DISSERTATION

ROLE OF RHIZOSPHERE BACTERIA AND ROOT EXUDATES ON THE ASSIMILATION OF PHOSPHORUS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2022

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ABSTRACT

ROLE OF RHIZOSPHERE BACTERIA AND ROOT EXUDATES ON THE ASSIMILATION OF PHOSPHORUS

Deficient phosphorus (P) bioavailability in soils is a major challenge for sustainable food production as effective strategies to access unavailable P are limited. Solubilizing-bacteria and root exudate metabolites that solubilize P are promising approaches to increase available P for plants. We hypothesized that compounds in root exudates could elicit the P-solubilization activity of bacteria. To test this hypothesis, the root exudates of *Arabidopsis* grown *in vitro* under sufficient and deficient P conditions were characterized using GC-MS. We tested the ability of previously screened root exudates to solubilize plant-unavailable P *in vitro*. In parallel, potential P-solubilizing bacteria were isolated from the rhizosphere of wild potatoes using conventional microbiology techniques. The bacteria strains were tested, both individually and in consortia, for their ability to solubilize organic (phytin) and inorganic (calcium) P sources *in vitro* and *in planta*. Lastly, selected root exudate compounds were incubated together with P-solubilizing bacteria, and bacterial growth, P solubilization activity, and plant growth were evaluated. Our results demonstrate that malic, nicotinic, and 3-hydroxypropionic acids improved solubilization of P as compared to a control. Likewise, the bacterial strains *E. cloacae*, *P. pseudoalcaligenes*, and *B. thuringiensis* enhanced plant growth and P content with additions of plant-unavailable P. Furthermore, we found that threonine and 4-hydroxybutyric acid elicit P solubilization in all bacteria, under both organic and inorganic sources, independent of bacterial growth. Subsequent exogenous application of threonine to soils improved plant root growth, enhanced nitrogen and

phosphorus content in roots and increased available levels of potassium, calcium, and magnesium in soils. Altogether, our findings expand on the function of exuded specialized compounds and suggest approaches to effectively recover residual P from soil, improving crop growth and nutrition.

ACKNOWLEDGMENTS

This dissertation project would not have been possible without the support of many people.

First, I would like to extend my sincerest gratitude to my graduate advisor Dr. Jorge Vivanco for his support at every stage of the research project and for providing funding throughout my graduate studies at Colorado State University. I am very grateful to the members of my graduate committee for their invaluable intellectual input, guidance, and support. Special thanks to Dr. Steven Fonte for his encouragement and funding for the last semester of my graduate studies, to Dr. Daniel Manter for his technical advice and training, and to Dr. Jessica Davis for her assistance and supervision.

I am very thankful to past and present members of the Vivanco Laboratory including Yanhui He and Mike DiLegge for being remarkably stimulating team members, and to Charlie Vollmer, Marcia Leite, and Juan Castillo for their motivation. To Sam Monohon, Derek Newberger, and Carley Rohrbaugh for brightening up the last stage of my time in graduate school.

Lastly, I am deeply indebted to my older brother Hugo F. Pantigoso and my mother Patricia Guevara for their unwavering support during my entire career. To members of my family Pantigoso and Guevara, to my friends Cybill Rejas, Luis Huezo and Yvan Delgado, and to the diverse communities that welcomed me, and provided me with comforting and inspiring outlets outside school, these include LASSO (Latino American Students and Scholars Organization), AGEAP International (International Alumni Association - Zamorano University), Fort Collins Running Club, and The Growing Food Security Project.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER 1 RECOVERING PHOSPHORUS FROM SOIL: RHIZOSPHERE BACTERIA AND ROOT EXUDATES INTERACTION 1

Introduction 1

Phosphorus in soils: overview 1

Phosphorus acquisition by plants 2

Plant phosphorus uptake by non-symbiotic rhizosphere bacteria 5

Interplay of root exudate and rhizosphere microbiome for nutrient acquisition 7

Manipulating rhizosphere microbes and root exudates to recover P from soils 9

REFERENCES 13

CHAPTER 2 ROLE OF ROOT EXUDATES ON ASSIMILATION OF PHOSPHORUS IN YOUNG AND OLD ARABIDOPSIS THALIANA PLANTS 20

Synopsis 20

Introduction 20

Materials and methods 23

Plant growth conditions, phosphorus fertilization and collection of root exudates 23

Gas Chromatography-Mass Spectrometry (GC-MS) of root exudates 24

Statistical analysis of total root exudates data 25

Qualitative analysis of phosphate solubilizing ability of compounds derived from root exudates 25

Quantitative analysis of phosphorus solubilizing ability of water-soluble compounds (individually and combined) 26

Statistical analysis for quantitative phosphate release 27

Results 27

Effect of phosphate levels on *A. thaliana* root exudates at different plant developmental stages 27

Differences in compound-levels in the vegetative and bolting stages due to phosphate fertilization 28

Root-exudate metabolites solubilize calcium phosphate in soil and liquid media 29

The plant mediates changes in secretion of solubilizing compounds in response to phosphorus status and developmental stage 30

Discussion 31

Conclusion 36

Tables 38

Figures 39

REFERENCES 43

CHAPTER 3 PHOSPHORUS SOLUBILZING BACTERIA ISOLATED FROM THE RHIZOSPHERE OF WILD POTATOES ENHANCE GROWTH OF MODERN POTATO

Synopsis 49

Introduction 50

Materials and methods 52

Soil and growing conditions for wild potato type 52

Rhizosphere soil collection and bacteria isolation 53

Bacterial isolate identification 54

Qualitative and quantitative determination of phosphorus solubilizing ability 54

Potato growth response to P amendments and bacteria consortia 55

Calculations 56

Data analysis 57

Results 57

Differences in relative abundance from wild versus cultivated potatoes 57

Bacteria isolates solubilized plant-unavailable phosphate 58

Effects if phosphorus source and bacterial consortia on plant growth 58

Plant P content, P uptake efficiency and P use efficiency responses 59

Effects of phosphorus source and bacterial consortia on soil pH 59

Discussion 60

Conclusion 66

Tables 67

Figures 69

REFERENCES 71

CHAPTER 4 ROOT EXUDATES STIMULATE PHOSPHORUS SOLUBILIZATION ABILITY OF BACTERIA 76

Synopsis 76

Introduction 76

Materials and methods 80

Phosphorus solubilizing bacteria and root-exudate derived compounds 80

Quantitative analysis of the effect of root exudates on bacteria solubilization 80

Quantitative analysis of the effect of root exudates on bacteria growth 81

Application of root exudates to the soil 82

Data analysis 83

Results 84

Effects of root exudates on growth of phosphorus solubilizing bacteria 84

Effects of root exudate in enhancing the phosphorus solubilization ability of bacteria 85

Effects of root exudate soil amendment in plant biomass 85

Effects of root exudate on plant and soil nutrient content 85

Discussion 86

Conclusion 91

Figures 93

REFERENCES 99

SUGGESTION FOR FUTURE RESEARCH 105

APPENDICES 106

LIST OF TABLES

Table 1-1 Five top compounds from root exudates identified by Principal Component Analysis (PCA) 38

Table 2-1 Two-way ANOVA for total dry biomass and root, shoot and tubers individually and P concentration 71

Table 2-2 Phosphorus use efficiencies of potato plants amended with four P sources in greenhouse conditions with and without PSB consortia application 72

LIST OF FIGURES

Figure 1-1. Root exudate compounds diverge in response to plant developmental stage and fertilization rate 39

Figure 1-2. Qualitative phosphate-solubilization analysis of compounds using a calcium-phosphate based medium (NBRIP). (A) 3-Hydroxypropionic acid (B) malic acid and (C) nicotinic acid 40

Figure 1-3. Quantitative phosphate-solubilization analyzed by coupled plasma-Optical Emission Spectrometer (ICP-OES) in 13 identified compounds 41

Figure 1-4. Phosphate-solubilizer compounds showing changes in cumulative root secretion levels at three distinct developmental stages ($p<0.05$) in response to increasing phosphate addition 42

Figure 2-1. Mean phosphate-solubilization of individual bacteria isolates and consortium incubated in NBRIP phytin and calcium phosphate solutions 73

Figure 2-2. Mean biomass (oven-dry weight) for potatoes grown in a greenhouse under of various P sources, both with and without the inoculation of a consortia of P solubilizing bacteria 74

Figure 3-1. Maximum specific growth rate (μ_{max}) of phosphorus solubilizing bacteria incubated with different root exudates under inorganic calcium phosphate media 102

Figure 3-2. Maximum specific growth rate (μ_{max}) of phosphorus solubilizing bacteria incubated with different root exudates in phytin organic media 103

Figure 3-3. Effect of individual root exudates on dissolved P by phosphorus solubilizing bacteria 105

Figure 3-4. Root fresh weight (g) of corn plants after periodic addition of root exudates 106

Figure 3-5. Nutrient content (%) in corn plant roots for nitrogen and phosphorus 107

CHAPTER 1 RECOVERING PHOSPHORUS FROM SOIL: RHIZOSPHERE BACTERIA AND ROOT EXUDATES INTERACTION

Introduction

Phosphorus in soils: overview

Phosphorus (P) is present in a small amount in the Earth's crust (0.09 wt%). After P is liberated from minerals during weathering, it is sequestered into several soil mineral complexes, and organic matter limiting its accessibility to organisms (Filipelli et al. 2008). Despite this limitation, P is essential to all life forms for its structural role and energetic function. Phosphate backbones provide structural support to DNA, and phosphate stores and transfers energy in the adenosine triphosphate (ATP) molecules in all biological systems. The major source of P for human use and most P-bearing mineral is fluorapatite, $Ca_5(PO_4)_3F$. All apatite minerals contain phosphate oxyanions linked by Ca^{2+} and, after its dissolution, P is transformed into several forms that are less available to plants. P and other plant nutrients have been released from parent materials in the soil over centuries by various weathering processes including CO_2 from biochemical respiration, increasing acidity from degrading organic matter and organic acid exudates from plant roots (Jenny et al., 1994). However, the need to increase food production driven by population growth, along with more nutrient demanding plant cultivars, has led to an increase in the rate of P movement through terrestrial and aquatic systems (Schipanski and Bennett, 2012).

The modern P cycle is dominated by agriculture and human activity. The P extracted from rocks is now primarily used in the oxidized form, phosphate, in fertilizers. Growing consumption

of inorganic P fertilizer has contributed to major increases in crop yield since 1959 (Tilman et al., 2001). Between 1960 and 1995, global P use increased 3.5-fold and is expected to increase another 3-fold by 2050. However, the substantial increase in fertilizer application to agricultural land is no longer reflected in a significant increase in yield per unit of added P (Sattari et al., 2012). This is because approximately 80% of the applied P accumulates in the soil as residual and unavailable P (White, 2009). Sattari et al. (2012) showed that 71% of the global crop land area experiences P surpluses. Investigation of agronomic inputs and outputs associated with production of 123 crops globally found that inputs of P fertilizer (14.2 Tg of $P.y^{-1}$) and manure (9.6 Tg of $P.y^{-1}$) exceeded P removal by harvested crops (12.3 Tg of $P.y^{-1}$) (Van Vuuren et al., 2010). Although a variety of practices that contribute to enhance crop efficiency have been implemented through the years (Smil, 2000; Cassman et al., 2002; MacDonald, 2011), P accumulation in soils and its impacts in ecosystems remain problematic (Sattari et al., 2012). There are emerging strategies that aim to modify plant traits without modifying the plant genes. One of these approaches aims to harness plant root exudates and the rhizosphere microbiome to enhance nutrient use efficiency. Mobilization and availability of P within the rhizosphere occurs in response to changes in chemical and physical characteristics that can be induced by either plants or microbes. Despite the promising future of this technology, more research is needed to understand and manipulate plant-microbe interactions to improve crop nutrition. Consequently, the following chapters focus on the role of rhizosphere microorganisms and plant root exudates to enhance P availability for plants.

Phosphorus acquisition by plants

In soils, P can be found in diverse organic and inorganic forms with varying degrees of solubility. Most soils contain important amounts of P ranging between 200 and 3000 mg P/kg

soil. A very small fraction of this P (<1%) is immediately available to plants (Richardson et al., 2009). After natural weathering or fertilizer incorporation, most of the inorganic P is absorbed to soil constituents such as clays and organic matter or precipitates with other elements (Sanyal and De Datta, 1991). Soil pH drives P mineral complexation, for instance, phosphate ions (PO_4^{3-}, $H_2PO_4^-$, or HPO_4^{2-}) react with calcium ions (Ca^{2+}) to form calcium phosphate $Ca_3(PO_4)_2$ under alkaline conditions (pH>7), decreasing its bioavailability. A considerable portion of P in soil is also found in various organic complexes (Jungk et al., 1993). Soil P organic complexes are comprised mostly comprised of monoesters, diesters, and phosphonates (Condron et al., 1990). Up to 38% of the total organic P in some soils may be comprised of monoesters such as phytate (Hayes et al., 2000). To be available to plants, inorganic P must be desorbed, or solubilized, and organic P must be mineralized from pools of total P to release orthophosphate anions (PO_4^{3-}) into the soil solution.

The limited capacity for P replenishment in the soil solution, slow P diffusion rates in soil, and temporal low concentrations of orthophosphate in the soil solution are the major factors contributing to P deficiency in plants (Bieleski, 1973). The uptake of P from soil depends on the ability of the plant to intercept P by exploring soil nutrient-rich patches and increasing the overall density of root hairs near the surface of the soil where P is often applied. This plant strategy can be genetically enhanced by developing ‘topsoil foraging plants’ through greater production of axial roots, shallower axial root growth angles, greater lateral root density, and greater root hair length and density (Lynch, 2019). However, traits that reduce the metabolic cost associated with soil-root exploration need to be targeted and incorporated together with anatomical traits (Lynch, 2019). Furthermore, when plants reach the P rich patches, the encountered P is yet in plant unavailable forms. Consequently, other physiological and internal

biochemical complementary strategies need to be utilized. These include the reduction of above ground growth rate, remobilization of internal P from storage organelles, the induction of high-affinity phosphate transporters located in root hairs, the release of P mobilizing compounds and modulation of the root microbiome by plant molecules (Shen et al., 2011; Sharma et al., 2013; Alori et al., 2017; Pascale et al., 2020). The release of low-molecular weight organic acids and other metabolites from roots can also affect the P availability by several different mechanisms (Jakobsen et al., 2005; Jones, 2011).

The two main strategies used by plants to enhance the dissolution of P containing minerals or mineralize organic P complexes differ. First, the exudation of low molecular weight organic molecules is proposed as a key mechanism for increasing plant inorganic P uptake in soils. The mechanism of action involving these low molecular weight organic acids include: 1. directly promoting the dissolution of sparingly soluble minerals containing inorganic P; 2. shifting pH and chemical equilibrium in soil solution; 3. altering the surface characteristics of mineral particles or occupying ligand exchange surfaces; and 4. competing with P for absorption sites on soil colloids, thereby releasing precipitated and absorbed P (Wang et al., 2015; Menezes-Blackburn et al., 2016). Although pH reduction is often correlated with P solubilization, some authors have not shown such relationship (Asea et al., 1988; Park et al., 2009). Plants can also secrete phosphatases to mineralize organic P: however, this mechanism is deemed to be less effective as a plant strategy to acquire P from soils compared to microbially-mediated organic P mineralization. Some organic acids released by plants and microbes, such as oxalic acid and citric acid have also been found to destabilize soil organic matter together with hydrolytic enzymes to release P (Clarholm et al., 2015).

Many of these root-exudate derived compounds are also used for plant-microbe beneficial communication in the rhizosphere to deal with biotic or abiotic stressors at different growth stages. For instance, plants can modulate the root exudate profile to selectively release compounds at varying developmental stages of growth when demands of P are higher. Pantigoso et al. (2020; see Chapter 2) showed that certain root exudate derived metabolites such as nicotinic acid, malic acid, and 3-hydroxypropionic acid were released under low P status and later stages of growth, when demands of P were higher. Further, these compounds were found to dissolve P. It should be noted that the root exudate profile is also influenced by other factors including soil mineralogy, pH, and rhizosphere microorganisms which use these substrates as a carbon, signaling or energy source (Huang et al., 2014).

Plant phosphorus uptake by non-symbiotic rhizosphere bacteria

Rhizosphere free-living microorganisms actively mediate P acquisition for plants by participating in all three major components of the soil P cycle: dissolution-precipitation, sorption-desorption, and mineralization-immobilization. Among the most commonly isolated bacteria are members of the genus *Bacillus, Pseudomonas*, and *Enterobacter*; however, a considerable number of other gram-negative (e.g., *Burkholderia spp*.) and gram-positive (e.g., *Arthrobacter spp*.) bacterial species have been found to promote solubilization (Bhattacharyya et al. 2012; Sharma et al. 2013). Microorganisms mineralize, immobilize, and solubilize various organic and inorganic fractions of P in soils. The magnitude of phosphorus solubilization and mineralization is highly dependent on the source of P. For instance, Wan et al. (2020) assessed the solubilization of eight Phosphorus Solubilizing Bacteria (PSB) genera and five P different sources including $Ca_3(PO_4)_2$, phytate, $FePO_4$, $AlPO_4$ and elemental P, finding variable performance of bacteria depending on the strain type and P source. Furthermore, the ability of

PSB to solubilize and mineralize P has been observed to co-exist in the same bacterial strains (Guang-Can et al., 2008; Wan et al., 2020).

Two main strategies used by PSB for enhancing plant P availability in soils are: 1. the enhanced dissolution of P-containing minerals through soil acidification and/or the release of metal complexing agents such as organic anions, siderophores, protons, hydroxyl ions and CO_2 (Jones and Oburger, 2011). 2. the enzymatic breakdown of organic P through the synthesis and release of phosphatases, phytases (a major component of organic P in soil), and phosphonatases and C-P lypases that break the C-P bond of organo-phosphanates by microorganisms (Rodriguez et al., 2006; Richardson and Simpson, 2011). Phosphatase production has been shown to be repressed by added P (Nannipieri et al., 2011); thus, the former strategy may be the most relevant under highly fertilized agricultural soils with low organic matter content. Additionally, beneficial rhizobacteria enhance the nutrient status for its host plants in different ways by secreting phytohormones such as indole-3-acetic acid, ACC deaminase, cytokinin, and gibberellic acid to promote root growth (Vessey et al., 2003; Rouphael and Colla, 2018). Many of these modes of action can be present in one strain (Cattelan et al., 1999; Wan et al., 2020). For instance, bacteria that solubilize forms of unavailable phosphate can enhance growth and yield but not necessarily P content of the host plant (De Freitas et al., 1997). Furthermore, it has been suggested that PSB do not mobilize sufficient P to change the crop nutritional status under field conditions. This is due to available P releases to the soil solutions from microbial solubilization being inadequate to supply microbial and plant demands (Raymond et al., 2021). However, highly effective PSB inoculants tested under soil and field conditions have shown opposing evidence (Baas et al., 2016, He et al., 2019, Afkairin et al., 2021). Nonetheless, broad effectiveness of microbial

inoculants under a large range of field, soils, and weather conditions remains limited (Salomon et al., 2020).

The inconsistent effectiveness of soil microbial inoculants is often attributed to establishment and survival traits (e.g., motility, fast growth, and quick dispersal). Introducing inoculant strains to soils expose them to competition for nutrients with indigenous microbes that can be better adapted to local environmental conditions. Furthermore, microbial colonization of the rhizosphere is hampered by other various abiotic factors including pH, moisture, texture, nutrients, and salinity (Thilakarathna et al., 2017). Inoculants need to thrive in different environments, thus their survival and functionality in foreign conditions is key for its effectiveness (Kaminsky et al., 2019). Alternative approaches to potentiate microbial inoculants are urgently needed.

Interplay of root exudates and rhizosphere microbiome for nutrient acquisition

Plants produce a plethora of chemically diverse primary and secondary metabolites many of which exert bioactive effects on microorganisms, affecting their composition and function. Plant root exudation, which represents one-third of photosynthesized carbon, plays a major role in determining the outcome of individual and community level chemical interactions (Pausch and Kuzyakov et al., 2018; O'Banion et al., 2019). Plant exudates are the primary form of communication of the plant with their biotic surroundings which facilitate a number of responses such as nutrient absorption, resource competition, same species signaling, attraction of beneficial microorganisms, along with many other interactions. Major organic compounds include sugars, amino acids, organic acids, phenolic compounds, and other secondary metabolites including coumarins, glucosinolates, benzoxazinoids, camalexin, and triterpenes. (Jacoby et al., 2020). By providing a diverse carbon rich environment, plant species harbor a distinctive microbial

community in their rhizosphere which, in turn, functions as driver of plant productivity (Badri and Vivanco, 2009; Mönchgesang *et al*., 2016; Trivedi et al., 2020). Major outcomes of plant-microbe signaling through root exudates are the recruitment, shaping, maintenance, control of microbial activity, the induction of plant immunity and biocontrol from plant pathogens, the support of plant nutrition and stress tolerance, and the modulation of above and below ground interactions (Venturi and Keel, 2016).

The major type of signaling mechanisms known to occur in the rhizosphere can be divided into three categories according to the direction of the communication and players involved: 1. Microbe-to-microbe: microbial intraspecies and interspecies signaling driven by quorum-sensing (QS) signal molecules allowing microbial communities to form and synchronize their behavior; 2. Microbe-to-plant: signaling from plant to microorganisms via plant-secreted molecules, shown to participate in several reported plant beneficial interactions, and 3. Plant-to-microbe interactions: signaling from microorganisms to plants affecting plant gene expression (Badri and Vivanco, 2009; Venturi and Keel, 2016). This interaction with the plant can happen at the single-strain or at the microbiome level. Individual microbes and the communities can form beneficial, neutral, or detrimental interactions. However, the mechanisms and signaling molecules associated with given groups and pathways for this communication are not fully known (O'Banion et al., 2019).

Recent research proposes that bacterial root microbiota, stimulated by specific chemical compound groups (i.e., coumarins), is an integral mediator of plant adaptation to nutrient-limiting soils (Harbort et al., 2020; Voges et al., 2019). These studies have shown that root secreted coumarins are inducible under iron starvation and mediate an interaction between the host and the microbial commensals that improve host iron nutrition. This genotype-environment

interaction suggest that the root microbiota is an integral component of plant edaphic adaptation to growth in iron-limiting soils. Similarly, a study from Koprivova et al., (2019) used a loss of function mutant (expressing pathway gene) to show that root-specific camalexin biosynthesis controls the plant growth-promoting effects of multiple bacterial strains; however, no nutritional component was shown in this study. Brisson et al. (2021) showed that amino acids, shikimic and quinic acid, increased under phosphate stress are preferentially absorbed by microorganisms that were positively correlated with root growth (Zhalnina et al., 2018). Other studies have demonstrated the direct integration between the plant phosphate status, associated root microbiota, and the soil P content (Castrillo et al., 2017; Finkel, et al., 2019). This may suggest that the plant modulates its root exudation profile to stimulate the proliferation of groups of microorganisms that aid plants with nutrient acquisition.

Manipulating rhizosphere microbes and root exudates to recover P from soils

Several strategies to manage and manipulate the rhizosphere microbiome for plant health are proposed: however, our ability to successfully apply them on a broader scale are limited (Chaparro et al., 2012; Wallenstein, 2017). Altering the rhizosphere by directly inoculating bacteria or fungal strains in soils near plant roots is becoming a widely used strategy, although fine-tuning aspects of root colonization, competition with native microbiota, and functionality under highly managed agricultural settings have not been fully achieved (Kaminsky et al., 2019; Salomon et al., 2022). This can be partially explained by the spatial and temporal variability that the microbes experience in the field after application and the technical challenges encountered in the selection, formulation, and production process (Kaminsky et al., 2019). In order to be fully implemented an effective plant-specific inoculant must be able to establish associations with the

resident microbiome in order to deploy its functional potential. This and other alternative approaches are discussed below.

Structure and activity of microbial communities are strongly influenced by the availability and composition of organic materials in soils. Different organic materials with often undefined biochemical composition such as organic amendments (e.g., manure) are used to increase diversity under the expectation that this would lead to enhanced functionality (Griffiths and Philippot, 2013). A more targeted approach proposes the use of identified and known organic amendments to steer desired outputs in an advantageous direction stimulating functional groups of bacteria via management practices (Chaparro et al., 2012; van Agtmaal et al., 2015). Adding organic materials containing precursors for metabolic pathways could lead to the production of biocontrol, nutritional and anti-stress compounds from indigenous soil bacteria (Garbeva and Weisskopf, 2019). For instance, volatiles emitted belowground by bacteria differentially impact plant nutrient content indicating that volatiles emitted belowground can affect the nutritional status of plants (Martin Sanchez et al., 2020).

Research in synthetic biology has begun to explore optimization of engineered plant growth promoting rhizobacteria to develop strains which do not suffer the ecological shortfalls of their natural progenitors. Transfer of plant growth promoting bacterial traits on mobile genetic elements into selected bacterial ‘chassis’ or whole communities can be used to customize effective rhizosphere bacteria with desirable traits for specific purposes (Haskett, Tkacz and Poole, 2021). Many mechanisms of nutrient mobilizing bacteria including nitrogen, P solubilization and phytohormone biosynthesis have been elucidated in a fine detail to be genetically engineered (Haskett, Tkacz and Poole, 2021). For instance, a recent study used combinatorial synthetic biology-based approach to generate 82 biochemically diverse phytase

enzymes which were integrated into the genomes of three bacterial strains. In this study, a fraction of the enzymes was able to mineralize phytate and promoted increased Arabidopsis growth (Shulse et al. 2019). Similarly, other important advances have been made recently toward developing application of N-fixing rhizobacteria that are associated with cereals (Ryu et al., 2020), phytohormones to enhance biomass and tolerance to abiotic stresses, and improving colonization by resident bacteria (Zuniga et al., 2018; Guo et al., 2019). However, an important prevalent challenge is to engineer rhizobacteria with large quantities for plant assimilation (e.g., N-fixing, P-solubilizing) without impacting the energetic balance in the plant (Haskett, Tkacz and Poole, 2021).

Root exudates have been proposed as a plausible mechanism for fine tuning the plant microbiome, because of its chemically diverse composition with signaling properties and its capacity to influence the composition and function of the root microbial community (Jacoby et al., 2020). Earlier research has explored genetic variation to induce traits that increase the exudation of organic acids or positive associations with symbiotic or non-symbiotic soil organisms that favor plant nutrition. For instance, P-efficient crop lines using natural and induced genetic variations for compound groups such as carboxylates (Pearse et al., 2007) and phosphatases have been developed and tested (George and Richardson, 2008). Although its effectiveness is evident under controlled conditions, it showed variable success when evaluated in soils. An important limitation of combining multiple P-efficiency traits into elite cultivars by conventional breeding is that they need to meet other agronomic and commercial characteristics before their release.

More recently, research on root exudate traits from wild relatives of modern crops offer opportunities to reduce the use of fertilizers and pesticides (Preece and Penuelas, 2020; Lannucci

et al., 2017). Because wild plant species often thrive in nutrient-poor soils and are able to successfully grow, reproduce, and maintain adequate nutrition in plant tissues, it is hypothesized that wild types may produce different extracellular enzymes and a higher proportion of organic anions resulting in more efficient solubilization of P. Differential microbial composition patterns in the rhizosphere of modern crops relative to their wild progenitors have been reported in potato and maize (Pantigoso et al., 2020; Schmidt et al., 2020). For instance, the distinct rhizosphere microbiome of wild potatoes was correlated with the higher capacity of wild types to uptake and utilize P. The tight association between root exudates and rhizosphere microbiome and the latest discoveries on the heritability component of the plant microbiome support the efforts to harness root exudates from wild relatives to improve functional features of modern crops (Peiffer et al., 2013; Ruger et al., 2021). Recent evidence shows that the manipulation of root exudate composition from root apices enriching certain bacterial communities throughout the root system is feasible (Kawasaki et al., 2021). The advantage of this approach is that exudates are deposited at the root-soil interface where they are most likely to impact microbial growth. Additionally, the release of substrates along the host life cycle can maintain selection pressure on a given established community. Alternative approaches encourage the identification, selection and use of certain root exudates to directly mobilize nutrients in soils and use them in combination with elicitors to encourage inoculant proliferation (Pantigoso et al., 2022, see Chapter 4).

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CHAPTER 2 ROLE OF ROOT EXUDATES ON ASSIMILATION OF PHOSPHORUS IN YOUNG AND OLD ARABIDOPSIS THALIANA PLANTS[1]

Synopsis

The role of root exudates has long been recognized for its potential to improve nutrient use efficiency in cropping systems. However, studies addressing the variability of root exudates involved in phosphorus solubilization across plant developmental stages remain scarce. Here, we grew *Arabidopsis thaliana* seedlings in sterile liquid culture with a low, medium, or high concentration of phosphate and measured the composition of the root exudate at seedling, vegetative, and bolting stages. The exudates changed in response to the incremental addition of phosphorus, starting from the vegetative stage. Specific metabolites decreased in relation to phosphate concentration supplementation at specific stages of development. Some of those metabolites were tested for their phosphate solubilizing activity, and 3-hydroxypropionic acid, malic acid, and nicotinic acid were able to solubilize calcium phosphate from both solid and liquid media. In summary, our data suggest that plants can release distinct compounds to deal with phosphorus deficiency needs influenced by the phosphorus nutritional status at varying developmental stages.

Introduction

Phosphorus is an essential element for plant growth and development (Lynch, 2007), and a non-renewable resource (Cordell et al., 2009; Gilbert, 2009). Despite the fact that the total

[1] This research was originally published in PloS one Journal by Pantigoso, H. A., Yuan, J., He, Y., Guo, Q., Vollmer, C., & Vivanco, J. M. (2020). Role of root exudates on assimilation of phosphorus in young and old Arabidopsis thaliana plants. *PloS one*, *15*(6), e0234216.

amount of phosphorus is high in most agricultural soils, crop yields are often limited by low availability due to the non-soluble form and low mobility of this nutrient (Torri et al., 2017; Wei et al., 2016). It has been estimated that residual phosphorus fertilizer known as 'phosphorus legacy' in soil can be sufficient to sustain crop yield for the next century and could alleviate expected phosphorus shortages in the next 50 years (Zhu et al., 2018). Hence, studies addressing potential solutions to exploit soil phosphorus reserves are needed.

Plants have developed several strategies for acquisition of phosphorus in low nutrient environments mainly by modifying root structure and changing the soil chemical properties in the rhizosphere (Neumann & Romheld, 2000). These mechanisms include longer root formation and increases of root:shoot ratio allowing the transport of phosphorus from the roots to the shoots (Bates & Lynch, 2001; Heydari et al., 2019). Certain plants such as *Lupinus* sp. can promote the formation of cluster roots to secrete phosphorus solubilizers such as citrate and malate in sufficient quantities to lower the rhizosphere pH, thus enhancing the movement of phosphorus and consequently plant uptake (Braum & Helmke, 1995; Hocking & Jeffery, 2004). The secretion of phosphorus solubilizers is not restricted to cluster root-forming plants. Several other species such as alfalfa, spinach and radish have also been documented to increase the efflux of organic anions as a result of a lack of phosphorus available in the soil (Lipton et al., 1987; Gerke, 2015; Zhang et al., 1997).

Increasing phosphorus solubility can also be achieved by modifying the rhizosphere chemistry. Root-secreted phosphorus solubilizers are capable of increasing solubility of a variety of insoluble phosphorus forms in the soil such as organic phosphorus, inorganic phosphorus like calcium phosphate, and humic substances bonded to phosphorus anions such as Al (III) and Fe

(III). They can be classified as protons or OH^-/HCO_3^- equivalents, redox equivalents and di- and tricarboxylic acid anions (Gerke, 2015). The mechanisms used by plants to solubilize phosphorous vary according to the plant species, nutritional status of the plant, and soil and environmental conditions (Hinsinger, 2001). However, organic acids such as oxalate, citrate, and malate are recurrent in a variety of plant species and thereby are the most studied means used by plants to solubilize phosphorous (Gerke, 2015). Considerably less research has been performed to explore the total root exudate profile and to identify other compounds exerting similar and complementary functions in the rhizosphere.

All plants share a similar need for phosphorus, but this need differs broadly based on the crop type and its developmental stage (Marschner, 1998; Marschner, 1995). In general, most crops require early phosphorus supplementation for optimum yield (Grant et al., 2001). Nevertheless, higher amounts of phosphorus in later growth stages are required proportional to increases in the biomass of the plant (Marti & Mills, 1991a; Marti & Mills, 1991b; Awonaike et al., 1991; White & Veneklaas, 2012). Due to the variation of phosphorus demand during the plant's lifetime, it becomes necessary to fully understand fluctuations of root exudates as a means to solubilize the phosphorus present in the of the soil.

In this study, we tested the effect of three phosphate fertilization levels on root exudate composition of *A. thaliana* at distinct developmental stages. We hypothesized that phosphate status will promote a shift in the relative concentrations of certain root exudates with inorganic phosphate solubilizing properties and certain metabolites involved in phosphate solubilization will be inhibited under high phosphate concentrations. The results showed that the total exudates changed in response to the addition of phosphate, and that certain metabolites were reduced under increasing phosphorus amendments at varying growth points. As a proof of concept, some

of the metabolites that decreased in quantity to the phosphorus addition were tested and four of them were found to solubilize phosphate.

Materials and methods

Plant growth conditions, phosphorus fertilization and collection of root exudates

Arabidopsis thaliana (L.) Heynh wild-type Columbia seeds were obtained from Lehle Seeds (Round Rock, USA) and surface sterilized with Clorox bleach (Sodium Hypochlorite, 8.25%) for 1 min. Seeds were rinsed with distilled water 3 times and plated on different phosphorus levels of Murashige and Skoog (MS) agar (1.5%) (supplemented with 3% sucrose) in square plates. The plates were placed vertically in a growth chamber (Percival Scientific) at 25 ± 2 °C with a photoperiod of 16 h: 8 h, light: dark for germination. The germinated seedlings were grown in three phosphate levels: full strength (100%, 1.25 mM), half (50%, 0.625 mM) and a quarter (25%, 0.3125 mM) in solid MS medium as described above. The three phosphate levels used in this study did not stimulate the plant starvation response, which is generally activated at values below 2 μM phosphorus in the soil solution (Fohse et al., 1988). Instead, we followed a low and high fertilization regime used commercially in agriculture (Sattari et al., 2012). Phosphate concentration was adjusted with Na_2HPO_4 and NaH_2PO_4, and phosphate -free MS medium was used as basal medium. After seven days of growth, the seedlings were transferred to six well plates, each well containing 5 mL of liquid MS with 1% sucrose with one individual seedling per well containing distinct phosphate levels as described above. The seedlings grown in solid MS at the particular phosphate level were placed under the same phosphorus level under liquid M conditions. All the plates were placed on an orbital shaker at 90 rpm under 25 ± 2 °C and lit by cool white fluorescent light (45 μmol m^{-2} s^{-1}) with a photoperiod of 16 h: 8 h, light: dark. The

nutrient solutions were replaced every week by transferring the plants to new six well plates with 5 ml of fresh liquid MS medium under the same phosphate levels as stated above.

Root exudates were collected as follows: 1–3 days after transplanting for seedling stage, 8–10 days after transplanting for vegetative stage, and 15–17 days after transplanting for bolting stage. Prior to the collection of root exudates, plants were removed and washed mildly with sterile water and subsequently placed in new wells containing 5 ml of sterile water for two days. We used sterile distilled water to prevent the interference of exogenously supplemented salts and sucrose present in the Murashige and Skoog media in subsequent GC-MS analyses of the root exudates. The solution in which the plants were floating was collected and considered as the root exudate. The solution of one plate containing six wells with six individual plants was pooled and considered as one replicate. We used three replicates for each stage under three phosphorus levels. The root exudates were filtered through a 0.45 μm (Millipore, MA) to remove root sheathing and root border-like cells and were freeze-dried and stored at -20 °C for further analyses.

Gas Chromatography-Mass Spectrometry (GC-MS) of root exudates

To characterize the chemical composition, root exudates were processed as described by Chaparro et al, (2013) and subjected to gas chromatography—mass spectrometry GC-MS analyses at the Genome Center Core Services, University of California Davis. Extracts were briefly dried under nitrogen gas and then methoximated and trimethylsilylated. The derivatives were analyzed by an Agilent 6890 gas chromatograph (Santa Clara, CA) containing a 30-m-long, 0.25-mm inner diameter rtx5Sil-MS column with an additional 10 m integrated guard column. Metabolites were detected using the BinBase algorithm (Fiehn et al., 2008) and identified by

comparing the retention index and mass spectrum of each analyte against the Fiehn mass spectral library from the West Coast Metabolomics Center, University of California Davis.

Statistical analysis of total root exudate data

To discover the differential expression levels of compounds across the plant's growth stages and fertilizer levels, R statistical software (R Core Team, 2017) was used to perform principal components analysis (PCA) on all annotated compounds. For each of the following analyses, we performed a PCA by first separating the total root exudate data by plant growth stage. Within each plant growth stage (e.g. bolting), we performed a centered and scaled PCA. By verifying that the first two principal components explained a sufficient amount of the variance in the compound(s) expression levels, we were able to determine which compounds had the highest correlation with these components through the magnitude of the variance. The variances are representative of a compound at each growth stage and at a particular phosphate level. The largest variance represents the highest correlation to the principal components. This method allowed us to determine which compounds explained most of the variance across the fertilizer levels and for each of the plant's growth stages.

Significant differences between phosphorus amendment and compound counts level per developmental stage were analyzed using a one-way ANOVA. Tukey HSD test was used to identify significance ($p < 0.05$) among phosphorus treatments.

Qualitative analysis of phosphate solubilizing ability of compounds derived from root exudates

From a list of selected compounds, only 13 of them were diluted in ddH_2O at the desired concentration (100 mM) (Table 1-1). We used the reported concentration of organic acids in the rhizosphere (1 μM to 100 mM) as reference to select the concentration of the compounds tested

in this study (Jones, 1998; Veneklaas et al., 2003; Strom et al., 2005). These 13 compounds were qualitatively evaluated for their phosphate solubilizing abilities on National Botanical Research Institute phosphate growth medium (NBRIP) solid medium containing: 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 0.2 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.03 g $MnSO_4$, 0.003 g $FeSO_4 \cdot 7H_2O$, 12 g agarin 1 L water, pH: 7.0–8.0 (Nautiyal, 1999). All compounds used in this experiment were purchased from Thermo-Fisher Scientific. The specific solution (100 µL) of each compound was placed on NBRIP solid medium. The plates were inoculated at room temperature and let to sit overnight. Phosphate solubilizing ability was visually judged as a clear halo around every drop of solution containing the given compound. Briefly, the test of the relative efficiency of isolated metabolites was carried out by selecting the metabolites that were capable of producing a halo or clear zone in the surrounding medium by the dissociation of inorganic minerals such as calcium phosphate.

Quantitative analysis of phosphorus solubilizing ability of water-soluble compounds (individually and combined)

For quantification, 35 µL of the same concentration previously tested (100 mM) of the 13 compounds were added to 5 mL liquid NBRIP medium resulting in a final concentration of 7mM. The tubes were then placed at a continuous agitation at 150 rpm on a rotary shaker for 72 h. Afterwards, the solution was centrifuged at 6000 rpm for 5 min, and the supernatant was filtered with 0.2 µm filter (Thermo-Fisher Scientific). Liquid NBRIP medium without compound addition was used as control. The concentration of phosphorus in the supernatant was analyzed according to with the protocol of Soltanpour et al., (1983) and measured by means of inductive coupled plasma-optical emission spectrometer (ICP-OES) (Perkin Elmer 7300DV) at the Soil,

Water and Plant Testing Laboratory of Colorado State University. This experiment and analysis were repeated twice with 3 repetitions.

In order to determine the potential cumulative effect of 3-hydroxypropionic acid, malic acid, nicotinic acid, and glutamic acid, they were combined and the available phosphorus (mg l^{-1}) was determined by OES-ICP. A compound mixture that included the previously tested concentration (7 mM per compound) was assayed in order to compare if the combination of compounds would equal or surpass the effect of a single compound. Briefly, 35 µL (100 mM) of each compound was added to 5 mL liquid NBRIP medium resulting in a final concentration of 7mM. Each compound added to the pool had a concentration of 7 mM. Thus, the combination effect of four compounds were tested in a liquid NBRIP medium.

Statistical analysis for quantitative phosphate release

Significant differences between phosphorus content measured in NBRIP liquid media was analyzed using a one-way ANOVA. Tukey HSD test was used to identify significant treatments in multiple comparisons. P-value were considered significant below 0.05. Assumptions of normality and homogeneity of the data were confirmed prior to the analysis.

Results

Effect of phosphate levels on* A. thaliana *root exudates at different plant developmental stages

The effect of increasing phosphate at three concentrations on the root exudates of *A. thaliana* was analyzed at various developmental stages. In total, 456 compounds were detected by GC-MS among the treatments. The data set was reduced to 201 annotated compounds, and only these were kept for statistical analysis. The grouping of the compounds in

the plot maintained the same pattern even after subtracting the non-annotated compounds (Supplementary1-1 Figure), suggesting that all of the differentially expressed compounds were indeed annotated by the GC-MS analyses.

The variability in our data, after subtracting the non-annotated compounds, was analyzed using a principal component analysis (PCA) where variability of component 1 (PC1) accounted for 29.8%, while component 2 (PC2) accounted for 21.7%. We determined that the plant's developmental stage was responsible for the separation of the compounds in three marked groups: seedling, vegetative and bolting (Figure 1-1A) as previously reported (Chaparro et al., 2013; Chaparro et al., 2014; Yuan et al., 2015). When analysis included fertilizer levels segregation was observed in certain developmental stages and phosphate levels (Supplementary 1-2 Figure). Overall, phosphate levels did not cause a significant separation on the root exudate patterns at the seedling stage, but the separation was observed in the vegetative and particularly in the bolting phases (Figure 1-1A). In the vegetative stage, a clear parting was observed at 25% phosphate compared to the 50 and 100% treatments (Figure 1-1B). In the bolting stage, there was a clear division between the three fertilizer levels, but the 50% level was the most distant rate (Figure 1-1C).

Differences in compound-levels in the vegetative and bolting stages due to phosphate fertilization

A separate analysis was performed to determine correlations between different and highly expressed compounds for a specific fertilization level and developmental stage. We focused our analysis on just the 25% phosphate at the vegetative stage, and all three treatments at the bolting stage because these treatments had the highest dissimilarity in the PCA. In addition, the 50% and

100% treatment at the vegetative stage were grouped because they were clustered in the PCA. For each of these treatments, we found the five top compounds that explained the largest proportion of the variance in the principal components (Table 1-1). In addition, the abundance of the compounds based on phosphate level and growth stage were determined (Supplementary 1-3 and Supplementary 1-4 Figures). We found some interesting patterns, such as that some compounds decreased expression upon increased fertilization (e.g., 3-hydroxypropionic acid, malic acid, galactinol), while other compounds showed a positive correlation with phosphate amendment (e.g., guanine, glutamic acid, sophorose).

Root-exudate metabolites solubilize calcium phosphate in solid and liquid media

We then tested the ability of some of the selected compounds irrespective of their abundance upon fertilization to solubilize phosphate solubilization. We found that 3-hydroxypropionic acid, malic acid, and nicotinic acid formed a clear halo when tested at a concentration of 100 mM, indicating the ability of this compound to release free phosphate from calcium phosphate (Figure 1-2). At 100 mM, none of the remaining tested compounds solubilized phosphate detectably.

The phosphate -solubilizing effect of the selected compounds was further tested using a more sensitive technique (OES-ICP) where the compounds where tested at a final concentration of 7 mM in liquid NBRIP medium. The results showed that in addition to 3-hydroxypropionic acid, malic acid, and nicotinic acid, glutamic acid also had phosphate solubilizing ability. Using this method, glutamate, malate, and nicotinic acid solubilized approximately ten times the amount of phosphorus present in the control (5.34 mg L^{-1}) whereas 3-Hydroxypropionic acid solubilized almost fifteen times more (Figure 1-3).

Further analysis aimed to test the combined effect of all the four compounds on NBRIP medium at 7 mM concentration of each compound resulting in a twenty times higher available phosphate (101.21 mg L^{-1}) compared to the control (Figure 1-3).

The plant mediates changes in secretion of solubilizing compounds in response to phosphorus status and developmental stage

We further analyzed the exudation level upon phosphate fertilization of 3-hydroxypropionic acid, malic acid, nicotinic acid, and glutamic acid in the different developmental stages. Depending on the compound, our results showed that the cumulative secretion levels of the compounds increased, decreased or remained statistically similar ($p>0.05$) as a function of phosphate amendment (Figure 1-4). At the seedling stage, changes in cumulative secretion of the four solubilizers was not related to phosphate level significantly which agrees with the PCA analysis (Figure 1-1A). Interestingly, at the vegetative stage, nicotinic acid and 3-hydroxypropionic acid showed higher abundance at the lowest phosphorus level (0.3125 mM) and decreased significantly ($p<0.05$) for the two higher levels (0.625 and 1.25 mM). Malic and glutamic acid followed a similar pattern; however, their changes were not statistically significant. At the bolting stage, differences of compound cumulative secretion were observed for glutamic acid, malic acid and nicotinic acid, but not for 3-hydroxypropionic acid which did not increase or decrease following an incremental phosphorus level. Cumulative secretion of malic acid was reduced significantly from 0.3125 to 0.625 mM phosphate treatments, and then incremented its cumulative secretion for the highest treatment (1.25 mM). However, secretion levels of malic acid for the two highest phosphate treatments (0.625 and 1.25 mM) were below the value for the lowest rate (0.3125 mM) (Figure 1-4A). Similarly, cumulative secretion of nicotinic acid increased significantly from 0.3125 to 0.625 mM but dropped for 1.25 mM of phosphate (Figure

1-4C). Lastly, glutamic acid consistently increased upon higher phosphorus fertilization reaching its peak at 1.25 mM of phosphate (Figure 1-4B).

Discussion

For the most part, the influence of phosphorus fertilization on root secretion has been studied at specific developmental stages (Carvalhais et al., 2011; Keerthisinghe et al., 1998), among genotypes of the same plant species (Vengavasi & Pandey, 2018) or between different species (Li et al., 2007). For instance, Vengavasi et al., (2018) found cultivar-dependent differences in root exudation of soybeans grown under phosphorus -sufficient versus phosphorus -deficient conditions. It is worth noting that these plants were sampled at the reproductive stage of growth, a metabolically active stage with higher demand for energy and phosphorus nutrition. In a different study, the authors found differences in root exudation in maize seedlings grown in phosphorus -sufficient and phosphorus -deficient conditions (Carvalhais et al., 2011). In contrast, here we studied the interplay of increasing phosphorus fertilization on root exudation at different plant developmental stages (seedling, vegetative and bolting) in *A. thaliana*.

Our results showed that the root exudate profiles were similar within the seedling stage across all phosphate fertilization treatments (Figure 1-1A). Thus, we hypothesized that at this growth stage roots did not respond to phosphorus fertilization. *A. thaliana* is considered a plant that can thrive in marginal soils where optimum nutrient conditions are limited (Mitchell-Olds & Schmitt, 2006), and at early developmental stages the plant does not require high amounts of phosphorus as a mechanism to cope with poor soil conditions (White & Veneklaas, 2012). In fact, it has been shown that the reserves of phosphorus in the seeds of several plants, including *Brassicaceae* species, can support seedling growth in a medium lacking phosphorus

for at least four weeks after germination (Nadeem et al., 2011; Nadeem et al., 2012; Bolland & Baker, 1988; White & Veneklaas, 2012).

In contrast, maize, a monocotyledonous plant, possesses two genes induced by phosphate starvation in its genomes compared to five in eudicots such as *A. thaliana* (Yang et al., 2007; Hasan et al., 2016); suggesting a difference in phosphate responsiveness between these two plant groups. In addition, signs of phosphorus scarcity in eudicots (e.g. *A. thaliana*) is often observed at later stages of growth (Johnson et al., 1996). In the vegetative stage, the root exudates at 50% and 100% phosphate showed greater visual similarity in the PCA than the exudates at 25% evincing an initial sensing from the plant in response to its phosphorus demand. At the bolting stage, the three levels of phosphate fertilization had distinct root exudation patterns. These results may suggest that as the plant ages the demand for phosphorus increases, as evidenced by the differential root exudation profiles (Neumann et al., 1999). This result is in accordance with Tawaraya et al., (2014), who showed that phosphorus content increased in shoot and root-dried soybean tissues as the plant developed, and that root exudate content, collected on day 1, 5, 10 and 15 of growth, increased for phosphate -depleted compared to phosphate -sufficient treatments.

Our results suggest that plants in the vegetative stage sense only the 25% phosphate treatment as being low, whereas plants at bolting stage sense both 25% and 50% phosphate as low. This pattern of incremental phosphorus requirement during progression in plant development is common in annual plants such as *A. thaliana* (White and Veneklaas, 2012). For instance, *Brassica napus* L. requires an incremental supply of phosphorus at flowering onset, which is critical for protein and oil synthesis, and the development of seeds (Lickfett et al., 1999; Rose et al., 2008). It is worth noting that the intermediate phosphate rate (50%) at bolting stage

was largely separated (in the PCA plot) compared to 25% and 100% treatments, indicating a higher dissimilarity in root exudation composition. This could be due to the fact that the functions of plant metabolites are diverse and are not restricted only to nutrient acquisition. For instance, root exudates can be substrates, chemotactic or signaling molecules that regulate plant root and microbial interactions (Venturi & Keel, 2016). Such plant modulation can be specific to developmental stages (He et al., 2019).

The PCA data allowed for the visual determination of changes in root exudation composition between plant developmental stages and phosphate fertilization levels. We then developed a list of compounds based on those differences observed in the PCA in response to phosphorus nutrient status; and four of those compounds (i.e. 3-hydroxypropionic acid, nicotinic acid, glutamic acid and malic acid) were confirmed as phosphate solubilizers. Three (3-hydroxypropionic acid, nicotinic acid, and malic acid) out of the four compounds were significantly more abundant at the lowest phosphate rate and reduced in concentration as the amendments was elevated.

Our findings agree with a variety of studies evaluating the exudation of organic acids (e.g. malic acid) in various plant species under various phosphate availabilities (Wang & Lambers, 2019; Gerke, 2015). Malic acid is a primary compound released by roots under phosphorus deficiency, but often not the most effective (Wang & Lambers, 2019). In contrast, nicotinic acid and glutamic acid are less abundant than malic acid or oxalic acid in plant species (Tamaraya et al., 2014; Tamaraya et al., 2013). 3-Hydroxypropionic acid has not been previously associated with phosphate solubilization, however it has been described as a natural product of a plant endophytic fungus (Schwarz et al., 2004; Gunatilaka, 2006). Further, the knowledge of the secretion of these compounds throughout plant phenology is scarce mainly because these studies

are often performed in hydroponic systems limiting the root exudate collection to early stages of plant development for a variety of plant crops (Wang & Lambers, 2019; Oburger & Jones, 2018). In our study, we observed that the cumulative secretion of malic acid significantly increased during low phosphate availability, but it was limited at the bolting stage. 3-Hydroypropionic acid and nicotinic acid followed the same pattern, however they were significantly secreted above control level only in the vegetative stage. Conversely, glutamic acid and nicotinic acid increased in abundance at bolting stage when phosphate levels increased. Interestingly, nicotinic acid changed cumulative secretion depending on the developmental stage. It has been reported that nicotinic acid induced flowering in *Lemna* plants (Fujioka et al., 1986), and that nicotinic acid can alleviate abiotic plant stresses by increasing hormone levels such as indole-3-acetic acid and gibberellic acid (El-Bassiouny, 2005).

Based on these observations we hypothesize that plant developmental stage modulates root exudation to deal with phosphorus deficiency by three potential mechanisms: (1) Plants secrete synergistic phosphate-solubilizing compounds in stages of high phosphorus demand. In this study nicotinic acid, a moderate phosphate -solubilizer, was released in combination with 3-hydroxypropionic acid, a stronger phosphorus-solubilizer. This agrees with a recent study showing synergistic association of citrate and phytase to improve acquisition of plant unavailable phosphorus in tobacco in the vegetative stage (Giles et al., 2017). However, this study was performed under soil conditions and not using liquid NBRIP media. (2) Plants prevent the degradation of phosphate-solubilizing compounds such as malic acid, rapidly degraded by soil microbes (Jones, 1998), by releasing a different compound such as 3-hydroxypropionic acid preceding a growth stage of high phosphorus demand. It has been shown that certain plants, such as lupin, can release compounds that inhibit microbial activity to reduce organic acid degradation

prior to the release of organic acids (Weisskopf et al., 2006). Lastly, (3) plants secrete specific compounds to mediate either direct nutrient solubilization or the proliferation of distinct microbial taxa (with phosphate solubilizing activity) at specific growth stages (e.g. vegetative, bolting). In support of this hypothesis, root exudates can promote the activity of symbiotic microbes, such as phosphate solubilizing bacteria and siderophore-releasing bacteria and exert mobilization of non-available plant nutrients in soils at a single growth stage (O'Banion et al., 2020; Rolfe et al., 2019; Voges et al., 2019).

It has been estimated that organic acids constitute 5 to 10% of the total organic carbon in the soil solution. The concentration of organic anions measured in the soil solution usually ranges from 100 nM to more than 580 μM in the rhizosphere of cluster roots (Jones, 1998). However, millimolar concentrations of organic anions are likely required in the soil solution to effectively increase soluble phosphate concentration especially in calcareous soils (Strom et al., 2005; Ryan et al., 2001). Strom et al., (2005) tested three organic acids (citrate, malic and oxalate) and a wide range of concentrations (1 mM to 100 mM) to evaluate its effects on the mobilization of phosphorus in calcareous soil. The results showed that the phosphorus mobilization of the tested compounds had a low efficiency and its effect varied depending on the type of organic acid, compound concentration, and pH. Further, due to the low phosphorus mobilization efficiency of those compounds it is still argued if the benefit of releasing large amounts of organic acids into the soil will exceed the cost of carbon lost by the plant, which can be seen as an unnecessary trade-off (Strom et al., 2005). However, low efficiency organic acids can be particularly important in phosphate mobilization for calcareous soils with a limited phosphorus availability for plants. Finally, our evidence supports the above-mentioned hypothesis, that plants release a

combination of compounds with different phosphorus-solubilizing efficiencies, at specific stages of growth, to deal with particular phosphorus needs.

Lastly, root exudates from liquid culture systems allow the determination of exudation rates unaltered by the soil matrix or microbial decomposition if performed under sterile conditions as we did in this study (Oburger & Jones, 2018). However, the quality and quantity of the root exudate profile may be impacted by the nutrient solution culture method (also known as hydroponic methods) (Oburger et al., 2013). Soil-hydroponic hybrids methods for root exudation collection are not exempt of potential physical/physiological perturbances. Thus, sterile nutrient solution culture methods remain especially important to assess temporal dynamics of root exudates.

In summary, the significance of these findings relies on the potential of customizing specific metabolites to be utilized as soil amendments under the most limiting phosphorus conditions and most demanding stage of plant growth. The role of secondary metabolites in phytoremediation efforts has been previously investigated (Singer et al., 2003) as well as the use of customized synthetic bacteria communities to modify plant phosphate accumulation (Paredes et al., 2018; Awasthi, 2019). However, the use of customized metabolites for phosphorus acquisition remains unexplored.

Conclusions

The data collected indicate that root exudate patterns change as a response to the supply levels of phosphorus, and this change was accentuated as the plant reached maturity, when phosphate demands are higher. 3-Hydroxypropionic acid and nicotinic acid accumulated significantly at the vegetative stage under lower phosphate supplementation and was found to

solubilize phosphate under both solid and liquid medium. This study sheds light on the influence of plant nutrient status and plant phenological growth stages driving the composition of plant root exudates. Future research should focus on understanding the effects of metabolites at a particular developmental stage of growth under phosphorus depleted soil, as well as to test the potential of these phosphate-solubilizing compounds in making phosphorus available for plants grown in soils saturated in unavailable phosphorus forms.

Table 1-1. Five top compounds from root exudates identified by Principal Component Analysis (PCA).

Growth Stage	P level (%)	Compound name	
Seedling	N/A	N/A	
Vegetative	25	Nicotinic acid	*
		4-hydroxybutyric acid	*
		3-hydroxypropionic acid	*
		1-monostearin	
		1-monopalmitin	
Vegetative	50 and 100	Threonine	*
		Proline	*
		O-acetylserine	*
		Leucine	*
		Alanine	*
Bolting	25	Threonic acid	
		Octadecanol	
		Malic acid	*
		Glycine	*
		Galactinol	*
Bolting	50	Scopeletin	
		phenylacetamine	
		5-aminovaleric acid	*
		1-monopalmitin	
		1-monoheptadecanoyl glyceride NISTT	
Bolting	100	Sophorose	
		Guanine	
		Glutamic acid	*
		Adenine	
		1-Kestose	

Compounds are divided by phosphate level and plant developmental stages. Compounds diluted in ddH2O at 100 mM are indicated (*).

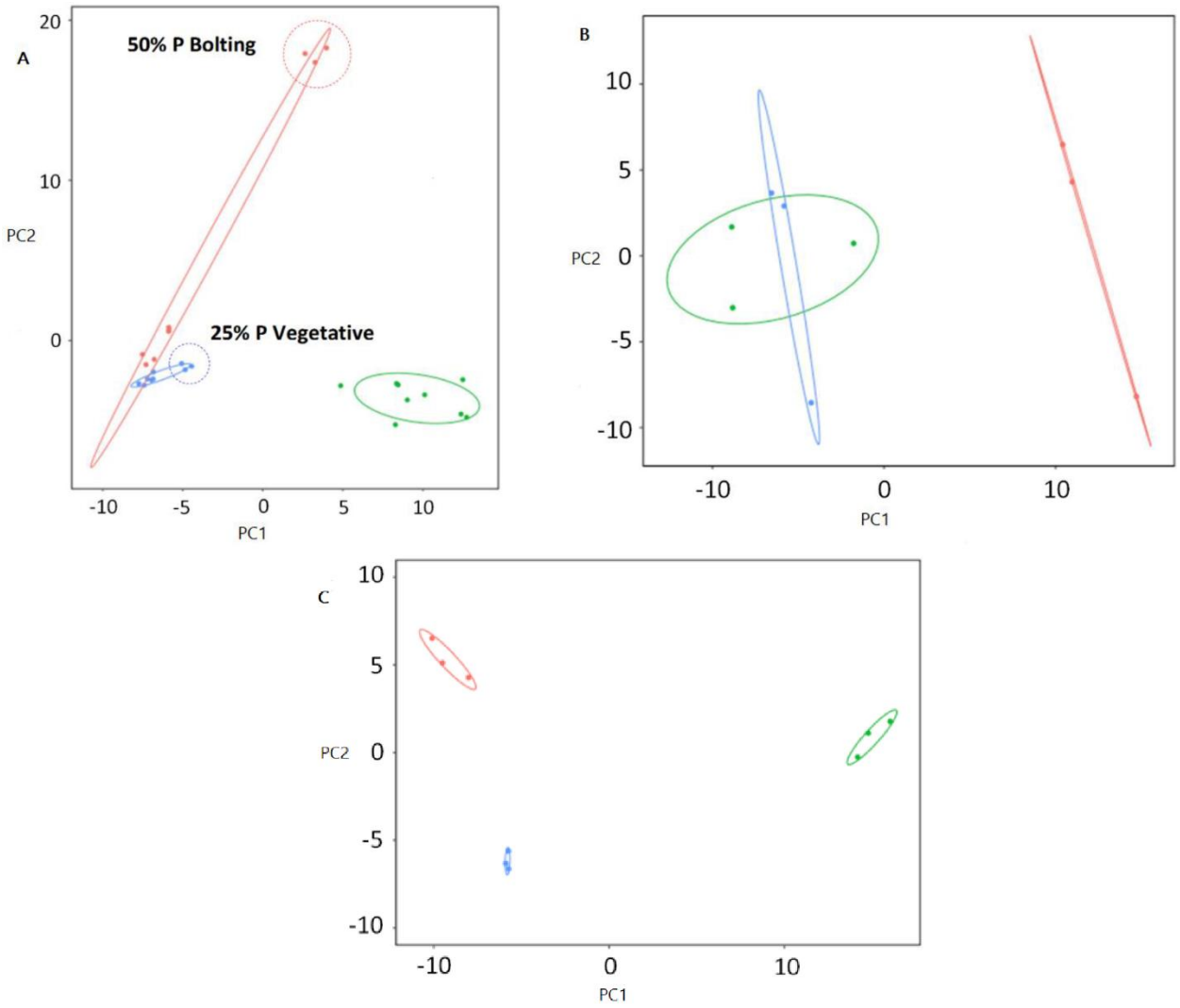

Figure 1-1. Root exudate compounds diverge in response to plant developmental stage and fertilization rate.
(A) 201 annotated compounds with proper identification detected using GC-MS was analyzed using a Principal Component Analysis (PCA) graph. PCA show dissimilarity of metabolite expression profiles between plant growth stages where PC1 explained 29.8% and PC2 21.7% of the variability. All phosphorus levels (25%, 50% and 100%) are present in each of the plant growth stages shown; seedling (green), vegetative (blue), and bolting (red). Dotted circle highlight clusters of particular fertilization levels. (B) Plot of PCA for vegetative stage only where PC1 explained 43.6% and PC2 14.6% of the variability. Compounds grouped by phosphorus treatments: 25% P (red) fertilization clusters separates from 50% P (blue) and 100% P (green). **(C)** Plot of PCA for bolting stage only where PC1 explained 64.3% and PC2 12.9% of the variability. Compounds grouped by phosphorus treatments: 50% phosphorus (green) separated from 25% phosphorus (red) and 100% phosphorus.

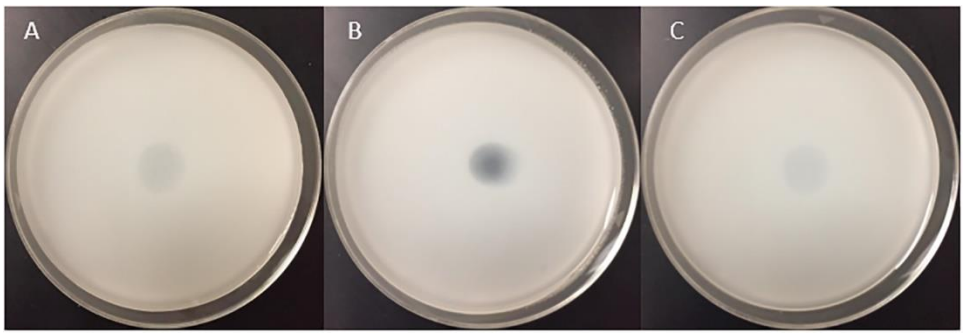

Figure 1-2. Qualitative phosphate-solubilization analysis of compounds using a calcium-phosphate based medium (NBRIP). (A) 3-Hydroxypropionic acid (B) malic acid and (C) nicotinic acid.

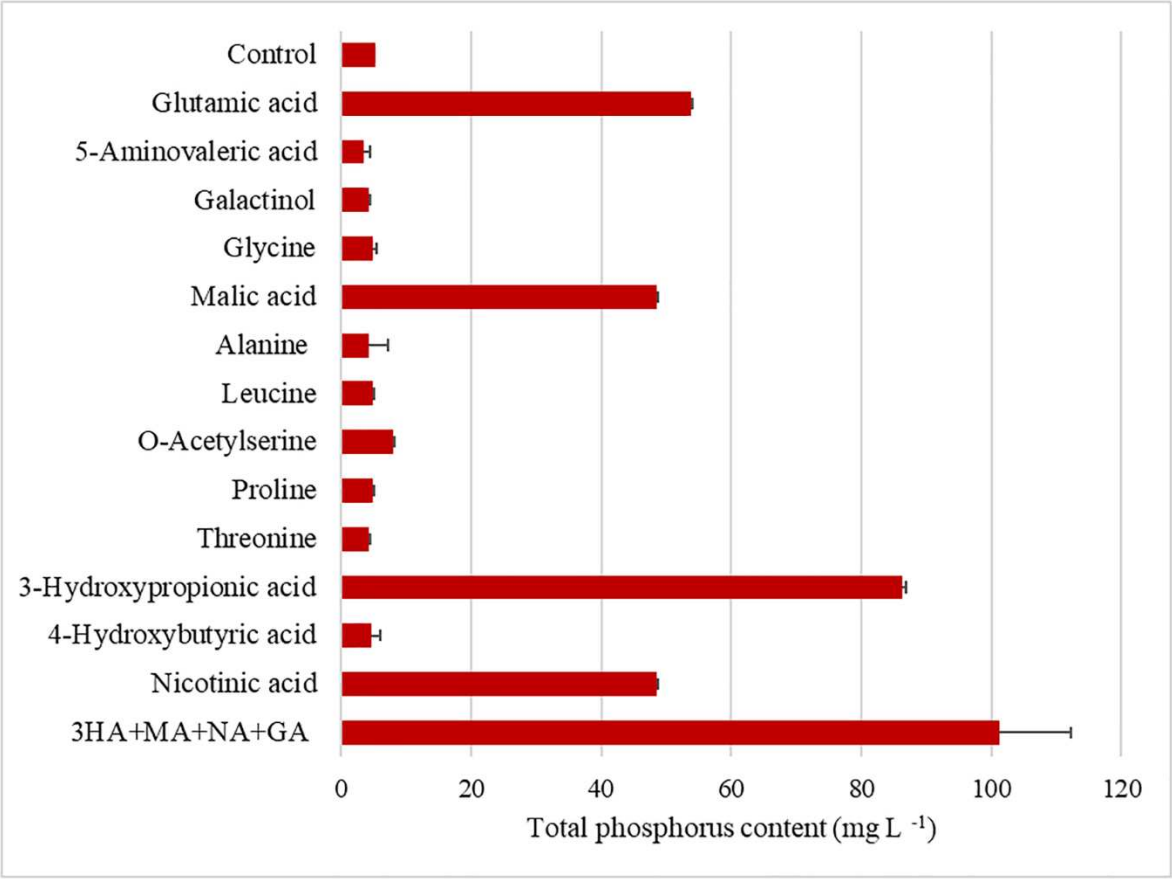

Figure 1-3. Quantitative phosphate-solubilization analyzed by coupled plasma-Optical Emission Spectrometer (ICP-OES) in 13 identified compounds.
Available phosphorus content in NBRIP liquid media after incubation of 72 hours with each of the 13 compounds at 7mM concentration. 3HA+MA+NA+GA treatments is the combination of 3-hydroxypropionic acid (3HA), malic acid (MA), nicotinic acid (NA) and glutamic acid (GA). Each compound added to the pool had 1.75 mM, 7 mM and 28 mM concentration.

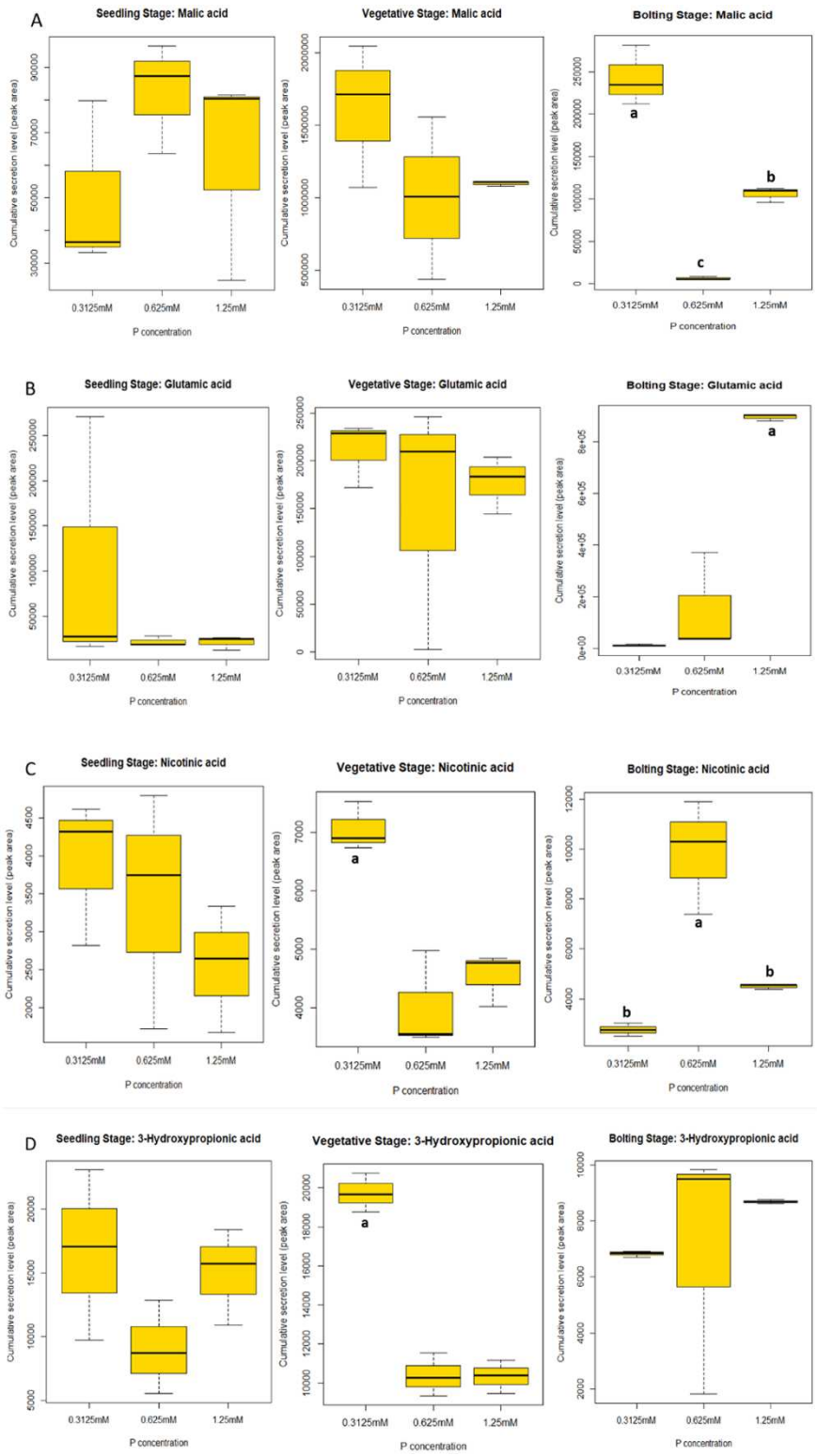

Figure 1-4. Phosphate-solubilizer compounds showing changes in cumulative root secretion levels at three distinct developmental stages ($p<0.05$) in response to increasing phosphate addition (0.312, 0.625 and 1.25 mM). Malic acid (A), Glutamic acid (B), Nicotinic acid (C), 3-hydroxypropionic acid (D).

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CHAPTER 3 PHOSPHORUS SOLUBILIZING BACTERIA ISOLATES FROM THE RHIZOSPHERE OF WILD POTATOES ENHANCE GROWTH OF MODERN POTATO VARIETIES

Synopsis

Wild potato species harbor a distinctive rhizosphere microbiome than its modern counterparts providing a competitive advantage for acquiring phosphorus (P) in their native habitats. However, the influence of phosphorus solubilizing bacteria (PSB), recruited by wild potatoes rhizosphere, on modern potato varieties performance is not well understood. Here, it was hypothesized that PSB isolated from wild potatoes could enhance plant growth and solubilization of various P forms when co-inoculated to commercial potatoes (*Solanum tuberosum*). To test this hypothesis, three bacteria *Enterobacter cloacae*, *Bacillus thuringiensis*, and *Pseudomonas pseudoalcaligenes* were isolated from the rhizosphere of the wild potato *Solanum bulbocastanum* grown under greenhouse conditions and characterized for their P-solubilizing activities. It was found that both, individual bacterial species and the consortium of the three bacteria, dissolved organic (i.e. phytin) and inorganic P (i.e. calcium phosphate) *in vitro*. The bacteria consortium increased dissolved P by 36-fold for calcium phosphate and 6-fold for phytin compared to a sterile control and surpassed the effect of each individual PSB strain. To further evaluate the effect of the PSB consortia on plant growth and P use efficiency, the bacteria were co-inoculated on a commercial potato cultivar and amended separately with phytin, calcium phosphate, commercial P fertilizer, or a combination of the three P sources. The results showed an overall increase in total dry biomass and shoot P content in treatments co-inoculated

with PSB. Our findings indicate that PSB isolated from wild potatoes and inoculated to modern potato varieties have the potential to enhanced yield and nutrient uptake.

Introduction

Phosphorus (P) is an essential element for all living organisms and for food, fiber, and fuel production (Bennett and Schipanski, 2013). The availability of P in soils for plants is typically constrained due to continuous soil sorption and by the formation of insoluble P complexes by binding with aluminum, iron oxides and hydroxides, and calcium; or through the formation of organic complexes such as orthophosphate esters, phosphonates and anhydrides (Condron et al. 2005; Shen et al. 2011). The relatively low amount of available P in agricultural soils typically leads to substantially higher P fertilizer rates than required by the crop for normal growth (Sattari, 2012). Accumulated, and often unavailable P, in agricultural soils represents a financial loss for farmers, while excess P in runoff and leaching has important environmental implications for aquatic habitats and soil biodiversity (George et al. 2016). It is estimated that residual and unavailable P in some soils would be sufficient to sustain crop yield for the next century and could alleviate expected P shortages in the next 50 years (Zhu et al. 2018). Hence, there is a widespread interest to find ways to utilize accumulated soil P for crop nutrition (Richardson, 2001; Withers et al. 2014). Current management strategies to utilize this soil residual P tend to focus on the adjustment of the soil pH to the optimal range of P availability (between 5-6 pH) (Barrow, 2017). However, due to the high amounts of input required to make meaningful, long-term changes to soil pH, this is often not economically feasible for farmers. Other, not fully adopted, strategies to enhance P mobilization include the addition of phosphate-solubilizing microorganisms, phosphatase enzymes, enzyme activators, low molecular weight organic acids, crop residues, lignin, humic acids, and zeolites (Richardson, 2001). Considering the importance

of the two major P fractions existing in soils, effective strategies to recover organic and inorganic P sources are urgently needed. Organic P in the soil accounts for 30-65% of the total P and is mainly found as inositol phosphates (commonly called phytate) (Condron et al. 2005). Inorganic P comprises 35-70% of total soil P content in topsoil (Harrison et al. 1987), where calcium-phosphate is the primary mineral source under moderately weathered soils with neutral to alkaline pH (Bennett and Schipanski, 2013).

Microbes play an important role in altering P availability in soils by increasing plant available P through the solubilization of mineral P and mineralization of organic P, but also by immobilizing and competing with plants for available P (Richardson, 2001; Oliverio et al. 2020). Microorganisms accomplish these tasks through the release of mineral dissolving compounds (e.g., organic acids anions), and the secretion of extracellular enzymes (i.e., phosphatases) (Jones and Oburger, 2010). These strategies are bacterial strain and P source dependent. For instance, Tao et al. (2008) showed that species of the genera *Bacillus* and *Pseudomonas* had phosphate-solubilizing and -mineralizing abilities co-existing in the same bacterial strain. In addition, a strain of *Enterobacter agglomerans* isolated from the rhizosphere of wheat exhibited significant abilities to solubilize hydroxyapatite and to hydrolyze organic phosphate (Kim et al. 1997). Although certain PSB could solubilize organic or inorganic P, it is not clear if this solubilization could affect P use efficiency in commercial crops.

Wild relatives of modern crops typically occupy low-nutrient soil environments in their natural habitats, and unlike managed crops, they are not subjected to frequent additions of synthetic fertilizers. In fact, wild plant species often thrive in nutrient-poor soils and are able to successfully grow, reproduce, and maintain adequate nutrition in the plant tissues (Chapin, 1987; Porter and Sachs, 2020). Wild and cultivated plants are known to distinctly modulate their

rhizosphere microbiota promoting a host-specific community to facilitate plant nutrient acquisition (Berendsen et al. 2012). Increasing evidence suggests that domesticated plants exert a relatively limited selection of their microbiota compared to their wild counterparts (Porter and Sachs, 2020). For instance, Schmidt et al., (2020) demonstrated that teosinte appears to have greater effect than modern corn cultivars on microbial rhizosphere recruitment. Similarly, Pantigoso et al. (2020) showed correlations between enrichment of PSB taxa in the rhizosphere of wild types of potato as compared to commercial varieties (Pantigoso et al. 2020). However, it is unclear if specific PSB isolated from wild potatoes (*Solanum bulbocastanum*) could have a direct contribution on P solubilization, and furthermore if these PSBs could be utilized in commercial potato varieties to enhance P use efficiency.

In the present study, we hypothesized that PSB could be isolated from *S. bulbocastanum* Additionally, we hypothesized that these PSB could increase P use efficiency and biomass gain when applied to commercial potatoes (*Solanum tuberosum*). Accordingly, bacteria were isolated from the rhizosphere of *S. bulbocastanum*, characterized, and assessed for their potential phosphate-solubilizing ability under *in vitro* and greenhouse conditions.

Methodology

Soil and growing conditions for wild potato type

Botanical seeds of the wild potato *Solanum bulbocastanum* (PI 275184) were obtained from the Potato Gene Bank, Sturgeon Bay, Wisconsin, USA. Potato seeds were pre-treated with gibberellic acid at 2000 ppm for 2 hours after being surface disinfected with 5% of sodium hypochlorite for 5 minutes and left soaking for 24 hours in distilled water. Seeds were

germinated on wet filter papers and moved to Murashige and Skoog nutritional media (4.33 g L^{-1}) for two weeks.

One seedling of *S. bulbocastanum* was transplanted to pots (15 cm diameter by 14.5 cm deep) containing a mix of two-part sand and one-part soil. The soil was collected in August 2019 from a depth of 15 cm in an organic field at the Agricultural Research, Development, and Educational Center (ARDEC) of Colorado State University, near Fort Collins, CO. The soil had sandy clay loam texture, pH of 8.1, and an organic matter content of 2 %. The phosphorus content of the mix was > 11 ppm, which is considered high level for AB-DTPA (ammonium bicarbonate-diethylenetriaminepentaacetic acid) test. Levels of P in the mixed used to growth wild potato plants were consistent with Pantigoso et al., (2020). Wild potato plants were grown in benches in the Greenhouse Facility at Colorado State University for approximately 45 days under standard light and temperature conditions receiving irrigation as needed.

Rhizosphere soil collection and bacteria isolation

Rhizosphere soil, was sampled by carefully excavating the roots of *S. bulbocastanum*, removing soil particles attached to the root, and collecting this material in a plastic bag. For isolation of soil bacteria, 1 g of rhizosphere soil was vigorously stirred in 20 mL of sterile water. An aliquot of this soil extract was placed in NBRIP (National Botanical Research Institute Phosphate) liquid medium containing tricalcium phosphate ($Ca_3(PO_4)_2$) as the phosphate source (Nautiyal 1999). The NBRIP medium is comprised of glucose (10.0 g), $Ca_3(PO_4)_2$ (5.0 g), NaCl (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $(NH_4)_2SO_4$ (0.5 g), KCl (0.2 g), $MnSO_4$ (0.03 g), $FeSO_4 \cdot 7H_2O$ (0.003 g) with a pH of 7.0–8.0. Five mL of NBRIP media was inoculated with 10 µL of soil extract and incubated in a rotary shaker at 170 rev min^{-1} at room temperature for 48 hours. The culture media was then diluted 10^{-6} and a 1 mL aliquot was plated on NBRIP solid media (1.2%

Bacto agar) and incubated at 30°C overnight for 12 hours. PSB candidates that differed in size, shape, and color were separated into different petri dishes containing NBRIP several times to purify single colonies. Eight selected bacteria candidates were subsequently grown in Luria-Bertani liquid media (Bertani, 1951) before its use and/or mixed with glycerol and frozen for storage.

Bacterial isolate identification

Eight bacterial isolates were identified to the genus level using a 64-well Vitek 2 GN card containing biochemical tests measuring C source utilization, inhibition, and resistance; enzymatic activities were processed in the Veterinary Diagnostic Lab at Colorado State University, Fort Collins, Colorado. For species-level identification, 526 base pairs of the 16S rRNA amplicon sequencing were amplified using the universal primers: 0005F (5' – TGGAGAGTTTGATCCTGGCTCAG – 3′) and 0531R (5' - TACCGCGGCTGCTGGCAC – 3'). The 16S identification analysis was performed in the MIDI labs, Inc., Newark, DE. Three isolates out of the eight were different species.

Qualitative and quantitative determination of phosphorus solubilizing ability

Using a 2.5 mm platinum wire loop, a streak of bacteria culture obtained from pure cultures of each of the three selected isolates were dipped into liquid Luria-Bertani medium (Bertani, 1951) and incubated separately in a rotary shaker at 170 rev min^{-1} at room temperature overnight until reaching the mid-exponential growth phase. An aliquot of diluted (OD_{600} = 1; 1×10^8) cultured bacteria was placed on NBRIP solid media containing calcium phosphate or phytin as the P source to quantitatively evaluate their phosphate solubilizing ability. Then, the bacterial

isolates were qualitatively evaluated such that a clear halo observed around the bacteria colonies was interpreted as evidence of phosphate solubilization ability.

For the quantitative experiment, a 50 µL diluted ($OD_{600} = 1$; 1×10^8) aliquot from each pure culture of *E. cloacae*, *B. thurigiensis*, and/or *P. pseudoalcaligenes* was added to 5 mL liquid NBRIP medium separately and incubated in a rotary shaker for 72 hours. For the inoculation of bacterial consortium, a proportion of each strain was prepared and mixed at the same final concentration ($OD_{600} = 1$; 1×10^8) and incubated, also for 72 hours. After incubation and as preparation for downstream analysis, the solution was centrifuged at 6000 rpm for 20 minutes to remove the suspended bacteria cells and the remaining calcium phosphate/phytate. Liquid NBRIP media without the addition of bacteria was used as a control. The concentration of phosphate in the supernatant was analyzed according to the protocol of Soltanpour et al. (1983) and measured with an inductive coupled plasma-optical emission spectrometer (ICP-OES; Perkin Elmer 7300DV) at the Soil, Water and Plant Testing Laboratory of Colorado State University.

Potato growth response to P amendments and bacteria consortia

Certified tubers of the commercial potato (*S. tuberosum*) cultivar "Defender" were pre-sprouted for a week and whole tubers of similar size were planted in plastic pots (15 cm diameter by 14.5 cm deep) containing three parts of sand and one parts peat moss for a total weight of 555 g. To reduce the population of microbes and to assess the sole effect of P-solubilizing activity of the co-inoculated bacteria, sand and peat moss were heat sterilized on an autoclave for three 30 minutes-cycles at 121 °C and thoroughly mixed prior to P amendment and tuber planting. A sample of this substrate mixture was analyzed for Olsen P resulting on 16.4 ppm of available P. The soil amendments used in this experiment were composed of four P sources 1) fertilizer Triple Super Phosphate (P_2O_5), 2) calcium phosphate ($Ca_3(PO_4)_2$), 3) calcium phytate

($C_6H_6Ca_6O_{24}P_6$), and 4) a combination of the three previous phosphate sources. Triple Super Phosphate contain 43%, calcium phosphate 25%, and phytin 20% of phosphate, respectively. All four treatments received approximately 1 g of phosphate per kg of soil in two separate applications; at planting and after 30 days. A fifth P un-amended treatment was included as a control. Each of the five P fertilization treatments were grown with and without the inoculation of PSB, in a full factorial and fully randomized design, with five replicates per treatment. The PSB applied was the same co-inoculation of bacteria *E. cloacae*, *B. thurigiensis*, and *P. pseudoalcaligenes* mentioned above. A proportion of each strain at the same final concentration (OD_{600} = 1) was mixed and 1 mL aliquot of the consortium was applied to the soil near the main stem of potato plants at 2, 3, and 4 weeks after planting. One soil sample per each treatment was collected for pH and nutrient analysis at harvest time and sent to the Soil, Water and Plant Testing Laboratory of Colorado State University for analysis (Supplementary table 1, 2).

This study was performed under greenhouse conditions from August to October 2020 in the Plant Growth Facility (PGF) at Colorado State University, Fort Collins, Colorado. Plants were harvested two weeks after the final inoculation, and 60 days after tuber planting. Fresh biomass of shoot, roots and tubers were recorded separately, and then each plant component was oven-dried for 4 days at 80 °C and weighed. Total P in the aboveground plant shoot tissue was analyzed by digesting the material in a block digester with HCl and HNO_3 and cleared with H_2O_2. Then the sample was brought to a volume of 50 mL and total P was read on an ICP-OES.

Calculations

Phosphorus utilization efficiency (g tuber g^{-1} P applied) by average potato plant in each pot was calculated as: P utilization efficiency = tuber biomass / P applied.

Phosphorus uptake efficiency (mg P g^{-1} P applied) by potato plants in each pot was calculated as: P uptake efficiency = P content in shoots / P applied.

Data analysis

One-way ANOVA was used to analyze differences in dissolved P between individual bacteria isolates and consortium incubated in NBRIP phytin and calcium phosphate solutions. Two-way ANOVA was used to examine the effect of P amendment type and PSB inoculation and their interaction on plant biomass and leaf P content. Homogeneity of variance and normality were assessed, and log transformations were applied as needed to meet these assumptions. A probability level of $p = 0.05$ was considered statistically significant.

Results

Differences in relative abundance from wild versus cultivated potatoes

16S rRNA sequencing provided species-level resolution for the three bacteria isolates. Phosphorus-solubilizing bacteria were identified in GenBank with 100% similarity with *Enterobacter cloacae* GU191924, 99% similarity to *Pseudomonas pseudoalcaligenes*, and 99% similarity to *Bacillus thuringiensis.* Phylogenetic trees for the three isolates at genus level are provided in Figure S1.

To further investigate differences in relative abundance of the three bacteria genera isolated from *S. bulbocastanum,* and to compare their abundance with cultivated relatives, the 16S rRNA sequenced data from Pantigoso et al. (2020) was analyzed. The analysis showed a greater relative abundance for Enterobacteriaceae and Pseudomonadaceae in wild potato species when compared to cultivated potato cultivars (Figure S2).

Bacteria isolates solubilized plant-unavailable phosphate

The phosphate-solubilizing effect of the bacteria isolates were qualitatively tested using phytin and calcium phosphate NBRIP solid media. Individual bacteria and a consortium of the three isolates formed a clear halo around the colony indicating their ability to release free phosphate from calcium phosphate and phytin complexes (Figure S3).

The phosphate-solubilizing activity of the bacteria isolates was analyzed by ICP-OES. This method allowed us to evaluate the amount of dissolved P (plant available phosphate) after incubation of bacteria strains in calcium phosphate and phytin solutions. The results showed that individual isolates and the bacteria consortium significantly increased the content of dissolved P under both P solutions relative to the control ($p < 0.001$; Figure 1).

Absolute values for dissolved P were lower under calcium phosphate (Fig. 1A) than under phytin solutions (Fig. 1B), but the relative difference between the bacteria inoculated treatments and the control for calcium phosphate were greater than differences for phytin. Under calcium phosphate media, the bacterial consortium resulted in a 36-fold increase in dissolved P relative to the control, while increases for individual strains were: *P. pseudoalcaligenes* 26-fold, *B. thuringiensis* eight-fold, and *E. cloacae* 13-fold. In the phytin solution, the consortium increased dissolved P approximately six-fold more than the control, *P. pseudoalcaligenes* four times, *B. thuringiensis* two-fold, and *E. cloacae* 1.44-fold.

Effects of phosphorus source and bacterial consortia on plant growth

The effect of different P sources and PSB consortia inoculation on total potato biomass revealed clear treatments differences due to P source ($p < 0.001$), such that the treatments containing TSP generally produced the most biomass, while those amended with CaP and phytin

were the lowest. We also observed the PSB consortia to yield a significant increase in total biomass relative to the uninoculated control ($p = 0.043$), but there was no significant interaction between P source and PSB inoculation (Fig. 2). Similarly, P source had a significant effect on each of the plant components (shoots, roots and tubers; $p < 0.002$). Inoculation with the PSB consortia significantly increased shoot ($p = 0.031$) and root ($p = 0.014$) biomass, on average, across treatments, but no effect was observed for tubers. There were no significant interactions between P source and PSB consortia for any of the plant components (Table 1). When analyzing P concentration of the aboveground biomass (leaf tissue) statistical differences between treatments were observed for P source ($p < 0.001$), but not for PSB consortia inoculation ($p = 0.859$; Table 1).

Plant P content, P uptake efficiency and P use efficiency responses

Aboveground P content, P uptake efficiency and P utilization efficiency revealed clear treatment differences due to P source ($p < 0.0001$; Table 2). Treatments containing Triple Super Phosphate representing the highest value and those with CaP and phytin the lowest. PSB inoculation significantly increased P content ($p = 0.039$), and had a marginally significant effect on P uptake efficiency ($p = 0.085$) and P utilization efficiency ($p = 0.051$). No significant interaction between P source and PSB inoculation was observed.

Effects of phosphorus source and bacterial consortia on soil pH

To evaluate the effects of P amendments and bacterial inoculation on soil pH, soils from all the treatments were collected and analyzed at harvest. Overall, soil pH from bacterial inoculated treatments were similar across P source, compared to those without bacterial inoculation. Soil pH remained relatively stable for treatments that received calcium phosphate (6.9), phytin (6.9), the

combination of P sources (6.1), and the control compared to the P fertilizer treatment which pH decreased to 5.4. A similar pattern was observed under bacterial co-inoculated treatments where soil pH for calcium phosphate (6.6), phytin (6.8), the combination of P sources (6.1), and the control (6.7) were higher relative to the P fertilizer treatment (5.6) (Supplementary table 2).

Discussion

Wild potato (*S. bulbocastanum)* recruits a distinct rhizosphere microbiome compared to its modern relative when grown under the same soil conditions. Recent studies suggest that differences in rhizosphere microbiome structure between wild types and cultivated crops significantly correlate with nutrient uptake favoring the wild type (Brisson et al., 2019; Schmidt et al., 2020; Brisson et al., 2021). In this study, we grew wild potato (*S. bulbocastanum)* to isolate PSB from its rhizosphere

Three P solubilizing bacteria strains were identified as *Enterobacter cloacae*, *Pseudomonas pseudoalcaligenes*, and *Bacillus thuringiensis*. Each bacterial strain increased dissolved P under calcium phosphate and phytin P sources *in vitro*, when applied individually and together as consortia. Further, based on 16S sequencing reads we showed a higher relative abundance of Enterobacteracea and Pseudomonadacea, and a marginally lower abundance of Bacillaceae families in the rhizosphere of *S. bulbocastanum* when compared to the same families present in the rhizosphere of modern relatives. These observations agree with mounting evidence suggesting that domesticated plants either exert a relatively limited selection or select for different microbial communities with less obvious functionality, in terms of P acquisition, compared to their wild counterparts (Martin-Robles et al. 2018; Escudero-Martinez and Bulgarelli, 2019, Porter and Sachs, 2020; Jaiswal et al. 2020). Several studies between wild accessions, ancestral and modern varieties have shown evidence of significant shifts in

composition of their rhizosphere microbiota in different crops such as maize, rice and bean (Peiffer et al. 2013; Edwards et al. 2015; Perez-Jaramilo et al. 2017; Walters et al. 2018). We have recently shown effects on the rhizosphere microbiome upon phosphorus fertilization on cultivated and non-cultivated potatoes. These effects revealed differences in bacterial community structure and phosphorus absorption favoring non-cultivated potatoes (Pantigoso et al. 2020). However, it is unknown if the differential plant recruitment of microbes observed in microbiomes of modern and wild types constitute a functionally important component for plant nutrition (Cordovez et al. 2019). In addition, the selection relaxation hypothesis predicts that germplasm bred under more intensive agricultural conditions will exhibit greater functional trait disruption than germplasm bred under less intensively managed conditions (Porter and Sachs 2020). Consistent with this hypothesis, numerous crop taxa have evolved a reduced ability to associate with mycorrhizae under high levels of P fertilization (Hetrick et al. 1993; Xing et al. 2012; Martin-Robles et al. 2018). Similarly, De la Torre-Hernandez et al. (2020) investigated bacteria community composition and plant growth promoting rhizobacteria (PGPR) traits associated with wild and cultivated cactus plants. Interestingly, the bacteria isolated from the wild types showed a higher number of PGPR with functional traits including a higher number of P solubilizing strains. Genera *Bacillus* and *Pseudomonas* were found in both samples, but *Pseudomonas* was particularly abundant in the wild cactus type. These findings are further supported by Coleman-Derr et al. (2016) and Hilton et al. (2013) who reported a marked reduction in microbial diversity in rhizosphere soils of cultivated agave compared to native agave plants.

In a previous study, we showed a correlation between P rate supplied to soils, P content in shoot tissues, and differential abundance of bacterial taxa for *S. bulbocastanum* when compared

to modern potato relatives grown under the same conditions (Pantigoso et al. 2020). In this study, we postulate that effective PSB recruitment of wild potato type enhances P solubilization improving plant growth. Our findings are supported by recent studies including Schmidt et al. (2020) who demonstrated that teosinte appears to have greater effect than modern corn cultivars on rhizosphere recruitment and individual plant-microbe interaction related to nitrogen uptake. Brisson et al., (2021) showed that root exudate metabolite profile and rhizosphere microbial communities were distinct between teosinte and modern maize and shifted in response to phosphorus availability. In addition to the known plant genotype effect on differential bacterial recruitment, the soil environment from which microbial inoculants were collected significantly contributes to the effect on plant growth-promotion in terms of plant biomass and assimilation of key nutrients (Gu et al. 2020). Most PSB species identified in the literature are ubiquitous in soils and their occurrence have been reported in numerous geographic areas, in a wide range of soil conditions, and under variable nutrient environments, highlighting the importance of wild type and its specific root exudation signature for specialized microbial recruitment across soil types (Johri et al. 1999; Rodriguez and Fraga, 1999; Chen et al. 2006; Perez et al. 2007; Oliveira et al. 2009; Aranda et al. 2011).

The most common PSB strains isolated so far belong to the genera *Pseudomonas, Bacillus, Enterobacter, Rhizobium, Arthrobacter, Flavobacterium and Azospirillum* (Rodriguez and Fraga, 1999). Numerous reports from *in vitro*, greenhouse and field studies have shown that soil microbial inoculants comprised by *Bacillus*, *Enterobacter*, *Pseudomonas* enhance productivity in horticultural crops when applied alone or in combination with commercial fertilizer treatments (Baas et al. 2016; Richardson, 2001). In addition, *Bacillus*, *Pseudomonas* and *Enterobacter* strains have also shown abilities for solubilizing and mineralizing organic and inorganic P forms

(Kim et al. 1997; Guang-Can et al. 2008; Afkairin et al. 2021). In agreement with this, we showed that a single PSB strain can solubilize more than one plant unavailable form of P, and that soils inoculated with a consortium of PSB species increase potato plant biomass and P content.

Under *in vitro* conditions, we observed that the increase of dissolution of P by the bacteria consortium was significant for both forms of P tested, but more effective for calcium phosphate than for the organic form (phytin) suggesting a higher efficiency of the consortia to solubilize calcium phosphate (Adnan et al. 2017). This is consistent with Guang-Can et al. (2008) who showed greater abilities of P-solubilization under inorganic P sources, and with Bass et al. (2016) that demonstrated the increase P uptake and productivity in various plant crops by a phosphorus mobilizing consortia. The affinity of bacteria isolates to utilize a given P form can also be attributed to their specific metabolism or temporal requirements of P. In addition, under our study conditions, calcium phosphate was the primary form of plant-unavailable P used for isolation of bacteria from soils slurries of wild potato rhizosphere. This supports the idea that PSB may be more effective depending on the type of plant-unavailable P used for its isolation as well as the type of P source that PSB encountered after its inoculation to soils.

In our greenhouse experiment, potato plants were grown under heat sterilized soil to lower the population of microbes and the potential positive or negative influence of the residing microbial community on the newly introduced bacterial strains and its P-solubilization activity. A global assessment of biofertilizer performance showed significant yield responses and improved P uptake for various crops inoculated with P-solubilizing bacteria under field conditions. Thus, evidencing that PSB have the potential to be effective with or without a competing community after being introduced to soils (Schütz et al. 2018). In agreement with

this, we observed an overall significant effect of PSB inoculation in total plant biomass. However, the increase in plant biomass was absent for phytin treatment, despite bacterial inoculation. This contradicts our *in-vitro* studies where it is shown that PSB significant dissolved P under both calcium phosphate and phytin. These discrepancies could be explained by the potential insufficient bacterial phosphorus solubilization from phytin to supply the demand for plant growth (Raymond, et al. 2021). In addition, our *in vitro* study showed that the bacterial consortium was more effective solubilizing calcium phosphate, producing several folds more dissolved P than under phytin medium. Furthermore, our initial isolation process used primarily a calcium phosphate source, which further explains why the bacterial isolates prefereds the mineral P over the organic source (Kim et al. 1998).

The highest availability of P in soils for plants is within the range of 5 to 6 pH units (Barrow, 2017). P availability decreases as it approaches neutrality and beyond. We observed that soil pH from calcium phosphate, phytin and the combination of P sources treatments remained stable. However, P fertilizer treatment reduced the pH from 6.7 to 5.6 in the control. This reduction in pH could have further increased the availability of P in soils for plants, affecting both, PSB inoculated and non-inoculated treatments equally. Conversely, non-inoculated treatments with plant unavailable P (calcium phosphate and phytin) did not substantially increase or decrease the pH. However, these same plant unavailable P treatments reduced the plant growth when compared to non-inoculated non-amended control treatment. This is due to the binding properties of calcium phosphate and phytin on the existing orthophosphate in the substrate. Interestingly, upon inoculation, PSB counteracted the negative effect of soil calcification by increasing plant growth under calcium phosphate treatment but not under phytin. This observation agrees with

Adnan et al. (2017) in which PSB inoculation nullify the antagonistic effect of soil calcification on bioavailable P under both mineral and organic P sources.

Co-inoculation of *Enterobacter* sp. *Bacillus* sp. and *Pseudomonas* sp. increased P content across treatments. This result agrees with previous research indicating a greater P availability and plant crop uptake after soil inoculation of P solubilizing microorganisms (Alori et al. 2017; Bargaz et al. 2018). Further, the efficiency of the plant to uptake P and to transform this P into biomass in response to bacteria inoculation was evaluated. Impacts of PSB on P uptake and P utilization were only marginally significant, however, our findings suggest PSB inoculation can improve P use efficiency after only 60 days of plant development. These findings are consistent with several reports documenting the improvement in nutrient use efficiency in potato under controlled and field conditions with PSB addition (Munda et al. 2015, 2018; Adesemoye, Torbert, and Kloepper, 2008; Naqqash et al. 2016; Rodriguez and Fraga, 1999). Lastly, in our study we demonstrate the efficacy of soil microbial inoculation on the potato's ability to acquire and utilize various forms of P. However, it is important to consider that PSB can have multiple mechanisms other than P solubilization. These include secretion of phytohormones, antibiotics used as biocontrol, or the substances that chelate specific nutrients, which were not tested under our experimental conditions (Etsami & Maheshwari, 2018; Ahmed and Holmstrom). Most importantly, we showed that PSB inoculation is effective under soils containing insoluble P forms such as calcium phosphate, a prevalent form of plant unavailable P in agricultural soils. Thus, higher P bioavailability of sparingly soluble P can be translated into reduced P-fertilizer inefficiencies and potentially greater profit for farmers. In summary, these results demonstrate the ability of PSB isolated from wild plants rhizosphere soil to enhance crop P nutrition and their

potential to increase availability of sparingly soluble forms of P in soils, thereby increasing crop yield and reducing P fertilizer inefficiencies.

Conclusion

In this study we show that wild potato *Solanum bulbocastanum* effectively recruit phosphorus solubilizing bacteria with the ability to assimilate organic and inorganic phosphorus forms under *in vitro* and *in planta* conditions in modern potato cultivars. Furthermore, we found that co-inoculation of *Enterobacter cloacae*, *Pseudomonas pseudoalcaligenes*, and *Bacillus thuringiensis* have the potential to increase yield and phosphorus nutrient uptake in potatoes.

Table 2-1. Two-way ANOVA for total dry biomass and root, shoot and tubers individually and P concentration.

Total biomass	**p value**
P_source	<.0001
PSB	0.0431
P_source*PSB	0.2065

Shoot biomass	**p value**
P_source	<.0001
PSB	0.0314
P_source*PSB	0.3043

Root biomass	**p value**
P_source	0.0012
PSB	0.0148
P_source*PSB	0.4865

Tuber biomass	**p value**
P_source	<.0001
PSB	0.2135
P_source*PSB	0.2177

P concentration	**p value**
P_source	<.0001
PSB	0.8593
P_source*PSB	0.0554

Table 2-2. Phosphorus use efficiencies of potato plants amended with four P sources in greenhouse conditions with and without PSB consortia application.

	P content (mg P plant^{-1})		P uptake efficiency (mg P shoot g^{-1} P applied)		P utilization efficiency (g tuber g^{-1} P applied)	
Treatments	**with-PSB**	**without-PSB**	**with-PSB**	**without-PSB**	**with-PSB**	**without-PSB**
Calcium phosphate	0.956	0.666	2.078	1.448	3.509	0.000
Phytin	0.856	0.726	1.861	1.578	0.223	1.448
Fertilizer	4.268	3.484	9.278	7.574	19.162	16.967
Mix	1.749	1.852	3.803	4.026	12.886	9.520
No P	1.023	0.836				
Statistical significance			***p* value**			
P_source	<.0001		<.0001		<.0001	
PSB	0.0395		0.0854		0.0518	
P_source*PSB	0.7695		0.6263		0.2981	

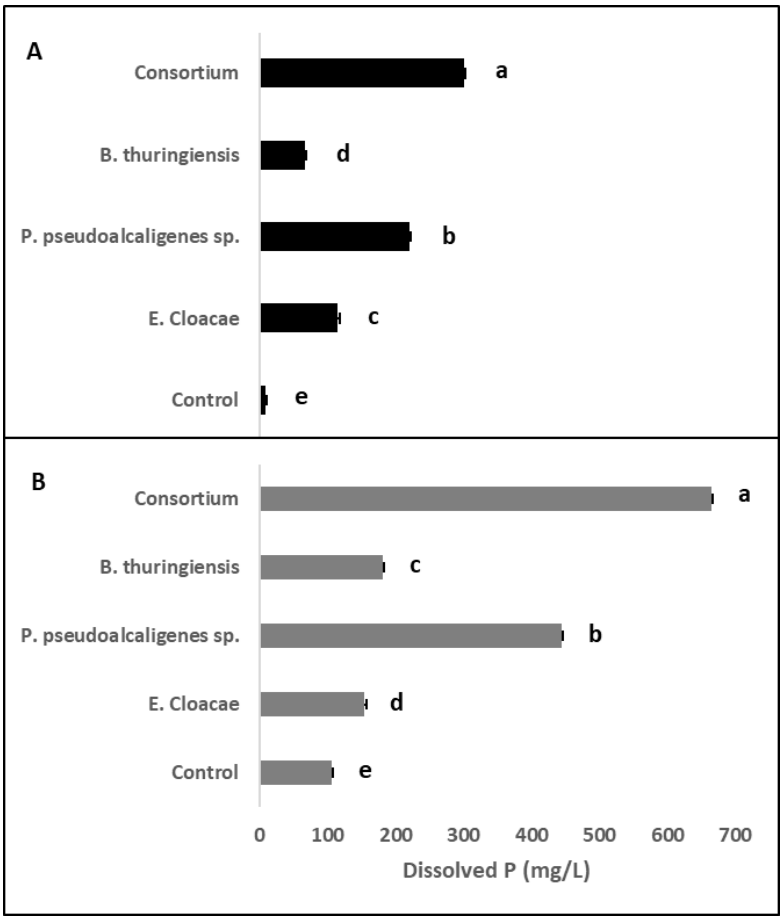

Figure 2-1. Mean phosphate-solubilization of individual bacteria isolates and consortium incubated in NBRIP phytin and calcium phosphate solutions. Samples were analyzed by coupled plasma-Optical Emission Spectrometer (ICP-OES). Figure shows dissolved P (mg L^{-1}) released from (A) calcium phosphate and (B) phytin media. Different letters indicate significant differences (p <0.05) and error bars the standard error of the mean.

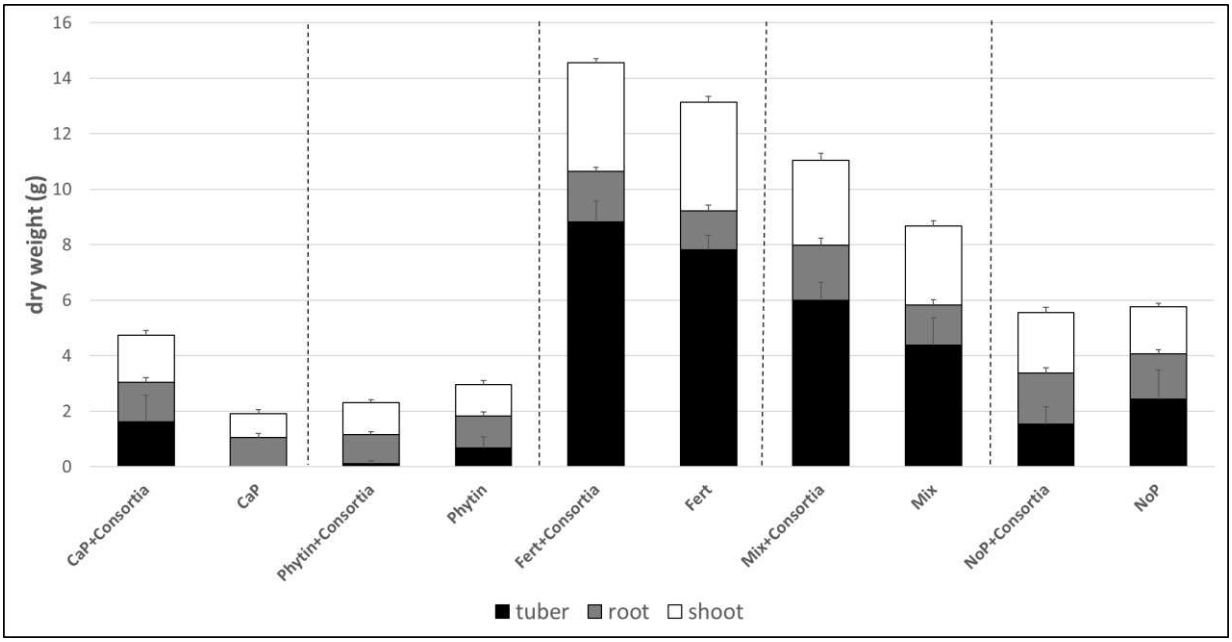

Figure 2-2. Mean biomass (oven-dry weight) for potatoes grown in a greenhouse under of various P sources, both with and without the inoculation of a consortia of P solubilizing bacteria. Potato plant biomass is divided into three components (shoot, root and tubers). Error bars represent the standard error of the mean for each plant component.

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CHAPTER 4 ROOT EXUDATES STIMULATE PHOSPHORUS SOLUBILIZATION ABILITY OF BACTERIA

Synopsis

Low phosphorus (P) availability in soils is a major challenge for sustainable food production, as most soil P is often unavailable for plant uptake and effective strategies to access this P are limited. Solubilizing-bacteria and root exudate compounds that solubilize P are promising approaches to increase plant-available P. Here, we studied the ability of root exudates (galactinol, threonine, and 4-hydroxybutyric acid) induced under low P conditions to stimulate the ability of bacteria to solubilize P. Galactinol, threonine, and 4-hydroxybutyric acid were incubated with the P solubilizing bacterial strains *Enterobacter cloacae*, *Pseudomonas pseudoalcaligenes*, and *Bacillus thuringiensis* and either inorganic (calcium phosphate) or organic (phytin) forms of plant-unavailable P. We found that the addition of individual root exudates generally no supported bacterial growth rate. However, root exudates provided together with the different bacterial inoculum did appear to enhance P solubilizing activity and overall P availability. Threonine and 4-hydroxybutyric acid induced P solubilization in all bacteria. Subsequent exogenous application of threonine to soils improved the root growth of corn, enhanced nitrogen and P concentration in roots and increased available levels of potassium, calcium and magnesium in soils. Thus, it appears that threonine might promote the bacterial solubilization and plant-uptake of a variety of nutrients.

Introduction

Most of the existing phosphorus (P) in soils globally is locked in primary minerals, absorbed on soil particle surfaces, or in organically complexed forms (Syers, 2008; Richardson et al. 2009). Although P fertilizer is readily available for plants, once applied to soils, it faces constraints such as poor diffusion, limited solubility, and fixation on mineral surfaces; thus, increasing the pool of plant unavailable P in soil.

Phosphate fertilizer originates from rock phosphate minerals, a non-renewable resource and predicted to become scarce in the following decades (Cordell et al. 2009; Cordell and White, 2015). It has been estimated that unlocking residual P pools in soils can play an important role in reducing global P fertilizer demand by up to 50% by 2050 (Sattari et al. 2012). Current strategies to access unavailable soil P and nutrient management practices to supply P to crops are often inefficient. To overcome soil P fixation processes and to maintain P in the soil solution at optimal levels for plant growth excessive applications of phosphate fertilizer to agricultural soils is common (Menezes-Balckburn et al. 2018). Overapplication of P leads to increased pollution and decreased farm profitability. Finding widely applicable and sustainable solutions to the inefficiencies in agricultural P use and its bioavailability offers great promise to support long-term productivity and the sustainability of agricultural systems.

The desire to increase P bioavailability in soils has encouraged the study of phytochemicals and beneficial microbes in the plant rhizosphere to enhance P uptake and plant yield (Richardson et al. 2009). Plants roots can exude a considerable amount of photosynthates that contributes to a unique zone of soil called the rhizosphere (Badri and Vivanco, 2009). The release of carbon compounds into this zone leads to the proliferation of microorganisms within, on the surface, and outside the roots. The diverse chemical composition of root exudates plays a major functional role that includes the direct solubilization and acquisition of non-soluble nutrients from the soil

and regulation of plant-microbe interactions involved in nutrient acquisition (Dakora and Phillips, 2002; Badri and Vivanco, 2009).

Plants possess the ability to modulate the chemical composition of root exudates, that in turn, influence members of the rhizosphere microbial community by discriminating between mutualist, commensal, and pathogenic root-microbe interactions (Chaparro et al. 2012; Zhalnina et al. 2018). For instance, plants associate with symbiotic and free-living organisms that help mediate plant P uptake; these organisms can be multicellular such as mycorrhizal fungi or single-cell bacteria such as those from the genera *Enterobacter* spp., *Bacillus* spp., or *Pseudomonas* spp. (Richardson and Simpson, 2011). Plants initiate these interactions when there is a soil P shortage (Pantigoso et al. 2020), and such interactions are affected by soil type and abiotic factors (Venturi and Keel, 2016; Sasse et al. 2018).

The main mechanisms by which plants deal with P scarcity include changes in root morphology by modifying root branching, increasing root length, forming of root hairs, and generally investing more in belowground allocation to increase the root surface for P uptake (Hermans et al. 2006; Lynch and Brown, 2008). However, even when plant roots can physically reach the immobile P in soils, this P is often in non-soluble forms that cannot be taken up. The root then switches to complementary strategies to improve solubilization such as the release of selected root exudates to improve P mobilization (Jones and Darrah, 1995; Gerke, 2015). Some of the major chemical groups of P-mobilizing root exudates is organic acids, including amino acids and fatty acids with a range of reported biological functions in the plant rhizosphere (Menezes-Blackburn, 2016; Zhalnina et al. 2018). P dissolution rates can be greatly accelerated in soil in the presence of organic acids leading to 10-1000-fold higher P concentration in the soil

solution, depending on soil type and organic acid concentration (Gerke, 1994; Jones and Darrah, 1994b).

Root exudates can induce the growth of microorganisms, act as chemo-attractants to motile microbes and are a source of carbon for numerous microbes (Walker et al. 2003a, Kawasaki et al. 2018). Some bacteria dominate the rhizosphere of certain plants based on specific metabolites secreted by a plant species. For instance, *Burkholderia* species that metabolize citrate and oxalate are highly present in the rhizosphere of densely packed lateral roots of lupine (Weisskopf et al. 2011). The artificial addition of phytochemicals to soils have also been shown to affect the composition and functions of soil microbiota (Badri et al. 2009; 2013; Huang et al. 2019). Recent studies have shown that coumarins present in root exudates increase the abundance of single microbial strains or whole microbial communities present in the soil (Voges et al. 2019; Koprivova et al. 2019). Similarly, the supplementation of soil with organic acids can change the phosphatase enzymatic activity and shift the community composition including beneficial rhizobacteria (Macias-Benitez et al. 2020). In addition, tricarboxylic acids such as malic acid selectively signal and recruit free-living beneficial bacteria *Bacillus subtilis* (Rudrappa et al. 2008). Testing the potential enhancement of root exudate-molecules on PSB offers a promising means to increase efficiency of commercial microbial inoculants already in use in farming systems as well as to improve P use efficiency by unlocking legacy P in soils.

In a recent study, Pantigoso et al. (2020) found that certain molecules were exuded in high amounts by *Arabidopsis thaliana* roots grown under deficient P conditions. Some of those molecules containing organic acids directly solubilized non-soluble P under *in vitro* conditions. In the same study, a second group of molecules such as galactinol, threonine, and 4-hydroxybutyric acid were equally enriched but did not increase P solubilization directly. It was

hypothesized that these compounds were involved in signaling with P solubilizing bacteria (PSB).

The objective of these study is to determine the role of specialized metabolites, previously screened, on the growth and activity of rhizosphere beneficial bacteria. Here we hypothesize that galactinol, threonine, and 4-hydroxybutyric acid exuded by plants under P deficiency can be used to stimulate the growth and/or activity of specific PSB, thus improving the effectiveness of the bacterial inoculum. Further, we tested the possibility that those root exudate derived and specialized metabolites could positively stimulate the native PSBs contained in a natural soil; thus, facilitating nutrient acquisition for the plant.

Materials and Methods

Phosphorus solubilizing bacteria and root-exudate derived compounds

This study used bacterial strains *Enterobacter cloacae*, *Bacillus thuringiensis*, and *Pseudomonas pseudoalcaligenes* isolated from wild potato, *Solanum bulbocastanum,* previously screened for their ability to solubilize P and tested *in vitro* and *in planta* experiments (Pantigoso et al. 2020a; Pantigoso et al., 2021). Similarly, this study employed three root exudate-derived compounds: galactinol, threonine, and 4-hydroxybutyric acid, that were identified earlier to occur in high concentrations in the root exudation profile of *Arabidopsis thaliana* grown under low P conditions (Pantigoso et al., 2020b).

Quantitative analysis of the effect of root exudates on bacteria solubilization

Using a 2.5 mm platinum wire loop, a streak of bacteria culture obtained from pure cultures of each of the three selected isolates was dipped into liquid Luria-Bertani medium (Bertani, 1951), and incubated separately in a rotary shaker at 170 rev min^{-1} at room temperature overnight

until reaching the mid-exponential growth phase. A 50 µL diluted ($OD_{600} = 1$; 1×10^8) aliquot from each pure bacterial culture and 50 µL of 10 mM concentration from a given compound (galactinol, threonine, and 4-hydroxybutyric acid) was added to 4.95 mL liquid NBRIP (National Botanical Research Institute Phosphate) medium and incubated in a rotary shaker for 72 hours (Nautiyal, 1999). One of each of the three dissolved compounds was combined (one-third part per each compound) and mixed at the same final concentration of 0.1 mM. For the inoculation of the bacterial co-inoculum, one of each of the three bacterial strain was prepared and mixed at the same final concentration ($OD_{600} = 1$; 1×10^8) and incubated for 72 hours. Two plant-unavailable sources of phosphate, calcium phosphate and phytin, were used to prepare NBRIP medium. The NBRIP medium is comprised of glucose (10.0 g), $Ca_3(PO_4)_2$ (5.0 g), NaCl (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $(NH_4)_2SO_4$ (0.5 g), KCl (0.2 g), $MnSO_4$ (0.03 g), $FeSO_4 \cdot 7H_2O$ (0.003 g) with a pH of 7.0–8.0. For phytin media preparation, calcium phosphate was replaced with 10 g of phytin ($C_6H_6Ca_6O_{24}P_6$). The pH of the initial phosphate media was near neutral for both media (~7 pH). After incubation, the solution was centrifuged at 6000 rpm for 20 minutes to remove both the suspended bacteria cells and the remaining calcium phosphate/phytate. Liquid calcium phosphate/phytin medium, with each compound separately, and without the addition of bacteria, were used as controls. The concentration of phosphate in the supernatant was analyzed according to the protocol of Soltanpour et al. (1983) and measured with an inductively coupled plasma-optical emission spectrometer (ICP-OES; Perkin Elmer 7300DV) at the Soil, Water and Plant Testing Laboratory of Colorado State University.

Quantitative analysis of the effect of root exudates on bacteria growth

A 10 µL diluted ($OD_{600} = 1$; 1×10^8) aliquot from each pure culture of *E. cloacae*, *B. thuringiensis*, and/or *P. pseudoalcaligenes* and 5 µL of each of the three compounds at 10 mM

concentration were combined with 150 µL calcium phosphate/phytin liquid medium separately (one bacterial strain per compound) and in combination (one strain combined with the compound mixture) in a 96 well-plate. Subsequently, the plate was incubated for 48 hours at 25 ℃ in a spectrophotometer, and growth, was monitored by optical density (660 nm). After incubation, the maximum specific growth rate for the culture (μ_{max}) was used to compare the effect of the compound on bacterial growth, based on the calculations of Maier and Pepper (2015). Liquid calcium phosphate/phytin medium without the addition of bacteria was used as a control. Deionized and DNA-free water was used to bring the controls to the same volume as the inoculated treatments.

Application of root exudates to the soil

Certified organic seeds of commercial corn (*Zea mays*) cultivar 'Natural Sweet F1' from Johnny's Selected Seeds (Windslow, Maine) were grown under greenhouse conditions at the Horticulture Center of Colorado State University, Fort Collins, CO. The average temperature in the greenhouse was 20 to 25 ℃ and the experiment lasted six weeks. Seeds were sown in squared pots containing 300 g pine forest soil, horizon O, collected to a depth of 30 cm from a natural area, Grey Rock Forest, Poudre Canyon, Bellvue, CO, (40.69 °N, 105.28 °W, 5,580 feet of elevation). The climate is semiarid, with an average annual precipitation of 409 mm (usclimatedata.com, accessed 2021). The soil is classified as a sandy clay loam with an organic matter content of 3.3%, nitrogen content of 0.4 ppm, phosphorus 26.7 ppm and a pH of 6.8. Pine soil forest with a history of no-fertilizer amendment were used because of its undisturbed conditions relative to highly managed agricultural soils. No fertilization or amendments were applied, and the corn plants were irrigated based on growth and demand keeping a relatively constant moisture in the soil. Ten posts with corn plants were assigned to each of five treatments,

10 repetitions per each treatment. The treatments consisted of pots receiving one individual compound and the three in combination, as well as the control. The compounds galactinol, threonine, 4-hydroxybutyric acid, and a combination were applied to the base of the corn plants twice a week. A volume of 1 mL at 1 mM concentration was added to each plant, except for the control, which received an equivalent amount of pure water. The treatment with the combination of compounds also received addition with a total concentration of 1 mM (0.33 mL of each compound).

Plants were harvested 6 weeks after emergence, roots were gently rinsed to removed soil particles, and the fresh weight of roots and shoots was recorded. Plants were oven dried at 90 °C for 72 hours, and the dry weight was also recorded. Total P in the plant shoot and root tissues were analyzed separately by digesting the plant tissue in a block digester with HCl and HNO_3 and cleared with H_2O_2. Then the sample was brought to a volume of 50 mL, and total P was read on an ICP-OES. Available P in the soil samples was identified using the Olsen P Method (Olsen, 1954). Both plant and soil P analysis were performed at the Ward Laboratories (Kearney, Nebraska).

Data analysis

One-way ANOVA was used to analyze the effects of root-exudate derived compounds on dissolution of P by individual bacterial isolates and co-inoculum incubated in calcium phosphate/phytin. One-way ANOVA analyzed the effects of root-exudate derived compounds on bacterial growth. One-way ANOVA was used to examine the effects of compound addition on plant dry biomass, and P content in soil and plant tissue. Homogeneity of variance and normality were assessed previously. A probability level of $p = 0.05$ was considered statistically significant.

Results

Effects of root exudates on growth of phosphorus solubilizing bacteria

The effect of the three-root exudate-derived compounds was assessed on bacteria growing in an organic and inorganic phosphate media. In the inorganic P calcium phosphate media, galactinol significantly increased the growth of *B. thuringiensis,* but threonine, 4-hydroxybutyric acid, and the combination of compounds did not influence the bacterial growth (Figure 1C). In contrast, the effect of threonine, 4-hydroxybutyric acid, and galactinol significantly decreased the growth of *P. pseudoalcaligenes* and *E. cloacae*, but applying a mixture of the compounds did not result in a significant change in growth (Figure 1A,B). Similarly, galactinol and 4-hydroxybutyric acid significantly decreased the growth of the bacterial consortia, but no effect was observed for threonine and the combination of the compounds (Figure 1D).

When examining bacterial growth in the organic phytin media, galactinol significantly increased the growth of *B. thuringiensis,* but threonine, 4-hydroxybutyric acid and the combination of compounds did not have an effect on *B. thuringiensis* (Figure 2C). Similar to what it was observed in the inorganic media threonine and 4-hydroxybutyric acid significantly decreased the growth of *P. pseudoalcaligenes* and *E. cloacae* in the phytin media, but the combination of compounds did not cause a significant change (Figure 2A,B). Galactinol decreased the growth of *P. pseudoalcaligenes* but did not affect *E. cloacae.* Threonine, 4-hydroxybutyric acid, and galactinol significantly decreased the growth of the bacterial consortia but no effect was observed with the combination of compounds (Figure 2D). In summary, only galactinol showed a significant increase in the growth of *B. thuringiensis* under both organic and inorganic media. *E. cloacae* and *P. pseudoalcaligenes* significantly showed reduced growth in

both P media to all compounds except for the mix, which had a lower concentration of each compound.

Effects of root exudates in enhancing the phosphorus solubilization ability of bacteria

The effect of three root-exudate derived compounds on the enhancement of P solubilization by bacteria was assessed. In the calcium phosphate inorganic media, threonine, 4-hydroxybutyric acid, galactinol, and the combination of compounds significantly increased dissolved P in the medium for *E. cloacae* and *P. pseudoalcaligenes* (Figure 3A,B). For *B. thuringiensis*, only threonine and 4-hydroxybutyric acid increased dissolved P (Figure 3C). In contrast, threonine, galactinol and the combination of compounds significantly increased dissolved P in the bacterial consortia (Figure 3D) and the uninoculated media with just compounds added showed no significant effects. However, the effect of the compound addition on the enhancement of phosphorus solubilization was not significant for any of the bacterial strains in phytin (organic phosphate) media (data not shown).

Effects of root exudate soil amendments on plant biomass

The impact of exogenous application of root-exudate on plant biomass was assessed after periodically adding compounds to corn plants growing in a nutrient-poor soil. Threonine addition significantly increased the fresh root weight of corn compared to the control treatment (Figure 4). No effects of threonine were observed for shoot or total plant biomass (shoots and roots). The other compounds, galactinol, 4-hydroxybutyric acid, and the combination of compounds, displayed no significant impacts on the corn root, shoot or total fresh biomass (Table S1).

Effects of root exudates on plant and soil nutrient content

Bi-weekly applications of threonine increased the concentration of N (*p-value*=0.023) and P (*p-value*=0.037) in plant roots related to the untreated control but did not significantly increase the levels of potassium, calcium, or magnesium (Figure 5). Similarly, 4-hydroxybutyric acid increased P (*p-value*=0.028) but not nitrogen concentration compared to the control. Galactinol and the compound combination did not affect the concentration of N and P in root tissues (Table S2).

The same applications of threonine increased soil available potassium, calcium, and magnesium, but N and P were not significantly altered. The compound 4-hydroxybutyric acid increased calcium and magnesium in soil. Galactinol and the compound combination did not significantly affect potassium, calcium, or magnesium levels. Galactinol, 4-hydroxypropionic acid, and the compound combination amended to the soil did not increase nitrogen and phosphorus content in soils (Table S3).

Discussion

We have previously reported that root exudates from *A. thaliana* exhibited distinct profiles under sufficient and deficient P conditions, and these exudates lead to an increase in dissolved P in a low P environment (Pantigoso et al. 2020). In the same Study, a second groups of compounds were found in high abundance under low P conditions, but no enhancement of P-solubilization in their own was observed. Thus, we hypothesized that they must act on P-solubilization via other means. This study investigates whether certain root-derived compounds, under P scarcity, modulate bacterial functional traits such as growth and P-solubilizing activity. Recent investigations have shown that the manipulation of root exudate composition from root apices enriches certain bacterial communities throughout the root system (Kawasaki et al. 2021). Here we found that the application of the amino acid threonine, the sugar galactinol, and the fatty

acid 4-hydroxybutyric acid modulated the growth and activity of PSB strains under *in vitro* conditions. In addition, the periodic exogenous amendment of threonine to a natural soil increased the growth of corn roots and increased the levels of plant available potassium, magnesium, and calcium in soils.

We observed bacterial specificity on the effect of the amended compounds. For instance, galactinol increased the growth of *B. thuringiensis* but decreased the growth of *E. cloacae* and *P. pseudoalcaligenes*. Galactinol and other RFOs (Raffinose Family of Oligosaccharides) are currently emerging as crucial molecules produced by plants during stress responses that provide relief against pathogen infection, drought, and high salinity stress (Sengupta et al. 2015, Kim et al. 2008). In addition, galactinol has been shown to be used by *Agrobacterium* as a nutrient source providing a competitive advantage to colonize the rhizosphere of tomatoes (Meyer et al 2018). The same mechanism to uptake RFOs is highly conserved in bacterial symbionts and pathogens from the Rhizobiaceae family (Meyer et al 2018); thus, diverse bacteria appear to have the capability to uptake and metabolize this group of compounds.

It has been reported that high sugar concentrations can inhibit bacterial growth, but lower levels of sugars can exhibit the opposite effect, which indicates that there is threshold-concentration upon which certain sugars act as growth inhibitors or as nutrient sources that stimulate growth (Mizzi et al. 2020). When assessing the effect of galactinol on PSB activity we observed that galactinol did not enhance the solubilization of P in *B. thuringiensis* but did increase P solubilization by *E. cloacae*, *P. pseudoalcaligenes*, and in the bacterial consortium. Sugar-like compounds such as galactose and galactosides have been reported to support microbial activity and growth of N-fixing *Sinorhizobium meliloti* before and during nodulation (Bringhurst et al. 2001). Zhang et al (2014) reported that free-living microorganisms in the

rhizosphere can use root exudates such as sugars, amino acids, and other compounds to promote colonization and functional traits that support plant growth and nutrition (Zhang et al. 2014). We note that galactinol increased P-solubilizing activity by *E. cloacae* and *P. pseudoalcaligenes,* but it reduced the growth of both bacteria. In contrast, galactinol increased the growth of *B. thuringiensis* while maintaining its P-solubilizing activity. Aforementioned comparisons between bacterial growth and P-solubilization were only made under calcium phosphate due to P-solubilization was not significantly affected under phytin. Galactinol has been shown to be involved as signal stimulating root colonization by *Pseudomonas chlororaphis* O6 in cucumber eliciting an induced systemic resistance against plant pathogen *Corynespora cassiicola* (Kim et al. 2008). When challenged by abiotic stresses such as drought and salinity, tobacco plants overexpressing galactinol synthase (*CsGolS1*) demonstrated tolerance, however bacteria meditation for abiotic stresses was not reported (Kim et al. 2008). In light of these findings, we hypothesize that galactinol could be involved in growth and P-solubilization activity of PSB and could be concentration specific. Other previous studies have demonstrated that adding carbon compounds such as glucose to the soil can increase P microbial utilization as compared to solubilization (Kuono et al. 2002; Zhang et al. 2018), influencing the enrichment of rhizosphere bacteria (Carvalhais et al. 2015).

Similar to galactinol, the effect of threonine on PSB growth was strain specific. Threonine at 0.1 mM concentration showed an inhibitory effect on *E. cloacae* and *P. pseudoalcaligenes* but did not decrease the growth of *B. thuringiensis* in both the organic and inorganic media. Interestingly, treatments with lower amounts of threonine (0.03 mM) from the compound combination did not decrease the growth of any of the PSB. Inhibitory effects of amino acids (i.e., cysteine) on *E. coli* at higher concentrations have been previously reported (Harris, 1981).

Despite the negative effect on growth, threonine consistently enhanced the P solubilization of all the bacterial strains tested, suggesting a broader effect on PSB strain activity. In support of this, amino acids such as threonine are constituents and important N, C or energy sources for growth and activity for a range of bacteria (Radkov et al. 2016). Further, several bacterial species from the genera *Bacillus*, *Pseudomonas* and *Enterobacter* have been shown to exhibit chemotaxis toward multiple amino acids, including threonine (Ordal and Gibson, 1977; Oku et al. 2012). Carvalhais et al. (2015) have shown that exudation of different amino acids, in lower amounts, such as asparagine, ornithine, and tryptophan increase abundance of rhizobacteria *Bacillus sp*. and *Enterobacter* sp. In addition, root exudation of amino acids in P-deficient roots stimulate the growth and activity of organism involved in nutrient acquisition (Carvalhais et al., 2011). However, the effect of amino acids on bacterial growth and activity are highly variable among bacterial species and are influenced by the environment and the physiology of the organism (Yang et al. 2015). Furthermore, bacterial growth inhibition, attraction and repellent responses is caused by certain amino acids, and this effect is reversed when the concentration decreases. Thus, suggesting the inability of some bacterial strains to metabolize higher concentrations of certain amino acids (Yang et al. 2015). For instance, Brisson et al. (2021) showed that shikimic and quinic acids were secreted by roots under phosphate stress and were preferentially absorbed by microorganisms and correlated with root growth (Zhalnina et al. 2018). Similarly, Harbort et al. (2020) showed that coumarins improve plant performance by eliciting microbe-assisted iron nutrition. These findings suggest that the plant selectively modulates its root exudation profile to stimulate the proliferation of groups of microorganisms that aid in phosphorus acquisition.

The effect of 4-hydroxybutyric acid (4-HA) on bacterial growth followed the pattern observed for threonine. 4-HA also reduced the growth of the bacterial consortia under calcium

phosphate media but positively impacted P solubilization in all three PSB strains except for the bacterial consortia. Hydroxy fatty acids such as 4-HA function as modulators of many signal transduction pathways in plants in response to different stresses (Macabuhay et al. 2021; Siebers et al. 2016). Recent studies evidenced that fatty acids from plant root exudates have the ability to participate in strong plant-microbe interactions stimulating N metabolism in rhizosphere bacteria (Sun et al. 2016). Lu et al. (2012) demonstrated stimulation of bacterial enzymatic-mediated denitrification by fatty acid oleamide and erucamide from duckweed root exudates. This evidence supports the hypothesis that compounds such as threonine and 4-HA could be acting as a signal rather than simple C source for certain plant beneficial bacteria. We also noted that exogenous application of threonine to soils resulted in an increase of fresh corn root weight, while the remaining compounds applied did not affect plant growth. We hypothesize that the effect of threonine on plant biomass is a response to its ability to trigger activity and chemotaxis on a wide range of microbes favoring positive nutritional feedback for plants. In support of this hypothesis, a study by Harbort et al. (2020) used data from plant fitness, coupled with elemental content and transcriptomic analysis, to confirm the benefits conferred by commensal microbes under iron limitation occur via coumarin signaling-molecule mechanism relieving iron starvation. It is commonly held that plants and rhizosphere microbes consume and compete for free amino acids in the rhizosphere (Owen and Jones 2001; Forsum et al. 2008). Plant roots are often outcompeted by microbes in the uptake of externally applied amino acids (Reeve et al. 2009; Moe, 2013). Early work has shown that amino acids can inhibit plant growth at high concentrations, a phenomenon referred as “general amino acid inhibition” which can affect plant root growth (Piryns et al, 1988; Pratelli et al. 2010). These observations have led to the speculation that amino acids may be taken up from the rhizosphere, where they are first rescued

and mineralized by bacteria, and then used as an inorganic N source by plants (Moe, 2013). In addition, under nutrient limited conditions bacterial survival strategies can increase their ability to catabolize amino acids (Zinser and Kolter, 1999). In addition, we found that threonine increased N and P concentration in plant root tissues, and the available calcium and magnesium in soils were higher as well.

We found that bacterial growth response was similar under organic and inorganic P, but the P-solubilizing activity varied. The three compounds tested impacted PSB activity under calcium phosphate but did not affect P solubilization under phytin. It has been reported that the ability of microbes to solubilize P is highly dependent on the source of P (Illmer and Schinner, 1995; Wan et al. 2021). Thus, it appears that threonine, galactinol and 4-hydroxybutyric acid are inducing mineral dissolving compounds such as organic acids that help the bacteria to solubilize inorganic P. This is in contrast to the mechanism used by bacteria to solubilize/mineralize organic P such as the secretion of phosphatases and phytases (Sinha, 1967).

Conclusion

Specialized metabolites, derived from root exudates, act as signals and sources for rhizosphere microorganisms with implications for P availability and uptake by plants. This study has described the effects of specialized root exudates, such as threonine, 4-hydroxybutyric acid and galactinol to stimulate P-solubilizing activity of bacteria and its effect on soil and plant nutrient content. Effect of specialized compounds on bacteria were found to be species and phosphorus source dependent. Under greenhouse conditions threonine was shown to stimulate root growth and significantly higher nitrogen and phosphorus content in root tissues. Our findings expand on the function of exuded specialized compounds and suggest alternative approaches to effectively recover residual P from soil. Further work should focus on identifying

and testing root exudate derived compounds aiming to efficiently promote biological activity, growth and functional features, leading to improvements in nutrient use efficiency, and the reduction of excessive applications of synthetic fertilization to croplands.

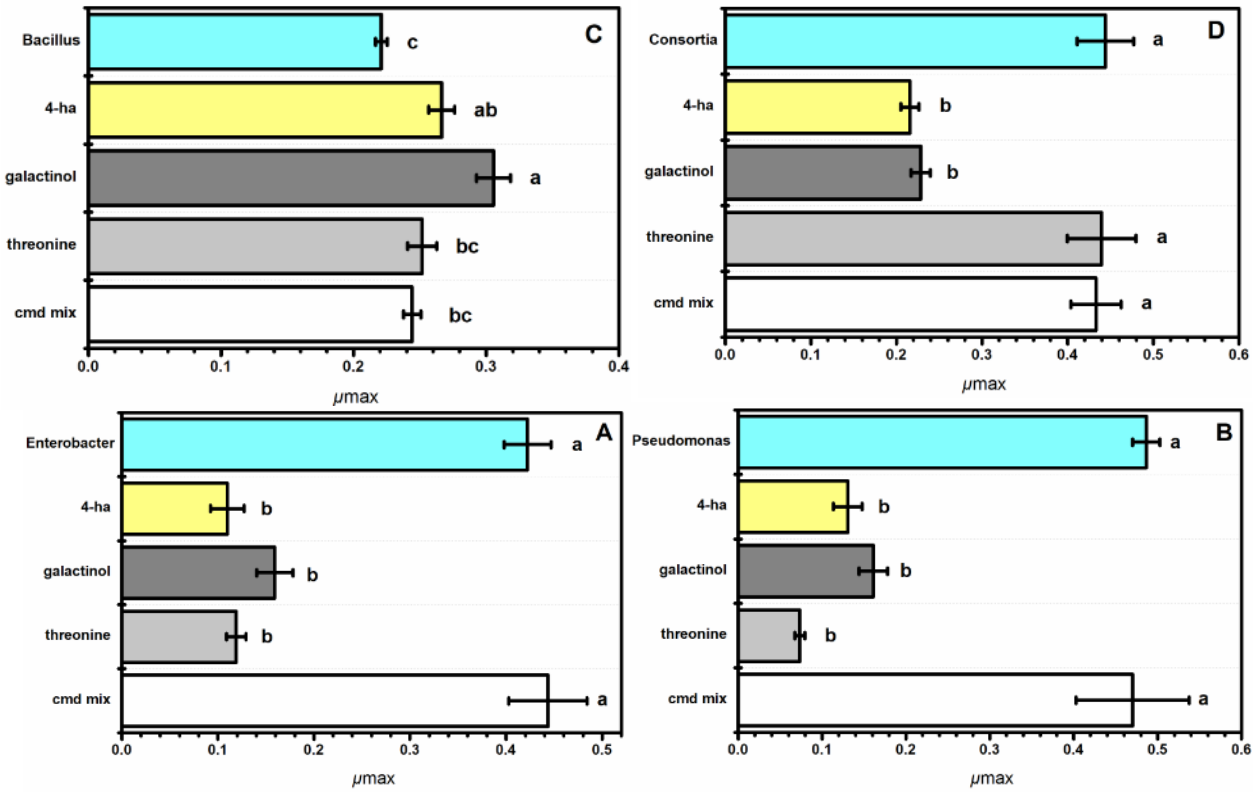


Figure 3-1. Maximum specific growth rate (μ_{max}) of phosphorus solubilizing bacteria incubated with different root exudates under inorganic calcium phosphate media. Each panel correspond to A. *Enterobacter cloacae*, B. *Pseudomonas pseudoalcaligenes*, C. *Bacillus thuringiensis*, D. bacteria consortium. Different letters denote statistical significance (*p-value*<0.05).

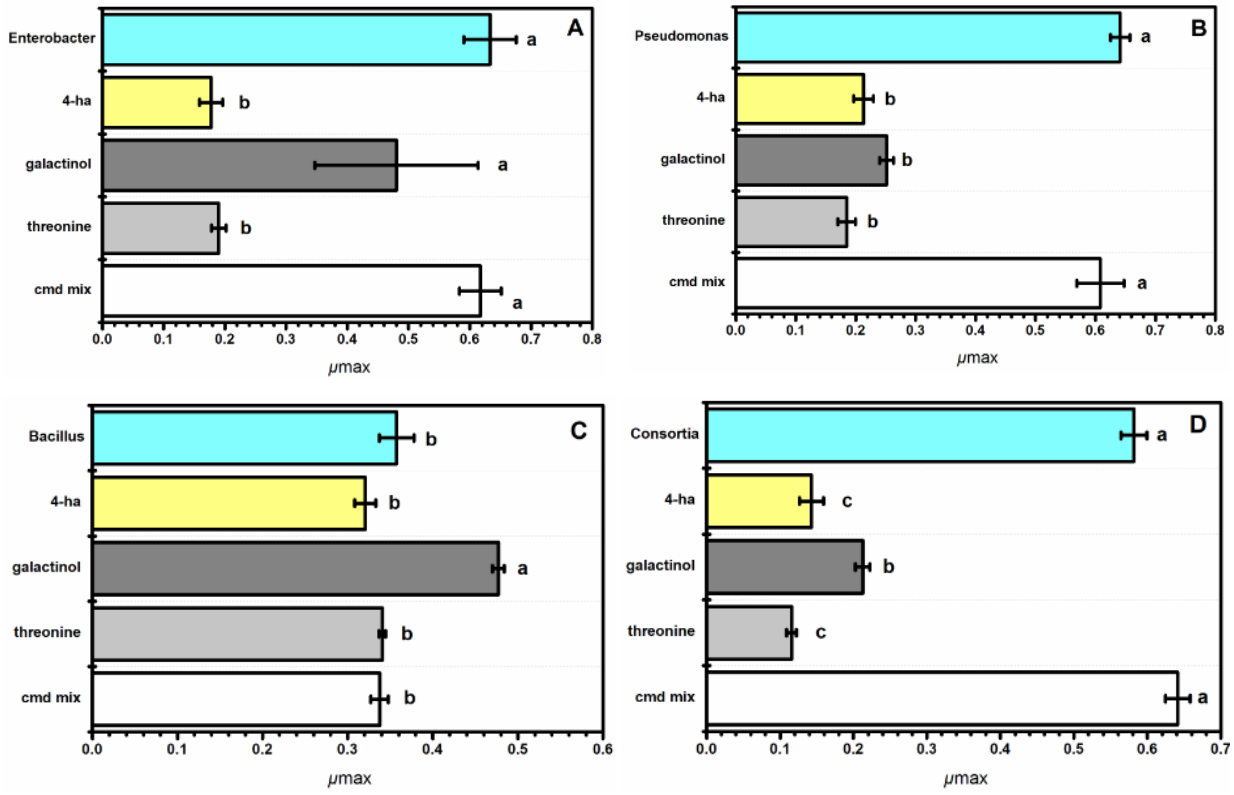
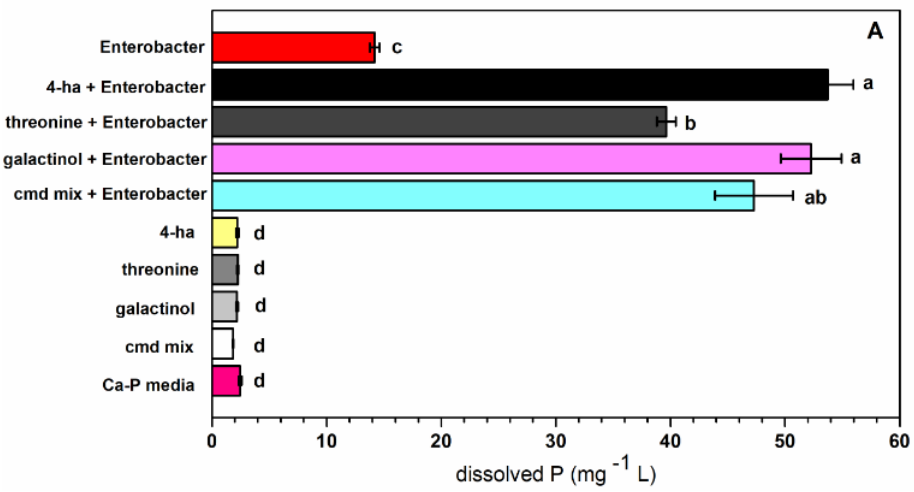


Figure 3-2. Maximum specific growth rate (μ_{max}) of phosphorus solubilizing bacteria incubated with different root exudates in phytin organic media. Each panel corresponds to A. *Enterobacter cloacae*, B. *Pseudomonas pseudoalcaligenes*, C. *Bacillus thuringiensis*, D. bacteria consortium. Different letters denote statistical significance (*p-value* < 0.05).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cmd mix + Enterobacter
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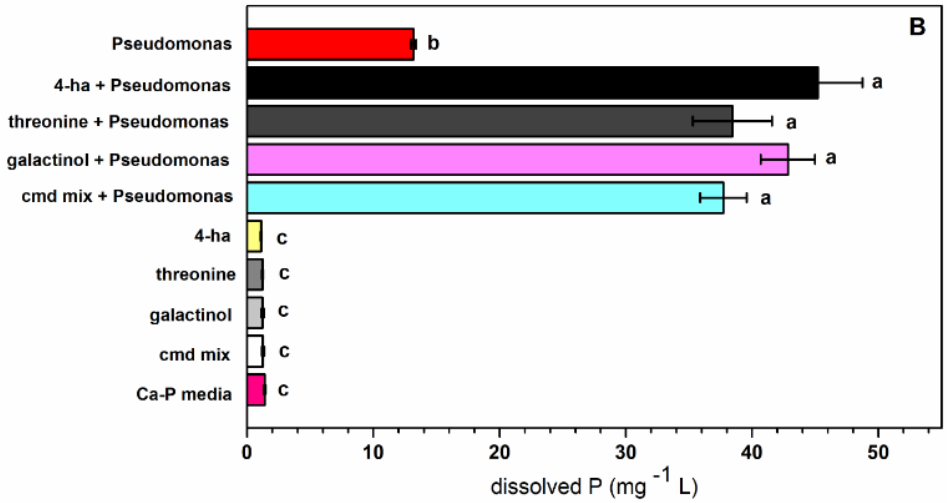
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galactinol + Pseudomonas
cmd mix + Pseudomonas
4-ha
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cmd mix
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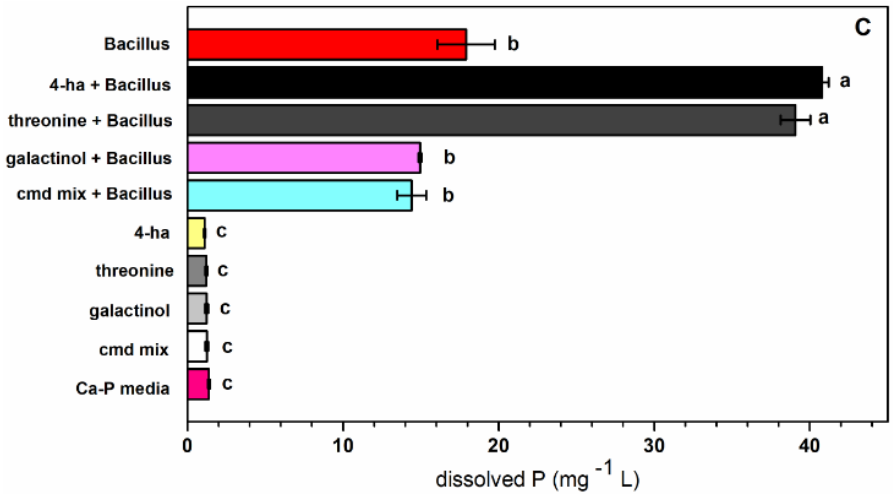
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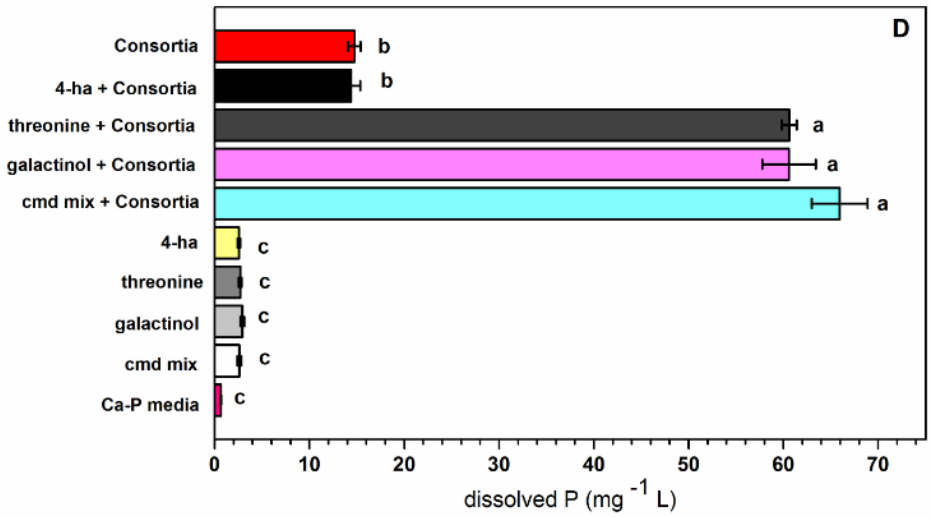

Figure 3-3. Effect of individual root exudates on dissolved P by phosphorus solubilizing bacteria. Each panel corresponds to A. *Enterobacter cloacae*, B. *Pseudomonas pseudoalcaligenes*, C. *Bacillus thuringiensis*, D. Bacteria consortium. Different letters denote statistical significance (*p-value*<0.05).

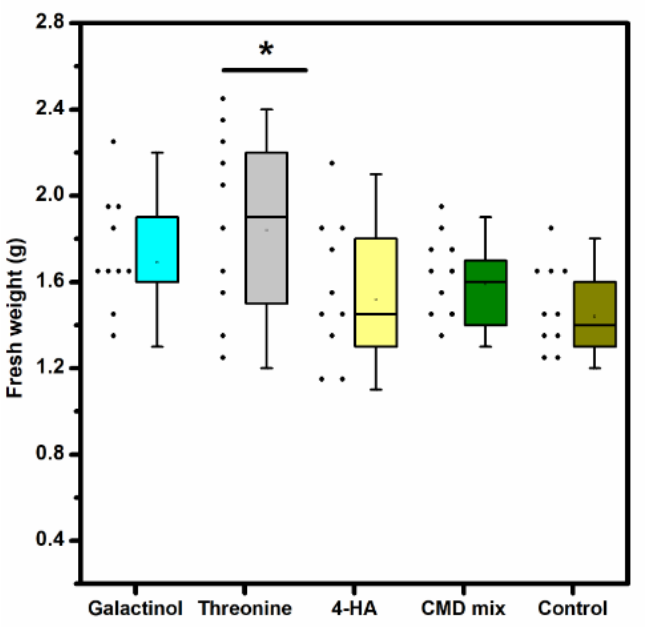

Figure 3-4. Root fresh weight (g) of corn plants after periodic addition of root exudates. Asterisk (*) denote statistical significance (*p-value* < 0.05) over the other treatments. Boxplot show distributions of n=10 samples where circles are data points.

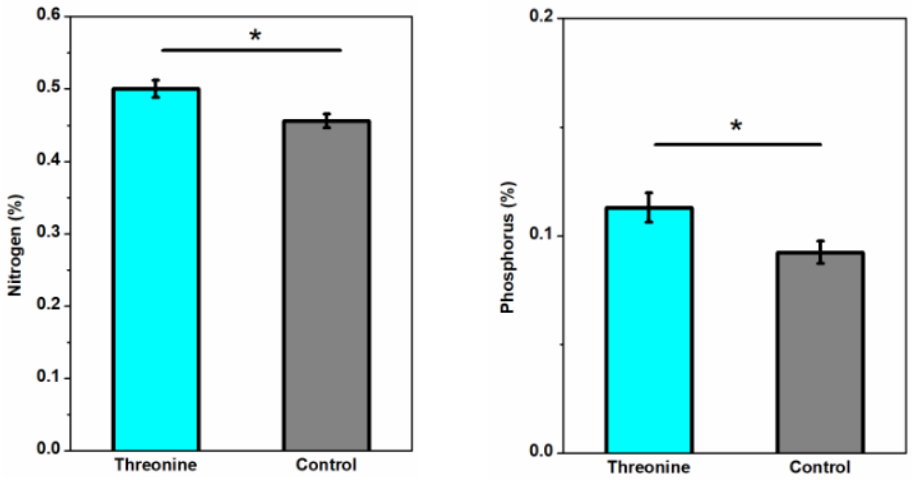

Figure 3-5. Nutrient content (%) in corn plant roots for nitrogen and phosphorus.

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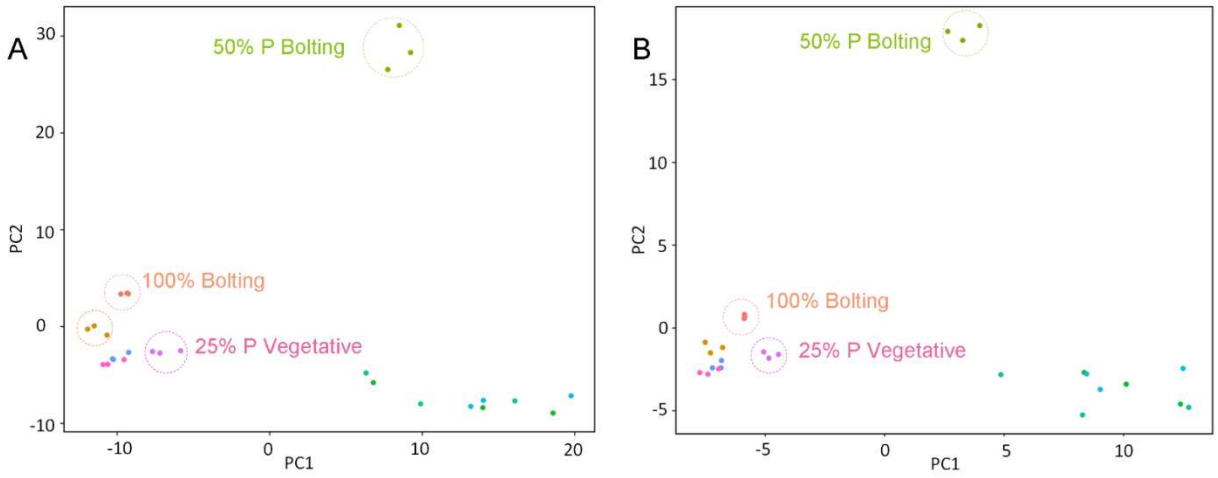
Suggestions For Future Research

Due to its multifunctional properties, root exudates are key to manipulate plant-microbial interactions to improve nutrient acquisition. Future research should focus on identifying specialized molecules with elicitation of microbial activities that could: 1. Improve the colonization and proliferation of commercial soil inoculants. 2. Target indigenous beneficial bacteria (involved in nutrient acquisition) already established in the rhizosphere of plant crops. To achieve the discovery of these specialized metabolites, we still need to develop optimal and universally standard methods of root exudate collection and analysis that resemble environmental soil conditions.

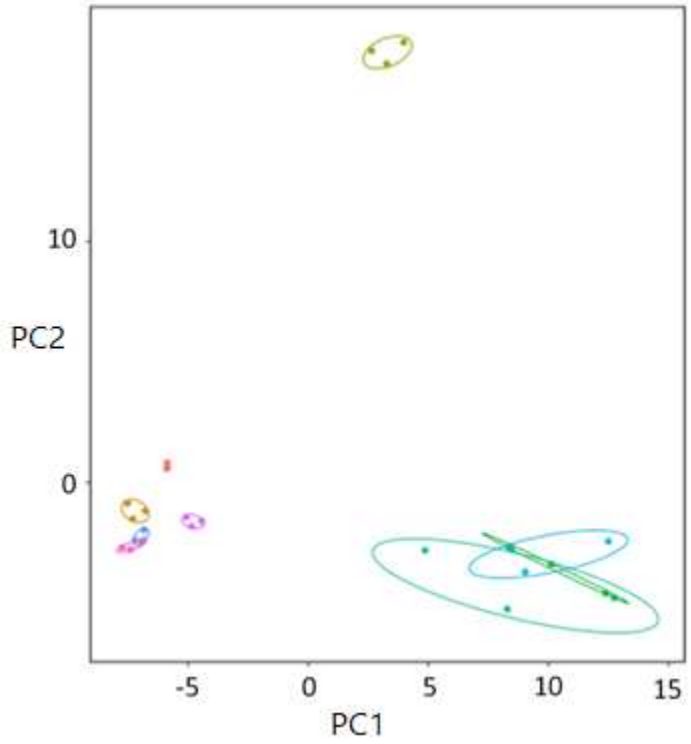
On the other hand, genomic information from soil microorganisms derived from cultivation-independent approaches is rapidly advancing our understanding of the phylogenetic of rhizosphere microbes. However, cultivation of soil microorganisms is still a complementary and necessary tool to fully understand functional attributes of plant microbiota. Thus, strategies that use next generation sequencing to identify conditions for effective cultivation followed by the isolation of microbes involved in nutrient solubilization, immobilization and mineralization will be critical.

Finally, identifying patterns in root exudate profiles and rhizosphere microbiome under wide range of environmental conditions, nutrient status and plant developmental stages will increase our understanding or plant-microbial interactions in the rhizosphere and translate this information into more efficient use of resources and crop nutrition.

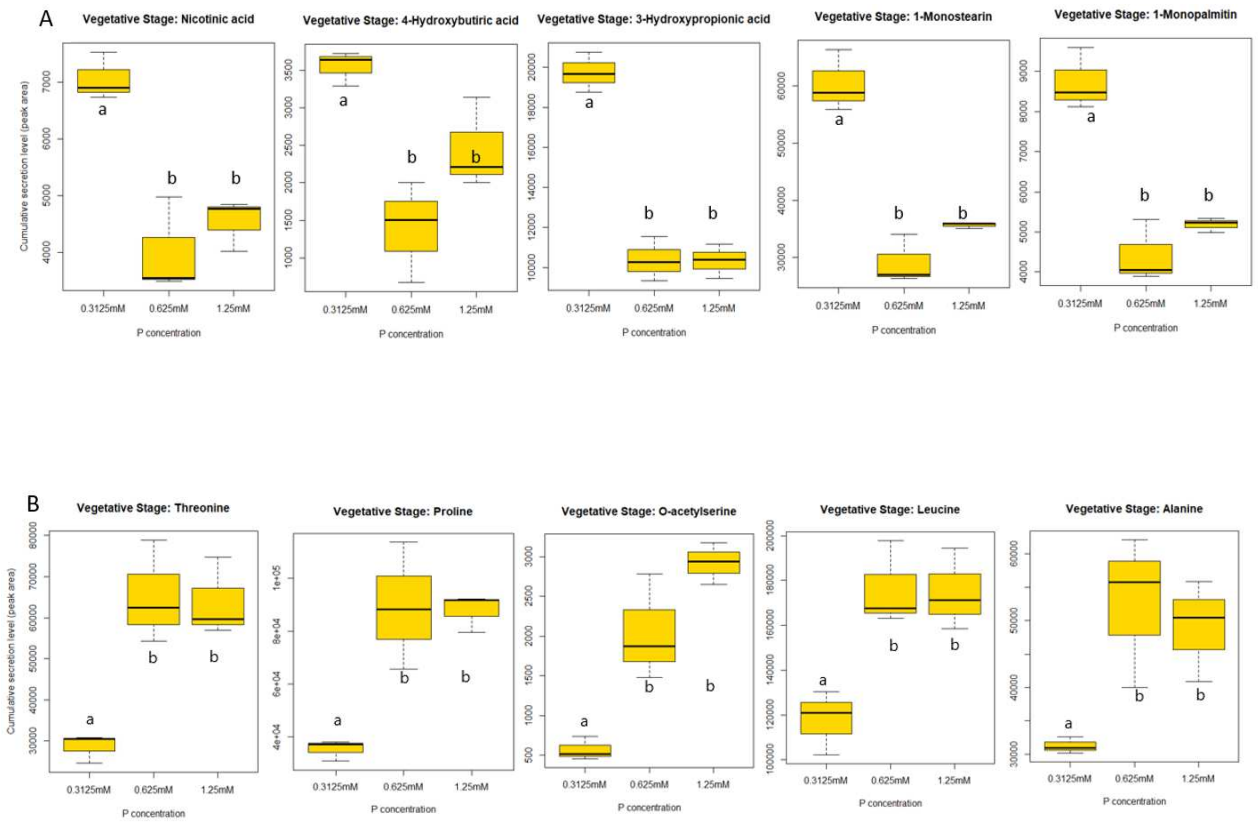
APPENDICES



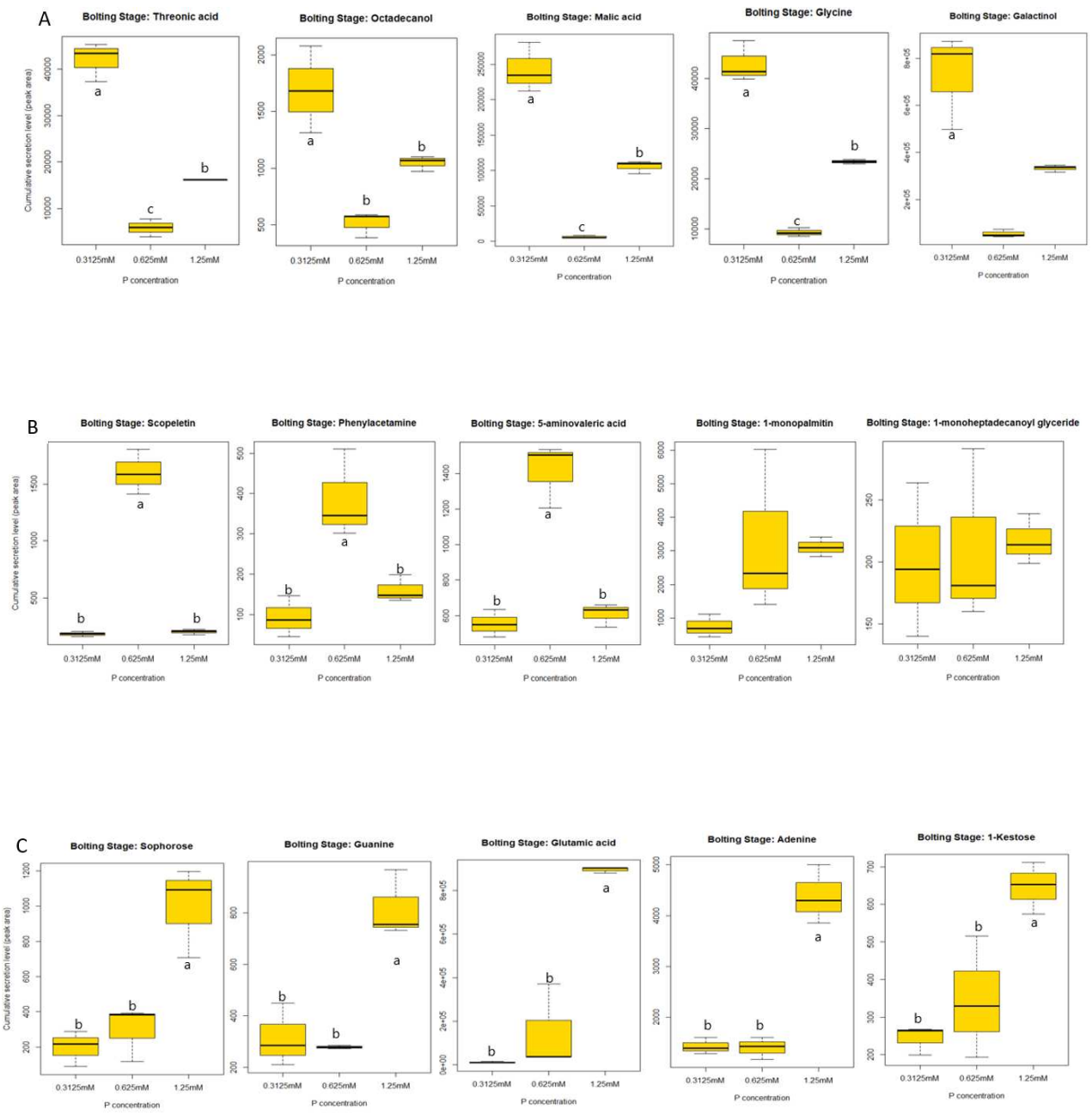
Supplementary figure 1-1. Root exudate compounds diverge in response to plant developmental stage and phosphate fertilization rate. (A) 456 compounds detected using GC-MS are plotted on the graph. PCA show dissimilarity among group of metabolites in the seedling stage at different fertilization levels: 25% (light green), 50% (light blue), 100% (green); vegetative stage: 25% (purple), 50% (pink), 100% (blue); and bolting stage: 25% (brown), 50% (olive), 100% (orange). (B) Data reduced to 201 annotated compounds with proper identification. PCA of compounds grouped by phosphate treatments in the seedling stage: 25% (light green), 50% (light blue), 100% (green); vegetative stage: 25% (purple), 50% (pink), 100% (blue); bolting stage: 25% (brown), 50% (olive), 100% (orange). The dotted circle indicates a cohesive group at a given fertilization level.



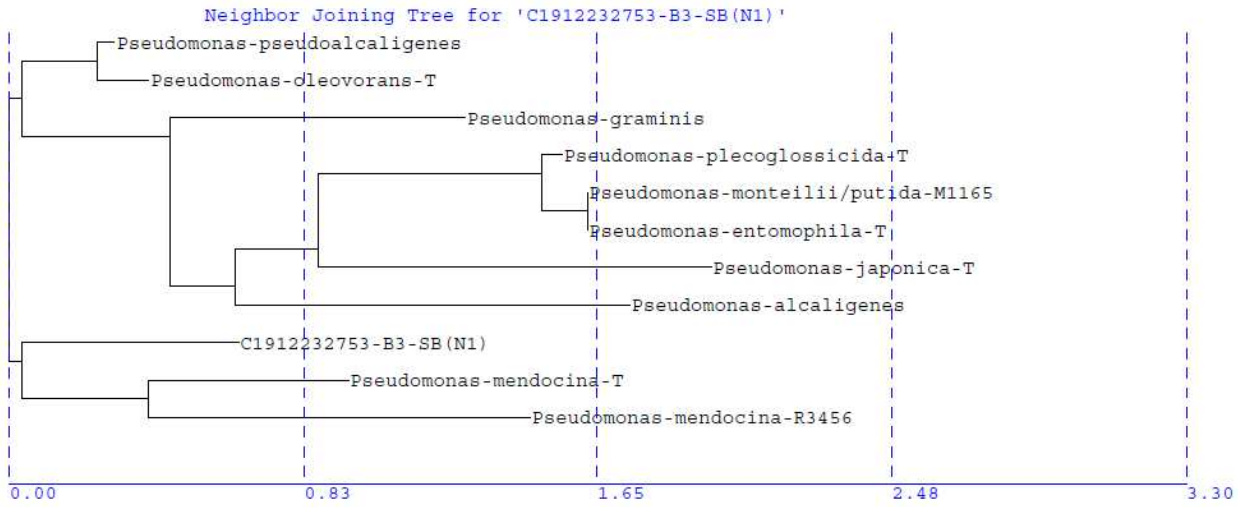
Supplementary figure 1-2. Root exudate compounds grouped by repetitions of fertilizer level. Treatments within plant developmental stages differ from one another, particularly the vegetative and bolting growth stages. Ellipses circle three repetitions of same fertilizer level. Color code correspond to seedling: 25% (light green), 50% (light blue), 100% (green); vegetative: 25% (purple), 50% (pink), 100% (blue); bolting: 25% (brown), 50% (olive), 100% (orange).

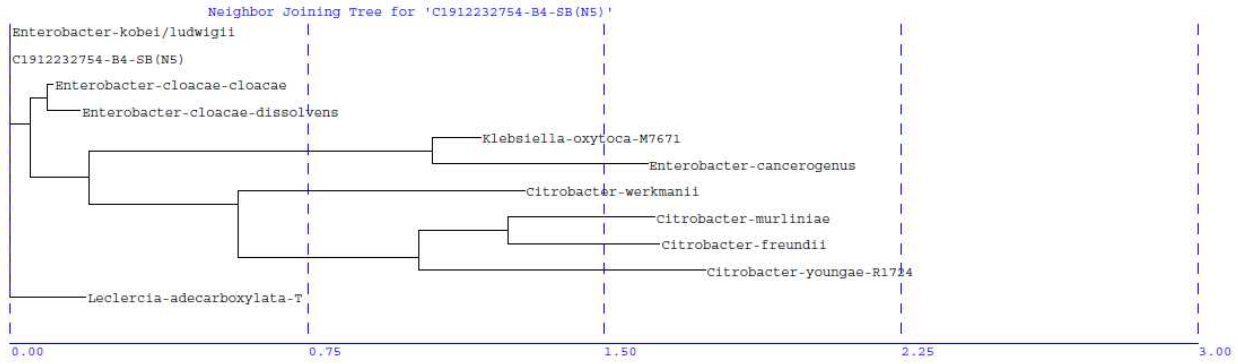


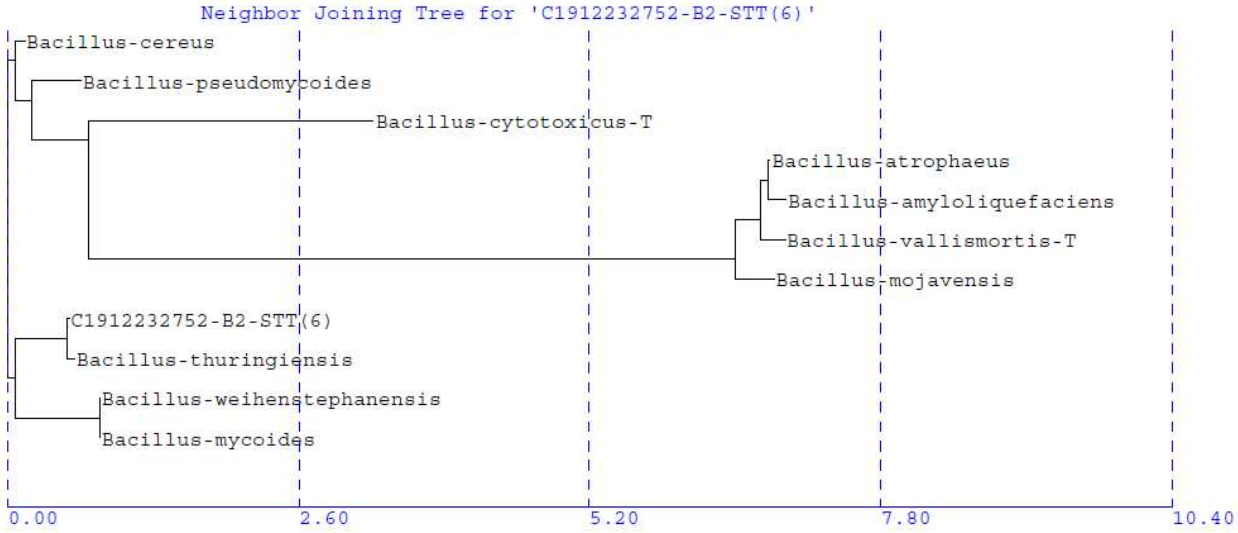
Supplementary figure 1-3. Top 10 compounds showing changes in cumulative secretion levels in the vegetative developmental stage ($p<0.05$) in response to increasing phosphate addition (0.312, 0.625 and 1.25 mM). Selected compounds based on PCA from vegetative 25% phosphate (A), and vegetative 50% and 100% phosphate (B).



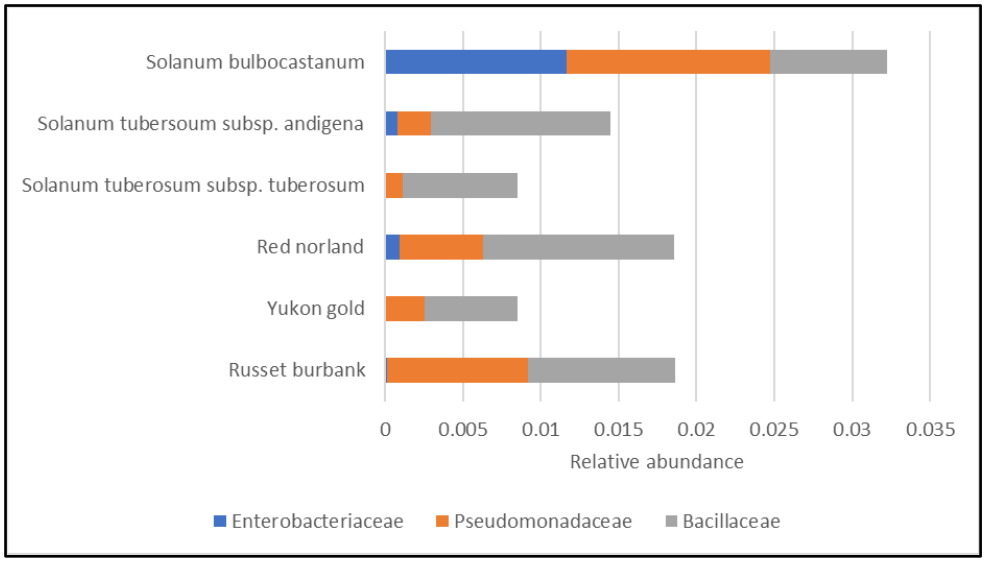
Supplementary figure 1-4. Top 15 compounds showing changes in cumulative secretion levels in the bolting developmental stage ($p<0.05$) in response to increasing phosphate addition (0.312, 0.625 and 1.25 mM). Selected compounds from bolting 25% phosphate (A), bolting 50% P (B) and bolting 100% (C).


Neighbor Joining Tree for 'C1912232753-B3-SB(N1)'
Pseudomonas-pseudoalcaligenes
Pseudomonas-oleovorans-T
Pseudomonas-graminis
Pseudomonas-plecoglossicida-T
Pseudomonas-monteilii/putida-M1165
Pseudomonas-entomophila-T
Pseudomonas-japonica-T
Pseudomonas-alcaligenes
C1912232753-B3-SB(N1)
Pseudomonas-mendocina-T
Pseudomonas-mendocina-R3456
0.00
0.83
1.65
2.48
3.30

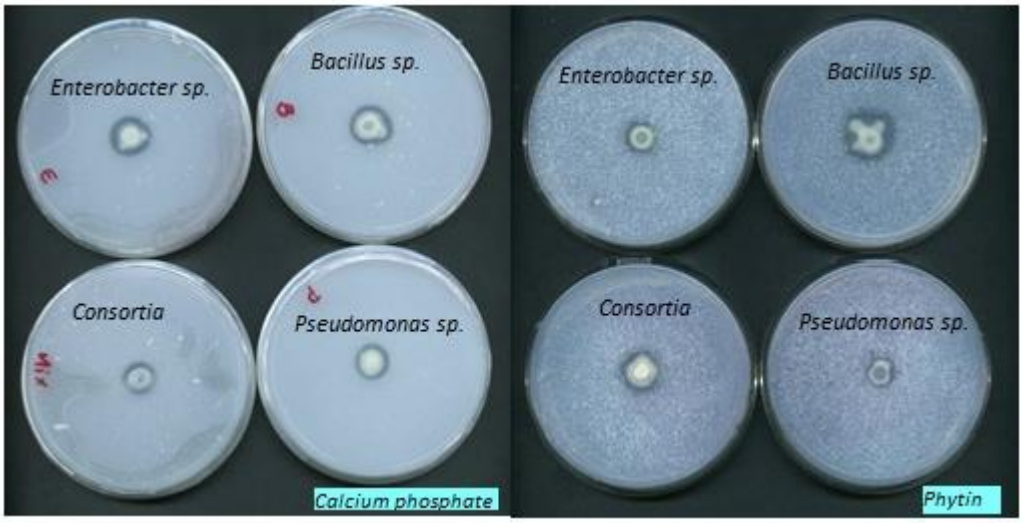

Neighbor Joining Tree for 'C1912232754-B4-SB(N5)'
Enterobacter-kobei/ludwigii
C1912232754-B4-SB(N5)
Enterobacter-cloacae-cloacae
Enterobacter-cloacae-dissolvens
Klebsiella-oxytoca-M7671
Enterobacter-cancerogenus
Citrobacter-werkmanii
Citrobacter-murliniae
Citrobacter-freundii
Citrobacter-youngae-R1724
Leclercia-adecarboxylata-T
0.00
0.75
1.50
2.25
3.00


Neighbor Joining Tree for 'C1912232752-B2-STT(6)'
Bacillus-cereus
Bacillus-pseudomycoides
Bacillus-cytotoxicus-T
Bacillus-atrophaeus
Bacillus-amyloliquefaciens
Bacillus-vallismortis-T
Bacillus-mojavensis
C1912232752-B2-STT(6)
Bacillus-thuringiensis
Bacillus-weihenstephanensis
Bacillus-mycoides
0.00
2.60
5.20
7.80
10.40

\



Supplementary figure 2-2. Relative abundances of 16S rRNA sequencing reads of three major microbial taxa including *Enterobacteriaceae* (blue), *Pseudomonadaceae (orange)* and *Bacillaceae* (grey). Relative abundances of non-cultivated *Solanum bulbocastanum* is compared to other cultivated potato relatives. The data used to generate this graph come from an experiment performed under the same experimental conditions detailed in Pantigoso et al., (2020).



Supplementary figure 2-3. Phosphate-solubilizing effects of bacteria isolates, individually and in consortium, in calcium phosphate media (left) and phytin media (right). For both pictures: *Enterobacter cloacae* (upper left), *Bacillus thuringiensis* (upper right), *Pseudomonas pseudoalcaligenes* (lower right), combination of the three bacteria (lower left).

Supplementary table 2-4. Soil nutrient content per treatments with different P source types. One group with inoculated PSB (phosphorus solubilizing bacteria), and a control treatment without PSB inoculation.

			Amomonium Acetate (ppm)				M-3
With-PSB	**KCL Nitrate (ppm N)**	**Phosphorus Olsen (ppm P)**	**K**	**Ca**	**Mg**	**Na**	**S**
Calcium phosphate	0.3	13.3	37	1190	161	51	17.4
Phytin	0.3	212.6	51	948	298	50	19.6
Fertilizer	0.4	274.2	20	920	129	46	40.5
Mix	0.3	230.3	20	920	223	54	26.6
No P amended	0.3	10.8	21	1035	146	37	16.2

			Amomonium Acetate (ppm)				M-3
Without-PSB	**KCL Nitrate (ppm N)**	**Phosphorus Olsen (ppm P)**	**K**	**Ca**	**Mg**	**Na**	**S**
Calcium phosphate	0.9	12.9	41	1017	140	30	26.6
Phytin	0.7	240.6	66	1021	291	38	15.4
Fertilizer	2	212.9	128	1693	329	26	43.4
Mix	0.3	177.4	22	992	222	49	36.7
No P amended	0.4	14.5	23	943	138	40	32.3

Supplementary table 2-5 Soil pH values per treatments with different P source types. One group with inoculated PSB (phosphorus solubilizing bacteria), and a control treatment without PSB inoculation.

With-PSB	Soil pH 1:1
Calcium phosphate	6.6
Phytin	6.8
Fertilizer	5.6
Mix	6.1
No P amended	6.7

Without-PSB	Soil pH 1:1
Calcium phosphate	6.9
Phytin	6.9
Fertilizer	5.4
Mix	6.1
No P amended	6.6

Supplementary table 3-1 Available nutrient content (ppm) in soils at harvest after periodic application of compounds to soil. Using ANOVA, statistical differences were analyzed between treatments for each nutrient found in soil. Different letters denote statistical significance (*p-value* < 0.05).

	N	P	K		Ca		Mg	
Galactinol	0.6	7	145.6	b	1530.2	b	184	b
Threonine	0.35	6.6	175.5	a	1731.25	a	208.25	a
4-Hydroxybutyric acid	0.44	6.96	161.4	ab	1689.2	a	203.8	a
Mix	0.52	6.48	152.6	ab	1617	ab	195.6	ab
Control	1.02	7.34	157.8	ab	1500.8	b	182.4	b
p-values	>0.05	>0.05	0.037		<0.001		<0.002	