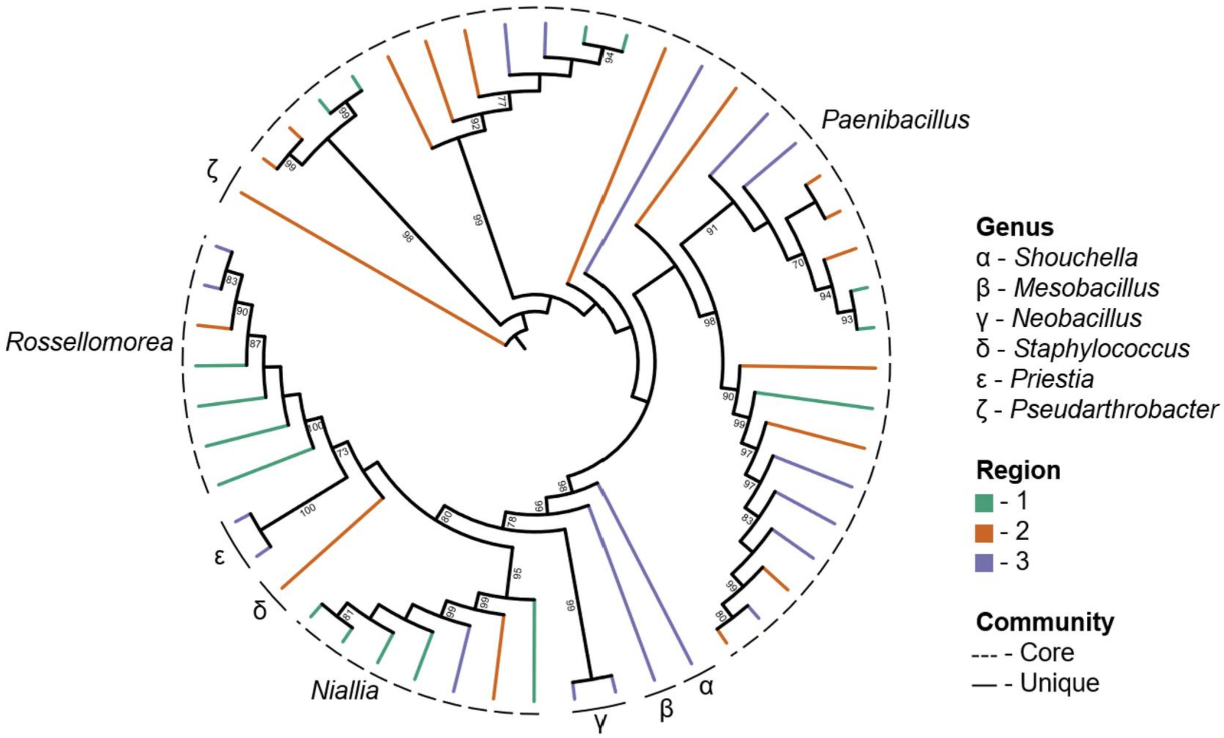


Figure 4.2: Phylogenetic tree of identified CDB strains. Phylogeny constructed with 16S rRNA sequences for 52 CDB strain. Branches are colored by the region from which each strain was isolated. Dashed lines indicate the respective strains are categorized as core and solid lines indicate the respective strains are categorized as unique.

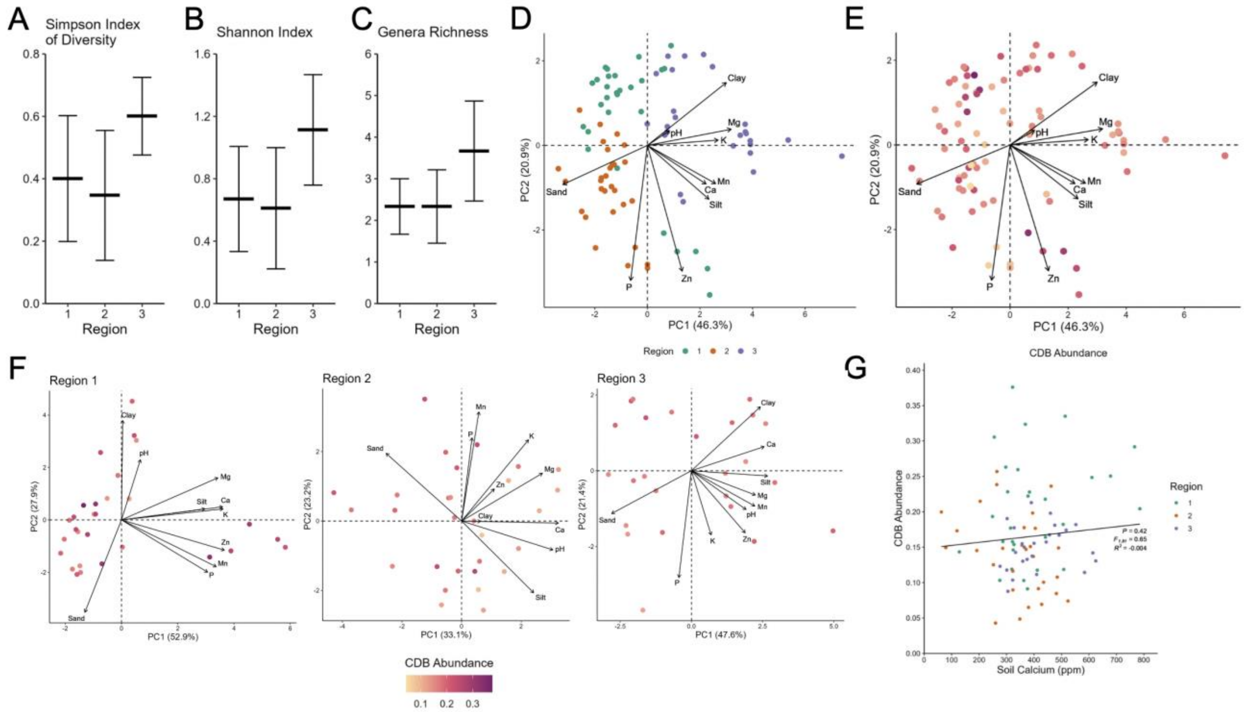


Figure 4.3: Principal components analysis (PCA) of soil characteristics and CDB relative abundance. (A) Simpson Index of Diversity calculated with 16S rRNA sequences at a region-specific level. (B) Shannon Index calculated with 16S rRNA sequences at a region-specific level. (C) Genera richness calculated with 16S rRNA sequences at a region-specific level. (D) PCA of soil characteristics data across all regions surveyed. Individual points are colored by region. (E) PCA of soil characteristics data across all regions surveyed. Individual points are colored by the abundance of CDB present in the soil. (F) PCA of soil characteristics data at region-specific levels. Individual points are colored by the abundance of CDB present in the soil. (G) Scatterplot of soil calcium levels and CDB abundance. (A-C) The bar represents the mean value of the respective alpha diversity metric for each region. The error bars represent the standard error. (D-F) Vectors in the PCA represent the strength each variable has on influencing variation for each principal component graphed.

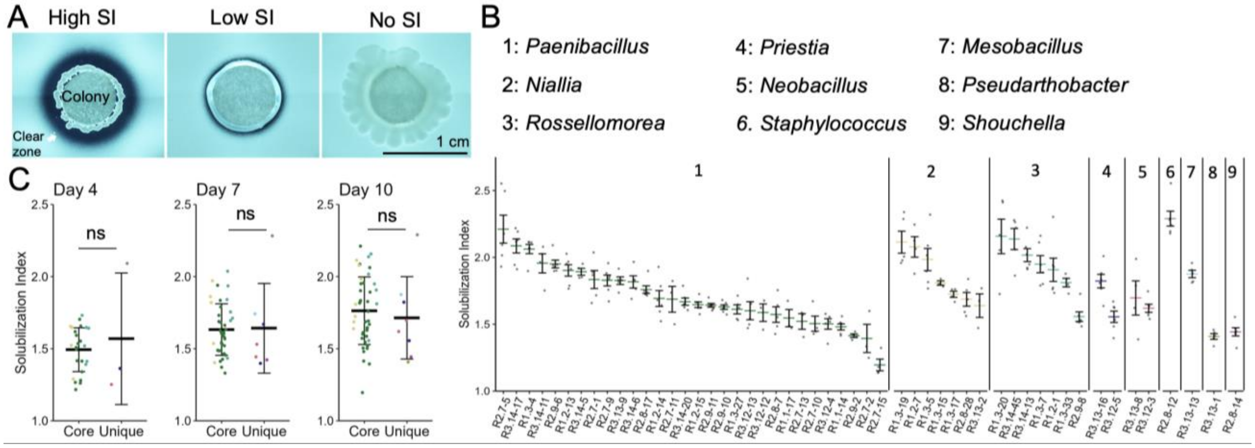


Figure 4.4: The Solubilization index of core and unique CDB strains. (A) Visual representation of CDB solubility range. (B) Solubilization Index for all 52 identified CDB, grouped by genus; measurements taken on day 10. (C) Comparison of the solubilization index, core and unique CDBs at 4, 7, and 10 days. ns: not significant based on unpaired student t-test.

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CHAPTER 5

CONCLUSIONS

Our research aimed to identify a novel method to reduce the use of mineral fertilizers and fungicides and provide a way to ameliorate calcium-deficient soils in peanut production. This project also furthers our understanding of the interaction among calcium nutrient cycling, peanut development, and soil. We have optimized the isolation, identification, and characterization of calcite-dissolving bacteria (CDB) through the refinement of the solubility index assay and soil extraction methods. We successfully identified CDB for the first time in peanut-producing fields. We also surveyed the geographic distribution and phylogenic diversity of CDB in the major peanut growing regions of Georgia. The mechanisms for CDB-mediated solubilization of calcite are currently unclear. A reduction in pH has been observed both on indicative media and in liquid culture, indicating that secretion of acids is a potential mechanism. After application of both individual and synthetic communities of CDB to field soil and sand, we found that CDB strains could increase the amount of available calcium. However, we also noticed that CDB only survived shortly in soil, indicating the current application method may not have long-lasting impact in field. The soil calcium level plays an important role in CDB survival and abundance. A negative correlation between innate soil calcium level was observed in the initial CDB study at Black Shank Farm, Tifton GA. Lastly the native soil microbiome can dramatically affect the viability and function of supplemented CDB community.

We conducted a survey of CDB in three major regions of peanut production in southern Georgia, with three distinct soil types. Through phylogenetic mapping, diversity analysis, and soil characterization, we determined that CDB were widespread throughout peanut producing regions. During the Survey of the peanut producing regions of Georgia, we did not observe the negative correlation between CDB abundance and innate soil calcium level. However, the average field calcium level was higher than the threshold (below 150 ppm) to observe a negative correlation between calcium level and CDB abundance. Only 4 sample sites out of the 120 collected samples were below the 150 ppm calcium threshold. The differences in CDB population and soil composition may also contributes to the discrepancy. The core CDB community that is present in all three regions consists of three genera *Paenibacillus, Niallia* and *Rossellomorea*. *Paenibacillus* is the dominant genus making up 30 strains of the 52 isolates collected. The unique CDB consisted of 4 genera which are uniquely present in only one field *Pseudarthobacter, Shouchella, Mesobacillus and Staphylococcus.* The identification of the CDB presents an opportunity to optimize a synthetic community, while also elucidating the interaction between different CDB and their impacts on the calcium nutrient cycle.

To assist in the development of synthetic communities and provide a new system to study peanut pod-specific responses, we developed the ITG system. The system supports the growth of individual peanut pods in a controlled soil environment separated from the peanut root. The system is suitable to characterize pod specific responses, while also reducing variance caused by using multiple plants compared to a single plant. The ITG system is a tool that various types of peanut researchers can utilize; primarily designed to determine optimal soil conditions for pod development. It also can be used for pathogenicity experiments, breeding and studying the efficacy of fungicides and fertilizers.

From this study we demonstrated that CDB are present in the Georgia peanut field and are most likely widespread through most agricultural systems. CDB can solubilize calcite on plates and in soil. The most abundant and widely distributed CDB genus found in the survey is *Paenibacillus*. The CDB phenotype is found in a wide variety of bacteria genera, however the molecular mechanism governing calcite dissolving function is still unknown. We have developed the ITG system to further peanut research and provided a versatile tool for studying various biotic and abiotic conditions. Overall, we characterized a functional group of bacteria that may assist in the reduction and use of fertilizers and fungicides in peanut production, while also providing a new route of study in optimization of Plant Growth-Promoting Rhizobacteria.