Preservation and Deployment of Biofertilizers to Mitigate Soil Phosphorous Loss from Agricultural Systems

By

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B.S. Mechanical Engineering, Texas A&M University, 2021

Submitted to the Department of Mechanical Engineering
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN MECHANICAL ENGINEERING

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2023

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ABSTRACT

The world is approaching peak phosphorus within the next 20 years, which poses a severe threat to the food security of a rapidly growing global population. Phosphorus is the second most essential macronutrient for plants after nitrogen, and its scarcity results in stunted growth, poor root development, and reduced crop agricultural crop yields. While phosphorus is naturally present in the soil, it is often not available in sufficient quantities or in a form that plants can synthesize. The use of phosphate-rich fertilizers has compensated for the low levels of phosphorus in agricultural systems, however, up to 95% of these fertilizers become "fixed" in the soil, causing environmental damage and reducing soil fertility. It is critical to find sustainable ways to manage our depleting phosphorus resources and minimize the environmental impact of phosphate fertilizers. To address these challenges, this research introduces a framework that leverages silk-based biopolymer encapsulation to preserve and deliver phosphate solubilizing microorganisms to the soil on naturally occurring phosphate rocks. By enabling the revival of phosphate solubilizing bacteria and initiating the solubilization of adjacent phosphate rocks, this approach not only improves the accessibility of untapped phosphate resources for plant roots but also facilitates the continual solubilization of various forms of insoluble phosphate, including legacy phosphorus from past fertilizer applications. This research showed phosphate solubilizing bacteria encapsulated in the biopolymer-coated phosphate rock remained viable after 30 days of storage and demonstrated effective solubilization of its host phosphate rock in solution. The addition of the biopolymer-coated phosphate rocks to chickpea seedlings showed a significant increase in the phosphorous content in chickpea leaves compared to the addition of uncoated rocks. Additional investigations can be undertaken to evaluate the potential of this framework as a controlled-release fertilizer by varying coating parameters such as material processing, biopolymer concentrations, and fertilizer amounts. The results of this thesis provide a foundation for further exploration and development of natural phosphate biofertilizers, bringing us one step closer to a more sustainable and resilient future in agriculture.

Thesis Supervisor: Benedetto Marelli

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ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to the individuals and organizations who have provided invaluable support and contributions throughout the research and writing of my master's thesis. First and foremost, I am deeply thankful to my thesis advisor, Benedetto Marelli, for your guidance and kindness. My sincere appreciation extends to my thesis reader, Kripa Varanasi, for all his support towards the realization of this work. I am grateful to UM6P for providing the funding that enabled me to pursue this study. I am grateful to my labmates, for their advice, support, and friendship. I express my gratitude to my past research advisors, Dr. Mansoor, Dr. Karaman, and Dr. Tafreshi, at Texas A&M, for their invaluable mentorship. My passion for STEM was ignited by my high school physics teacher, Mr. Shehouri, and it lives on throughout this work and many more to come. To Mom, Dad, Yazan, Maya, and Hourig, thank you for everything.

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Chapter 1. Introduction

1.1 Overview and Motivation

Phosphorous is an essential element for all forms of life, playing a critical role in many biological processes such as energy transfer, DNA synthesis, and cellular signaling. Phosphate forms the backbone of DNA and RNA molecules, which carry the genetic information that provides the instructions for the development, growth, and maintenance of all living organisms. Phosphate is also a critical component of ATP, the molecule that provides energy for cellular processes. In plants, ATP is essential for processes such as photosynthesis, respiration, and cell division, which are critical for plant growth and reproduction. It is also important for the development of root systems, the production of flowers and fruit, and the regulation of other nutrients in the plant (Kalayu, 2019). Without sufficient phosphorus, plants may exhibit stunted growth, poor root development, and reduced yields, especially agricultural plants.

Phosphorus is naturally present in the soil, but it is often not available to plants in sufficient quantities or in a form available for the plants to synthesize (Raymond et al., 2021). Plants obtain phosphorus by uptaking it in the form of soluble phosphate, most commonly PO3- or P2O4- from organic matter in the soil. Once the plants are harvested, the phosphorus is removed from the soil as well, decreasing the amount of plant-available phosphate. Due to the extensive amount of time it takes the phosphate to regenerate in the soil, most agricultural soils are phosphorus deficient (Wendimu et al., 2023).

To compensate for the low level of phosphorus in agricultural systems, farmers rely on the regular application of phosphate-rich fertilizers produced from phosphate rock (Cordell et al., 2009); however, around 75-95% of these fertilizers become “fixed” in the soil and have detrimental effects on soil fertility and the environment (Kalayu, 2019). Phosphate rock is a type of naturally occurring sedimentary rock and a non-renewable source of phosphorous. The countries that house the world’s largest phosphate reserves are Morocco, USA, and China (Wendimu et al., 2023). The depletion of the global commercial phosphate reserves is predicted to occur within the next twenty years, as we approach “peak phosphorous” (“Approaching Peak Phosphorus,” 2022; Cordell et al., 2009). Therefore, it is crucial to find more sustainable ways to continue to benefit from this limited resource while minimizing the environmental damage caused by phosphate fertilizers.

1.2 The Phosphorous Cycle

The Phosphorus Cycle describes how phosphorus transforms and moves through soil, water, and organic material, summarized in Figure 1-1. Inorganic and organic fertilizers, as well as the decomposition of plant and animal matter, contribute to the addition of phosphorus to soil. Plant uptake is the primary means by which phosphorus is removed from soil, although it can also be lost through surface runoff, erosion, or subsurface leaching (Mwangi et al., 2020; Prasad & Chakraborty, 2019). Sorption and desorption reactions occur on the surfaces and edges of hydrous oxides, clay minerals, and carbonates. Sorption involves the covalent bonding of phosphorus with iron and aluminum in acidic soils, and with calcium carbonate in alkaline soils. The availability of phosphorus is greatly affected by precipitation and dissolution reactions, which are slow processes that can be influenced by changes in red-ox potential caused by waterlogging or draining soil. The microbial cycling of phosphorus from soluble inorganic forms to insoluble organic forms

is referred to as immobilization, while the reverse process is known as mineralization, catalyzed by the phosphatase enzyme (Kalayu, 2019).

Phosphorus occurs in soil as inorganic and organic P compounds. Most soils contain a relatively low amount of total P, and only a small fraction of the total P is available to plants. Most P compounds in soils have low water solubility. Once in the soil solution, soluble P moves mainly by diffusion. Phosphorus in soils generally occurs as the anions H2PO4-or HPO42-. Phosphorus reacts with calcium (Ca2+), magnesium (Mg2+), iron (Fe3+), and aluminum (Al3+). Phosphorus reactions in soil are pH dependent. In acid soils, soluble phosphorus in the soil solution reacts with Fe and Al to form low solubility Fe and Al phosphates. In calcareous soils, soluble phosphorus in the soil solution reacts with Ca to form low solubility Ca phosphates (FAO, 2022; Rodríguez & Fraga, 1999).

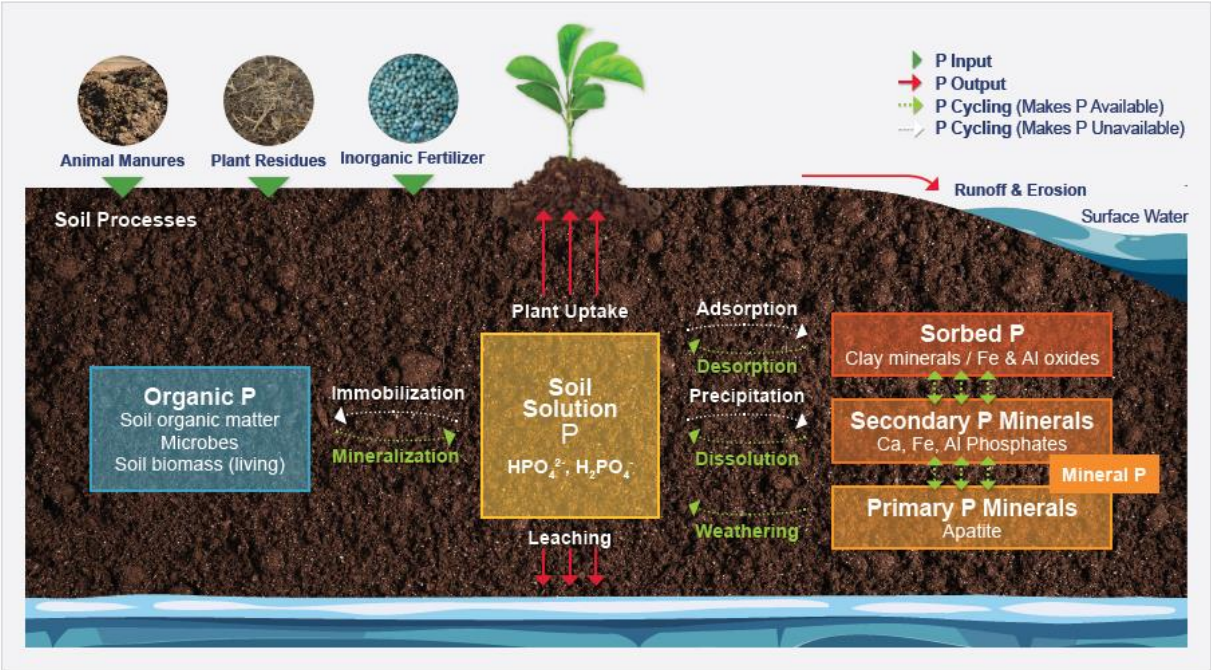

Figure 1-1 The Phosphorous Cycle in Soils (Prasad & Chakraborty, 2019)

1.3 Phosphate Fertilizers and Their Threats to the Phosphorous Cycle

The growth of human civilizations and the resulting need for agricultural production has put a tremendous strain on the natural phosphorous cycle. Repetitive cycles of planting and harvesting have depleted the soil of essential nutrients, such as nitrogen and phosphorus. The discovery of the Haber-Bosch reaction in 1914 allowed for the extraction of ammonia from the air, leading to the creation of nitrogen and phosphate fertilizers. While these fertilizers have enabled food production on a massive scale, they have also created a host of problems (Kyriakou et al., 2020; Vaccari, 2009).

1.3.1 Climate Change

Phosphate fertilizers are primarily made through a process called the "wet acid process." The wet acid process typically starts with the production of phosphoric acid, which is produced by reacting phosphate rock with sulfuric acid. The resulting mixture of phosphoric acid and gypsum is then filtered, and the phosphoric acid is further purified through a series of chemical reactions to remove impurities. The purified phosphoric acid is then reacted with ammonia to produce ammonium phosphate fertilizers, such as monoammonium phosphate (MAP) and diammonium phosphate (DAP). The wet acid process can also be used to produce other phosphate fertilizers, such as triple superphosphate (TSP) and single superphosphate (SSP), which are made by reacting phosphate rock with a mixture of sulfuric acid and water. TSP and SSP contain lower levels of phosphorus than MAP and DAP, but they are still widely used in agriculture (Vaccari, 2009).

The production of phosphate fertilizers through the wet acid process requires significant amounts of energy and generates large quantities of waste materials. The Haber-Bosch reaction used to extract ammonia from the air for fertilizer production is a significant source of greenhouse gas emissions. In fact, approximately 1.4% of global emissions are caused by this reaction alone (Kyriakou et al., 2020). In addition to the carbon emissions associated with the production of the sulfuric acid and ammonia used in the process, the production of phosphoric acid generates large amounts of gypsum waste (Vaccari, 2009).

1.3.2 Sustainability of Life on Water and Land

Phosphatic fertilizers have a significant impact on the environment, particularly through eutrophication. Eutrophication is the pollution of surface water caused by the leaching of agricultural fertilizers, farmyard manures, and the discharge of treated and untreated municipal sewage containing phosphates and nitrogen into water bodies. Leached phosphorus from fertilizers contributes to increased growth of aquatic plants and harmful algae blooms in water. The consequences of this include decreased water clarity, the production of potent algal toxins, the death of desirable fish species, and clogging of water treatment plant filters (Wendimu et al., 2023). Mitigating algal blooms and eutrophication relies heavily on reducing phosphorus inputs, which requires finding ways to decrease the excessive use of chemical phosphatic fertilizers without compromising crop yield.

On land, the depletion of phosphorus has led to the abandonment of agricultural land once it becomes unproductive. As a result, deforestation has become a widespread problem as land is cleared to make way for new farmland. By finding solutions to the problem of phosphorus depletion, we can reduce the amount of land that is abandoned and deforested, leaving more habitats open for animals and helping to maintain biodiversity. Moreover, phosphatic fertilizers may contain environmentally hazardous metalloids, including Cd, Pb, Hg, U, Cr, and As, which can accumulate in soils over time and render them toxic and uncultivable. These heavy metals can adversely affect plant growth, yield, physiological functions, and the distribution of beneficial microorganisms in the soil (Wendimu et al., 2023). By reducing our reliance on chemical phosphatic fertilizers, we can mitigate these problems and help sustain life in water and on land for the generations to come.

1.3.3 Peak Phosphorous

It is estimated that within the next 20 years, the world will reach peak phosphorus ("Approaching Peak Phosphorus," 2022; Cordell et al., 2009; Gupta et al., 2014). Figure 1-2 shows the dramatic increase in the use of phosphate rock for fertilizer production in the past few decades, which is accelerating the depletion of the world's phosphate reserves. It is predicted that within the next 20 years, the world will reach peak phosphorous, a term used to describe the point in time where phosphorous production will hit its maximum, then begin to decline, similar to the concept of peak oil (Figure 1-2). This impending crisis is all the more alarming given the crucial role that phosphorus plays in food production.

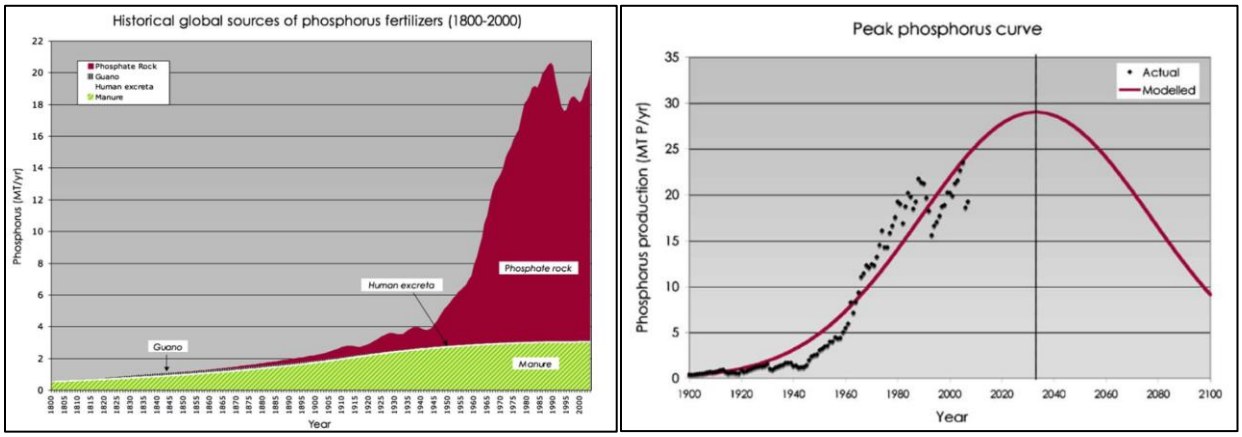

Figure 1-2 Left: Historical global sources of phosphorous fertilizers show that the use of phosphate rock has increased dramatically in the past few decades. Right: The world is approaching "peak phosphorous" by 2033(Cordell et al., 2009)

1.4 Sustainable Alternatives to Chemical Phosphate Fertilizer are Crucial

The problem of phosphorus depletion is a complex and urgent issue that requires immediate attention (Grantham, 2012). While the use of nitrogen and phosphate fertilizers has enabled food production on a massive scale, it has also created a host of problems that threaten our ability to sustainably feed the growing population. Finding new technologies to course-correct the damage done to the environment and protect our reserves of phosphorus is crucial if we are to sustainably address food security, life in water and on land, and climate change (Egan, 2023; Grantham, 2012; Wendimu et al., 2023).

The research focus needs to shift towards improving crop phosphorus use efficiency and enhancing the uptake of accumulated insoluble phosphorus by plants (Wendimu et al., 2023). This approach can reduce the amount of phosphatic fertilizers needed, benefit farmers in developing countries, minimize phosphate leaching into water bodies, and contribute to the conservation of phosphate rock reserves while preventing environmental catastrophes and sustainably feeding a growing population.

Chapter 2. Biofertilizers and Biopolymer Coatings in Agriculture

2.1 Phosphate Solubilizing Bacteria (PSB)

It has been shown that some plant growth promoting bacteria possess the ability to solubilize phosphate from a source of phosphorus (Sivasakthi et al., 2014) . Some of the most prominent bacteria species that can effectively solubilize phosphate are Bacillus, Pseudomonas, and Rhizobium(Sivasakthi et al., 2014) (Kalayu, 2019). These phosphate solubilizing bacteria (PSB) increase the amount of phosphorus in the soil that is readily available to plants, without altering the biochemical composition of the soil, thereby increasing the soil's fertility (Saeid et al., 2018).

The phosphate solubilization mechanism used by PSB is by lowering local soil pH, cation chelation, and ion exchange. PSB lower the local pH of the soil through production of organic acids and secondary metabolites or through the release of protons (Rodríguez & Fraga, 1999). The low soil pH allows naturally insoluble forms of phosphates, such as calcium phosphate, to become soluble. The organic acids produced also compete with phosphate in the soil for aluminum, calcium, and iron chelation sites, react with them, and fix them, preventing them from creating insoluble phosphate compounds (Kalayu, 2019).

Phosphorus use efficiency in agricultural lands can be improved through the inoculation of phosphate-solubilizing microorganisms (PSM). Inoculation with PSMs has been shown to enhance phosphorus uptake, as well as the uptake of other essential nutrients like potassium and nitrogen in plants. As PSMs do not infect the roots of plants, they can be used for various crops and are not host-specific. The use of PSMs has been shown to enhance growth, yield, and quality in a wide range of crops, including walnut, apple, maize, rice, mustard, oil palm, aubergine, chili, soybean, wheat, sugar beet, sugarcane, chickpea, peanut, legumes, and potatoes (Kalayu, 2019).

The use of PSMs as biofertilizers represents a potential substitute for inorganic phosphate fertilizers, promoting sustainable agriculture and offering ecological and economic benefits (Kalayu, 2019; Wendimu et al., 2023). Further research is needed to explore effective biofertilizers with multiple growth-stimulating attributes and to investigate the combination of rock phosphate as a phosphate source with PSM inoculum to minimize the risk of long-term phosphorus soil deficit (Kalayu, 2019).

It is important to note that upon the introduction of non-native bacterial strains into the soil, we run into the problem of competition, where the survival and establishment of the introduced strains may be limited by their ability to outcompete indigenous soil bacteria (Raymond et al., 2021; Rodríguez & Fraga, 1999). Therefore, the investigation of the stability and performance of PSB after they are introduced to the soil is crucial to assess their survivability and potential as biofertilizers. Directing research into the application methods of PSBs is needed not only to replace chemical phosphatic fertilizers but also to harness their multiple associated benefits that contribute to sustainable agriculture and overall environmental health (Raymond et al., 2021; Wendimu et al., 2023).

2.2 Biofertilizer Encapsulation in Silk Fibroin Biopolymers

Silk fibroin, derived from Bombyx mori silkworm cocoons, is a natural biopolymer that possesses the ability to form secondary structures such as gels, films, microneedles, scaffolds, and membranes that are flexible, conformable, transparent, and structural. The biomaterial has been used as medical sutures for many decades as it is non-immunogenic, non-toxic, and biocompatible (Sun et al., 2022). Silk fibroin has also been used by companies to coat meats and produce with a thin layer of the transparent biopolymer to keep food fresher for longer (Marelli et al., 2016). The silk film serves as a barrier for water and oxygen, the basic material properties required for the preservation of biomaterials. In addition, silk materials have successfully encapsulated microbes and antibiotics for their preservation (Li et al., 2015; Zvinavashe et al., 2019).

Trehalose is a versatile disaccharide that can serve as a stress protectant in organisms. According to the vitrification hypothesis, trehalose forms a glass-like matrix inside cells, preventing the denaturation, aggregation, and fusion of proteins. On the other hand, the water replacement hypothesis suggests that as cells become dry, trehalose replaces the hydrogen bonds between water and cellular components, thus preventing protein denaturation, aggregation, and membrane fusion (Zvinavashe et al., 2019).

A published study developed a silk-and-trehalose based seed coating that encapsulated a plant growth promoting bacteria, *Rhizobium Tropici*, on the surface of a seed by dip coating or spraying (Zvinavashe et al., 2019). The study investigated the synergistic preservation properties of silk fibroin and trehalose in a biomaterial formulation and found that at a 1:3 ratio of silk to trehalose, the coating was able to preserve and deliver *R. Tropici* to a bean plant, even after 4 weeks of preservation.

2.3 Polymer-Coated Fertilizers for Controlled Release of Nutrients

Release of nutrients refers to the process of transforming a chemical substance into a form that plants can readily absorb. Slow-release fertilizers gradually release nutrients at a slower rate compared to fertilizers that are quickly available to plants. To be classified as a slow-release fertilizer, it must meet specific criteria that is defined differently depending on the reference, for example, limited nutrient release within 24 hours and a substantial release at the designated release time. Controlled-release fertilizers offer a higher level of precision in fertilization by releasing nutrients at a predetermined rate over an extended period. Slow-release fertilizers (SRFs) and controlled-release fertilizers (CRFs) can be grouped into different categories, such as organic substances, inorganic compounds with low solubility, and water-soluble fertilizers that have physical barriers. Additionally, advancements have been made in developing biopolymer organic coatings, including coatings with starch-urea, chitosan, and alginate, for controlled-release fertilizers. These coatings allow for more controlled and targeted nutrient release (Fertahi et al., 2021; Ribeiro et al., 2020)

Coating techniques play a crucial role in the development of granular fertilizers. Several methods are commonly used, including immersion and spraying. Immersion involves dipping the fertilizer granules into a polymer coating solution, which adheres to the surface and is then dried. The fluidized bed method is suitable for coating solid granules by suspending them in a fluidized state and spraying solutions onto them. The coating pan technique is the simplest and most widely used as it employs a rotating drum where the coating solution is sprayed onto the granules, followed by drying (Fertahi et al., 2021).

Chapter 3. Proposed Solution: Biofertilizer-Phosphate Rock-Biopolymer Coating

3.1 Problem Statement

The world is approaching peak phosphorus within the next 20 years, which poses a severe threat to the food security of a rapidly growing global population. Phosphorus is the second most essential macronutrient for plants after nitrogen, and its scarcity results in stunted growth, poor root development, and reduced crop yields. While phosphorus is naturally present in the soil, it is often not available in sufficient quantities or in a form that plants can synthesize. The use of phosphate-rich fertilizers has compensated for the low levels of phosphorus in agricultural systems. However, up to 95% of these fertilizers become "fixed" in the soil, causing environmental damage and reducing soil fertility. To address this impending crisis, it is critical to find sustainable ways to manage phosphorus resources and minimize the environmental impact of phosphate fertilizers.

3.2 Research Question

How can the viability and functionality of phosphate solubilizing bacteria be preserved and demonstrated when introduced to soils and how effective are these preserved biofertilizers in increasing phosphate availability in plants while reducing soil phosphorous loss in plant-soil systems?

3.3 Thesis Statement

Preserving biofertilizers on phosphate rocks with a biopolymer coating offers a sustainable solution to increase phosphorus availability to crops in agricultural systems and minimizes soil phosphorus loss due to fixation and leaching.

Proposed Solution

This research proposes a novel approach that leverages the preservation properties of silk fibroin and trehalose to effectively protect phosphate solubilizing bacteria when coated on natural phosphate rock. By using biofertilizer-coated rocks as an alternative to conventional phosphate fertilizer granules, the aim is to enhance phosphorus availability for crop plants while minimizing negative environmental impacts. Once introduced into the soil, the biofertilizer-coated rocks facilitate the revival of phosphate solubilizing bacteria, which utilize the silk fibroin as a carbon source and initiate the solubilization of the adjacent phosphate rocks. This process not only enhances the accessibility of previously untapped phosphate resources for plant roots but also enables the continual solubilization of other forms of insoluble phosphate, including legacy phosphorus derived from previous fertilizer applications.

The research will focus on addressing three primary challenges: **a)** maintaining the viability of the phosphate solubilizing bacteria within the biopolymer-coated rocks, **b)** ensuring the bacteria's ability to solubilize phosphate after long-term storage on the rocks, and **c)** demonstrating the bacteria's efficacy in carrying out its function in a plant-soil system (Bashan et al., 2014; Raymond et al., 2021).

Chapter 4 of this thesis will present the main body, consisting of three sections with experimental approaches designed to address these challenges. Figure 3.1 provides a schematic representation of the organizational structure of this research. The findings and outcomes of this research will contribute to the development of sustainable agricultural practices that reduce reliance on chemical fertilizers, enhance phosphorus utilization efficiency, and mitigate the environmental impact associated with phosphorus loss from soils.

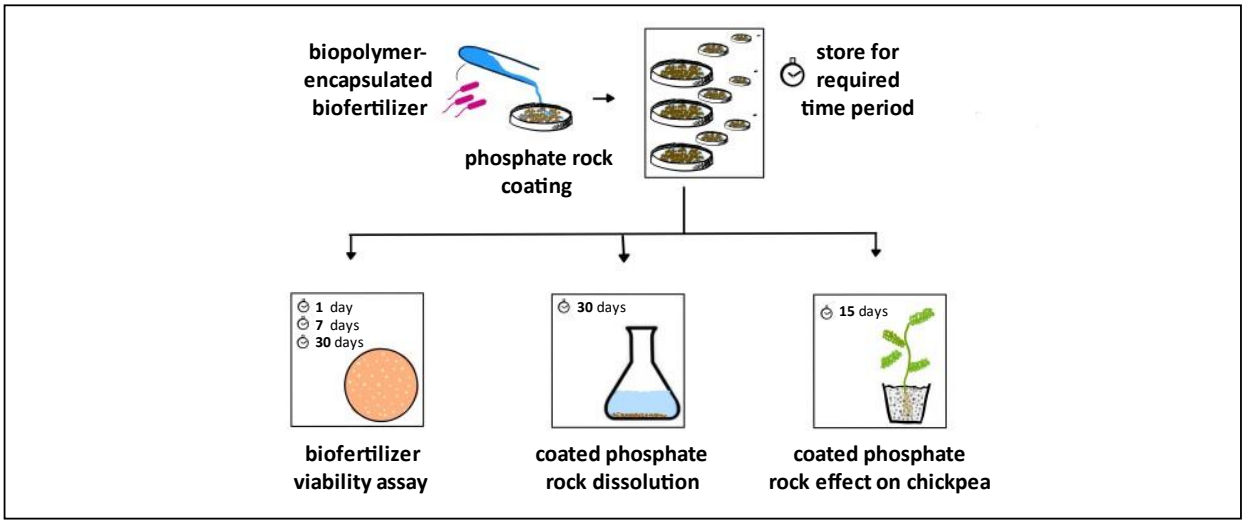

Figure 3-1 Overview of experiments in Chapter 4

Chapter 4. Development and Assessment of Biofertilizer-Biopolymer Coated Phosphate Rock as a Phosphate Fertilizer

4.1 Development and Preparation of Biofertilizer-Biopolymer Coated Phosphate Rocks

Pseudomonas Fluorescens

Pseudomonas Fluorescens is a well-established bacterial strain of the *Pseudomonas* genus that is classified as a PSB (Nautiyal, 1999; Sivasakthi et al., 2014). Throughout this work, *P. Fluorescens* is used as a PSB model as it is a ubiquitous strain and can be naturally found in the soil as well as on decaying plant matter, on water surfaces, and most recognizably in yogurt. They are rod-shaped, approximately 0.5-1 micrometer in length, and have numerous flagella attached to their cell wall, which looks like a tail, which gives them their motility (Scales et al., 2014). *P. Fluorescens* in the soil can colonize near the roots of plants and destroy pollutants found there (Sivasakthi et al., 2014).

The strain gets its name from its ability to secrete pyoverdine, a fluorescent pigment, which allows them to become colored under certain light conditions. They are obligate aerobes, which means that they must be in the presence of oxygen to survive, otherwise they would die from oxidative stress (Scales et al., 2014). Therefore, adequate aeration is provided to *P. Fluorescens* cultures throughout the experiments. *P. Fluorescens* is gram-negative, which makes it more resistant to antibiotics as they have a thin peptidoglycan layer outside their cell membrane (Scales et al., 2014). The antibiotic, Ampicillin, is used throughout the studies as P. Fluorescens possesses a natural antibiotic resistance to it.

Pseudomonas Fluorescens Culture Preparation

In the following sets of experiments, *P. Fluorescens* cultures were prepared in the same manner. *Pseudomonas Fluorescens* ATCC 13525 lyophilized pellets purchased from Microbiologics were rehydrated and streaked on Nutrient Agar (DIFCO 023400) plates. The plates were incubated at 26 °C for 48 hours, after which they are immediately used or stored at 4C for later use. One colony was picked from a plate and transferred to 20 ml of Nutrient Broth (DIFCO 023400) in a 250 ml flask and grown in a shaker incubator at 26 °C and 200 RPM for 24 hours. The culture was transferred into a 50 ml centrifuge tube and centrifuged at 4000 RPM for 15 minutes. The supernatant was discarded and replaced with 20 ml of 0.85% saline solution and homogenized. The bacterial solution was then diluted until its optical density at 600 nm (OD600) reached 1.0. Figure 4-1 shows a schematic of the P. Fluorescens culture preparation procedure described.

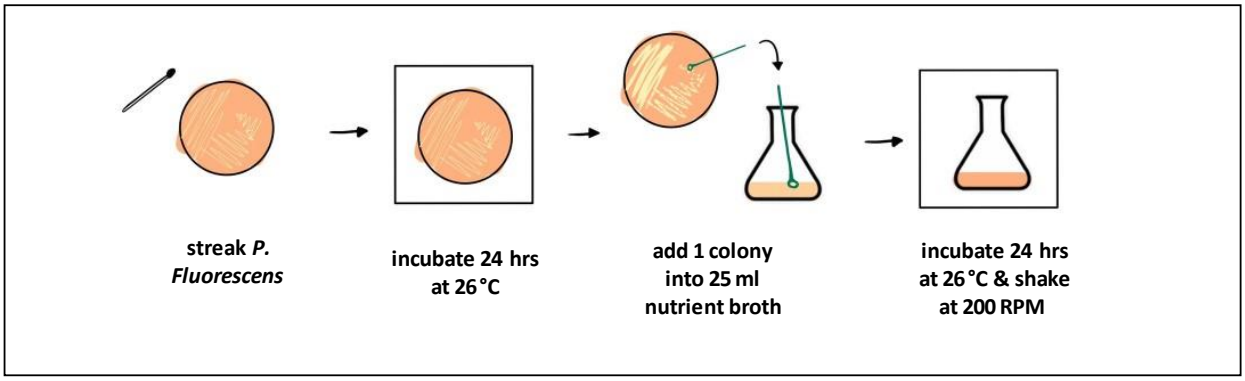

Figure 4-1 Pseudomonas Fluorescence Culture Preparation Procedure

Phosphate Rock Preparation

Natural 28% phosphate-available rock mined from Morocco was used throughout the experiments in this work. The rocks were sieved using a 120-mesh screen resulting in a rock size of [100 µm < x < 150 µm]. The rocks were then sterilized using dry heat sterilization at 170 °C in an oven for 1 hour. Figure 4-2 shows scanning electron microscopy (SEM) micrographs of the rocks after sieving and sterilization at increasing magnifications of 67x, 1.5kx, and 10kx, taken with a Zeiss-Merlin SEM. The images show the rough or porous surface texture of the rocks, as well as the range of variation in size.

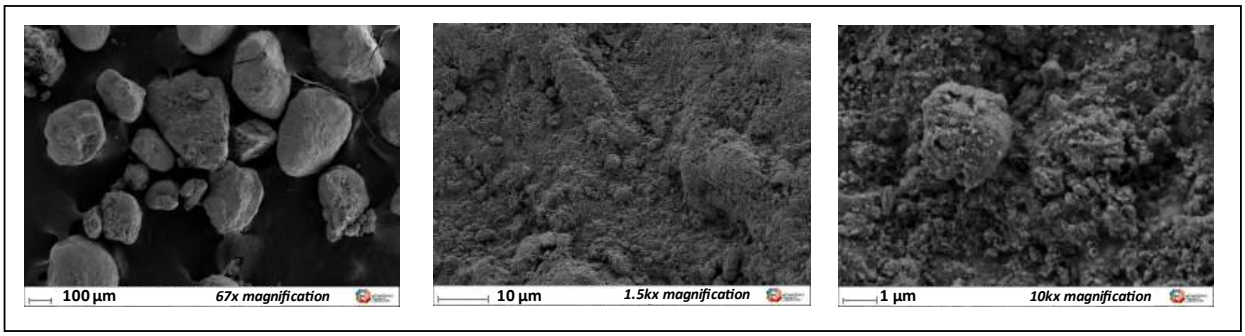

Figure 4-2 SEM micrographs of natural phosphate rock after sieving and sterilization. SEM settings: EHT=3.00kV, HE-SE2 detector

Phosphate Rock Coating Procedure

A 50 mg/ml stock solution of regenerated silk fibroin was prepared according to a defined protocol and sterilized by passing through a 0.2 µm filter (Rockwood et al., 2011). A 300 mg/ml stock solution of trehalose was also prepared and sterilized by filtration. To maintain the 1:3 ratio of silk to trehalose in the biopolymer coating defined in the literature, a silk and trehalose solution was prepared with working concentrations of 30 mg/ml and 120 mg/ml, respectively (Zvinavashe et al., 2019).

The previously prepared *P. Fluorescens* bacterial culture was transferred to several centrifuge tubes and centrifuged at 4000 RPM for 15 mins. The pellet was washed with 0.85% saline solution and centrifuged once more. The supernatant was discarded and replaced with 2 ml of the previously prepared biopolymer coating solution for each 1 ml of OD600 culture.

The solution was pipetted up and down in the centrifuge tube to disperse the bacteria until the solution was homogenized. 2 ml of bacteria-encapsulated coating solution was pipetted onto 1 g of sterilized phosphate rocks in a small sterile petri dish. The petri dish was placed on a shaker, and gently swirled at 100 RPM for 3 minutes to simulate the industrial process of a coating pan and ensure that the rocks are evenly coated. The coated rocks were left to dry inside a biosafety hood and stored until use. Figure 4-3 shows a schematic of the phosphate rock coating procedure described.

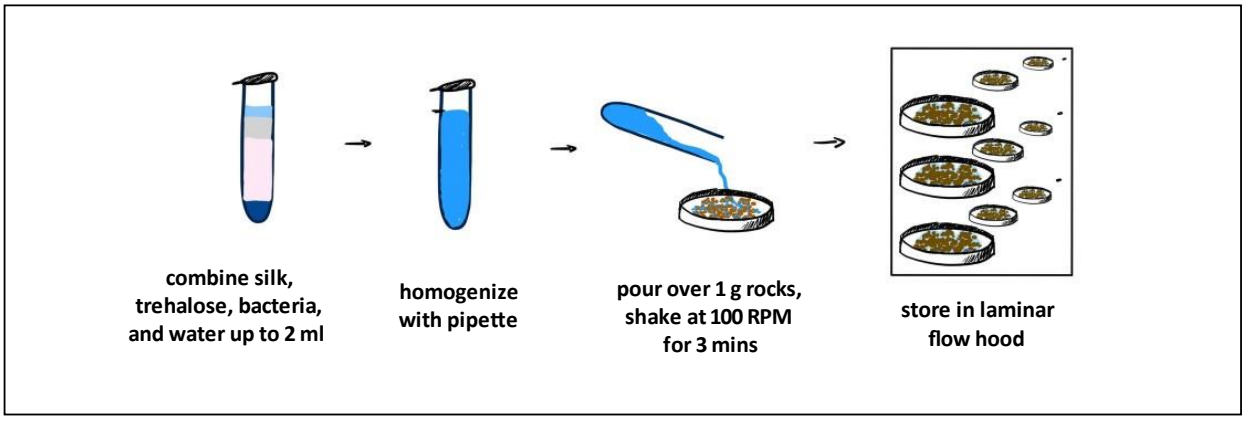

Figure 4-3 Preparation of biopolymer and biofertilizer coated phosphate rocks

Sample Descriptions

In addition to the silk-trehalose biopolymer prepared (labeled STB), three controls were prepared with only trehalose (TB), only silk (SB), and only water (B), maintaining the same concentrations as the original silk-trehalose biopolymer. The sample label descriptions are described in detail in Figure 4-4 for reference throughout this document.

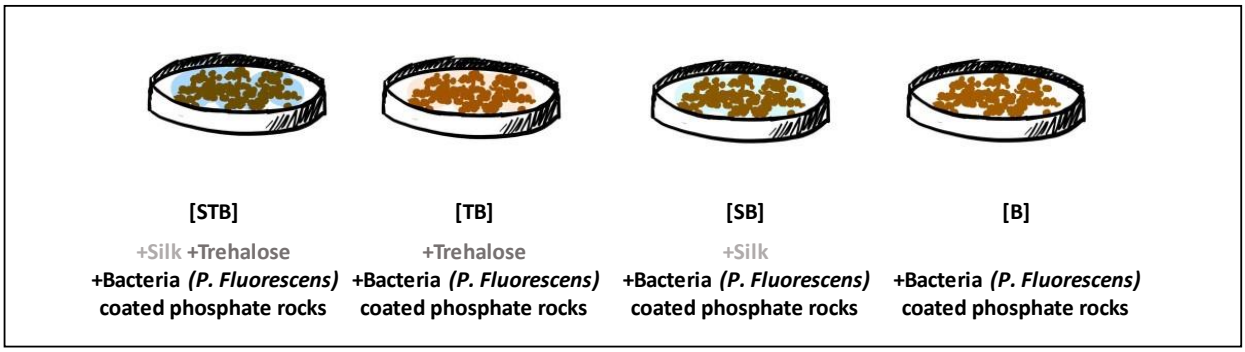

Figure 4-4 Sample label descriptions for biopolymer and biofertilizer coated phosphate rocks

After applying the silk-trehalose-bacteria coating on a sample of the rocks, they were stored in the laminar flow hood for 30 days, then SEM images were taken at magnifications of 2.5kx and 15 kx, shown in Figure 4-5. The SEM image at 15 kx magnification shows a *P. Fluorescens* colony establishing in one of the crevices in the surface of the phosphate rock.

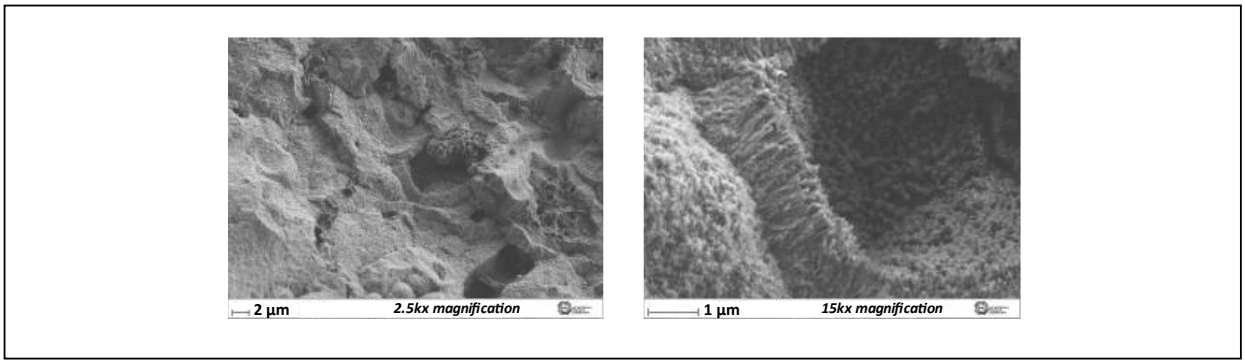

Figure 4-5 SEM micrographs of natural phosphate rock coated with a silk-trehalose biopolymer encapsulating P. Fluorescens. Images taken after storage of coated rocks for 30 days. SEM settings: EHT=3.00kV, HE-SE2 detector

4.2 Biofertilizer Viability in Phosphate Rock Biopolymer Coating

This work aims to develop biofertilizer-coated phosphate rocks as a sustainable alternative to chemically synthesized phosphate fertilizers currently used in agriculture. The chemically synthesized fertilizers have a shelf stability of 5-8 years, and some can last up to 10-20 years under the correct storage conditions; the liquid formulas on the market have a shorter shelf life of 1-3 years (Wendimu et al., 2023). Developing a bio-based solution that can effectively compete with current practices requires the biofertilizer to remain effective after shelf storage for extended periods of time. Therefore, the viability of phosphate solubilizing bacteria after the coating and storage of the phosphate rocks needs to be assessed to ensure their shelf stability.

The successful development of a stable and effective biofertilizer-coated phosphate rock that can remain viable for an adequate amount of time in storage before use in agricultural fields would be a significant advancement towards sustainable agricultural practices. The information gained from this study can be used to further optimize the bacteria and biopolymer-coated phosphate rock for optimal shelf stability and effectiveness in agricultural fields.

To assess the viability of the encapsulated P. Fluorescens on the biopolymer-coated phosphate rocks over time, a quantitative colony counting procedure was employed. This involves rehydrating the samples in a buffer solution, taking a sample of the solution, and transferring it to an agar plate. The colonies that grow on the plate are then counted to determine the number of re-culturable colonies in the original sample.

Experimental Procedure

Each viability measurement was performed on 3 different samples (n=3). Each coated rock sample was added to a sterile 50 ml centrifuge tube. 20 ml of sterile PBS were added to each tube and the solution was vortexed for three minutes to homogenize. The rocks quickly settle on the bottom of the tube, while the bacteria remain suspended in the solution. 1 ml was taken out of the solution and pipetted into 9 ml of PBS in a culture tube, and vortexed to homogenize. This serial dilution was repeated up to 4 times.

Nutrient agar plates supplemented with 100 µg/ml of Ampicillin were used for the colony counting procedure. The antibiotic was added to ensure that the only colonies growing are of P. Fluorescens and not any contamination that may have occurred during the plating process. 100 µl of each dilution was pipetted onto each plate and spread with an L-shaped spreader using the spread plate technique. The plates were incubated at 26 C for 48 hours, when colonies have fully formed and are visible enough to count clearly.

Comparing the plates to each other, the dilution with the most appropriate number of visible colonies (between 30-300) was selected and counted. In this experiment, every sample had three repetitions (n=3). The experiment was conducted at 4 different time points, after storing the rocks for 1 day, 7 days, and 30 days.

Results & Discussion

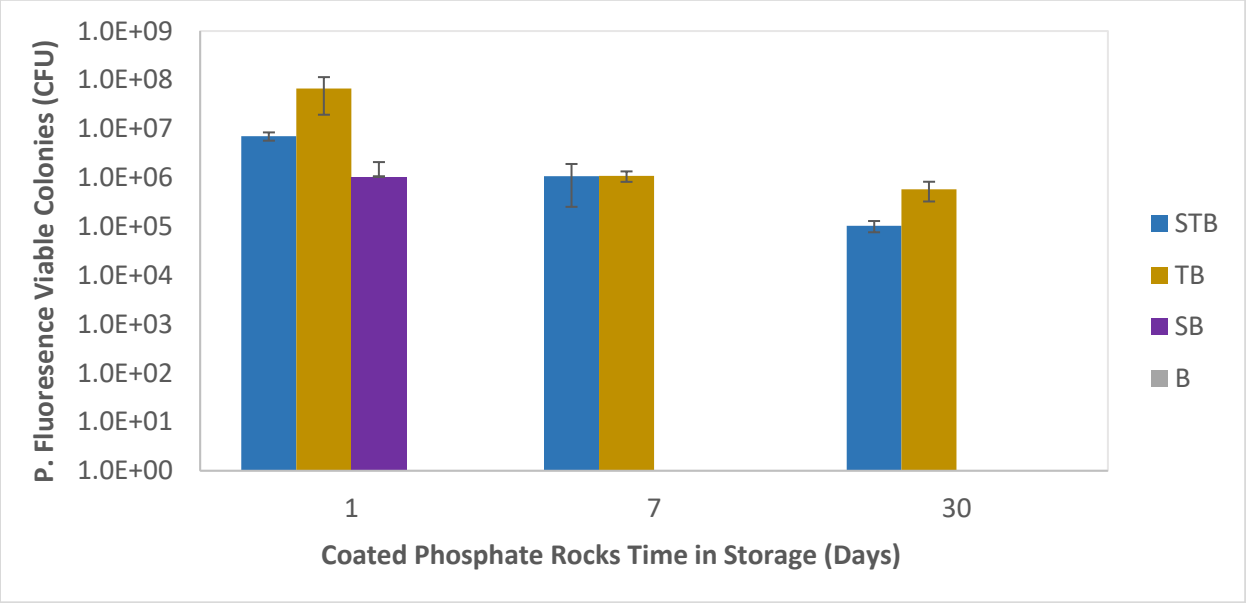

Figure 4-6 Viability of P. Fluorescens in biopolymer coating on phosphate rocks measured by colony counting. N=3

A one-way ANOVA test was conducted for the viability of the STB coated rock samples with the time in storage as the independent variable and the means were found to be significantly different ($p<0.05$). A post-hoc Tukey test was performed to investigate this significant difference between the groups. It was found that after 7 days in storage, the number of CFUs of *P. Fluorescens* decreased significantly from **$7.1x10^6$ CFUs** to **$1.1x10^6$ CFUs**. After 30 days in storage the mean number of viable colonies was **$1.0x10^5$ CFUs**, however, it was not found to be significantly different from the mean after 7 days in storage.

After 1, 7, and 30 days in storage, the rock samples coated with only a bacteria (B) solution contained no viable colonies. After 7 and 30 days in storage, the rock samples coated with only silk and bacteria (SB) contained no viable colonies.

A one-way ANOVA test was conducted for the STB, TB, and SB samples after 1 day in storage, and there was no significant difference found between the means ($p < 0.05$). The test was conducted with the STB and TB samples for 7 days and 30 days in storage respectively, and there was no significant difference found between the groups for both time points ($p < 0.05$).

To develop this coating for different strains of phosphate solubilizing bacteria, further investigations are needed to optimize the silk concentration while maintaining the 1:3 ratio of silk to trehalose, as described in the literature (Zvinavashe et al., 2019).

4.3 Biopolymer and Biofertilizer Coated Phosphate Rock Dissolution in Media

While the previous section was concerned with viability, this section explores whether the *preserved P. Fluorescens* colonies are able to solubilize phosphate rock after their resuscitation and to what extent. First, the solubilization ability of *P. Fluorescens* is confirmed. Then the solubilization of the coated phosphate rocks is assessed after their storage for 7 days and 30 days. Finally, the potential for the coated phosphate rocks to be used as a controlled-released fertilizer is explored. The experimental design in this section is inspired by previously published work (Saeid et al., 2018; Wyciszkiewicz et al., 2016).

4.3.1 Preliminary Test: Assessing Phosphate Solubilizing Ability of *P. Fluorescens*

To assess the phosphate solubilizing ability of the *P. Fluorescens* strain used in this work, an experiment was carried out following the screening methodology described in the literature (Nautiyal, 1999).

NBRIP

National Botanical Research Institute's Phosphate Growth Medium (NBRIP) is a defined microbiological growth medium designed to screen microorganisms capable of solubilizing phosphate due to its selective enrichment of phosphate solubilizing bacteria. NBRIP is more efficient in a liquid assay than other media, such as Pikovskaya medium (PVK), for assessing the phosphate solubilization potential of microorganisms in a liquid assay.

Experimental Design

NBRIP media was prepared by combining glucose, 10 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g, and the phosphate source, $Ca_3(PO_4)_2$, 5 g, in 1L liter of distilled water. The pH of the media was adjusted to 7.0 using a 5M NaOH solution, then autoclaved at 121C for 45 minutes for sterility. 10 sterile flasks (150 ml capacity) were filled with 10 ml of NBRIP and 100 µl of *P. Fluorescens* culture solution (OD600 = 1). The flasks were placed in a shaker incubator at 26C and shaken at 200 RPM for 4 days. At the relevant time points, a flask was removed from the incubator, the entire contents of the flask were centrifuged, and the supernatant was saved for phosphate and pH testing. A Mettler-Toledo pH probe was used to measure the pH of the samples taken out of solubilization experiments. The phosphate concentration in the samples were measured using a Malachite Green assay. The experiment was carried out in duplicate.

Determination of Released Phosphate

The malachite green phosphate assay was used to measure the phosphate concentration in samples, using the MAK308 Assay Kit from Sigma-Aldrich, according to the manufacturer's protocol. 50 µl of a supernatant sample was pipetted into clear 96-well plates and mixed with 100 µl of the malachite green solution. The well plates were incubated in the dark for 30 minutes to allow a green colored complex to form between free orthophosphate and malachite green.

Then, the samples are measured on a TECAN multi-well plate reader for absorbance at a wavelength of 620 nm. The absorbance values were correlated to phosphate concentrations using a calibration curve generated with a known phosphate standard, shown in Figure 4-7. Any sample that contained phosphate concentrations that fell beyond the assay's detection limit of 40 µM was diluted and its measurement was repeated. The malachite green phosphate detection assay was used as it is quick and robust, and more convenient for time point measurements compared to other analytical spectrometry methods.

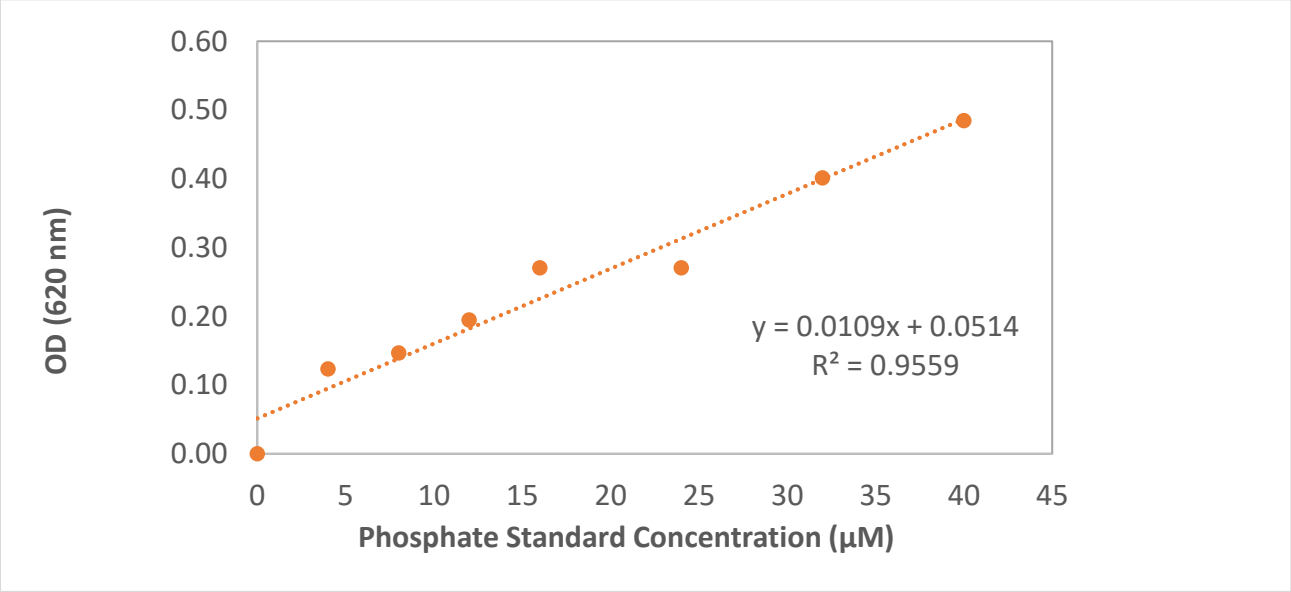

Figure 4-7 Normalized phosphate standard curve generated using a malachite green assay and a known phosphate standard. N=2

Results & Discussion

Figure 4-8 and Figure 4-9 show the phosphate concentration and pH in the NBRIP filled flasks that contain calcium phosphate and P. Fluorescens, over the course of 4 days. Within the first 24 hours, the phosphate concentration in the flasks increased to **10,246 µM**. By the 4th day in solution, the phosphate concentration in the flasks further increased to **19,379 µM**. Within the first 24 hours, the pH of the solution decreases from 6.3 to 4.2, then continues to slowly decrease to a pH of 4.0 by day 4.

It was previously reported that there exists a correlation between the total concentrations of organic acids released by applying phosphate solubilizing bacteria to a phosphate source and the total amount of released phosphorus (P2O2) (Saeid et al., 2018). An increase in the production of organic acids by the phosphate solubilizing bacteria explains the general decrease in pH observed during the solubilization process (Chen et al., 2006; Wyciszkiewicz et al., 2016).

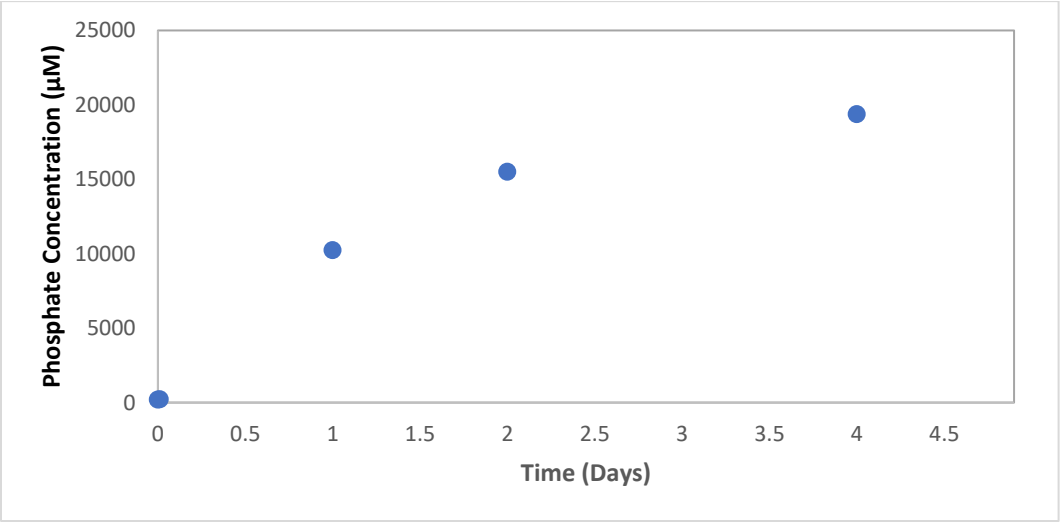

Figure 4-8 Concentration of solubilized phosphate from calcium phosphate by P. Fluorescens. N=2

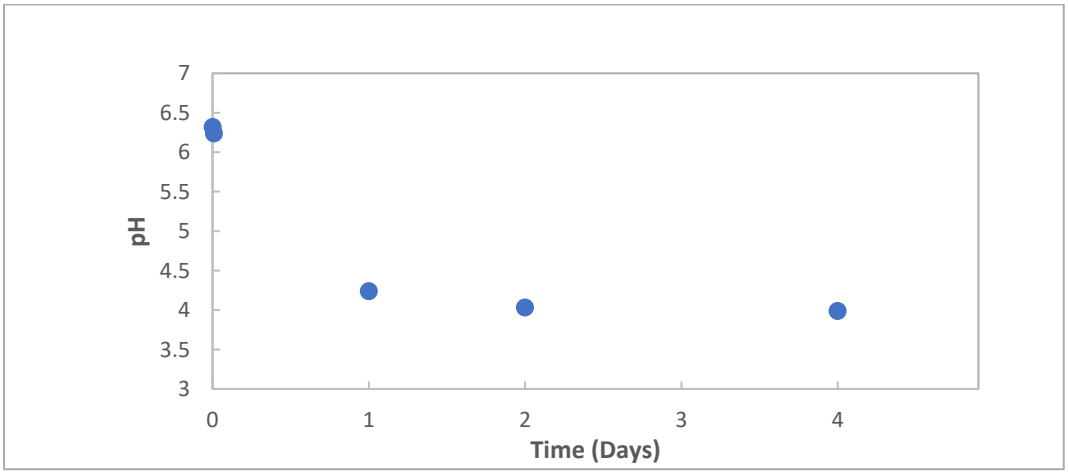

Figure 4-9 pH of NBRIP during solubilization of phosphate from calcium phosphate by P. Fluorescens. N=2

4.3.2 Solubilization of Phosphate Rock by P. Fluorescens

Modified NBRIP Media

NBRIP uses calcium phosphate (Ca3(PO4)2) as the phosphate source for solubilization. In this study, we are concerned with the solubilization of phosphate rock. Maintaining the same concentrations of each chemical in 1 L of distilled water, NBRIP was prepared as previously described, however, calcium phosphate was omitted. In this way, NBRIP was utilized as a supportive medium for the solubilization of phosphate rock with *P. Fluorescens*. The media was supplemented with 50 ug/ml of the antibiotic, Ampicillin, to ensure that the only colonies growing are of *P. Fluorescens* and not any contamination that may occur during the handling of the flasks.

Preliminary Test: Freshly Inoculated P. Fluorescens vs. only P. Rock

Experimental Design

The rocks were sieved using a 120-mesh screen resulting in a rock size of [100 μm < x < 150 μm]. The rocks were then sterilized using dry heat sterilization at 170 °C in an oven for 1 hour. 1 g of phosphate rocks were added to sterile 250 Erlenmeyer flasks and 100 ml of modified NBRIP was added. 1 ml of a *P. Fluorescens* culture (OD600 = 1) was pipetted into 5 flasks. As a control, 5 flasks were left without *P. Fluorescens* inoculation. The flasks were placed in an incubator at 26 °C and shaken at 150 RPM for 5 days. 1 ml samples of the solution were taken out at relevant time points and replaced with sterile water to maintain the same volume in the flasks. The samples were centrifuged at 10,000 RPM for 10 minutes to pellet any bacteria and rocks. The supernatants were saved for phosphate and pH testing. Mettler-Toledo pH probe was used to measure the pH of the samples taken out of solubilization experiments. The phosphate concentration in the samples were measured using a Malachite Green assay. The experiment was carried out with 5 repetitions per sample type.

Results & Discussion

Figure 4-10 and Figure 4-11 show the phosphate concentration and pH in the flasks over 5 days. A one-way ANOVA test for the phosphate rock sample with the independent variable being time in solution showed no significant difference between the means ($p>0.05$).

The same test for the inoculated sample showed a significant difference between the phosphate concentrations at different time periods in the solution. A subsequent Tukey test was used to investigate=te the differences between the means. It was found that there was an increase in concentration between days 1 and 2, 1 and 3, 1 and 5. There is no significant difference between day 3 and 5, indicating that solubilization is complete, with a mean concentration of **5,358 μM** on day 5. These results serve as a baseline for the next experiments in this section and are referenced later in the section.

Using a different phosphate raw material as a source of phosphorus, such as bones or ashes, influences the amount of phosphate solubilized by PSB (Wyciszkiewicz et al., 2016). This trend is seen when comparing the maximum amount of phosphate solubilized by using calcium phosphate (Figure 4-8) and phosphate rock (Figure 4-10). It can be seen that the PSB is able to solubilize significantly more phosphate from calcium phosphate compared to phosphate rock.

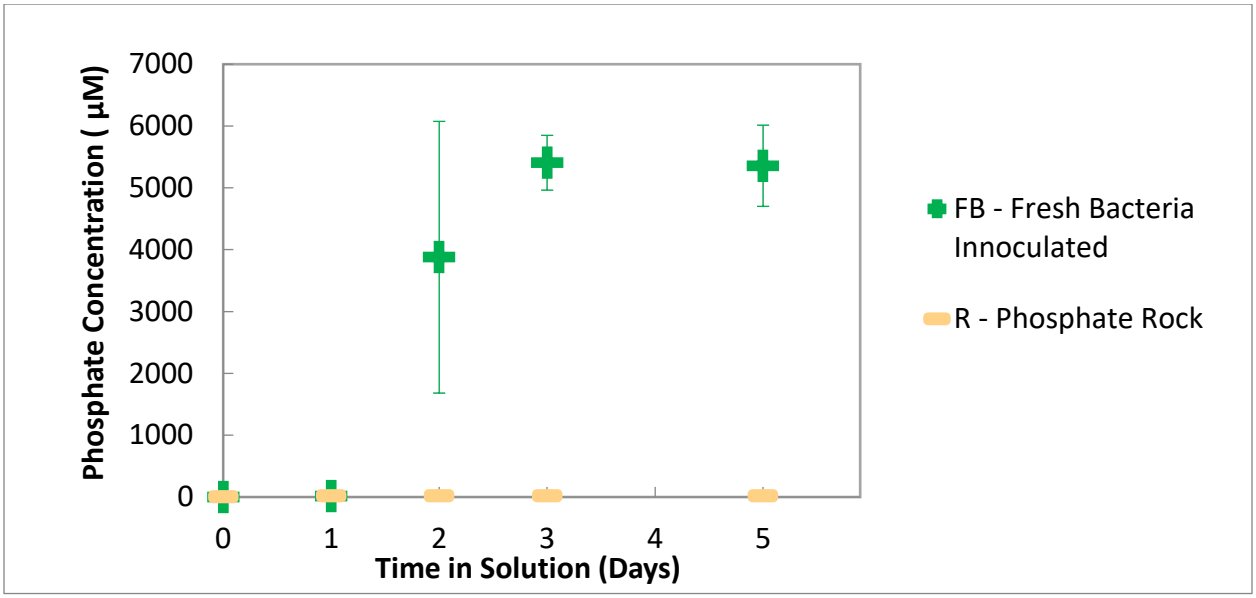

Figure 4-10 Phosphate concentration in 100 ml NBRIP and 1g of phosphate rocks. FB sample was inoculated with 1 ml of OD600=1 bacteria. N=5

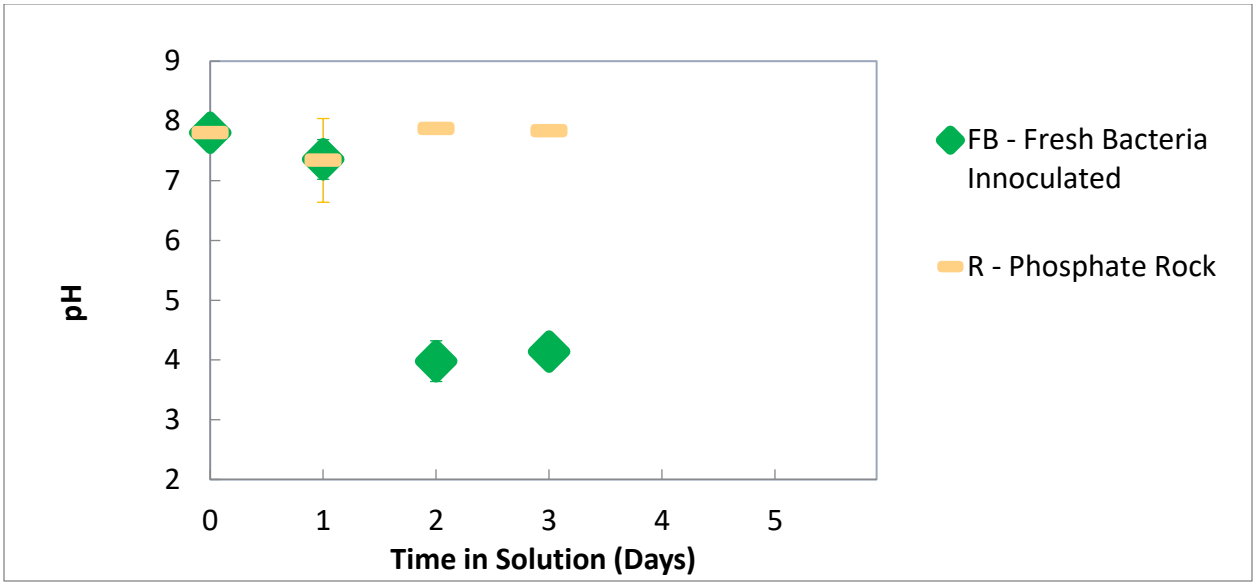

Figure 4-11 pH in 100 ml NBRIP and 1g of phosphate rocks FB sample was inoculated with 1 ml of OD600=1 bacteria. N=5

Biofertilizer-Coated Phosphate Rock Dissolution

Experimental Procedure

To measure the solubilization of phosphate by the preserved P. Fluorescens on the coated phosphate rocks, a dissolution experiment was conducted. Once the rocks were coated and stored, 5 different samples were taken out of storage after 30 days (n=5). The rocks were placed in 250 ml flasks and 100 ml NBRIP media was added. The flasks were placed in an incubator and shaken at 150RPM for 5 days. 1 ml samples of the solution were taken out at relevant time points and replaced with sterile water to maintain the same volume in the flasks. The 1 ml samples were centrifuged at 10,000 RPM for 10 minutes to pellet any bacteria and rocks. The supernatants were saved for phosphate and pH testing. Mettler-Toledo pH probe was used to measure the pH of the samples taken out of solubilization experiments. The phosphate concentration in the samples were measured using a Malachite Green assay.

Results and Discussion

Figure 4-12 shows the phosphate concentrations inside the flasks containing coated phosphate rocks in NBRIP solution, after the coated rocks were left in storage for 30 days. A one-way ANOVA test was conducted for the phosphate concentration in the flask containing the STB coated rock samples with the time in solution as the independent variable and the means were found to be significantly different ($p<0.05$). A post-hoc Tukey test was performed to investigate this significant difference between the groups. For the first two days, there is no significant difference between the phosphate concentrations in the STB flasks, remaining **below 30.0 µM**. On day 3 in solution, the concentration increases to **217.4 µM**, however this increase is not statistically significant compared to the first two days. On day 5 in the solution, the concentration increases to **5,964 µM**, and this increase is statistically significant compared to the first three days.

The phosphate concentration in the flasks containing SB coated and B coated rocks remained **under 30 µM** for the 5 days in solution and there was no significant difference when performing an ANOVA analysis with the time in solution as the independent variable. Comparing the phosphate concentration between the SB coated rocks and uncoated phosphate rocks containing no bacteria (from Figure 4-10), there is no significant difference, and similarly for the B coated rocks. This result indicates that the *P. Fluorescens* encapsulated in the coating was not able to solubilize the phosphate from the rocks after 30 days in storage. This result can be explained by the viability study shown in Figure 4-6, as the bacteria encapsulated in the SB and B coating did not remain viable after 30 days in storage.

A one-way ANOVA test was performed for the phosphate concentrations between the flasks containing STB coated rocks and TB coated rocks at the different time points in solution, with the independent variable being the presence of the silk. On the first day, there was no significant difference between the phosphate concentrations in the STB and TB flasks. On the second and third day, the concentration in the TB flask was significantly higher (**105.2 µM, 2803 µM**) than the concentration in the STB flask (**15.7 µM, 217.4 µM**). However, on day 5, there was no significant difference between the TB and STB flask concentrations, at **5133 µM** and **6098 µM**, respectively.

These results indicate that the TB coated rocks were able to solubilize the phosphate rock at a faster rate than the STB coated rocks, while solubilizing the same amount of phosphate from the rocks by the end of 5 days in solution. The viability results in Figure 4-6 showed that there is no significant difference between the number of colonies that survive in the STB coated rocks compared to the TB coated rocks after 30 days in storage. Therefore, the slower rate of phosphate release in the STB coated sample can be accounted for by the time it takes for the silk to degrade in solution (Zvinavashe et al., 2022). Although the trehalose in the coating is the critical preservation component due to its vitrification properties, the silk is integral as it provides the mechanical strength and robustness to the coating, in addition to adhesion during industrial coating process such as rotating pan coating (Fertahi et al., 2021; Zvinavashe et al., 2019).

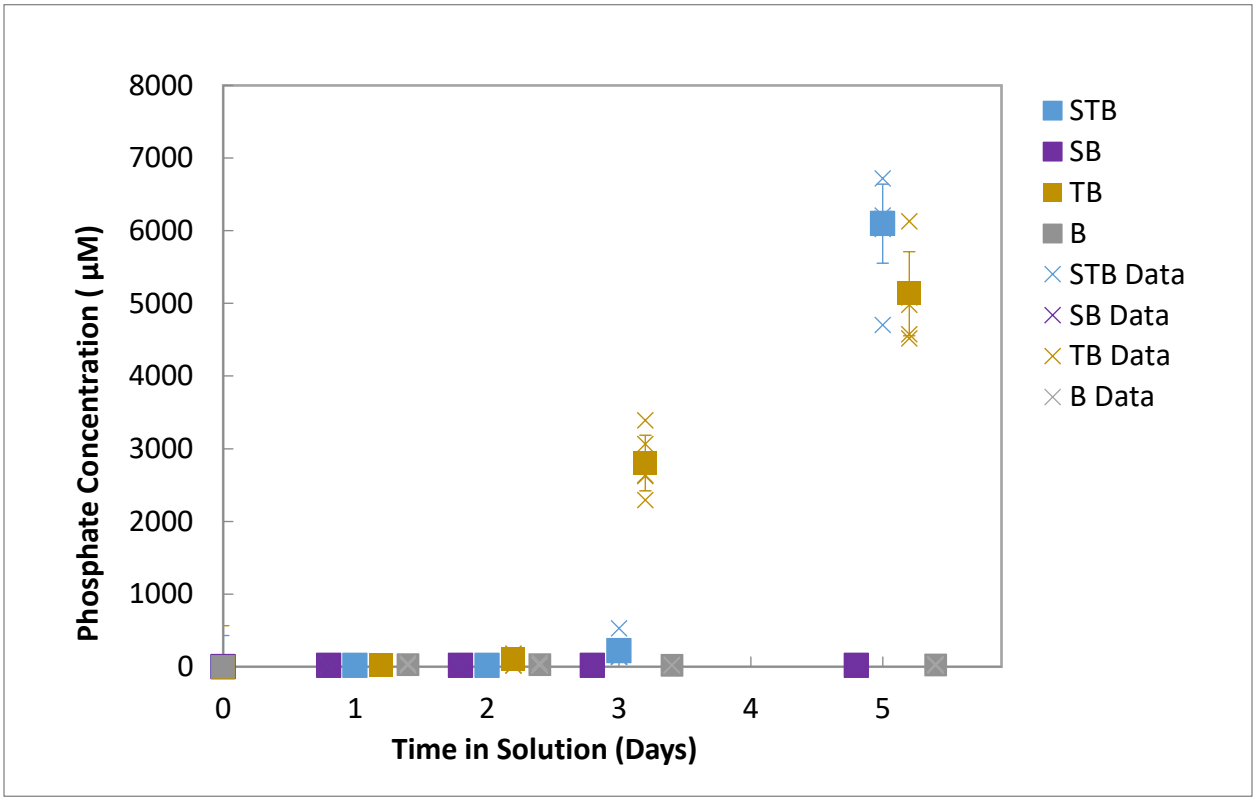

Figure 4-12 Phosphate concentration in 100 ml NBRIP and 1g of coated phosphate rocks after 30 days in storage. The sample labels represent the coating formulation, S=silk, T=trehalose, B=bacteria. Xs represent the individual measurements in each flask (N=5) and squares represent the means.

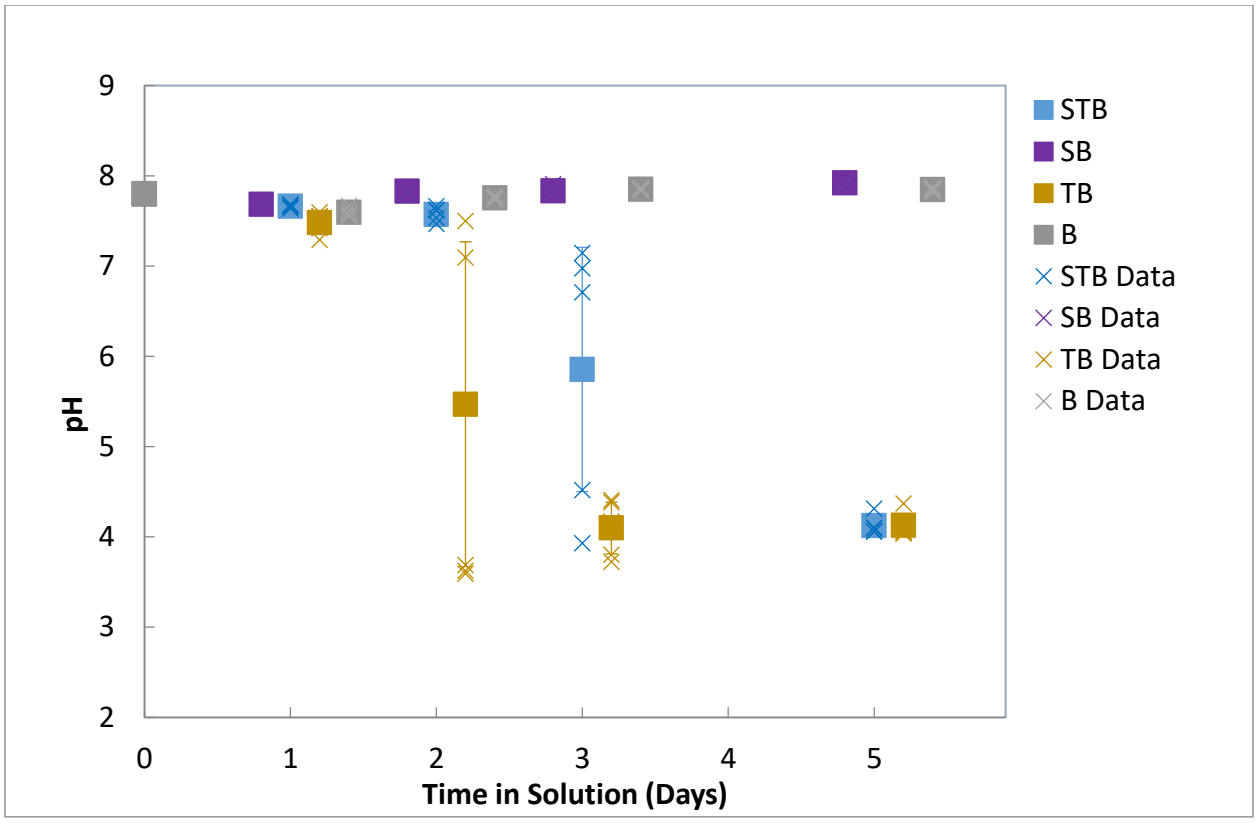

Figure 4-13 pH of NBRIP solution containing 1g of coated phosphate rocks after 30 days in storage. The sample labels represent the coating formulation, S=silk, T=trehalose, B=bacteria. Xs represent the individual measurements in each flask (N=5) and squares represent the means.

4.3.3 Potential for Biofertilizer-Coated Phosphate Rock as a Controlled Release Fertilizer

Experimental Design

Phosphate rock fertilizer granules were purchased and prepared in the same manner as described earlier. 1g of the rock granules were coated with a 2 ml solution of 35 mg/ml silk fibroin and left under a laminar flow hood to dry overnight. The next day, a solubilization experiment was conducted, maintaining the same experimental conditions as the solubilization experiments described earlier.

Figure 4-14 shows the rate of soluble phosphate release of the silk-coated phosphate rock fertilizer granules compared to the uncoated control. It was found that after 8 days in solution, the concentration of phosphate in the flasks containing the silk-coated fertilizers was significantly lower than that of the uncoated fertilizer. This indicates that the rate of dissolution of the fertilizer was slowed down by the silk, achieving a "slow release" effect.

Results & Discussion

As described in Section 2.3, fertilizer granules can be coated with polymers to control the rate of nutrient release, called controlled-release fertilizers (Fertahi et al., 2021). To use silk fibroin coatings to control the rate of phosphate release in soil, its degradation needs to be controlled. The degradation of silk fibroin has been previously studied in water, soil, and marine environments (Zvinavashe et al., 2022); it was found that microbial activity was a key driver of silk fibroin degradation and incorporating biofertilizers into silk films further accelerated their degradation.

In combination with the degradation study, the data generated in this work (Figure 4-12, Figure 4-14) indicates the potential of the silk-trehalose-biofertilizer coated phosphate rocks to be used as a controlled-released fertilizer, however, further investigation needs to be performed by varying different factors such as the amount of silk and trehalose in the coating, material processing of the silk, and amount of bacteria loaded onto the rock. In addition, in-vitro phosphate release tests in soil as well as field tests can be performed to validate this potential.

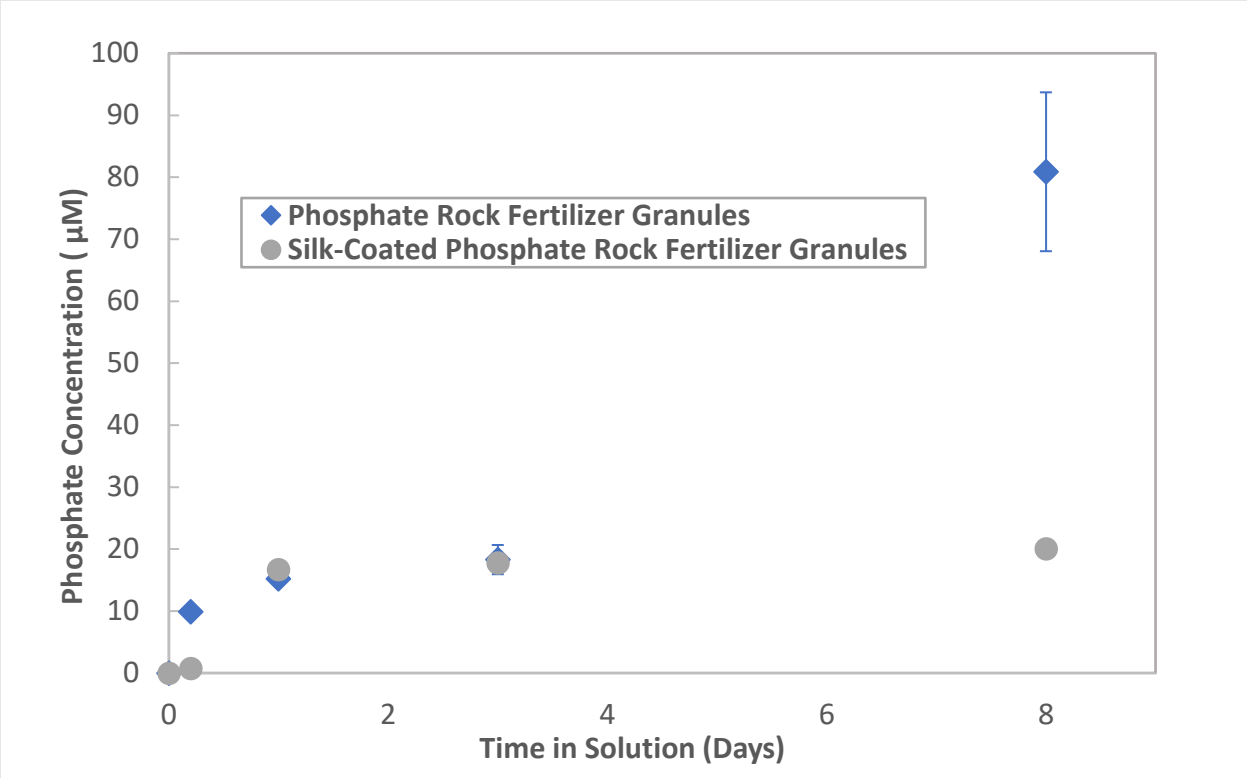

Figure 4-14 Phosphate concentration in 100 ml NBRIP of coated and uncoated phosphate rock fertilizer granules. N=5.

4.4 Investigating Biopolymer and Biofertilizer Coated Phosphate Rock Effect on Chickpea Plants

Materials and Methods

Vermiculite is a hydrous phyllosilicate mineral commonly used as a soil amendment due to its ability to improve water retention and soil aeration, however, it does not contain significant amounts of nutrients. Hoagland solution is a well-known plant nutrient solution that contains the necessary macronutrients and micronutrients required for their growth and development. Substituting soil for Hoagland solution allows for more precise control of plant nutrient availability and ratios, therefore, it is commonly used for plant research, hydroponic agriculture, and plant tissue culture (Hoagland & Synder, 1933).

To control the amount of nutrients being accessed by the plants in this study, chickpeas were planted in vermiculite-filled containers supplemented with nutrients in the form of a modified Hoagland solution. The solution was modified by excluding the phosphate source, potassium nitrate, to ensure that any changes in the plant physiology is attributed to the addition of phosphate rocks. The modified Hoagland solution (at 20% strength) was prepared according to the published concentrations in 1 L of DI water and sterilized by autoclaving at 121 C for 45 minutes (Hoagland & Synder, 1933).

Sample Types

Figure 4-15 visually explains the different sample types implored in this experiment. Sample [A] and [B] will be compared to explore the effect of the addition of nutrients (excluding P) on the plant's ability to synthesize phosphate. Sample [B] and [C] will be compared to investigate the effect of the addition of phosphate rock as a P source on the phosphorous uptake of the plant. Finally, Sample [C] and [D] will be compared to determine whether the biofertilizer coating is able to increase the P solubilization of the phosphate rock and increase the uptake of phosphorous by the plant.

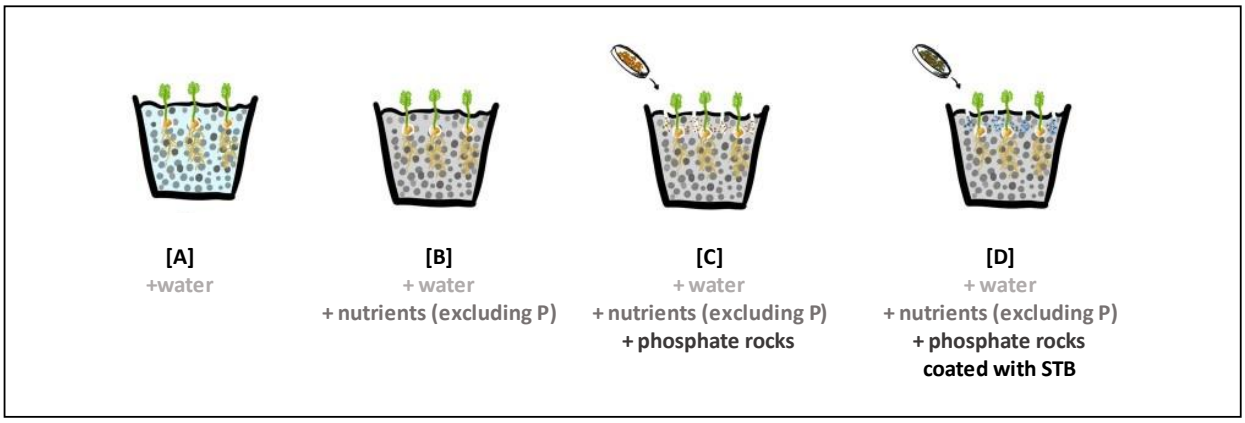

Figure 4-15 Sample type descriptions - investigating the effect of STB coated rock on chickpea plants

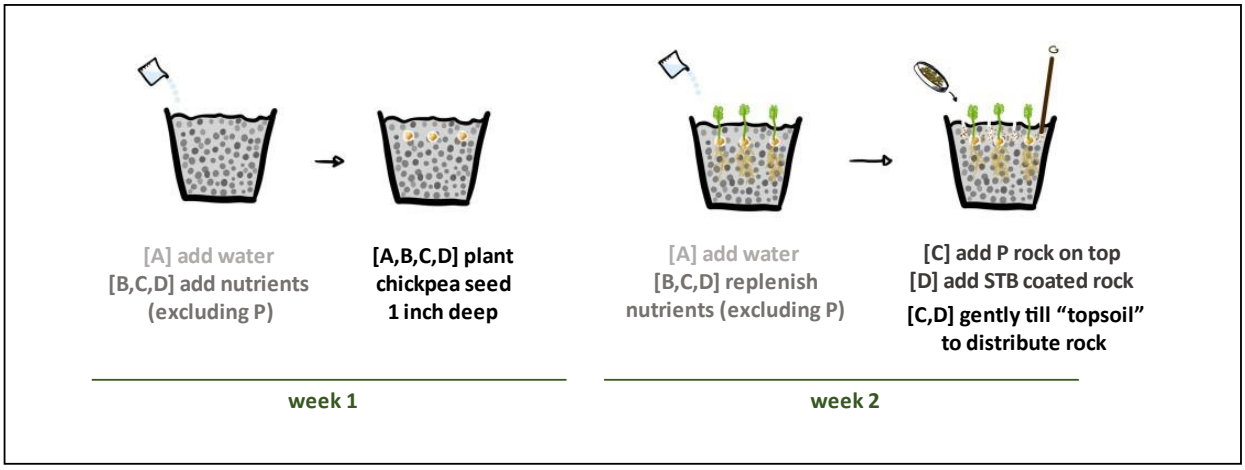

Figure 4-16 Experimental design - investigating the effect of STB coated rock on chickpea plants

Figure 4-16 summarizes the experimental design and setup. 15 planters (12 cm in height) were filled with 150 g of dry vermiculite. 200 ml of 20% strength modified Hoagland (P-deficient) solution was added to each pot, which was previously determined to be the amount of liquid required for vermiculite saturation. 3 planters were filled with only water as a control. The moist vermiculite was tilled with a wooden stick for aeration before planting the seeds. 6 chickpeas were sown in each planter, evenly spaced and 1 inch under the vermiculite. The containers were placed in a light and humidity-controlled plant-growth room to limit variability in growth conditions. 25 milliliters of sterile DI water were added to the pots every day for the first week of growth to prevent the seeds from drying out. The amount of water was determined by the average difference in the weight of the planters between the first two days due to water evaporation.

Preliminary experiments determined that the chickpea seeds germinate in 7-9 days under these conditions. 9 days after sowing, the plant shoots were assessed and the planters which contained plant shoots that were similar in height, leaf density, and appearance were selected for the experiment. 1 planter was selected with only water as a control. [Figure 4-15, Sample Type A]. 2 planters supplemented with P-deficient nutrient solution were selected as another control [Figure 4-15, Sample Type B]. 100 mg of sterile phosphate rocks were added to the surface of 2 planters supplemented with P-deficient nutrient solution and gently mixed into the top inch of vermiculite using a thin wooden stick [Figure 4-15, Sample Type C]. Similarly, 100 mg of silk-trehalose-P. Fluorescens coated phosphate rocks were added to the surface of 2 planters supplemented with P-deficient nutrient solution [Figure 4-15, Sample Type D]. Samples B, C, and D were replenished with 50 ml P-deficient nutrient solution. Sample A was replenished with 50 ml DI water. For the next 10 days, 50 ml of sterile DI water was added every other day to maintain moisture levels in the pots. At this stage, the plants have developed a root system that can access the water in the lower parts of the planter once the upper parts begin to dry and require less frequent watering.

Data Collection

In this experiment, data were collected on the growth and development of chickpea plants over a period of 3 weeks. After the seeds germinated, the plant shoots were observed every other day for symptoms of phosphorous deficiency. At the end of the 3-week period, the following method was employed to assess the nutrient content of the leaves and to determine the effectiveness of the bacteria and biopolymer-coated phosphate rocks in promoting phosphate uptake by the chickpea plant, shown in Figure 4-17.

The leaves were picked off the plants and placed in a 60°C oven until they were fully dry, which was indicated by a constant weight. To prepare the leaves for chemical analysis by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), the leaf samples had to undergo a chemical digestion procedure, similar to a published protocol (Pequerul et al., 1993). After drying, the leaves were ground into a fine powder using a mortar and pestle. 20 mg of the ground dry leaves from each plant were weighed and placed into 15 cm tubes. To digest the leaves, 500 µl of concentrated nitric acid was added to the flask and mixed, followed by the addition of 250 µl of 33% hydrogen peroxide. The tubes were left at room temperature overnight. The following day, the tubes were placed in a 50°C water bath for 15 minutes to complete the digestion. The samples were diluted up to 5 ml with 2% nitric acid and filtered using a 0.2 µm filter. Phosphate standards were prepared in a nitric acid matrix. The analysis was performed using an Agilent 5100 VDV ICP-OES. The recommended wavelength chosen for determination of the element Phosphorous was 178.22 nm. The analysis was carried out with 4-6 repetitions per sample type (n=4-6).

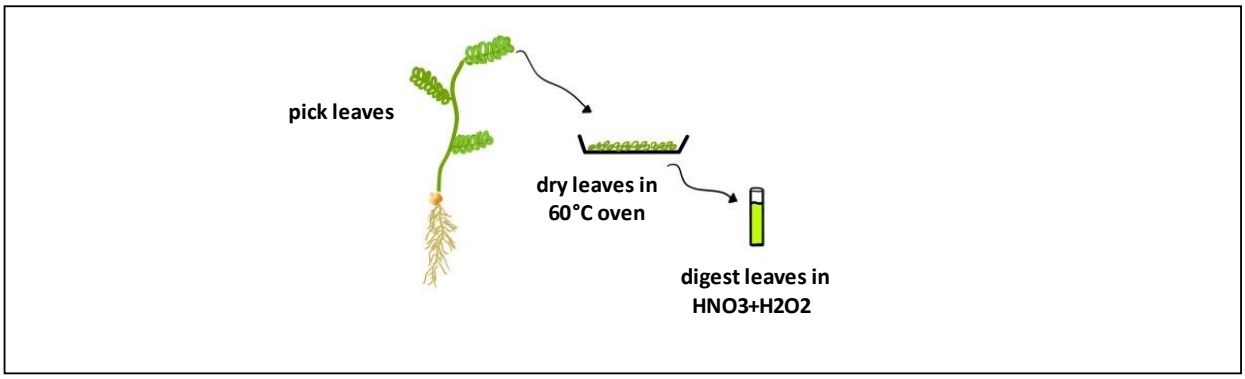

Figure 4-17 Data collection – digesting chickpea leaves for ICP OES analysis to determine P concentration - investigating the effect of STB coated rocks on chickpea plants

Results and Discussion

As early as 9 days after adding the phosphate sources to the planters, phosphate deficiency symptoms begin to appear in Samples [B] and [C] in the form of thin, oval shaped, and curled leaves that are beginning to yellow (Figure 4-18) (Long et al., 2019). Sample [D] containing the biofertilizer coated P rocks shows the darkest overall leaf color, indicating a higher chlorophyll content and healthier leaves, with no phosphate deficiency symptoms showing up yet.

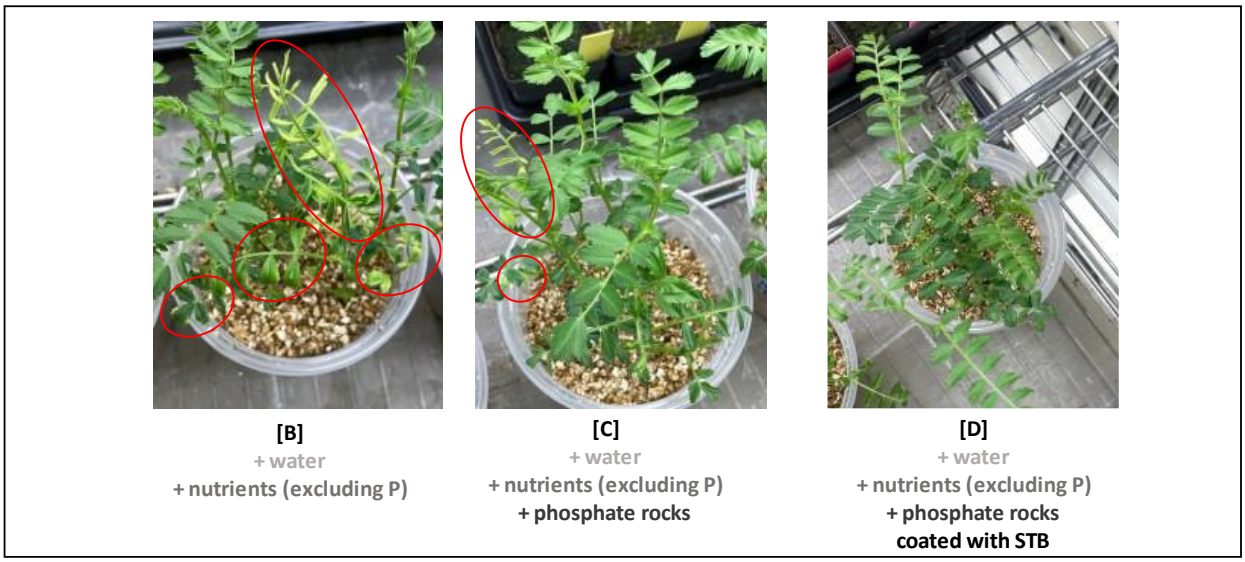

Figure 4-18 Chickpea Plants 17 days after planting

Figure 4-19 shows the concentration of P in the leaves as measured by ICP-OES at a wavelength of 178.222 nm;

Table 4-1 summarizes the raw data and its statistical analysis. A t-test assuming equal variances was performed between groups where 1 independent factor was changed.

When comparing Sample [C] to Sample [B], the independent variable explored is the presence of phosphate rock and it was found that there is no significant difference between the mean concentrations of P in the leaves ($p>0.05$). This result indicates that the addition of phosphate rock on its own did not provide a significant increase in availability of phosphate for the plant to uptake.

When comparing Sample [D] to [C], the independent variable being explored is the presence of coated biofertilizers. It was found that Sample [D] containing the phosphate rock coated with biofertilizers had a statistically significant ($p<0.05$) higher mean phosphorous content than Sample [C] containing only phosphate rock. This indicates that the biofertilizer had a positive effect on the uptake of P by the chickpea plant by solubilizing insoluble phosphates from the phosphate rock.

When comparing Sample [A] to Sample [B], the independent variable being tested is the addition of nutrients, excluding P. It was found that there was a significant increase in the uptake of P by the plant upon the addition of nutrients ($p<0.05$), despite neither sample being supplemented with phosphorous. This can possibly be explained by the synergistic relationships that plant nutrients have with each other.

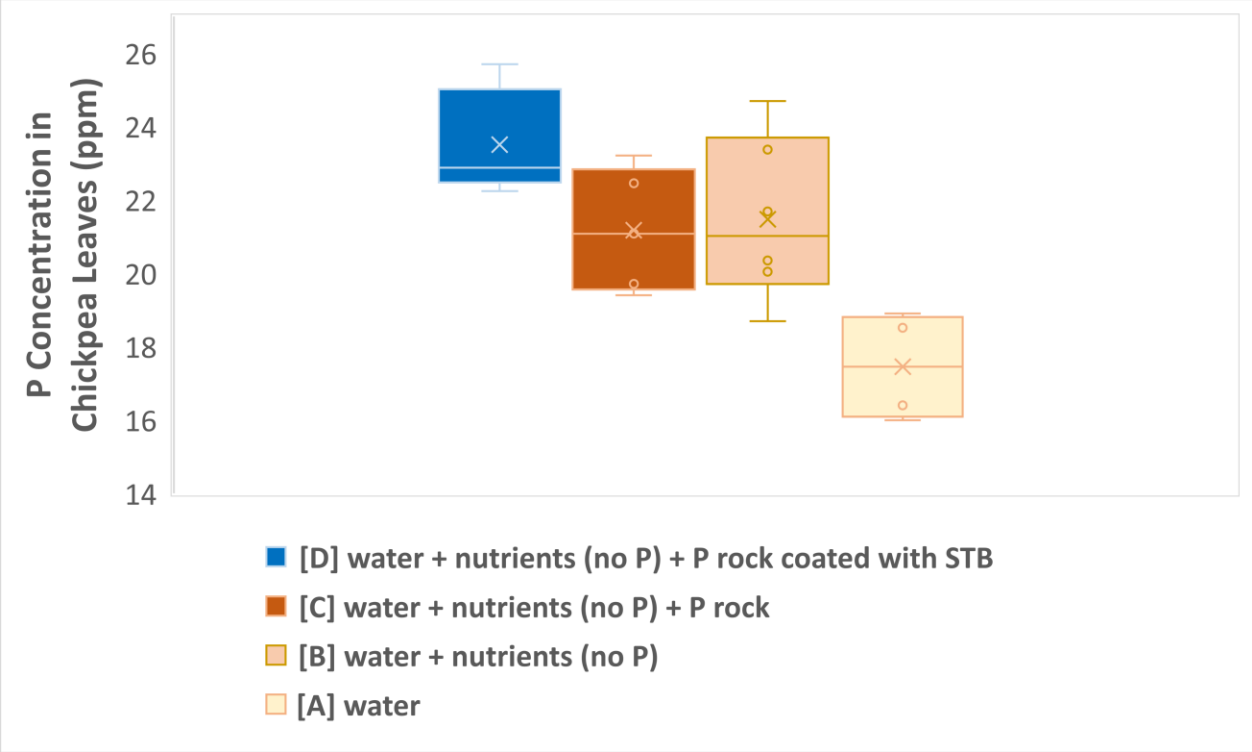

Figure 4-19 P concentration in chickpea leaves measured using ICP OES at a wavelength of 178.222 nm

Table 4-1 ICP OES Analysis of Digested Chickpea Leaves

	P Concentration in Chickpea Leaves at λ=178.222 nm			
	D	**C**	**B**	**A**
1	23.05	22.45	18.69	18.9
2	25.7	19.71	24.69	18.51
3	22.71	23.21	23.37	16.4
4	22.55	19.4	21.68	15.99
5	22.24	21.08	20.04	
6	24.79		20.35	
Mean	**23.51**	**21.17**	**21.47**	**17.45**
SD	1.28	1.49	2.04	1.27
P	**P=0.03209**			
P<0.05?	significant difference			
P		**P=0.75999**		
P<0.05?		no sig. difference		
P			**P=0.00994**	
P<0.05?			significant difference	

Chapter 5. Conclusions and Future Work

This work presents a promising solution to the impending global phosphorus scarcity and the environmental issues associated with chemical fertilizers. By leveraging the unique properties of silk fibroin and trehalose, this study proposes the development of a biofertilizer and biopolymer-coated phosphate rock that can effectively replace harmful chemical fertilizers in agricultural systems.

The biopolymer-coated phosphate rocks, designed to preserve phosphate solubilizing bacteria, hold the potential to enhance the availability of phosphorus in the soil, leading to improved plant growth and increased crop yields. The coating mechanism allows for the preservation of the bacteria and their subsequent resuscitation upon rehydration in the soil, initiating the solubilization of the phosphate rocks and rendering previously inaccessible phosphorus in the rock more available to plant roots. Encapsulating the bacteria in silk fibroin and trehalose provides it with better chances of survival and colonization in the soil.

While this proposed solution shows great promise, several challenges need to be addressed. Maintaining the viability of phosphate solubilizing bacteria within the biopolymer-coated rock in more realistic conditions that include temperature and humidity fluctuations, ensuring their ability to solubilize phosphate after longer-term storage, and demonstrating their functionality within plant-soil systems in the field are critical areas that require further investigation. To evaluate the potential of this solution as a controlled-release fertilizer, further tests are proposed that include varying the coating parameters, such as the amount of silk and trehalose, material processing of silk, and bacterial load, to investigate their effect on the phosphate release and uptake by plants. Additional field tests are also proposed to validate the findings and evaluate the efficacy of the biofertilizer-coated rocks in practical agricultural applications.

By overcoming these challenges and demonstrating the feasibility and effectiveness of the proposed solution, this research opens new possibilities for sustainable phosphorus management and environmentally friendly agricultural practices. By transitioning from harmful chemical fertilizers to natural and sustainable biofertilizers, we can ensure the long-term food security of our growing global population while minimizing the negative impact on the environment. The results of this thesis provide a foundation for further exploration and development of biofertilizers, bringing us one step closer to a more sustainable and resilient future in agriculture.

Chapter 6. References

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