SCREENING FOR CALCIUM PHOSPHATE SOLUBILIZING

RHIZOBIUM LEGUMINOSARUM

A Thesis

Submitted to the Faculty of Graduate Studies and Research

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

In the

Department of Soil Science

University of Saskatchewan

Saskatoon, SK

By

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ABSTRACT

Rhizobium leguminosarum are well known for their ability to fix nitrogen (N). In addition, their capacity to solubilize phosphate has been receiving attention in recent years. The work presented in this thesis examined two aspects of screening and evaluating dicalcium phosphate (Pi) ($CaHPO_4$) solubilizing *R. leguminosarum*. The objectives of this study were to: 1) identify a medium that is sensitive and effective as a screening tool for phospahte solubilizing *R. leguminosarum* ; 2) determine the effect of N and carbon (C) on growth and P solubilization of *R. leguminosarum* isolates; 3) determine the relationship between the ability to solubilize $CaHPO_4$ by *R. leguminosarum* isolates on solid medium and in liquid broth of same composition; and 4) assess and compare the ability of *R. leguminosarum* isolates to solubilize different P sources in soil under growth chamber conditions.

In this study, 30 *R. leguminosarum* isolates were evaluated for phosphate solubilization in broth and solid formulations of three different media, Yeast Mannitol Extract (YEM), Botanical Research Institute Phosphate Nutrient medium (MNBRI) and Pikovskaya Phosphate medium (PVK). All media contain $CaHPO_4$ as the only phosphorus (P) source. The *R. leguminosarum* isolates were selected on the basis of their different plasmid profiles, indicative of genetically distinct isolates.

All 30 isolates increased the Pi concentration in solution to varying degrees in liquid cultures but performance varied from one medium to another. The highest average solution Pi concentration achieved by the 30 *R. leguminosarum* isolates was obtained from PVK cultured broth. $CaHPO_4$ solubilization by *R. leguminosarum* isolates in liquid was associated with a decrease in pH. Among the three tested media, the lowest pH by the thirty *R. leguminosarum* isolates was obtained in PVK. Ability of the isolates to solubilize $CaHPO_4$ on the solid media was not comparable to the performance of the isolates grown in liquid because only fewer *R. leguminosarum* isolates showed visible P solubilization on the solid media.

The composition and formulation of medium influence the ability of the *R. leguminosarum* isolates to solubilize $CaHPO_4$. Effects of N and C concentrations on the

growth and $CaHPO_4$ solubilization by nine *R. leguminosarum* isolates were examined in liquid formulation. Ammonium N had a greater influence on the growth and $CaHPO_4$ solubilization by *R. leguminosarum* isolates than C at the tested levels. The growth of isolates was inhibited by ammonium N at 0.5 g L^{-1} as $(NH_4)_2SO_4$ meaning there were less viable cells in this N concentration than were of ammonium N at 0.1 g L^{-1}. The ability of isolates to solubilize Pi however was not affected by ammonium N at 0.5 g L^{-1} as $(NH_4)_2SO_4$. The media containing low N (0.1 g $(NH_4)_2S0_4$ L^{-1}) both Pi solubilization and growth of *R. leguminosarum* isolates were not affected.

R. leguminosarum isolates were tested for their effects on growth and P uptake of canola plants in P-deficient soils amended with different P sources. *R. leguminosarum* isolates were selected separately based on their ability to solubilize $CaHPO_4$ from the three screening media. A quadrant model was used based on the ability of the 30 *R. leguminosarum* isolates to solubilize $CaHPO_4$ on both solid and liquid formulations within a medium. The effect of *R. leguminosarum* on canola dry mass, tissue Pi content and total Pi uptake varied from one isolate to another, but was not different from the controls. The quadrant model failed to correlate isolates able to solubilize $CaHPO_4$ in laboratory screening to isolates able to solubilize P in the growth chamber. Despite the influence of the medium composition and formulation, none of tested media predicted Pi solubilization ability by the *R. leguminosarum* isolates in soils under growth chamber conditions, from their Pi solubilization of laboratory screenings.

The work of this thesis demonstrates that phosphate solubilization is a complex process that depends on both organism and soil. Growth condition is an important factor for a *R. leguminosarum* isolate to express its ability to solubilize $CaHPO_{4.}$ Liquid media screenings illustrate an isolate's ability to solubilize $CaHPO_4$ under nonstressful conditions, but solid media screenings demonstrate the P solubilization result of an isolate under more stressful conditions. The lack of relationship in P solubilization ability by *R. leguminosarum* isolates, between laboratory methods to soil test, means neither liquid or solid media can provide a definitive selection process. Additional parameters should be investigated to modify the soil bioassay protocols and ultimate selection procedures. These include pH conditions, isolate colonization, growth, and survival on plants and rhizosphere.

ACKNOWLEDGMENTS

I would like to acknowledge and thank my supervising professor Dr. Diane Knight for her support, encouragement and commitment to this project. I gratefully acknowledge the support, encouragement and guidance provided from the members of my Advisory committee, Drs. Mary Leggett, Fran Walley, and Bing Si. I also would like to thank my external examiner Dr. Karen Bailey.

To my family, Del, Becky and Jordan, thank you for your unconditional support, understanding, encouragement and patience. Thank you for being there always!

To my friends and colleagues at Novozymes BioAg (formerly Philom Bios Inc), thank you for the support and encouragement, especially Sarah Vick, Alissa Dearborn, Maureen Troesch, Shelagh Steckler, Ute Pastor, Amanda Yuskiw and Heather Meshka, Kari Priest, thank you for your great assistance in my laboratory and growth chamber work; Greg Holloway, Johnny Xavier and Cathy Kuiack, thank you for your encouragement and expertise.

A special thanks to Erin Burton and Darren Richman for helping me with soil samples. I would also like to acknowledge and thank the staff and fellow graduate students of the Department of Soil Science for making my time as a graduate student a truly wonderful experience.

I sincerely thank Novozymes BioAg for funding the project.

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LIST OF ABBREVIATIONS

C	carbon
cfu	colony forming unit
Ca	calcium
DCP	dicalcium phosphate ($CaHPO_4$)
DCPD	dicalcium phosphate dihydrate
MNBRI	modified National Botanical Research Institute's phosphate growth medium
N	nitrogen
NH_4-N	ammonium nitrogen
P	phosphorus
Pi	phosphate
PSM	phosphate solubilizing microorganisms
PVK	modified Pikovskaya's phosphate medium
SYE	sucrose yeast extract
TCP	tricalcium phosphate
YEM	modified yeast extract mannitol

1.0 INTRODUCTION

Phosphorus (P) is the major nutrient after nitrogen (N) that limits plant growth (Gyaneshwar, et al., 2002; Fernandez, et al., 2007). Chemical P fertilizer is the main source of plan available P in the agriculture soils, but almost 75 to 90% of added P fertilizer is precipitated by iron, aluminum and calcium complexes present in the soils (Gyaneshwar, et al., 2002; Turan et al., 2006). Soils in the agricultural region of Saskatchewan are dominantly calcareous. In calcareous soils, pH ranges between 7.3 and 8.5 depending on the amount of $CaCO_3$ present in the soil (Lindsay, 1979). With high levels of exchangeable Ca, available P ions react with solid phase $CaCO_3$ and precipitate on the surface of these particles to form Ca-P minerals (Lindsay et al., 1989).

Many soil bacteria and fungi have the ability to solubilize phosphate (Pi) minerals and make it available to plants (Oberson et al., 2001; Egamberdiyeva et al., 2003). They are capable of using inorganic and organic forms of P (Tarafdar and Jungk, 1987; Chen et al., 2002). The population of phosphate solubilizing microorganisms (PSM) varies from soil to soil and ranges from less than 10^2 colony forming units (cfu) g^{-1} of soil to 3 x 10^6 cfu g^{-1} of soil (Peix et al., 2001; Chabot et al., 1993). Total number of microorganisms is significantly increased in the rhizosphere which can be as much as 5 to 20-fold compared to soil outside of the rhizosphere (Brown and Rovira, 1999). Phosphate solubilizing microorganisms represented 0.1 to 0.5% of total bacterial and fungal populations in 29 Alberta soils (Kucey, 1983). Phosphate solubilizing microorganisms occur in both fertile and P deficient soils (Oehl et al., 2001).

Phosphate solubilizing microorganisms can grow in media containing insoluble calcium phosphate as the sole source of P. They utilize the phosphate mineral in order to release high amounts of Pi in the soil. Phosphate solubilization by PSM via the organic acids and H^+ excreted (Asea et al., 1988; Kucey, 1988; Cunningham and Kuiack, 1992; Illmer and Schinner, 1995; Takeda and Knight, 2006).

Laboratory screening for PSM typically is accomplished by an assay that uses either precipitated phosphate agar plates assay or liquid media/culture broth. Precipitated phosphate agar assays have been used widely in the initial screening for PSM (Pikovskaya, 1948; Halder et al., 1991; Abd-Alla 1994; Wenzel et al., 1994). Microorganisms capable of solubilizing phosphate minerals are grown on an agar medium with insoluble phosphates as the only P source. They produce a visible clearing zone around their colonies. The precipitated phosphate agar assay is a fast and simple method. However, despite the popularity of precipitated phosphate agar assays, doubts have been raised regarding the applicability of the precipitated phosphate agar method for a wide range of microorganisms. Many isolates that do not produce a clearing zone on the agar plates can solubilize various types of insoluble inorganic phosphates in liquid media (Louw and Webley, 1959; Gupta et al., 1994; Nautiya, 1999). In contrast to the precipitated phosphate agar plate assays, the liquid method is considered more sensitive for detecting P solubilization by microorganisms because a measurable Pi concentration can be detected from more microorganisms (Gupta et al., 1994; Nautiyal, 1999; Sangeeta and Nautiyal, 2001). The liquid media/culture broth method measures Pi released into the liquid culture from the initial insoluble phosphate substrate. Unfortunately, the liquid media method is labor intensive and time consuming. Given the differences in detecting PSM by these two methods, a direct comparison of solid to liquid formulations within a medium has rarely been conducted.

Media compositions, especially of N and carbon (C), and the buffering capacity of the medium, greatly influence P solubilization (Cunningham and Kuiack, 1992; Whitelaw, 2000; Sangeeta and Nautiyal, 2001; Pradhan and Sukla, 2005). Both ammonium salts and nitrate salts have been used as individual N sources or as a combined N source in P solubilization studies: ammonium N was best in reducing medium pH and promoting P solubilization (Cunningham and Kuiack 1992; Zhao, 2002; Pradhan and Sukla 2005). In other words, ammonium N concentration has an impact on P solubilization by microorganisms (Asea et al., 1988; Nautiyal, 1999).

The C source is considered the most influential factor for organic acid production. The metabolic pathway and the types of organic acids produced by microorganisms are either a result of the regular metabolic routes or induced by the type of sugar used (Nahas, 2007). For example, Nautiyal (1999) found that Pi concentration in a broth containing calcium phosphate increased with an increase in glucose with *Pseudomonas* sp. Thus, various sources of N and C, and different concentrations of N and C in combination with other components in the media affect P solubilization.

Phosphate solubilization is a complex phenomenon which depends on many factors such as nutritional, physiological and growth conditions of the microorganisms (Reyes et al., 1999). Soybean benefited from the co-inoculation of *B. japonicum* and the P solubilizing bacterium *Pseudomonas striata* based on the dry weight of nodules, dry matter of plants, and yield (Wasule et al., 2003). *Bacillus subtilis* increased rice root length and yield significantly from the control in both pot and field experiments in a Himalayan soil (Trivedi et al., 2003). However, according to Gyaneshwar et al. (2002), it is common to obtain PSM under laboratory conditions, while field performance by the PSM are highly variable - no increase in crop yield or P uptake was found in 70% of field experiments. To find a highly efficient PSM that performs well under laboratory conditions and in soil remains a challenge. To find a medium that predicts the ability of PSM to perform in soil from the laboratory screening is an importment step to approach that challenge.

The work presented in this thesis examined two aspects of screening and evaluating $CaHPO_4$ solubilizing *R. leguminosarum bv. viciae*. The objectives of this research were to: 1) identify a medium that is sensitive and effective as a screening tool for $CaHPO_4$ solubilizing *R. leguminosarum*; 2) determine the effect of N and C on growth and P solubilization of *R. leguminosarum* isolates in liquid media; 3) determine the relationship between the ability to solubilize $CaHPO_4$ by *R. leguminosarum* isolates on solid medium and in liquid medium of samilar composition; and 4) assess and compare the ability of *R. leguminosarum* isolates to solubilize different P sources in soil under growth chamber conditions.

2.0 LITERATURE REVIEW

2.1 Phosphorus in the Soil System and Its Availability to Plants

Phosphorus is importment for plant growth because it stimulates growth of young plants, promotes a vigorous start and hastens maturity. Consequently, plant growth is diminished, maturity is delayed and yield reduced when an inadequate supply of P is present (Sawyer and Creswell, 2000).

Phosphorus exists in soil in organic and inorganic forms. Each form is a continuum of many P compounds, existing in different phases and in equilibrium with each other. Availability of P ranges from soluble P (plant available) to very stable (plant unavailable) compounds (Fig. 2.1). There is a dynamic and complex relationship among the different forms of P involving soil, plants and microorganisms. Organic P compounds are found in humus and other organic materials including decayed plant, animal and microbial tissues. Organic P is also the principal form of P in manure. Organic P is usually combined with oxygen to form ester compounds (Thompson and Troch, 1978). These esters make up about 50 to 70% of identified organic P (McGill and Cole, 1981). In the Chernozemic top soils in western Canada, organic P was estimated at 25 to 55%, which could be available for plant growth after mineralization (Stewart et al., 1980). Phosphorus in labile organic compounds can be slowly mineralized (broken down and released) as available inorganic phosphate or it can be immobilized (incorporated into more stable organic materials) as part of the soil organic matter (Tate, 1984; Mckenzie and Roberts, 1990). The process of mineralization or immobilization is carried out by microorganisms and is highly influenced by soil moisture and temperature. Mineralization and immobilization are most rapid in warm, well-drained soils (Busman et al., 2002).

Approximately 70 to 80% of P found in cultivated soils is inorganic (Foth, 1990). Phosphorus fertilizers are the main input of inorganic P in agriculture soils. Despite

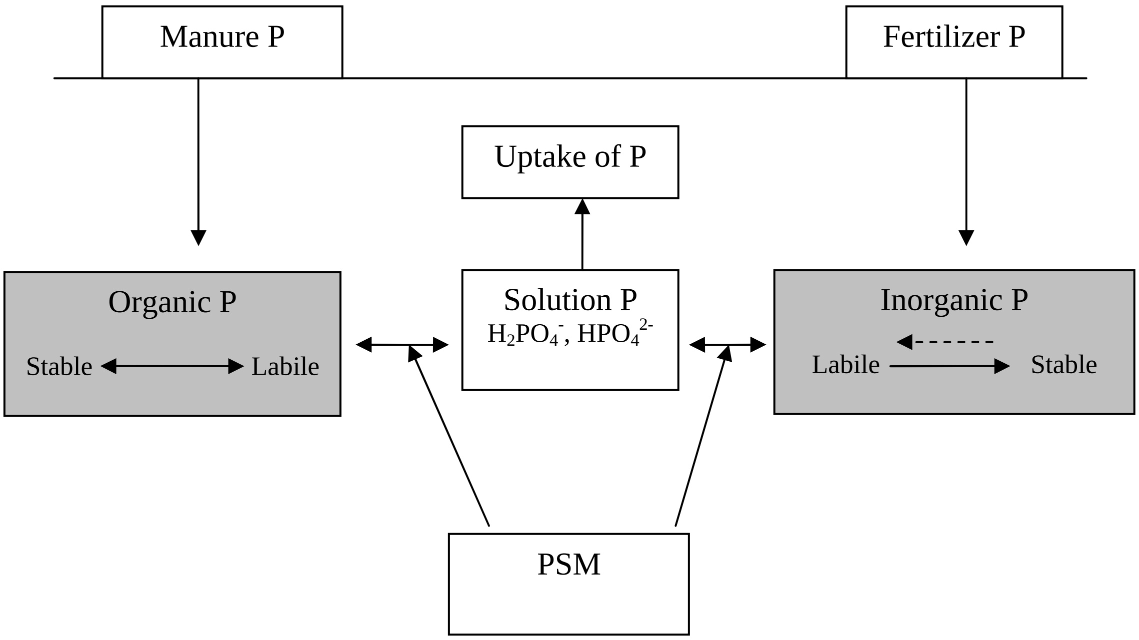

Figure 2. 1 The soil phosphorus cycle (adapted from Sharpley, 2006), solid line indicates the conversion process. The dashed line means very slow conversion.

its wide application, after N, P is the major nutrient limiting plant growth (Gyaneshwar, et al., 2002; Fernandez, et al., 2007). Worldwide, 5.7 billion hectares contain too little available P for sustaining optimal crop production (Hinsinger, 2001). Phosphorus ion concentration in most soils ranges from 0.1 to 10 μM; P required for optimal growth ranges from 1 to 5 μM for grasses and 5 to 60 μM for high demanding crops such as tomato and pea (Raghothama, 1999; Hinsinger, 2001). Plant available P in 29 southern Alberta soils was approximately 1% of total soil P (Kucey, 1983).

Phosphorus in fertilizers is converted to water-soluble Pi as orthophosphate ions $H_2PO_4^-$ and HPO_4^{2-} in soil within a few hours after application (Schulte and Kelling, 1996). As the fertilizer enters the soil, moisture from the soil begins to dissolve the fertilizer particles. The concentration of Pi in solution increases around the dissolved fertilizer particles and diffuses a short distance from the fertilizer particles (Busman et al., 2002). In most soils, orthophosphate ions $H_2PO_4^-$ and HPO_4^{2-} dominate at pH below 7 and above 7.2, respectively (Hinsinger, 2001). These negatively charged P ions attach strongly to the surfaces of minerals containing positively charged ions such as iron (Fe^{3+}) and aluminum (Al^{3+}) in acidic soils via sorption/desorption processes. Fe^{3+} and Al^{3+} act as the sorption sites for the negatively charged P (Sato and Comerford, 2005). These P anions also precipitate with the calcium (Ca^{2+}) in calcium carbonate minerals in calcareous soils forming relatively insoluble compounds. Both processes result in P being fixed or bound, thus removed from the soil solution and unavailable for plants (Banik and Dey, 1982; Foth, 1990; Schulte and Kelling, 1996).

The conversion from stable P to labile P is a slow process and does not occur over the course of one growing season (Guo and Yost, 1998). However, the conversion from labile P to plant available P is a rapid process (Tate and Salcedo, 1988). Soil inorganic P exists as many compound species and the species distribution is controlled mainly by solution pH and the concentration of cations (Lindsay, 1979). In most soils, maximum P availability occurs between pH 5.5 to 7. Within this pH range, P is fixed by hydrous oxides of Fe, Al, and Mn. Between pH 6 to 8 and pH 6.5 to 8.5, P is fixed by silicate minerals and Ca, respectively. As a result, the most efficient use of P in neutral and calcareous soils occurs between pH 6 to 7 (Sharpley, 2006).

In neutral and calcareous soils, soil pH is between 7.3 and 8.5 depending on the amount of $CaCO_3$ presenting in the soil (Lindsay, 1979). With high levels of exchangeable Ca, available P ions react with solid phase $CaCO_3$ and precipitate on the surface of these particles to form Ca-P minerals: $Ca(H_2PO_4)_2$ (monocalcium P), $CaHPO_4{\cdot}2H_2O$ (dicalcium phosphate dihydrate, DCPD, brushite), $CaHPO_4$ (dicalcium phosphate, DCP, monetite), $Ca_3(PO_4)$ (tricalcium phosphate, TCP), $Ca_4H(PO_4)_3{\cdot}2.5H_2O$ (octacalcium P, OCP), $Ca_5(PO_4)_3{\cdot}OH$ (hydroxyapatite) and least soluble apatites (Lindsay et al., 1989). The finer the size of solid phase $CaCO_3$ the higher the fixation of P. The solubility of Ca-P minerals is generally accepted as DCPD > DCP > TCP > hydroxyapatite. In alkaline soils, the initial products of reaction of fertilizer triple superphosphate are mainly DCPD and DCP (Russell, 1980; Whitelaw et al., 1999). Different phases of Ca-P compounds are transferable and, at a given pH, can be dissolved from unstable phases to become precipitated as stable phases. For example, a relatively soluble brushite when applied as fertilizer to calcareous soils can be transformed to monetite and slowly to octacalcium P. Octacalcium P can be stable for years if fertilizer is applied continually. The formation of hydroxyapatite is the ultimate result (Sposito, 1989).

Soil solution Pi concentration increases when water soluble P fertilizer applied to soil is readily dissolved. Over time, the soil fixes P by processes such as precipitation, thereby reducing its concentration in the soil solution. As a result, Pi in the soil solution is general low. In the United States, an average 29% of P added in fertilizer and manure is removed by harvesting crops (Sharpley, 2006). The Pi content is usually greater at surface horizons than in subsoils due to its immobility. The Pi accumulation in topsoil can be a problem especially in a reduced tillage system because of minimal or no mechanical incorporation when fertilizer is applied (Sharpley, 2006). Phosphate fertilizers can increase P availability initially, but will promote the formation of insoluble P minerals and consequently lead to P buildup. Therefore, P management is important both environmentally and economically. Phosphate solubilizing microorganisms may be an answer for maintaining the supply of plant available P because PSM carry out the conversion from labile P to plant available P.

2.2 Phosphate Solubilization by Microorganisms

2.2.1 Phosphate solubilizing microorganisms

Many soil bacteria and fungi have the ability to solubilize P and make it available to growing plants (Antoun et al., 1998). Microorganisms are central to the soil P cycle and play a significant role in mediating the transfer of P between different inorganic and organic soil P fractions, subsequently releasing available P for plant acquisition (McLaughlin, 1988; Oberson, 2001). There are two aspects in microbial P solubilization: 1) P released by solubilization processes (Rodriguez and Fraga, 1999), and 2) P released from accumulated P in biomass of microorganisms (Oehl, 2001). Inorganic phosphate solubilizing microorganisms (PSM) constitute various portions of the soil microbial population and vary from soil to soil (Banki and Dey, 1982; Kucey et al., 1989). The populations of PSM are reportedly varied and ranged from very low (less than 10^2 cfu g^{-1} of soil) in a soil in Northern Spain to very high (3×10^6 cfu g^{-1} of soil) in Quebec, Canada (Chabot et al., 1993; Peix et al., 2001). Phosphate solubilizing microorganisms were isolated from rhizosphere soils of different crops (Ponmurugan and Gopi, 2006). The numbers of PSM are more important in rhizosphere than non-rhizosphere soil (Kucey et al., 1989). The PSM represented 0.1 to 0.5% of total bacterial and fungal populations in 29 Alberta soils (Kucey, 1983). PSM occur in both fertile and P-deficient soils and the fastest initial rates of P incorporation were observed in P-deficient soils (Oehl, 2001).

Phosphate solubilizing fungi are superior to their bacterial counterpart for P solubilization both on precipitated agar and in liquid (Kucey, 1983). Fungal hyphae in liquid culture were attached to P mineral particles shown by scanning electron microscopy, whereas bacteria were not (Chabot et al., 1993). Furthermore, because of their hyphae, fungi are able to reach greater distances more easily in soil than bacteria. JumpStart ® is the first P-solubilizing inoculant on the market and the active ingredient is the fungus *Penicillium bilaiae* formerly known as *Penicillium bilaji* and *Penicillium bilaii*. *P. bilaiae* is known for its superior ability in Ca-P solubilization (Kucey, 1988; Sanders, 2003). *P. bilaiae* had a high solubilization for Idaho rock phosphate in solution culture (Kucey, 1983; Asea 1988). In addition to *P. bilaiae*, *P. aurantiogriseum* and *Pseudomonas* species solubilized Ca-P (Illmer and Schinner, 1995), and *Pseudomonas striata* and *Penicillium oxalium* solubilized Al-P and Fe-P (Gadagi and Tongmin, 2002). *Penicillium regulosum* strains utilized rock phosphate and stimulated the growth of maize plants with 3.6 to 28.6% increase in dry matter yields in a low fertility soil at pH 6.25 (Reyes et al., 2002). *Penicillium* and *Aspergillus* sp. are the dominant P solubilizing fungi found in rhizosphere (Kucey, 1983).

In addition to P solubilizing fungi, P solubilizing bacteria are present in soil and plant rhizospheres. The populations of these bacteria are higher in rhizosphere than non-rhizosphere soils (Katznelson et al., 1962). The most important P solubilizing bacterial genera are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium and Erwinia* (Rodriguez and Fraga, 1999). According to Babenko et al. (1984), the phosphate solubilizing patterns of bacteria were grouped into two categories: 1) soluble P increased linearly along with the growth of the bacterial culture; 2) soluble P increased at different points of the growth stage but not throughout the whole incubation period, which the authors attributed to induction and repression of the enzyme systems responsible for solubilization. Rodriguez and Fraga (1999) also compared 13 bacterial strains of different genera for their solubilizing abilities on different insoluble mineral phosphate substrates and indicated that *Rhizobium*, *Pseudomonas* and *Bacillus* species were among the most powerful P solubilizers.

Rhizobium leguminosarum is of particular interest because of its dual function: its ability to fix N and to solubilize P (Wood and Cooper, 1984; Chabot et al., 1996; Hara and de Oliveira, 2004). Lettuce and maize inoculated with two strains of P solubilizing *R. leguminosarum* are better in root colonization and growth. Additionally, rhizobia exhibited an ability to promote plant growth for non-legumes (Chabot et al., 1996b; Chabot et al., 1998). The multi-functionality exhibited by *R. leguminosarum* makes it important in food production in terms of reducing cost and improving efficiency of P fertilization, especially in P-limited soils, particularly in countries such as Australia, Brazil and India where soil available P is generally low. Roychoudhury and Kaushik (1989) reported that phosphate rock deposits are estimated at approximately 40 million tons in India. The phosphate rock deposits could be an inexpensive source of phosphate fertilizer for crop production if these deposits became available for plant growth (Halder et al., 1990).

Despite the beneficial influences by the PSM, some cases of inconsistent results have been reported. *Bacillus megaterium var. phosphoricum* performed inconsistently in soils as inoculant in India, former Soviet Union and the United States (Rodriguez and Fraga 1999). Furthermore, instability of P solubilizing character was reported for some organisms (Halder et al., 1990; Illmer and Schinner, 1992).

2.2.2 Plant growth promotion by phosphate solubilizing microorganisms

The potential use of P solubilizing microorganisms as inoculants with rock phosphates to increase P availability to plants has been studied intensively (Kucey, 1988; Illmer and Schinner, 1995; Sanders, 2003). Phosphate solubilizing microorganisms have an important contribution to overall plant P nutrition and growth, and have increased yields of many crops (Rodriguez and Fraga, 1999; Whitelaw, 2000; Leggett et al., 2001). Indirect growth promotion by PSM is achieved by reducing pathogen infection via the antibiotic or siderophores which are synthesized and supplied by the bacteria (Antoun et al., 1998; Rosas et al., 2006). A rhizospheric bacterium *Pseudomonas fluorescents*, solubilizes P, and produces antibiotics such as pyoluteorin (Trujilo et al., 2003). *Pseudomonas putida* produced siderophore (13 μmol benzoic acid mL^{-1}) (Pandey et al., 2006). A very small percentage of *R. leguminosarum* produced hydrogen cyanide and cyanogens. Hydrogen cyanide produced by *Pseudomonas* was used as a biological control of black root rot of tobacco (Antoun et al., 1998). Direct growth promotion includes fixing N_2 (*Rhizobium* biological N fixation), increasing root surface area (mycorrhizal associations), enhancing root systems by branching roots and stimulating root hair development (phytohormones stimulation), and solubilizing inorganic phosphate (*Penicillium* fungi) (Rodriguez and Fraga, 1999; Richardson, 2003). *P. bilaiae*, the active organism in the JumpStart ® was initially selected to solubilize P. *P. bilaiae* also promotes root growth and enhances root hair production (Gulden and Vessey, 2000). Two strains of *Rhizobium leguminosarum bv. phaseoli* stimulated root colonization on maize and lettuce in soils which had different P availability and also increased P concentration significantly (Chabot et al., 1996). Phosphate solubilizing Rhizobacteria enhanced the growth and yield of canola (de Freitas et al., 1997).

2.3 Mechanisms of Phosphate Solubilization

Apart from fertilization, mineralization and enzymatic decomposition of organic compounds, microbial P solubilization is the main contributor increasing plant available P (Illmer and Schinner, 1992). Several theories exist explaining the mechanisms of microbial P solubilization (Kucey, 1983, Asea et al., 1988; Cunningham and Kuiack 1992; Illmer and Schinner, 1995): the sink theory (Halvorson et al., 1990), the organic acid theory (Cunningham and Kuiack, 1992), and the acidification by H^+ excretion theory (Illmer and Schinner, 1995).

In the sink theory, P solubilizing organisms are able to remove and assimilate P from the liquid and therefore stimulate the indirect dissolution of Ca-P compounds by continuous removal of P from broth (Halvorson et al., 1990). Illmer and Schinner demonstrated P content in the biomass of two P solubilizing organisms (*Pseudomonas* sp. and *P. aurantiogriseum*) were the same as that in non-P solubilizing organisms

(Illmer and Schinner, 1995). They further argued that only about 1% of total P was absorbed by organisms despite all of the P solublized in broth. The sink theory, however, can be used to explain mineralization of organic P compounds in which the P content in biomass of organisms is consistently correlated with the decomposition of P-containing organic substrates (Dighton and Boddy, 1989).

The organic acid theory is recognized and accepted by many researchers. In this theory, insoluble sources of inorganic P in liquid broth are solubilized by PSM either by lowering the pH or by enhancing chelation of the cations bound to P. Chelation involves the formation of two or more coordinated bonds between a molecule (the “ligand”) and a metal ion resulting in a ring structure complex. Chelation by an organic acid ligand occurs via oxygen contained in hydroxyl and carboxyl groups (Whitelaw, 2000). The solubilization of 837 mg L^{-1} $CaHPO_4$ by *P. bilaiae* was achieved at pH 4.5 in the presence of citrate, but no $CaHPO_4$ solubilization occurred at the same pH in the presence of the inorganic acid alone indicating that chelation involved citric acid (Cunningham and Kuiack, 1992). Gluconic acid or *P. radicum* inoculation alone solubilized more amorphous Al-P than HCl at the same pH (Whitelaw et al., 1999). The insoluble sources of inorganic P in liquid broth are solubilized by PSM accompanied by the production of organic acids: the action of organic acids synthesis and lowering the pH cause dissolution of P compounds (Banki and Dey, 1982; Kucey, 1988; Cunningham and Kuiack, 1992; Whitelaw, 2000; Pradhan and Sukla, 2005). The production of organic acid leads to acidification of microbial cells and their surroundings and, consequently, the release of P ions from the P mineral by H^+ substitution for Ca^{2+} (Goldstein, 1994). Organic acids produced by PSM were determined by methods such as high performance liquid chromatography (HPLC) and enzymatic methods (Whitelaw, 2000; Parks et al., 1990). Various organic acids are identified by the liquid cultures of PSM (Table 2.1), and can be associated with specific microbial groups, e.g., 2-ketogluconic acid and oxalic acid are commonly found in bacterial and fungal cultures, respectively. Gluconic, acetic and lactic acids have been observed from both types of microorganisms and gluconic acid seems to be the principal organic acid frequently found among PSM.

The impact of organic acid production on P solubilization has been established for a while. Halder et al., (1990) reported that the amount of P solubilized by *R.*

Table 2. 1 Organic acids accompanied with P solubilization

Organic acid	Microorganism		Reference
	Fungi	Bacteria	
Oxalic	+		Cunningham and Kuiack, 1992
Citric	+		Cunningham and Kuiack, 1992
Lactic	+	+	Banik and Dey, 1982
Tartaric			Banik and Dey, 1982
Gluconic	+	+	Illmer and Schinner, 1995
2-ketogluconic		+	Halder et al.,1990
Acetic	+	+	Illmer and Schinner, 1995

+ Type of organic acid was observed from the culture solutions.

leguminosarum was nearly equivalent to the organic acid obtained from the culture. They also showed that the P release capacity was not an enzymatic process. Goldstein (1994) proposed that the direct periplasmic oxidation of glucose to gluconic acid, often as 2-ketogluconic acid, formed the metabolic basis of mineral P solubilization in some Gram negative bacteria. Illmer and Schinner (1995) doubted the organic theory. They demonstrated that by using different concentrations of gluconic acid (0 to 5000 μM) tested at different pH values (pH 4 to 7), there was no effect of gluconic acid on Ca-P solubility at pH > 6. Although many researchers support the organic acid theory, the amount of solubilized P is difficult to correlate with the organic acid measured in liquid culture (Illmer and Schinner, 1995).

The acidification by H^+ excretion theory was introduced by Illmer and Schinner in 1995 to explain Ca-P solubilization accompanied by a decrease in pH. They investigated Ca-P solubilization by *P. aurantiogriseum* sp. and *Pseudomonas* sp. from a forest soil containing Ca-P (hydroxyapatite and brushite). These authors observed that the P concentration in liquid broth increased with consumption of apatite and brushite, and that the P concentration also peaked at several points. Based on their results, they concluded that P concentration at the peaks might be due to the formation and secondary solubilization of organic P compounds. They also inferred that the organic compounds were assimilated as nutrients by these two organisms when the liquid broth was low in inorganic substrates.

The H^+ release is thought to be associated with cation assimilation, such as ammonium ion (NH_4^+). H^+ excretion accompanying NH_4^+ assimilation is responsible for P solubilization. Illmer and Schinner (1995) demonstrated that *P. aurantiogriseum* and *Pseudomonas* sp. solubilized hydroxyapatite and brushite effectively without contact between the cells and the substrates, and concurrently lowered the pH. They attributed the P mobilization to H^+ excretion at the cell surfaces. The excreted H^+ accompanying the decrease in pH acted as a solvent agent for P solubilization (Illmer and Schinner, 1995). The NH_4^+-N had the lowest pH value among different N sources and was the most effective on P solubilization in liquid cultures by *P. bilaiae* (Cunningham and Kuiack, 1992).

2.4 Assessing Phosphate Solubilization by Microorganisms

2.4.1 Assessing techniques

There are two main techniques used for evaluating P solubilization by microorganisms. One uses a precipitated phosphate agar plate assay and the other uses a liquid media/culture broth. Precipitated phosphate agar assays are used widely in the initial selection for P solubilizing microorganisms (Pikovskaya, 1948; Halder et al., 1991; Abd-Alla 1994; Wenzel and Ashford, 1994). Microorganisms capable of solubilizing phosphate minerals are grown on an agar medium with insoluble-phosphates (such as $CaHPO_4$) as the only P source and produce a visible clear zone around their colonies. The production of a clear/halo zone on the plate is due to the excretion of organic acids into the surrounding medium (Pikovskaya, 1948). To improve the clarity of the clear/halo zone, dyes such as bromophenol blue and alizarin red S are often used in the agar media (Cunningham and Kuiack, 1992; Gupta et al., 1994). The precipitated phosphate agar assay is a fast and easy-to-use method. It can be used to screen large numbers of isolates quickly and simultaneously. Despite the popularity of the precipitated phosphate agar assay, reliability concerns have been raised because many isolates did not produce a halo zone on the agar plates, but could solubilize various types of insoluble inorganic phosphates in liquid media (Louw and Webley, 1959; Gupta et al., 1994; Nautiya, 1999). Moreover, correlations between the size of clear zones on the plates of precipitated phosphate agar and the more quantitative data of P solubilization in the liquid media vary from study to study (Gupta et al., 1994; Nautiyal et al, 1999; Whitelaw, 2000).

In contrast to the precipitated phosphate agar plate assays, a direct measurement of phosphate solubilization in liquid media is considered more accurate (Nautiyal, 1999; Bhadauria et al., 2000; Sangeeta and Nautiyal, 2001). The liquid media/culture technique measures P released into the liquid from the initial insoluble phosphate substrate used. The rate of P solubilization is typically estimated by subtracting the final culture solution P from the un-inoculated control of P substrate (Rodriguez and Fraga, 1999). Unfortunately, the liquid media method is labor intensive and time consuming.

2.4.2 Media composition

Solubilization efficacy of microorganisms is influenced greatly by medium composition, especially the N and C sources, and the buffering capability of the medium used (Cunningham and Kuiack, 1992; Whitelaw, 2000; Sangeeta and Nautiyal, 2001; Pradhan and Sukla, 2005). The impact of medium composition was often studied in liquid (Table 2.2). P solubilized and released from various Ca-P compounds by PSM varied greatly with growth media and incubation times (Table 2.2). *P. radicum* released more P in the presence of NH_4^+-N compared to NO_3^- -N (Whitelaw et al., 1999). A 27.1% reduction in P released in bacterial culture solution occurred when KNO_3 was used as a sole source of N compared to $(NH_4)_2SO_4$ (Nautiyal, 1999). *Aspergillus* sp. also preferred NH_4^+-N among NH_4^+-N, NO_3^- -N, urea and casein as different N source (Pradhan and Sukla, 2005). *P. bilaiae* however released more P from insoluble Ca-P in culture solution with NO_3^--N and sucrose as the C source (Cunningham and Kuiack, 1992). Furthermore, the concentration of NH_4^+-N also affects the amount of P

Table 2. 2 Soluble P released from various Ca-P compound by PSM in culture solution

Microorganism		Soluble P (mg L^{-1})			Reference
Fungi	Bacteria	$CaHPO_4$	$Ca_3(PO_4)_2$	$Ca_5(PO_4)_3 \cdot OH$	
P. bilaiae		837			Cunningham and Kuiack, 1992
P. radicum		475			Whitelaw et al., 1999
P. radicum			360		Whitelaw et al., 1999
P. radicum		186			Whitelaw et al., 1999
P. radicum			213		Whitelaw et al., 1999
Aspergillus sp			480		Pradhan and Sukla, 2005
Penicillium sp			275		Pradhan and Sukla, 2005
	Pseudomonas sp	30			Illmer and Schinner, 1995
	Pseudomonas sp		8		Nautiyal, 1999
	Pseudomonas sp		35		Nautiyal, 1999
	Bacillus sp		8		Nautiyal, 1999
	Pseudomonas sp		26		Nautiyal, 1999
	Pseudomonas sp		90		Nautiyal, 1999
	Bacillus sp		21		Nautiyal, 1999
	Bacillus sp		268		Alikhani et al., 2007
	Bacillus sp			7.5-20	De Freitas et al., 1997
	Pseudomonas sp		52		Illmer and Schinner, 1992
	Pseudomonas striata		156		Rodriguez and Fraga, 1999
	Pseudomonas striata			22	Halder et al.,1993
	R. leguminosarum			356	Halder et al.,1993
	R. leguminosarum		88-197		Alikhani et al., 2007
	R. meliloti			165	Halder et al.,1993

solubilization; higher concentrations promote P solubilization (Nautiyal, 1999). Nautiyal also suggested that with 2.5g $(NH_4)_2SO_4$ L^{-1} P solubilization by *Pseudomonas* sp. was promoted (Nautiyal, 1999).

The identity of the C source is considered the most influential factor for acid production. Sugars in media are converted by enzymes into intermediate metabolites including organic acids. Enzyme systems vary from microorganism to microorganism. Hence, the metabolic pathway and the types of organic acids produced by microorganisms are either a result of the regular metabolic routes or the type of sugar used (Nahas, 2007). Glucose and maltose decreased culture solution pH and resulted in the highest P solubilization, whereas minimal pH change and P solubilization occurred in the absence of a C source (Pradhan and Sukla, 2005). Nautiyal (1999) also found that not only was glucose necessary, but its concentration was important for bacterial P solubilization in liquid. Soluble P concentration increased with an increase in glucose (Nautiyal, 1999). *P. radicum* favored higher sucrose concentration (e.g. 30g L^{-1}) for Pi solubilization (Whitelaw et al., 1999).

In soil, high C concentration in the rhizosphere supports and enhances microbial P solubilization activities (Lynch and Whipper, 1990), while decomposition of plant residues replenishes the C source. The solubilization of two types of rock P increased significantly during decomposition of wheat straws and cattle urine (Singh and Amberger, 1991). In addition to the C and N source, certain mineral elements are important; K and Mg concentration is critical for optimal P solubilization by soil bacteria (Nautiyal, 1999). Phosphate solubilization is a result of microbial activity under different growth conditions. Fungi are considered to be superior to bacteria both on precipitated agar and in liquid culture in their ability to solubilizie Pi (Kucey, 1983).

3.0 COMPARISON OF MEDIA USED FOR EVALUATING *R. leguminosarum* PHOSPHATE SOLUBILIZATION EFFICACY

3.1 Introduction

Phosphorus content in soil solution ranges from 100 to 400 g ha^{-1}(Turan et al., 2006). Despite its wide distribution in nature, plant available P is deficient in most soils due to precipitation or adsorption of P to ions such as Ca and iron (Turan et al., 2006). Phosphate concentration in soil solution has been shown to increase in the presence of low molecular weight organic acids (Gerke, 1992; Vassilev et al., 1997). Additionally, organic acids may solubilize P either by decreasing the soil pH or by chelating the cation bound to P (Fox et al., 1990; Whitelaw et al., 1999). Microorganisms excrete organic acids and hence these microorgnisms have been the focus of agricultural researchers for sometime (Whitelaw, 2000; Turan et al., 2006).

Rhizobium leguminosarum is well known for its N fixation capability. Its capacity to solubilize phosphate has been receiving more attention in recent years. Some rhizobial strains have the ability to solubilize inorganic forms of P and release available P to plants (Halder et al., 1990; Abd-Alla, 1994; Rodriguez and Fraga, 1999). Ability to solubilize P by *R. leguminosarum* varies between strains (Chabot et al., 1996a). For example, one strain of *R. leguminosarum* biovar *trifolii* showed a low ability for P solubilization in EL Chaco Arido soils in Argentina, whereas other strains of this biovariety showed a high ability to solubilize P in the root zone of rice in Egypt (Abril et al., 2003).

The availability and nature of nutrients is important to the mechanism of P solubilization because the concentration of organic acid production is influenced greatly by the substrates (Nahas, 2007). Media composition and formulation are important for evaluating P solubilizing ability of *R. leguminosarum* in the laboratory (Cunningham and Kuiack, 1992; Whitelaw, 2000; Sangeeta and Nautiyal, 2001; Pradhan and Sukla 2005).

A C source is required for synthesis of cell materials and oxidation of C compounds. The utilization of C by an organism is dependent on its enzyme system, specifically whether or not the enzyme system is naturally present or induced (Brock et al., 1994). The type and concentration of sugar also affects acid production (Gupta et al., 1976; Cunningham and Kuiack, 1992). *Aspergillus niger* solubilized 78% of fluorapatite in culture medium containing fructose as opposed to 59 to 69% solubilization in glucose-, xylose-, and sucrose-based media (Cerezine et al., 1988). *Aspergillus niger* solubilized more $CaHPO_4$ with maltose and manitol than with sucrose in liquid medium (Barroso et al., 2006). Solubilization of tricalcium P by *Pseudomonas* sp. increased with the inclusion of 20 g L^{-1}glucose but decreased when only 5 g L^{-1}glucose was included in the same liquid media (Nautiyal, 1999).

Phosphate solubilization is related to proton (H^+) excretion accompanying NH_4^+ assimilation or respiration (Illmer and Schinner, 1995). Given that P-containing compounds are dissolved by acidification, a bacterium capable of acidifying its external medium is indicative of P solubilization (Goldstein, 2003). Ammonium addition to bacterial cultures increased more P solubilization (Asea et al., 1988; Whitelaw, 1999). Microorganisms behave differently with different types of N sources. Furthermore, ammonium N was important and necessary for *Aspergillus* sp. and *Penicillium* sp. to solubilize tricalcium P in liquid culture (Pradhan and Sukla, 2006). Solubilization of fluorapatite rock by *A. niger* was enhanced more with ammonium N than with nitrate source in the liquid culture (Cerezine et al., 1988). Cunningham and Kuiack (1992) reported that citric acid production by *P. bilaiae* was promoted under N-limited conditions, while oxalic acid production was promoted under C-limited conditions. Citric acid was produced in both growth and stationary phases, whereas oxalic acid production was measured only in stationary phase (Cunningham and Kuiack, 1992).

Ammonium concentration also had an impact on P solubilization by microorganisms. Nautiyal (1999) found that 2.5 g ammonium L^{-1} inhibited P released by *Pseudomonas* sp. in liquid medium.

The goals for this study were to: 1) identify a solid medium that is sensitive and effective as a qualitative screening tool for $CaHPO_4$ solubilizing *R. leguminosarum* isolates; 2) determine the relationship between solubilization ability for $CaHPO_4$ on three solid media and their liquid formulation of same composition by *R. leguminosarum* isolates; and 3) determine the effect of C and N on the growth of *R. leguminosarum* isolates and their abilities to solubilize $CaHPO_4$. Three experiments were conducted to address the objectives; screening $CaHPO_4$ solubilizing *R. leguminosarum* isolates, and effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$.

The reasons for choosing $CaHPO_4$ as the insoluble Ca-P source in this study are as follows: 1) $CaHPO_4$ is the first product of Ca-P precipitation and is commonly seen in calcareous soils as the labile P reserve (Kumar and Narula, 1999); 2) triple superphosphate, a commonly used form of P fertilizer, contains enough Ca^{2+} to precipitate half of its P when applied to soil - there are more Ca-P precipitates in fertilized soils (Chabot et al., 1996b); and 3) utilization of soil $CaHPO_4$ is cheaper than applying P fertilizer, and it also reduces the potential for P run-off from P fertilizer.

3.2 Materials and Methods

3.2.1 *R. leguminosarum* isolates

3.2.1.1 Screening $CaHPO_4$ solubilizing *R. leguminosarum* isolates experiment

Thirty isolates of *Rhizobium leguminosarum bv. viciae* were selected from the Philom Bios, Saskatoon, SK, Canada culture collection and utilized in this study. All isolates were initially collected from the rhizospheres of *Vicia* sp. and *Lathyrus* sp. in Saskatchewan, Canada. Isolates used in this study were selected based on their plasmid profiles which are indicative of genetically distinct organisms (Table 3.1). Determination of the plasmid profiles were conducted using Eckhard analysis by Dr. M. Hynes, University of Galgary, Galgary, AB, Canada.

R. leguminosarum isolates were grown at 25^{o}C in 50 mL sucrose yeast extract (SYE) broth for 48 h on a rotary shaker at 200 rpm. After incubation, the isolate cultures were centrifuged at 4500 rpm for 10 min. The supernatant of the centrifuged cultures was discarded, and the pellets were re-suspended in 15 mL sterile SYE broth. The re-suspended cultures were mixed on a vortex mixer to obtain homogeneous mixtures prior to storage at -85^{o}C.

Table 3. 1 Genetically distinct (indicated by plasmid profile) *R. leguminosarum* isolates screened for ability to solubilize $CaHPO_4$

Strain	Plasmid profile†	Strain	Plasmid profile†
S001B-1	7	S014A-1	6
S002A-3	17	S014B-3	14
S003A-4	6b	S015B-1	12a
S006A-2	22	S016B-1	10
S008A-4	8a	S016B-2	11
S008B-2	9	S018A-1	5
S010A-2	23a	S018A-4	3
S010A-4	24	S019A-1	1
S011A-2	18	S019B-5	27
S011B-4	15b	S020A-1	16
S012A-2	19	S022A-2	2
S012B-3	20	S023A-4	1
S013A-2	25	S027A-1	4
S013B-2	26	S028A-4	21
S013B-3	25	S030A-4	13a

† Plasmid profile system for the 30 *R. leguminosarum* isolates is used exclusively by Dr. M. Hynes and indicative of genetical differences.

3.2.1.2 Effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ experiment

In the experiment of determination of effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$, nine *R. leguminosarum* isolates were selected based on their abilities to solubilize $CaHPO_4$ in PVK and MNBRI media and used. The method and procedure for growing the *R. leguminosarum* isolates are described in section 3.2.1.1.

Selection methods for the nine isolates are described in section 3.3.2.

3.2.2 Media Preparation

3.2.2.1 Screening $CaHPO_4$ solubilizing *R. leguminosarum* isolates experiment

The 30 *R. leguminosarum* isolates were evaluated for their abilities to solubilize P in liquid and solid formulations of three different media (Table 3.2): Pikovskaya's phosphate medium (PVK) (Pikovskaya, 1948); National Botanical Research Institute's phosphate growth medium (MNBRI) (Nautiyal, 1999); and yeast extract manitol (YEM) (Vincent, 1970). Pikovskaya's phosphate medium is routinely used for the screening of microorganisms with P solubilizing abilities. National Botanical Research Institute's phosphate growth medium was developed based on PVK in 1999 by C. S. Nautiyal of the National Botanical Research Institute, India (Nautiyal, 1999). Yeast extract manitol is the medium routinely used to culture *R. leguminosarum.* $CaHPO_4$ was used as the sole P source in the tested media (Table 3.2).

There is a similarity in chemical composition between the PVK and the MNBRI, and *R. leguminosarum* solubilization of $CaHPO_4$ from these two formulations was expected to be similar. These two media share the basic salt components. The PVK contains more micronutrients and has a higher N salt concentration. YEM is not a standard medium for screening P solubilizing microorganism. Furthermore, it contains mannital as C source whereas glucose is the source of C for the PVK and MNBRI.

For solid media preparation, all ingredients (Table 3.2) were added to an Erlenmeyer flask containing a stir bar, to which 1L of distilled water was added. The mixture was adjusted to pH 7.0 with 2M HCl or 2M NaOH prior to autoclaving. The 1-L mixture was autoclaved at 121^{o}C and 15 psi for 30 min on the liquid cycle, then allowed to cool in a water bath at 50-60^{o}C for at least 30 min. Plates were made by dispensing approximately 20 mL of medium into each sterile Petri plate.

For broth preparation, all ingredients except agar were added to an Erlenmeyer flask containing a stir bar (Tables 3.2 and 3.3). The 1-L mixture was autoclaved using the same procedure for solid media. Aliquots (10-mL) of sterile broth were then dispensed into 26-mm sterile test tubes.

Table 3. 2 Composition of the media used to evaluate $CaHPO_4$ solubilization efficacy by the thirty *R. leguminosarum* isolates.

Media components	Amount g L^{-1}			Source
	PVK	MNBRI	YEM	
Mannitol	-	-	10	EMD MX0214-3
Glucose	10	20	-	Fisher D16-500
Agar	15	15	15	EMD 1.01614.1000
$CaHPO_4$	5	5	5	Fisher C135-500
$(NH_4)_2SO_4$	0.5	0.1	0.1	Fisher A938-500
NaCl	0.2	-	0.2	
$MgSO_4$-$7H_2O$	0.1	0.25	0.25	Fisher M63-500
$MgCl_2$-$6H_2O$	-	10		EM MX0045-1
KCl	0.2	0.2	0.2	BDH ACS 645
Yeast extract	0.5	0.1	2.0	Amberex 1003AG
$MnSO_4$-H_2O	0.002	-	-	Fisher M113-500
$FeSO_4$-$7H_2O$	0.002	-	-	BDH ACS 354

3.2.2.2 Effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ experiment

Four liquid media based on MNBRI phosphate growth medium were used to study the effect of C and N on $CaHPO_4$ solubilization by nine selected *R. leguminosarum* isolates in broth culture with different concentrations of glucose (C) and $(NH_4)_2SO_4$ (N) (Table 3.3).

The broth preparation of this experiment is as same as the one described in section 3.2.2.1.

3.2.3. Study design and laboratory analysis

3.2.3.1 Screening $CaHPO_4$ solubilizing *R. leguminosarum* isolates experiment

Solubilization efficacies for $CaHPO_4$ by 30 *R. leguminosarum* isolates were evaluated and compared separately on solid and in broth formulations of three media using Completely Randomize Design (CRD). Treatments were arranged in a factorial design with two factors: 1) isolates and 2) media. Treatments were replicated three times.

Table 3. 3 Composition of MNBRI broth used to determine the effects of C and N on $CaHPO_4$ solubilization by t nine selected *R. leguminosarum* isolates

Media components	Amount g L^{-1}				Source
	HC & LN	LC & LN	HC & HN	LC & HN	
Glucose	20	10	20	10	Fisher D16-500
$CaHPO_4$	5	5	5	5	Fisher C135-500
$(NH_4)_2SO_4$	0.1	0.1	0.5	0.5	Fisher A938-500
$MgSO_4$-$7H_2O$	0.25	0.25	0.25	0.25	Fisher M63-500
$MgCl_2$-$6H_2O$	10	10	10	10	EM MX0045-1
KCl	0.2	0.2	0.2	0.2	BDH ACS 645
Yeast extract	0.1	0.1	0.1	0.1	Amberex 1003AG

The ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ on solid media was evaluated as follows: frozen *R. leguminosarum* isolates were removed from the -85^{o}C freezer and thawed at room temperature (24 $\pm$ 2^{o}C). For each of the 30 *R. leguminosarum* isolates, 20 μL cultures was dispensed with a sterile pipette in the center of the plate and incubated at 24 $\pm$ 2^{o}C. The ability of isolates to solubilize $CaHPO_4$ was assessed 16 d after inoculation, by measuring the diameter of the zone of clearing on the solid media surrounding the developed colony using a ruler. The size of the clearing zone was calculated by subtracting the colony diameter from the clearing zone with the colony. The ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ on solid media was indicated by the width of the clearing zone surrounding the colony. It was assumed that the wider the clearing zone away from the colony the more efficient the isolate is at solubilizing $CaHPO_4$ on a particular solid medium.

The ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ in liquid culture was evaluated using 26-mm test tubes. Each tube containing 10 mL of sterile liquid formulation of media was inoculated with 20 μL of the *R. leguminosarum* isolate using a sterile disposable pipette. Autoclaved, un-inoculated broth served as a negative control. *Penicillium bilaiae* (Philom Bios Inc., Saskatoon, Canada) was used as a positive control. The inoculated test tubes were incubated at 24 $\pm$ 2^{o}C on a rotary shaker at 200 rpm for 12 d. Cultures were centrifuged at 4500 rpm for 10 min. The P concentration in the supernatant was measured using the QuantiChrom™ Phosphate Assay Kit (BioAssay Systems, 3423 Investment Boulevard, Suite11, Hayward, CA 94545, USA).

The QuantiChrom™ Phosphate Assay Kit uses a 96-well plate assay and measures phosphate ions directly in the sample without any pretreatment. It utilizes a malachite green dye and molybdate which form a stable colored complex specifically with inorganic phosphate. The intensity of color, measured at 620 nm by spectrophotometer, is directly proportional to the P concentration in the sample. The linear detection ranges from 0.0028 to 0.47 mg dL^{-1}. A series dilution is required if the P concentration in the culture is higher than the detecting range. Tween 80 (0.01%) is used as the dilutant.

The assay procedure for evaluating the solubilization of $CaHPO_4$ by *R. leguminosarum* isolates in liquid cultures was as follow: a 50 μL aliquot of each supernatant sample was transferred, in duplicate, to a clear bottom 96-well plate and 0.1 mL of the reagent added to each well. The plate was tapped slightly to ensure mixing and incubated at 22 $\pm$ 2^{o}C for 30 min. The optical density of each sample was recorded at 620 nm using SofeMAX Pro program and SPECTRA MAX 340 spectrophotometer, Molecular Device (Suuyvale, CA, 94089 USA). The P concentration for each isolate in each replicate was the average of duplicate recordings. The P concentration was calculated based on the following equation:

$$\mathrm{P}(mg/dL) = \left(\frac{\mathrm{OD}_{sample} - \mathrm{OD}_{blank}}{\mathrm{OD}_{standard} - \mathrm{OD}_{blank}}\right) \mathrm{x}\ 0.28 \qquad \text{(Eq. 3.1)}$$

where OD_{blank} , $OD_{standard}$, and OD_{sample} are optical densities at 620 nm values of the blank, standard, and sample respectively.

Acid production by *R. leguminosarum* isolates in broth culture was indicated by pH changes from the un-inoculated medium. The pH of the supernatant was measured using a pH/ion meter (Accumet 950, Fisher Scientific) after the cultured broth had been centrifuged at 4500 rpm for 10 min.

3.2.3.2 Effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ experiment

The effect of C and N concentrations in the medium, on the solubilization of $CaHPO_{4,}$ and growth of *R. leguminosarum* isolates were determined in liquid. Of the three media, MNBRI is the simplest medium with highest C content was choosen for this experiment. Glucose was used as the C source and $(NH_4)_2SO_4$ was used as the N source. Two concentrations each of C and N were tested: 10 and 20 g L^{-1} for C; and 0.1 and 0.5 g L^{-1} for N. Other components in the liquid broth were based on the MNBRI liquid formulation (Table 3.3). The treatments testing the effects of N and C on the ability of *R. leguminosarum* to solubilize $CaHPO_4$ were: HL & LN (20 g L^{-1} C and 0.1 g L^{-1} N), LC & LN (10 g L^{-1} C and 0.1 g L^{-1} N), HC & HN (20 g L^{-1} C and 0.5 g L^{-1} N), LC & HN (10 g L^{-1} C and 0.5 g L^{-1} N), (Table 3.3).

3.2.4 Statistical analysis

3.2.4.1 Screening $CaHPO_4$ solubilizing *R. leguminosarum* isolates experiment

Phosphate solubilization by *R. leguminosarum* was compared to the un-inoculated medium and among the 30 isolates in each formulation. Data were analyzed for analysis of variance using JMP 7.0.2 program (SAS Institute, SAS Campus Drive, Cary, NC 27513, USA). Suitability of medium for screening $CaHPO_4$ solubilizing *R. leguminosarum* was compared by comparing means of P concentration. Efficacy of *R. leguminosarum* isolates to solubilize $CaHPO_4$ in three media was compared and the means separation was conducted using Tukey's Honestly Significant Difference Test. The relationship between P concentration from $CaHPO_4$ solubilization and pH was examed using the regression analysis in the liquid cultures of the *R. leguminosarum* isolates. The relationship between P concentration in liquid culture and zone of clearing on solid media of the same medium by *R. leguminosarum* isolates was examed using the regression analysis. A model or criterion for $CaHPO_4$ solubilization by *R. leguminosarum* isolates was developed based on the results of both the solid and the liquid formulations of each medium.

3.2.4.2 Effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ experiment

Effects of different concentrations of N and C on P solubilization and growth by *R. leguminosarum* isolates were compared. Data were analyzed by analysis of variance using JMP 7.0.2 program (SAS Institute, SAS Campus Drive, Cary, NC 27513, USA). The Tukey's Honestly Significant Difference Test was used to compare P concentration and *R. leguminosarum* titer in various liquid formulations containing different N and C concentrations.

3.3 Results

3.3.1 Screening $CaHPO_4$ solubilizing *R. leguminosarum* isolates experiment

3.3.1.1 $CaHPO_4$ solubilization on three solid formulations

The efficacy of P solubilization by individual isolates differed among media (Fig. 3.1). The smallest mean size of clearing zones by *R. leguminosarum* isolates was observed on the PVK medium (Table 3.4). The largest clearing zone was observed for isolate S019B-5 grown on MNBRI although this medium had the fewest number of isolates exhibiting a clearing zone (Fig. 3.2). Twenty one isolates had clearing zones on the YEM medium (Fig. 3.2).

Individual isolates solubilized $CaHPO_4$ differently on the three solid media leading to three distinct patterns. The order of the solubilization of $CaHPO_4$ by isolates on solid media was arranged based on the solubilization on PVK medium (Fig. 3.2).

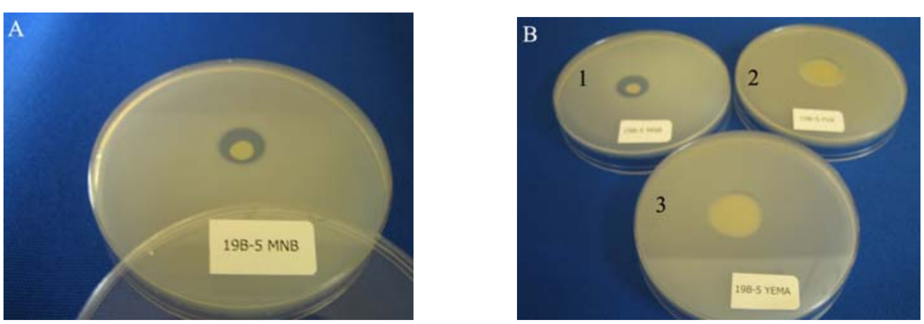

Figure 3. 1 $CaHPO_4$ solubilization by a *R. leguminosarum* isolate is indicated by the clearing zone (A). Different solubilization efficacies by a single isolate (S019B-5) cultured on the three different solid media (B), Plate 1 illustrates MNBRI, Plate 2 illustrates PVK and Plate 3 illustrates YEM.

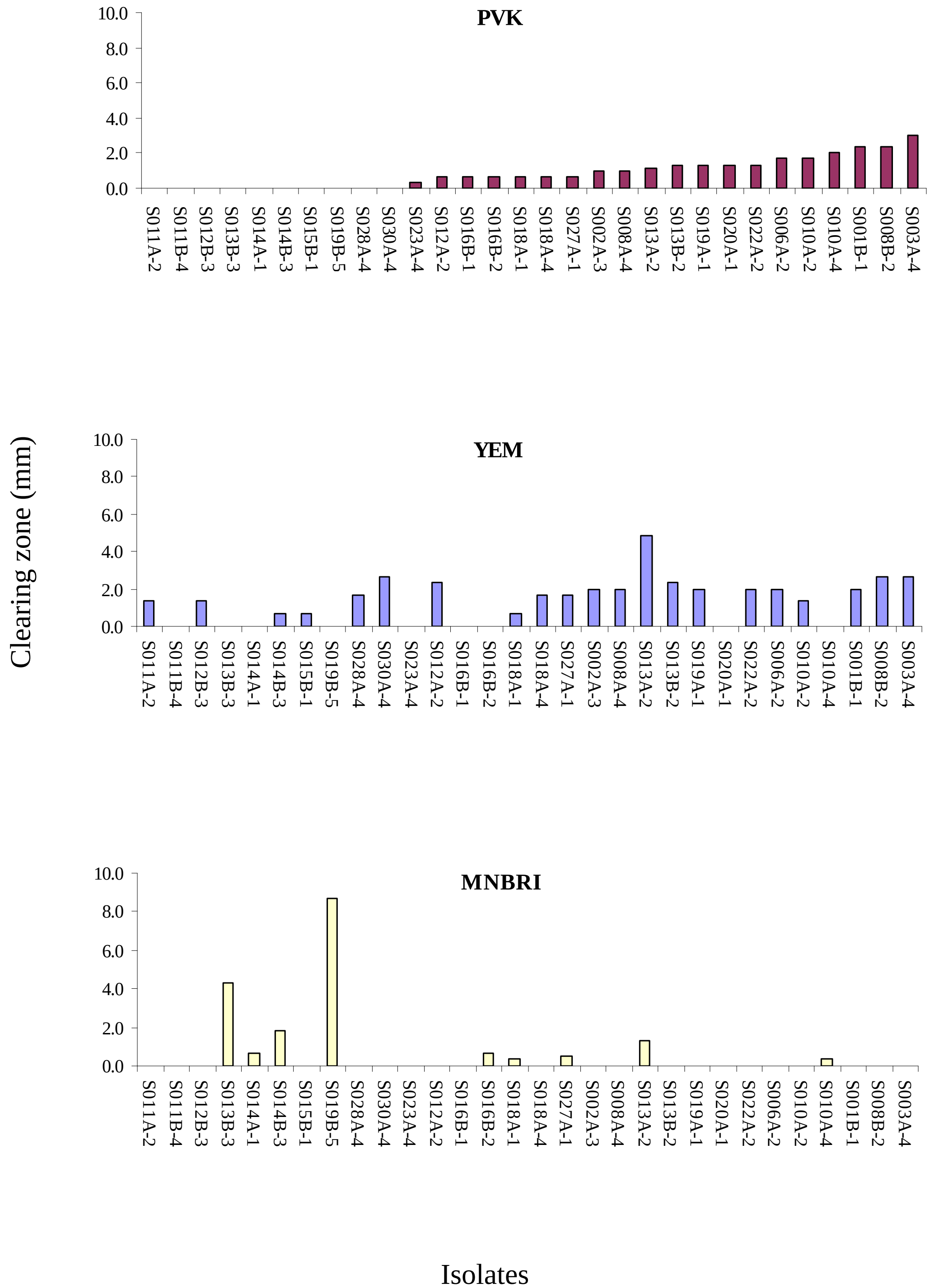

Figure 3. 2 Solubilization patterns of $CaHPO_4$ by *R. leguminosarum* isolates indicated by zones of clearing surrounding the developed colony grown on three solid formulations. Value of each isolate is the mean of three replicates.

Table 3. 4 $CaHPO_4$ solubilization on solid media by 30 *R. leguminosarum* isolates.

Solid formulation	Clearing zone (mm)		No. of isolates with clearing zone
	Range	Mean	
PVK	0.5-3.0	1.8 b	20
MNBRI	0.5-10.0	3.1 a†	9
YEM	1.0-7.0	2.7 a	21

† Means followed by the same letter are not significantly different ($p \geq 0.05$) according to Tukey's Honestly Significant Difference Test. Mean values are the *R. leguminosarum* isolates that showed clearing zones.

3.3.1.2 CaHPO4 solubilization in three liquid formulations

All 30 *R. leguminosarum* isolates grown in liquid solubilized $CaHPO_4$ and released more P than the controls (Fig. 3.3). The highest P concentration solubilized by *R. leguminosarum* isolates was observed in PVK in terms of maximum concentration and range (Table 3.5).

Table 3. 5 $CaHPO_4$ solubilization in liquid by 30 *R. leguminosarum* isolates.

Liquid formulation	P concentration from isolates (mg L^{-1})		No. of isolates with soluble P	P concentration *P. bilaiae*‡ (mg L^{-1})
	Range	Mean		Mean
PVK	207-372	276.1 a	30	1898
MNBRI	93-222	158.3 b†	30	2491
YEM	117-287	179.3 b	30	690

† Means followed by the same letter within the column are not significantly different ($p \geq 0.05$) according to Tukey's Honestly Significant Difference Test. Values are means of three replicates.
‡ *P. bilaiae* included as a positive control.

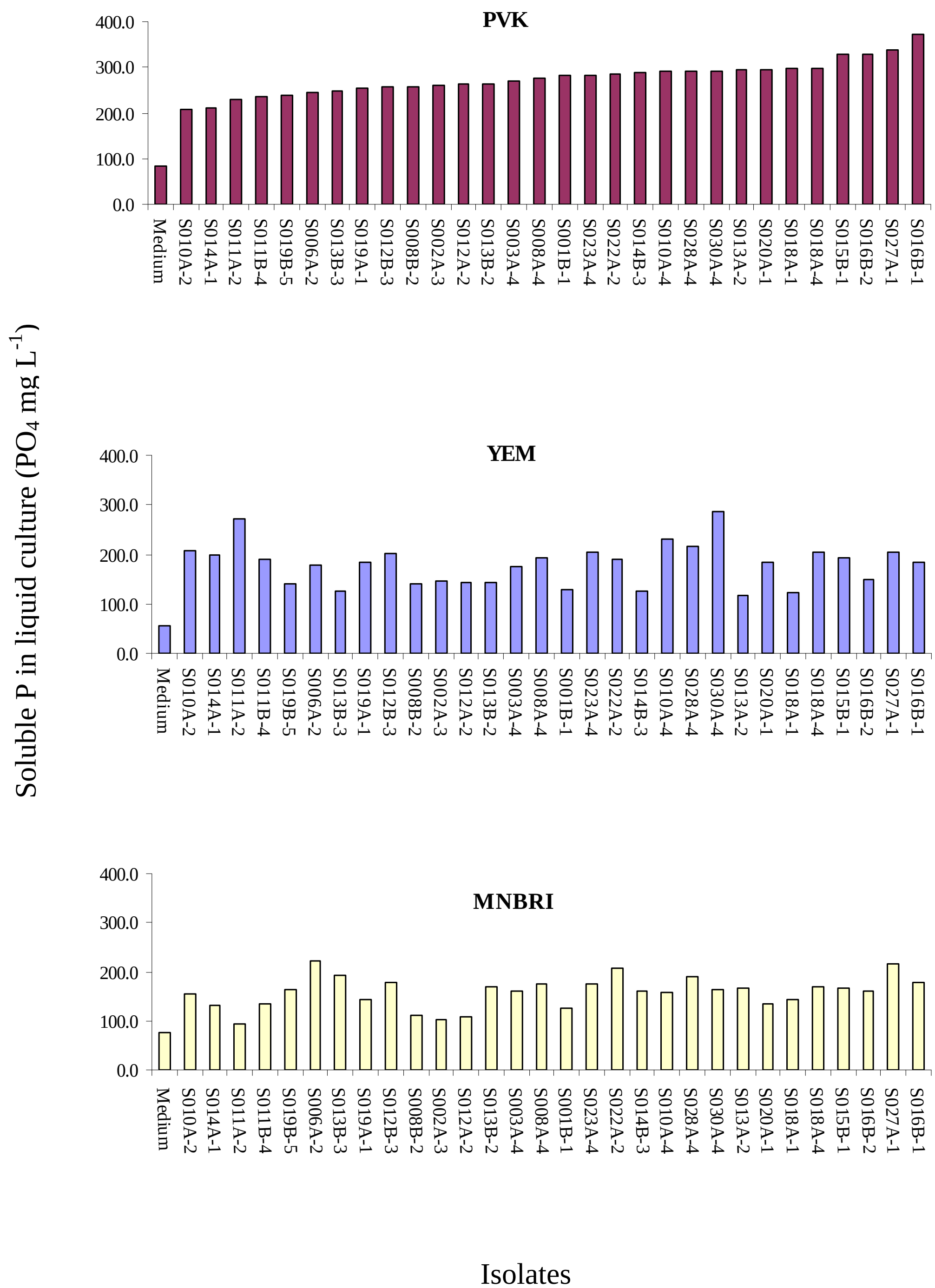

Figure 3. 3 Concentration patterns of P solubilized from $CaHPO_4$ by thirty *R. leguminosarum* isolates grown in three liquid formulations. The positive control organism (*P. bilaiae*) solubilized $CaHPO_4$ 1898 mg L^{-1} in PVK, 690 mg L^{-1} in YEM and 2491 mg L^{-1} in MNBRI. Value of each isolate is the mean of three replicates.

R. leguminosarum isolates solubilized different amounts of $CaHPO_4$ depending on the media (Fig. 3.3). Furthermore, the relative amounts of P solubilized by the isolates were not the same across all media (i.e., the pattern of P solubilization by an *R. leguminosarum* isolate differed for all three media). The amount of P solubilized by the positive control, *P. bilaiae*, was higher than any of the *R. leguminosarum* isolates, in the same liquid formulations (Table 3.5).

Phosphate solubilization by *R. leguminosarum* was accompanied by a decrease in pH in the liquid cultures (Table 3.6). A reduction in pH was observed in cultures of all 30 *R. leguminosarum* isolates and in all three liquid media. The largest pH decrease was obtained in the PVK formulation. The relationship between P concentration from $CaHPO_4$ solubilization and acidification by *R. leguminosarum* indicated by pH in the liquid culture was also examined. No significant relation was found between P concentration and pH in the cultured PVK and YEM liquid formulations incubated with the thirty *R. leguminosarum* isolates; however there was a significant relationship between P concentration and pH in the cultured MNBRI liquid formulation ($p < 0.001$) (Table 3.7).

Table 3. 6 The pH of the three liquid formulations after 12-day incubation with 30 *R. leguminosarum* isolates.

Formulation	pH		
	Uninoculated	Range†	Mean
PVK	6.3	4.4-5.0	4.8 c‡
MNBRI	6.3	4.5-5.9	5.0 b
YEM	6.6	4.8-5.9	5.2 a

† The end-point pH range of the 30 *R. leguminosarum* isolates

‡ Means followed by the same letter are not significantly different ($p \geq 0.05$) according to Tukey's Honestly Significant Difference Test. Values are means of three replicates.

Table 3. 7 Regression analysis for Pi concentration and pH after 30 *R. leguminosarum* isolates were cultured for 12 d in three different liquid formulations (n=30).

Broth	r^2	*p*
PVK	0.001	0.829
YEM	0.005	0.700
MNBRI	0.485	<0.001

3.3.1.3 Comparison of $CaHPO_4$ solubilization in solid and liquid media

A quadrant model was developed for each medium where P concentration in the liquid culture was plotted against the zone of clearing on the solid formulation for each isolate (Figs. 3.4-3.6). Lines separating the quadrants are the mean values for P in solution for the 30 isolates from each of the solid or liquid formulations of the same medium. True positive indicates that an isolate solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative indicates that an isolate solubilized less $CaHPO_4$ than the mean of that particular medium and formulation; false positive indicates that an isolate was better than average at solubilizing $CaHPO_4$ on solid medium but lower than average at solubilizing $CaHPO_4$ in liquid; and false negative indicates that an isolate was lower than average at solubilizing $CaHPO_4$ on solid medium with higher than average at solubilizing $CaHPO_4$ in liquid broth.

There was no correlation between P solubilization by the isolates on solid and in liquid formulations of the same medium (Table 3.8). For example, for the MNBRI medium, isolate SO19B-5 solubilized the most $CaHPO_4$ on the solid formulation, but rated 11th in the liquid formulation. Besides media composition, media formulations (solid vs. liquid) also affected the amount of P solubilized from $CaHPO_4$ by *R. leguminosarum*.

Table 3. 8 Regression analysis for Pi concentration in liquid cultures and zone of clearing on solid media of the same medium by 30 *R. leguminosarum* isolates (n=30).

Medium	r^2	*p*
PVK	0.006	0.893
YEM	0.009	0.602
MNBRI	0.018	0.479

3.3.2 Effect of C and N on the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ experiment

In order to select a subset of *R. leguminosarum* isolates to assess the effect of N and C on the solubilization of $CaHPO_4$, results from the liquid (Fig. 3.3) screening of the 30 *R. leguminosarum* isolates were plotted. Amounts of P in solution from the 30 isolates grown in PVK and MNBRI liquid formulations were plotted, and the relationship in solubilizing $CaHPO_4$ between these two media were determined by regression analysis (Fig. 3.7). Nine *R. leguminosarum* isolates were selected according to different abilities to solubilize $CaHPO_4$ in the PVK and the MNBRI. Among the nine selected isolates, three had a positive relationship between PVK and MNBRI, indicating that P concentration increased in the PVK and also increased in the MNBRI (S008b-2, S023a-4 and S028a-4), three had a greater than average ability to solubilize $CaHPO_4$ in both PVK and MNBRI (high in PVK & MNBRI) (S016b-1, S016b-2 and S027a-1), three were selected because they had a lower than average ability to solubilize $CaHPO_4$ in PVK (low in PVK) (S006a-2, S010a-2 and S019b-5).

There was no significant difference in P concentration among all four tested liquid media with different N and C combinations (p>0.05) by the nine *R. leguminosarum* isolates (Fig. 3.8). However, the growth of *R. leguminosarum* was affected greatly by the C and N concentrations in the liquid media especially the N concentration. Formulations of low N (0.1 g L^{-1} N) promoted *R. leguminosarum* growth whereas the formulations of high N (0.5 g L^{-1} N) inhibited *R. leguminosarum* growth regardless of the tested C concentrations (Fig. 3.9). Because high N media prevented *R. leguminosarum* growth, fewer viable cells were present in these media comparing to the low N media, meaning that the amount of P solubilized per colony forming unit (cfu) was higher in high N media than in low N media (Fig. 3.10).

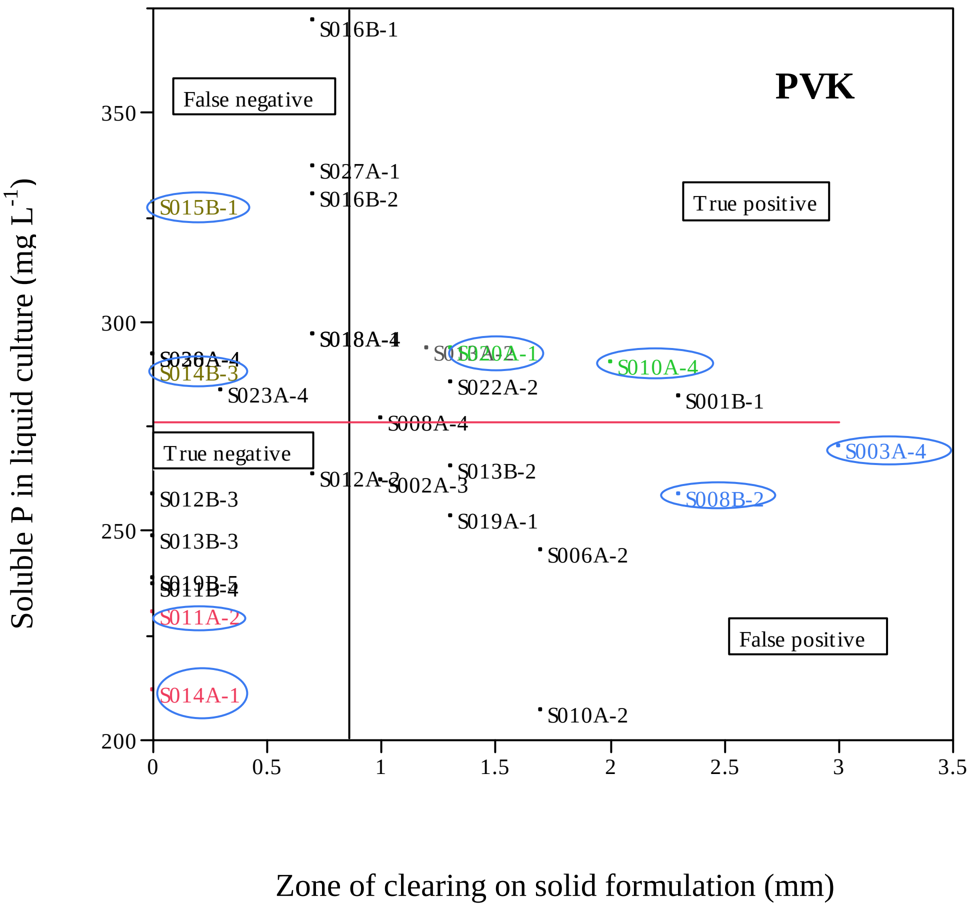

Figure 3. 4 Quadrant model illustrates the relationship between P concentration (mean = 276.1 mg L^{-1}) in the liquid culture and the zone of clearing (mean = 0.9 mm) on the solid formulation by *R. leguminosarum* isolates grown on solid and broth formulations of PVK medium containing $CaHPO_4$. Quadrants are separated by the mean of P solubilization of the 30 isolates from each of solid or liquid formulation. True positive indicates that an isolate has solubilized $CaHPO_4$ greater than the means for both the formulations; true negative indicates that an isolate has less $CaHPO_4$ solubilization than the means of both formulation; false positive indicates that an isolate is better than average at solubilizing P on solid medium but below average at solubilizing P in liquid broth; and false negative is indicative of a below average P solubilizing on solid medium but an above average P solubilizing in liquid. Isolates selected for the growth chamber study (described in Chapter 4) are circled.

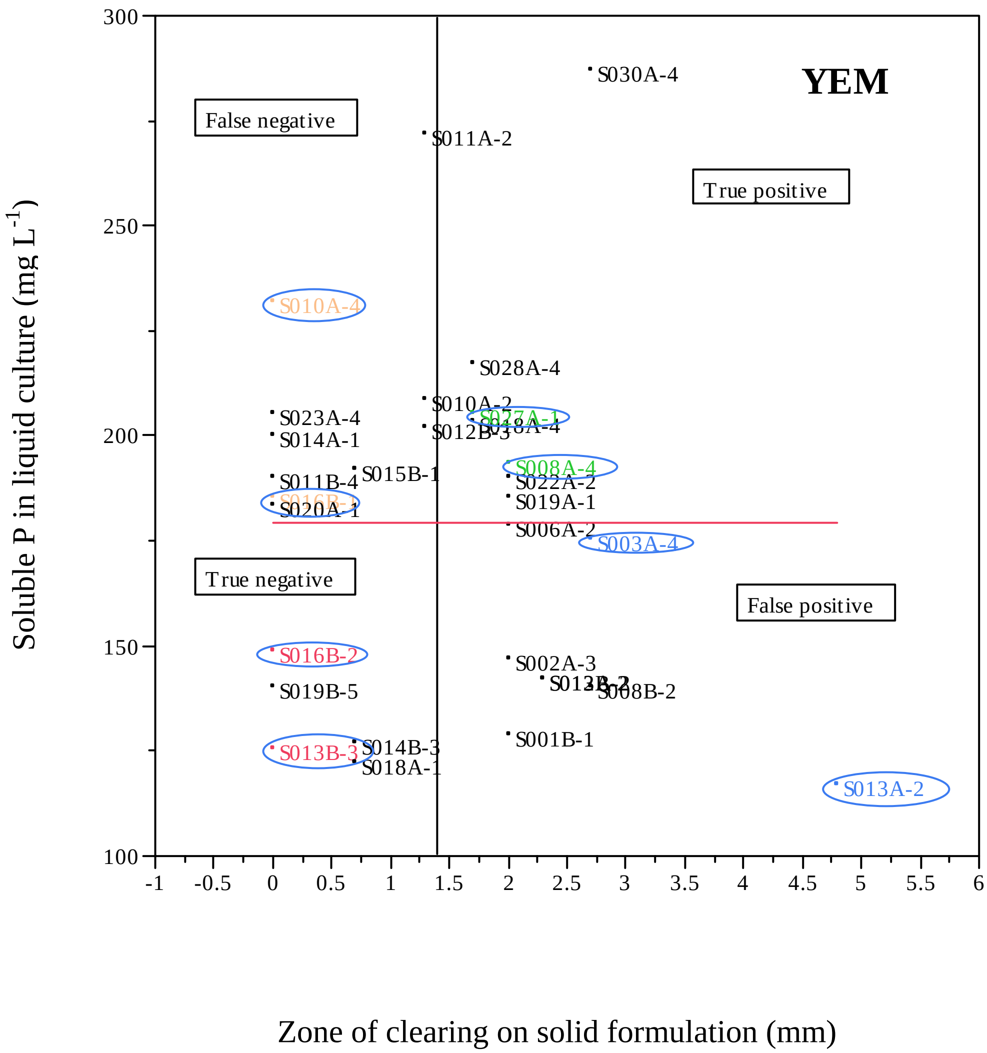

Figure 3. 5 Quadrant model illustrates the relationship between P concentration (mean = 179.3 mg L^{-1}) in the liquid culture and the zone of clearing (mean = 1.4 mm) on the solid formulation by *R. leguminosarum* isolates grown on solid and broth formulations of YEM medium containing $CaHPO_4$. Quadrants are separated by the mean of P solubilization of the 30 isolates from each of solid or liquid formulation. True positive indicates that an isolate has solubilized $CaHPO_4$ greater than the means for both the formulations; true negative indicates that an isolate has less $CaHPO_4$ solubilization than the means of both formulation; false positive indicates that an isolate is better than average at solubilizing P on solid medium but below average at solubilizing P in liquid broth; and false negative is indicative of a below average P solubilizing on solid medium but an above average P solubilizing in liquid. Isolates selected for the growth chamber study are (described in Chapter 4) circled.

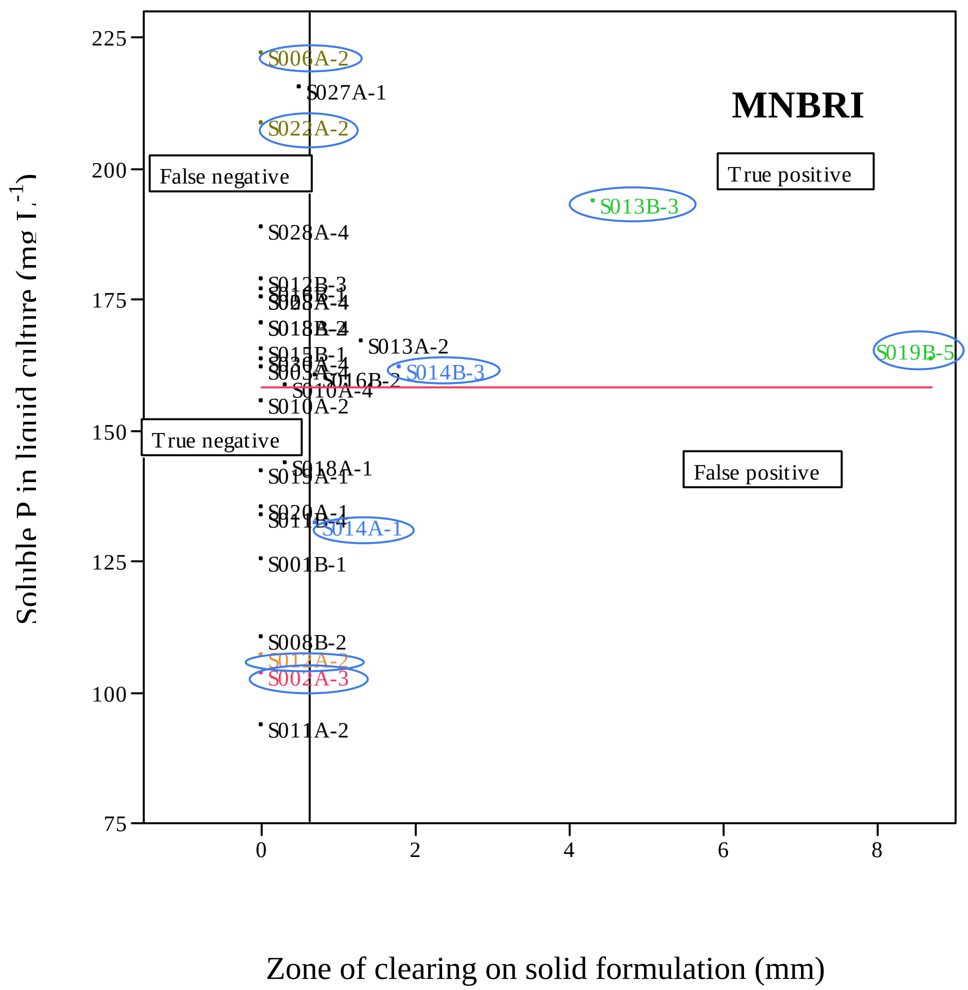

Figure 3. 6 Quadrant model illustrates the relationship between P concentration (mean = 158.3 mg L^{-1}) in the liquid culture and the zone of clearing (mean = 0.6 mm) on the solid formulation by *R. leguminosarum* isolates grown on solid and broth formulations of MNBRI medium containing $CaHPO_4$. Quadrants are separated by the mean of P solubilization of the 30 isolates from each of solid or liquid formulation. True positive indicates that an isolate has solubilized $CaHPO_4$ greater than the means for both the formulations; true negative indicates that an isolate has less $CaHPO_4$ solubilization than the means of both formulation; false positive indicates that an isolate is better than average at solubilizing P on solid medium but below average at solubilizing P in liquid broth; and false negative is indicative of a below average P solubilizing on solid medium but an above average P solubilizing in liquid. Isolates selected for the growth chamber study (described in Chapter 4) are circled.

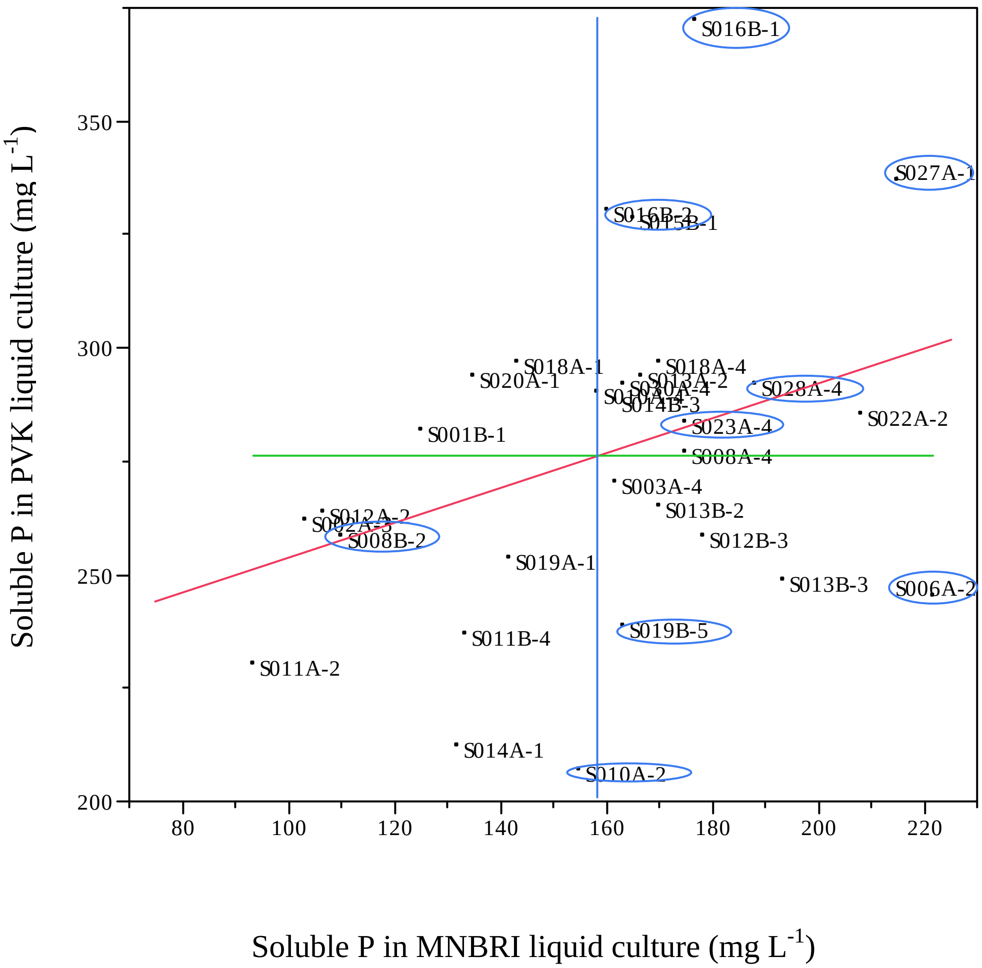

Figure 3. 7 Regression analysis for the amount of P in solution from 30 *R. leguminosarum* isolates grown in PVK and MNBRI liquid formulations (r^2 = 0.11). Nine *R. leguminosarum* isolates, circled, were selected based on their $CaHPO_4$ solubilization in PVK and MNBRI. Horizontal line is mean of $CaHPO_4$ solubilization in PVK by thirty *R. leguminosarum* (280 mg L^{-1}). Vertical line is mean of $CaHPO_4$ solubilization in MNBRI (158 mg L^{-1}).

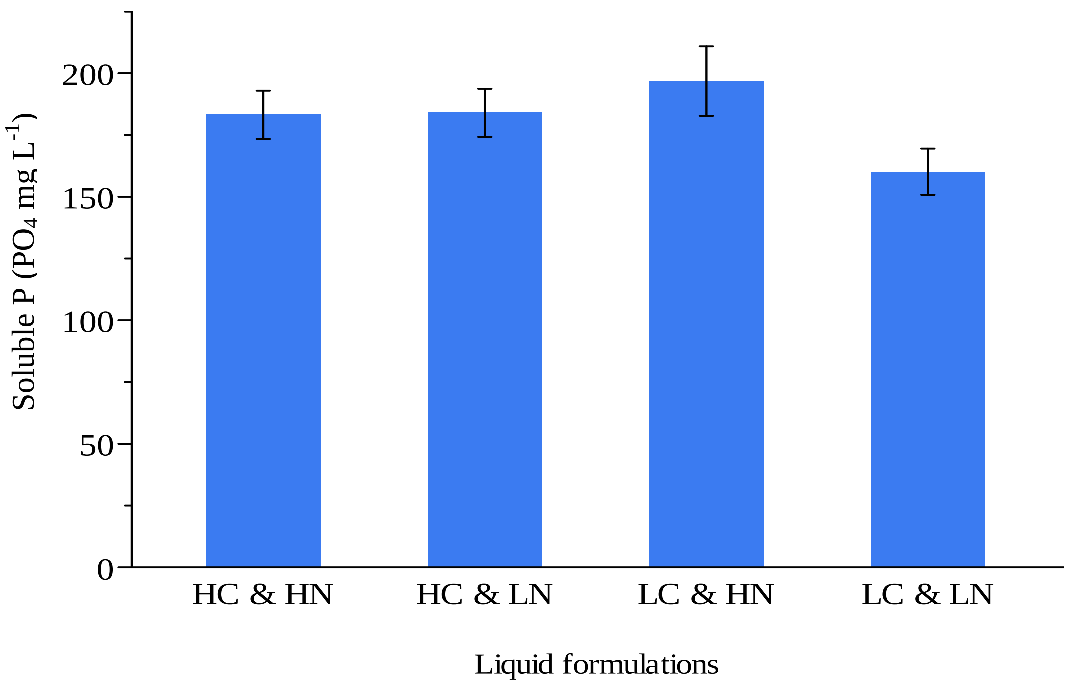

Figure 3. 8 Amount of P in solution after 12 d incubation of nine *R. leguminosarum* isolates in MNBRI media with different amounts of C and N according to Tukey's Honestly Significant Difference Test ($p \geq 0.05$). Values are means of three replicates. HC & LN (20 g L^{-1} C and 0.1 g L^{-1} N), LC & LN (10 g L^{-1} C and 0.1 g L^{-1} N), HC & HN (20 g L^{-1} C and 0.5 g L^{-1} N), and LC & HN (10 g L^{-1} C and 0.5 g L^{-1} N).

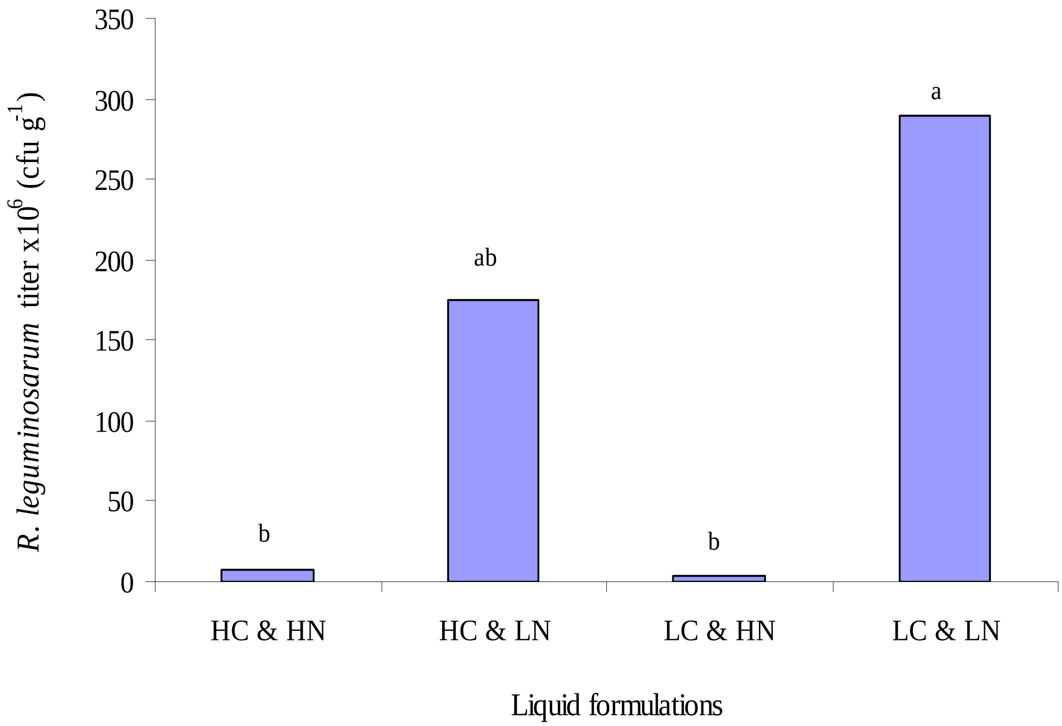

Figure 3. 9 Growth of nine *R. leguminosarum* isolates grown in MNBRI with different concentrations of C and N 12 d after incubation. Values are means of three replicates. Means followed by the same letters are not significantly different ($p \geq 0.05$) according to Tukey's Honestly Significant Difference Test. HC & LN (20 g L^{-1} C and 0.1 g L^{-1} N), LC & LN (10 g L^{-1} C and 0.1 g L^{-1} N), HC & HN (20 g L^{-1} C and 0.5 g L^{-1} N), and LC & HN (10 g L^{-1} C and 0.5 g L^{-1} N).

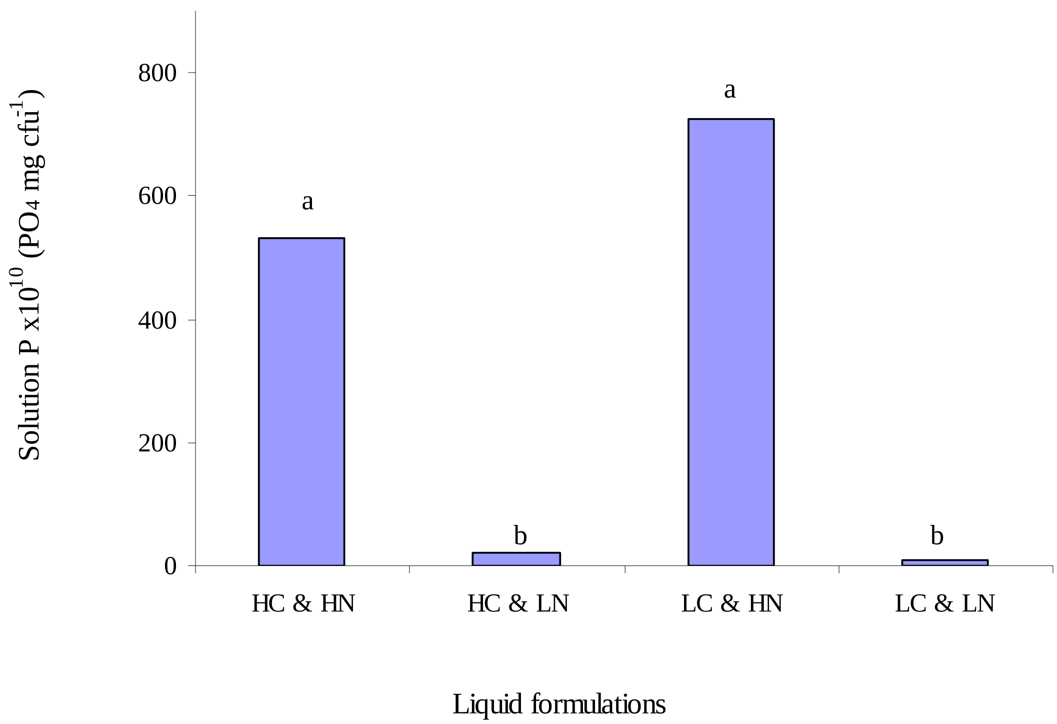

Figure 3. 10 Amount of P in solution on a colony forming unit basis after 12 d incubation with nine *R. leguminosarum* isolates in MNBRI with different amounts of C and N according to Tukey's Honestly Significant Difference Test ($p \geq 0.05$). Values are means of three replicates. Means followed by the same letter are not significantly different ($p \geq 0.05$) according to Tukey's Honestly Significant Difference Test. HC & LN (20 g L^{-1} C and 0.1 g L^{-1} N), LC & LN (10 g L^{-1} C and 0.1 g L^{-1} N), HC & HN (20 g L^{-1} C and 0.5 g L^{-1} N), and LC & HN (10 g L^{-1} C and 0.5 g L^{-1} N).

3.4. Discussion

3.4.1 $CaHPO_4$ solubilization in three liquid media

Microorganisms dissolve insoluble phosphates by the production of organic acids and/or by the secretion of H^+ in microbial cell culture. Consequently, Pi may be released from an insoluble source by proton substitution or complexing with Ca^{2+} (Fox et al., 1990; Illmer and Schinner, 1992; Whitelaw et al., 1999; Whitelaw, 2000; Takeda and Knight, 2006). Calcium phosphate solubilization is associated with organic acid production. In *P. bilaiae*, acid anions complex with Ca^{2+} and P is released into cultured solution (Takeda and Knight, 2006). The type of acid produced is dependent on the microorganism. Gluconic acid is produced as the principle organic acid by phosphate solubilizing bacteria such as *Pseudomonas* sp. (Illmer and Schinner1992) and 2-ketogluconic acid is produced by *R. leguminosarum* (Halder et al., 1990). Oxalic and citric acids are produced by *P. bilaiae* (Cunningham and Kuiack, 1992; Takeda and Knight, 2006). *Bacillus* and *Aspergillus* decreased pH from 6.8 to 4.0 and 3.5, respectively, in liquid culture (Turan et al., 2006). *Aspergillus niger* grown in maltose-based liquid medium also decreased pH from 7.0 to 3.8 and solubilized $CaHPO_4$ (Nahas, 2007). Acidification by *R. leguminosarum* is important in solubilizing $CaHPO_4$ in PVK, YEM and MNBRI liquid cultures. A decrease in pH was observed in all three test liquid media. The greatest pH decline was obtained in PVK. Similarly, the highest amount of P was measured in PVK, although there was no correlation between pH and P solubilization in PVK culture (Tables 3.5 and 3.6). According to Illmer and Schinner (1995) it is uncommon to find a correlation between an organism's ability to solubilize P and its ability to reduce media pH.

The highest amount of P solubilized in PVK by *R. leguminosarum* could be a function of the high N content in medium. It is likely that assimilation of NH_4^+ was promoted by high levels of $(NH_4)_2SO_4$ and yeast extract in PVK and accompanied with the excretion of H^+. The NH_4^+ was from two sources; readily available N salt and the product of mineralization from the yeast. Despite of high concentration of yeast extract in the YEM, the MNBRI and YEM are media with low N salt content, and amount of available NH_4^+ may be lower comparing to the PVK.

A decrease in pH in this study is probably associated with the excretion of H^+ or the production of organic acids by the *R. leguminosarum* isolates. A lack of correlation between solution P concentration and pH by *R. leguminosarum* grown in the liquid cultures of PVK and YEM suggests that solubilization of $CaHPO_4$ is not likely due exclusively to a decrease in H^+. It is likely that the organic acid produced by *R. leguminosarum* complex with the Ca^{2+} causing the solubilization of $CaHPO_4$ in PVK and YEM media. Organic acids were not measured in this study. However, a strong correlation between solution P concentration and pH by *R. leguminosarum* grown in the MNBRI liquid cultures suggests that the H^+ substitution for Ca^{2+} could be the mechanism by which *R. leguminosarum* solubilizing $CaHPO_4$ in the MNBRI medium. The mechanism of H^+ substitution for Ca^{2+} is not efficient because the P concentration obtained from the MNBRI is the lowest among the three tested liquid media. *Aspergillus niger* had direct correlation between the solubilization of tricalcium phosphate and pH after three weeks of incubation (Maurya and Kumer, 2006). Illmer and Schinner (1995) studied the solubilization of hydroxylapatite (Ca-P) by *Penicillium* sp. and *Pseudomonas* sp. These authors stated that protons might originate from NH_4^+ assimilation as microorganisms propagate and excrete H^+ causing the solubilization of Ca-P. Takeda and Knight (2006) concluded that solubilization of rock P by *P. bilaiae* was enhanced in a pH buffered media concurrent with organic acid production and a decrease in Ca^{2+}. Based on a lack of relationship between P concentration from solubilizing $CaHPO_4$ and pH exhibited in PVK and YEM, P solubilization by *R. leguminosarum* clearly is not caused solely by reduction of pH. The production of organic acids accompanied by complexing with Ca^{2+} is the most efficient mechanism for solubilizing $CaHPO_4$ by *R. leguminosarum*, and the secretion of H^+ accompanied by substituting with Ca^{2+} may also occur for solubilizing $CaHPO_4$ by *R. leguminosarum*.

3.4.2 Effect of C and N concentrations on $CaHPO_4$ solubilization

Both ammonium salts and nitrate salts have been used as individual N sources or combined N sources in P solubilization studies. Ammonium N was best in reducing medium pH and promoting P solubilization (Pradhan and Sukia 2006; Cunningham and Kuiack 1992; Zhao et al., 2002). However, high concentrations of ammonium N decreased P solubilization. Nautiyal (1999) found $(NH_4)_2SO_4$ (2.5 g L^{-1}) decreased tricalcium P solubilization by 30% with *Pseudomonas sp* in liquid culture comparing to the control (0.1 g L^{-1}).

In this study, various concentrations of C and N in the liquid media were tested for their effects on nine *R. leguminosarum* isolates ability to solubilize $CaHPO_4$. There were no differences in P concentration related to the nine *R. leguminosarum* isolates grown in liquid cultures with various C and N concentrations (Fig. 3.8). The growth of all nine isolates was inhibited greatly at high N (0.5 g $(NH_4)_2SO_4$ L^{-1}) (Fig. 3.9). Higher ammonium N prevented *R. leguminosarum* growth, but did not affect its ability to solubilize $CaHPO_4$ indicating that fewer number of cells with the high N treatments produced same amount of solution P comparing to more populated cells of the low N treatments. Therefore P concentration on a *R. leguminosarum* per cfu basis was significantly higher ($p<0.05$) in the high N media than those of the low N media (Fig. 3.10). High N formulations inhibited *R. leguminosarum* growth regardless of the test C concentration. High N in liquid media promotes *R. leguminosarum* to solubilize $CaHPO_4$ could be because it likely stimulates its NH_4^+ assimilation. High N also leads to low C/N concentration ratio in the medium which in turn affecting the growth of isolates. *R. leguminosarum* can be benefited in solubilizing $CaHPO_4$ from higher N but a higher level of N also inhibits its growth, suggesting that the solubilization of $CaHPO_4$ is associated with ammonium N and its concentration. High ammonium N content (0.5g $(NH_4)_2S0_4$ L^{-1}) promotes solubilization of $CaHPO_4$ on a per cfu basis suggesting that an enhanced ability to solubilize $CaHPO_4$ is a survival response by *R. leguminosarum* to the high ammonium N. Thus, the solubilization of $CaHPO_4$ by *R. leguminosarum* is influenced not only by ammonium N and its concentration, but equally regulated to the threshold of an isolate to ammonium N. Perhaps the solubilization of $CaHPO_4$ is a function of stressful survival of *R. leguminosarum*. Ability of *R. leguminosarum* to solubilize P remains high even its growth is poor because of the C/N concentration ratio. This knowledge is important when considering a potential P solubilizing organism as various N conditions can occure in soil.

Stress induced P solubilization by bacteria isolated from chickpea rhizosphere soils were reported by Nautiyal et al. (1999) where they found all four bacteria tested were capable of solubilizing more tricalcium P than controls. The test medium had higher pH (pH 8.0) and salt (2.5% NaCl) conditions compared to control medium where pH was 7.0 and 0% NaCl. The effect of $(NH_4)_2SO_4$ on $CaHPO_4$ solubilization and growth of *R. leguminosarum* could potentially explain the inconsistent results reported when $(NH_4)_2SO_4$ is used. A low concentration of ammonium N promoted P solubilization (Asea et al., 1988; Whitelaw et al., 1999) whereas a high concentration of ammonium N inhibited P solubilization (Nautiyal, 1999). Microorganisms have different thresholds for ammonium N. If an organism has a high threshold for ammonium N, more Pi can be solubilized with an increase in the concentration of ammonium N. The ability of *R. leguminosarum* to solubilize $CaHPO_4$ is influenced by ammonium N and its concentration.

Acid production and the P solubilization ability of an organism in a laboratory environment are influenced greatly by the C and N sources (Cunningham and Kuiack 1992; Rodriguez and Fraga; 1999Whitelaw, 2000; Pradhan and Sukia 2006). Solubilization of $CaHPO_4$ by *R. leguminosarum* in liquid cultures varies among media (Fig. 3.3). Solubilization of $CaHPO_4$ was the highest overall in the PVK liquid culture indicating that medium composition and concentration influenced $CaHPO_4$ solubilization. All three tested media contained inorganic N salt, $(NH_4)_2SO_4$, but at different concentrations. YEM is an organic-N rich medium (high yeast extract), PVK is an N salt rich medium, and MNBRI is a N-limited medium (Table 3.1). *R. leguminosarum* solubilized the largest amount of P in PVK suggesting that ammonium N was preferred by *R. leguminosarum* for P solubilization. The lowest amount of P solubilized by *R. leguminosarum* isolates occurring in MNBRI broth indicating that low N content limits $CaHPO_4$ solubilization. P solubilization by the isolates in YEM ranged between the PVK and MNBRI probably due to the high concentrations of yeast extract. Although YEM has the same amount of ammonium N as MNBRI it contains a large amount of yeast extract. Yeast extract is a complex and undefined nutrient source; it supplies mainly N, but it also provides vitamins and other micronutrients. Pradhan and Sukla (2005) found ammonium N was important and necessary for solubilization of tricalcium P with *Aspergillus* sp. and *Penicillium* sp. Ammonium N also proved important for an increase in tricalcium P solubilization by *Bradyrhizobium* (Halder et al., 1991). Illmer and Schinner (1995) suggested that media low in C were better for screening P solubilizing *Rhizobium*. The ability of 48 wheat rhizosphere bacteria to solubilize dicalcium P was influenced by the high glucose content (1%) in the medium (Harris et al., 2006). Results of different N and C concentrations in the media on *R. leguminosarum* abilities to solubilize $CaHPO_4$ suggest that P concentration is related more to the ammonium N and its concentration than the concentration of C. Media low in C (10 g glucose L^{-1}) promoted *R. leguminosarum* growth. Variation in the ability to solubilize $CaHPO_4$ among *R. leguminosarum* isolates within one medium suggests that there are differences in physiology and metabolic capability of the isolates; some isolates utilize N differently which lead to higher P solubilization.

3.4.3 $CaHPO_4$ solubilization on three solid media

The solid media were less sensitive in the detection of the ability of *R. leguminosarum* isolates to solubilize $CaHPO_4$ than liquid broth (Fig. 3.2.). Less than 30 isolates were able to solubilize $CaHPO_4$ as assessed by the zone of clearings on the three tested solid media as opposed to all 30 isolates solubilizing $CaHPO_4$ in all three liquid formulations. The limitation for P solubilization on the solid media could have been caused by the unavailability of water in the solid media and subsequently limited nutrient supply. Agar concentration affected the cell numbers and colony diameters of *Pseudomonas* sp. and *Bacillus* sp. (Mitchell and Wimpenny, 1997). In comparison with liquid broth, substrates were limited to the isolates due to their availability and solubility in the solid media. Thus, *R. leguminosarum* isolates grown on solid media might not utilize the same metabolic pathway as when they are grown in liquid culture. Utilization of substrates from the solid media varies from one isolate to another because of their genetic profile. The utilization of C by an organism is dependent on its enzyme system (Brock et al., 1994). Because a single isolate produces different degrees of P solubilization on three different solid media suggests substrates availability is also important in solubilizing $CaHPO_4$ by *R. leguminosarum*. Therefore, fewer *R. leguminosarum* isolates were capable of solubilizing $CaHPO_4$ on solid media. Furthermore, solubilizing $CaHPO_4$ by *R. leguminosarum* on solid media is influenced by individual isolates and the availability of nutrients in the media. Solubilizing $CaHPO_4$ by *R. leguminosarum* is meaningful only within the context of its growth conditions.

The lack of correlation in the solubilization of $CaHPO_4$ between liquid and solid medium found in this study indicates that there is a difference in ability to solubilize $CaHPO_4$ by *R. leguminosarum* isolates in different formulations. Therefore, an isolate can have measurable P solubilization in the liquid formulation, but fail to produce a visible clearing zone on the solid medium suggesting that solubilization in a liquid formulation by an isolate does not predict its result on the solid medium of the same composition. The solubilization of $CaHPO_4$ by *R. leguminosarum* isolates is influenced greatly by the tested medium composition and its formulation.

Both solid and liquid formulations are used in screening P solubilization microorganisms. Researchers use either solid or liquid, but rarely both formulations. The degree of P solubilization found in *R. leguminosarum* is related to the test media and their formulations. A prediction of P solubilization is true only in that particular formulation and medium, and should not be generalized.

Phosphate solubilization on the solid media is visible by the zone of clearing. The procedure is easy to perform. Furthermore, nutrient availability in solid media is somehow restricted because of water availability. Microorganisms in soil have to survive different nutrient availability conditions due to soil properties. Solid media, in comparison to the liquid media, have a closer representation to soil conditions. Solid media therefore may be suitable for an initial screening of PSM. Phosphate solubilization in liquid media can be measured and thus liquid media are useful in studying P solubilization mechanisms and comparing P solubilization efficacy between microorganisms. Even though the degree of P solubilized in liquid media can be measured the isolates that solubilize small amounts of P may not be meaningful in terms of practical applications. Ultimately phosphate solubilizing microorganism selected from media either from a solid or liquid medium will need to be tested in soil for confirmation.

4.0 GROWTH CHAMBER STUDY: EFFECT OF PHOSPHATE SOLUBILIZING *R. leguminosarum* ON CANOLA GROWTH AND P UPTAKE

4.1 Introduction

Soil microorganisms are important components of the dynamic P cycle that occurs within soil. They are involved in: 1) the transformation of different forms of P; 2) mineralization of organic P; and 3) solubilization and immobilization of inorganic P (McLaughlin, 1988; Oberson, 2001). A substantial number of these microorganisms, including fungi and bacteria, are associated with the plant rhizosphere (Katznelson et al., 1962). Interaction between plants and microorganisms in the rhizosphere can affect plant growth, nutrient management and yield (Whitelaw, 2000; Rodriguez and Fraga, 1999; Leggett et al., 2001). The bacteria that exhibit a beneficial effect on plant growth are categorized as plant growth promoting rhizobacteria (PGPR). When these bacteria are inoculated onto plants, they promote plant growth via different mechanisms including enhanced P uptake (Jakobsen et al., 2005).

Solubilization of inorganic P is one of the direct influences of PGPR (Rodriguez and Fraga, 1999; Richardson, 2003). The use of P solubilizing microorganisms (PSM) to increase plant nutrient availability and subsequently benefit plant growth has been reported for different crops and regions. Soybean benefited from the co-inoculation of *Bradyrhizobium japonicum* and the P solubilizing bacterium *Pseudomonas striata*; dry weight of nodules, dry matter of plant, and yield were increased significantly compared to an uninoculated control in a neutral pH Indian soil with 22 to 40 kg available P ha^{-1} (Wasule et al., 2003). *Bacillus subtilis* increased rice root length and yield significantly from the control in both pot and field experiments in a Himalayan soil (Trivedi et al., 2003). *R. leguminosarum* has been shown great ability to solubilize inorganic forms of P: Ca-P, Al-P and Fe-P and release available P to plants (Halder et al., 1990; Rodriguez and Fraga, 1999; Abd-Alla, 1994). According to Antoun et al. (1998), the ability of

Rhizobium and *Bradyrhizobium* to solubilize dicalcium P varied depending on the strain. Percentage solubilization was estimated by comparing the size of clearing zones on agar plates containing dicalcium P. *R. leguminosarum* bv. *trifolii* solubilized 4% of dicalcium P whereas *R. leguminosarum* bv. *viciae* solubilized 71%. Chabot et al. (1998) also reported that maize and lettuce grown in different available P soils, ranging from poor to very fertile, and inoculated with two strains of *R. leguminosarum bv. phaseoli*, showed superior root colonization.

Benefits of PSM on root growth and yield are not always correlated with plant tissue P content (Wakelin et al., 2007). *Penicillium* sp. strain KC6-W2 increased wheat biomass (ranging from 6.6% to 19%) in three different soils compared to the control. However, the benefit of this strain on total foliar P uptake was not consistently demonstrated in all three soils. Two strains increased foliar P uptake, but one strain decreased uptake compared to the control (Wakelin et al., 2007). Soybean growth was stimulated by the bacterium *Burkholderia* sp., but was not correlated with shoot P content under greenhouse conditions (Fernandez et al., 2007). de Freitas et al. (1997) observed that *Bacillus* and *Xanthomonas* increased canola plant height and biomass, but did not increase tissue P content.

In addition to enhancing P solubilization, some PGPR produce growth regulators and antibiotics. Antoun et al., (1998) tested 266 isolates from different genera and species, two strains of *R. leguminosarum* increased radish yields by 31 to 50%. Of the 266 isolates of *Rhizobium* and *Bradyrhizobium*, 58% produced the growth regulator indole-3-acetic acid; whereas 3% produced hydrogen cyanide which is associated with the biological control of black root rot of tobacco (Antoun et al., 1998).

Testing microorganisms for P solubilization in soil conditions is an important step for confirmation of laboratory results and necessary for any meaningful application. Laboratory screening for microorganisms that solubilize P can be achieved on either solid or liquid media (Abd-Alla 1994; Wenzel and Ashford, 1994). Some isolates incapable of solubizing P on solid media can solubilize P in liquid culture (Louw and Webley, 1959; Gupta et al., 1994; Nautiya, 1999) indicating that differences exist between media and formulations for inducing P solubilization by PSM. Selective media that can be used as a screening tool for evaluating PSM, and also predict PSM performance in soil, would be the ideal choice.

The purpose of this growth chamber study was to assess the ability of *R. leguminosarum* to solubilize different phosphate fertilizers in growth chamber conditions. *R. leguminosarum* isolates were previously selected from both solid and liquid formulations of each of the three media (YEM, PVK and MNBRI) (Chapter 3). Three growth chamber experiments were conducted separately for the isolates selected from each medium to determine: 1) which medium (PVK, YEM or MNBRI) was the best predictor of the ability of the *R. leguminosarum* isolates to solubilize P in soil in a growth chamber; and 2) the effect of the selected P solubilizing *R. leguminosarum* isolates on growth and P uptake of canola.

4.2 Materials and Methods

4.2.1 *R. leguminosarum* isolates

Eight isolates of *R. leguminosarum bv. viciae* were selected based on their ability to solubilize $CaHPO_4$ in the liquid or solid formulations of each medium (YEM, PVK, MNBRI) from the screening study conducted in Chapter 3. Efficacy of each isolate in solubilizing $CaHPO_4$ was classified in a quadrant model based on its solubilization on solid media: true positive, true negative, false positive and false negative (Figs. 3.4-3.6). Lines separating the quadrants are the mean of P (mg L^{-1}) solubilized by the 30 isolates from each of the solid or liquid formulations of the same medium. True positive indicates that an isolate solubilized $CaHPO_4$ in an amount exceeding the mean of that particular medium and formulation; true negative indicates that an isolate solubilized $CaHPO_4$ in an amount less than the mean of that particular medium and formulation; false positive indicates that an isolate solubilized $CaHPO_4$ in an amount exceeding the mean in solid medium, but in an amount less than the mean in liquid; and false negative indicates of in amount less than the mean in solubilizing $CaHPO_4$ by an isolate in solid medium. Two isolates were selected from the mid-point of P solubilization between solid and liquid formulations in each of the quadrants (Table 4.1).

Table 4. 1 Selected *R. leguminosarum* isolates based on the ability of the isolate to solubilize $CaHPO_4$ from the two formulations of the same medium.

Medium	*R. leguminosarum* isolate			
	True Positive	True Negative	False Positive	False Negative
YEM	S027A-1	S016B-2	S003A-4	S010A-4
	S008A-4	S013B-3	S013A-2	S016B-1
PVK	S010A-4	S011A-2	S003A-4	S015B-1
	S020A-1	S014A-1	S008B-2	S014B-3
MNBRI	S013B-3	S012A-2	S014A-1	S006A-2
	S019B-5	S002A-3	S014B-3	S022A-2

4.2.2 Soil characterization

Two soils were used in the growth chamber study, both of which had been pre-tested for their response to P fertilizer (triple super phosphate 0-45-0). Both soils were Brown Chernozemic. The A horizon (0-15 cm) from soil A, legal location SE 5 6 10 W3, was used in the experiments for PVK and YEM *R. leguminosarum* isolates. The A horizon from soil B (0-15 cm), legal location SE 5 7 10 W3, was used in the experiments for MNBRI *R. leguminosarum* isolates. The soils were air-dried and the physical and chemical characteristics of soils determined by ALS Group Agriculture Services, formerly Enviro-Test Laboratories (Saskatoon, Canada). The P concentration as PO_4-3 was determined using Kelowna method (Quain et al., 1994) (Table 4.2).

4.2.3 Growth chamber study design and data analysis

Efficacy of *R. leguminosarum* isolates to solubilize Evergrow rock phosphate (0-6-0) was tested and compared in a growth chamber experiment. The eight isolates in Table 4.1 were tested with three P source treatments. Rates were based on soil test recommendations for canola. Phosphate treatments were no added P (control), full rate of triple superphosphate, 0.07 g pot^{-1} (67 kg ha^{-1}), and full rate of Evergrow rock phosphate 0.1 g pot^{-1} (100 kg ha^{-1}).

Table 4. 2 Physical and chemical characteristics of soils

	Soil A	Soil B
Sample ID	287249	229353
Legal Location	SE 5 6 10 W3	SE 5 7 10 W3
Soil Climatic Zone	Brown	Brown
Previous Crop	Fallow, Cultivated	Fallow, Cultivated
Date Sampled	2-Jul-05	4-Sep-06
Soil water holding capacity [2]	31 %	30 %
Depth (cm)	0-30	0-30
Texture	Clay Loam	Clay Loam
pH [1]	7.8	7.5
Salinity Rating	Non Saline	Non Saline
Organic Matter (%)	1.4	nd[†]
NO_3-N (kg ha^{-1})	14.6	6.7
P (kg ha^{-1})	23.6	20.2
K (kg ha^{-1})	879	697
SO_4-S (kg ha^{-1})	69.6	20.2

[1] 1:2 soil: water extract
[2] Volumetric method
[†] not determined

Approximately 930 g of soil was added to four 10-cm square plastic pots. Nitrogen (NH_4NO_3, 120 kg ha^{-1}) and sulfur ($(NH_4)_2SO_4$, 23 kg ha^{-1}) fertilizers were applied in solution according to fertilizer recommendations for growing canola. Phosphate sources were suspended in water and applied as a "band" in which the P-treatment suspension was dribbled onto the surface of the soil. 100 g of soil was layered on top of the fertilizer band prior to seeding.

R. leguminosarum isolates were grown at 25^{o}C in sucrose yeast extract (SYE) broth for 48 hours on a rotary shaker (200 rpm). After incubation, the isolate cultures were centrifuged at 4500 rpm for 10 min. The supernatant of the centrifuged cultures were discarded. The pellets were re-suspended in fresh sterile SYE broth. The cultures were then agitated to obtain homogeneous mixtures prior to storage at -85^{o}C.

Canola seeds (*Brassica napus*) were inoculated by placing 100 g of seeds in Ziploc bags and adding the equivalent of 5x 10^3 colony forming units (cfu) of thawed *R.*

leguminosarum isolates per seed as the inoculation target. *R. leguminosarum* suspensions were injected into Ziploc bags, size 26.8 x 27.3 cm, containing the canola seeds. The seeds were agitated by shaking the bags for approximately 30 sec. Six treated seeds were sown in each pot. After emergence, plants were thinned to three plants of uniform appearance per pot. Each pot was placed in an open Ziploc bag, size 26.8 x 27.3 cm to minimize cross-contamination and excessive loss of moisture. The soil was maintained at 40% water holding capacity. Temperature was 20^{o}C and 15^{o}C for 16 h (day) and 8 h (night) cycles, respectively, to simulate early spring soil condition. The P source treatments were arranged in a completely randomized factorial design with four replicates.

Canola plants were harvested 5 wk after emergence by removing all plant material above the soil. Dry mass was determined after drying the plant material at 70^{o}C in an oven for 7 d. Plant tissue P concentration was determined using an inductively coupled plasma (ICP) emision spectroscopy by the ALS laboratory Group Agricultural Service, Saskatoon (Huang and Schulte, 1985). Total P uptake was calculated by multiplying plant dry mass by plant tissue P concentration.

Data was checked for normality and homogeneity and was transformed to log data. Analysis of variance was performed using the full factorial model of JMP 7.0.

4.3 Results

4.3.1 Evaluation of *R. leguminosarum* isolates using a P solubilization quadrant model

Neither plant dry mass nor tissue P content of canola were increased by inoculation with *R. leguminosarum* isolates from the true positive selection group of any media tested (Tables 4.3, 4.4 and 4.5) even though the isolates in the true positive group had superior ability to solubilize $CaHPO_4$ in the laboratory evaluations. Furthermore, none of the *R. leguminosarum* groups from any of the three tested media increased

Table 4. 3 Affect of *R. leguminosarum* isolates from four pre-screening quadrants on canola dry matter production under different P fertilized treatments

Quadrant group	Dry mass (g pot $^{-1}$)			
	0 P	Super P	Rock P	Mean
YEM				
True positive	2.43	3.09	2.50	2.68
True negative	2.70	3.18	2.64	2.84
False positive	2.66	3.17	2.65	2.83
False negative	2.70	2.86	2.46	2.67
Control	2.48	2.96	2.71	2.72
Mean	*2.59 b*	*3.06 a*	*2.59 b*	
	P rate * P quadrant (*p*=0.4)			
PVK				
True positive	2.78 d	3.24 abcd	3.17 abcd	3.06
True negative	2.86 cd	3.36 ab	2.86 d	3.03
False positive	2,99 bcd	3.51 a	3.15 abcd	3.21
False negative	2.96 bcd	3.16 abcd	2.79 d	2.97
Control	3.12 abcd	3.22 abcd	3.44 abc	3.26
Mean	*2.94*	*3.29*	*3.08*	
	P rate * P quadrant (*p*=0.016)			
MNBRI				
True positive	2.38	3.04	2.37	2.59
True negative	2.31	3.17	2.71	2.73
False positive	2.27	2.96	2.39	2.54
False negative	2.2	3.15	2.62	2.66
Control	2.26	3.13	2.57	2.65
Mean	*2.28 c*	*3.08 a*	*2.53 b*	
	P rate * P quadrant (*p*=0.5)			

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Values are the mean of four replicates. True positive indicates that an isolate has solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative indicates that an isolate has shown less $CaHPO_4$ solubilization than the mean of that particular medium and the formulation; false positive indicates that an isolate has good $CaHPO_4$ solubilization on solid medium but poor in liquid; and false negative is indicative of poor $CaHPO_4$ solubilization on solid medium. Control means no isolate addition.

Table 4. 4 Affect of *R. leguminosarum* isolates from four pre-screening quadrants on canola tissue P content under different P fertilized treatments

Quadrant group	Tissue P (mg g $^{-1}$ pot $^{-1}$)			
	0 P	Super P	Rock P	Mean
YEM				
True positive	1.6 d	2.5 a	1.8 bcd	1.9
True negative	1.7 bcd	2.7 a	1.7 bcd	2.1
False positive	1.7 cd	2.7 a	1.8 bcd	2.1
False negative	1.8 bcd	2.7 a	1.9 bc	2.1
Control	2.0 b	2.6 a	1.8 bcd	2.1
Mean	*1.7*	*2.6*	*1.8*	
	P rate * P quadrant (*p*=0.019)			
PVK				
True positive	2.1	2.1	2.3	2.3
True negative	2.1	2.6	2.1	2.2
False positive	2.1	2.4	2.1	2.1
False negative	2.1	2.6	2.2	2.2
Control	2.1	2.5	2.1	2.2
Mean	*2.1 b*	*2.6 a*	*2.1 b*	
	P rate * P quadrant (*p*=0.76)			
MNBRI				
True positive	2.3	2.4	2.4	2.4
True negative	2.1	2.4	2.1	2.2
False positive	2.3	2.6	2.4	2.4
False negative	2.2	2.4	2.2	2.3
Control	2.5	2.6	2.4	2.5
Mean	*2.3 b*	*2.5 a*	*2.3 ab*	
	P rate * P quadrant (*p*=0.87)			

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Values are the mean of four replicates. True positive indicates that an isolate has solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative indicates that an isolate has shown less $CaHPO_4$ solubilization than the mean of that particular medium and the formulation; false positive indicates that an isolate has good $CaHPO_4$ solubilization on solid medium but poor in liquid; and false negative is indicative of poor $CaHPO_4$ solubilization on solid medium. Control means no isolate addition.

Table 4. 5 Affect of *R. leguminosarum* isolates from four pre-screening quadrants on canola total P uptake under different P fertilized treatments

Quadrant group	Total P uptake (mg pot $^{-1}$)			
	0 P	Super P	Rock P	Mean
YEM				
True positive	3.8	7.8	4.4	5.1 B
True negative	4.7	8.6	4.6	5.7 A
False positive	4.4	8.5	4.7	5.6 A
False negative	4.8	7.6	4.5	5.5 AB
Control	5.0	7.7	4.8	5.7 AB
Mean	*4.5 b*	*8.0 a*	*4.6 b*	
P rate * P quadrant *p*=0.13)				
PVK				
True positive	5.8 d	8.7 a	7.3 abcd	7.2
True negative	5.9 d	8.8 a	5.8 d	6.8
False positive	6.2 cd	8.5 a	6.4 cd	7.0
False negative	6.1 cd	8.0 ab	6.0 d	6.7
Control	6.4 bcd	7.9 abc	7.3 abcd	7.2
Mean	*6.1*	*8.4*	*6.6*	
P rate * P quadrant (*p*=0.03)				
MNBRI				
True positive	5.4	7.3	5.9	6.2
True negative	4.8	7.8	5.8	6.1
False positive	5.3	7.7	5.7	6.2
False negative	4.9	7.7	5.9	6.1
Control	5.7	8.1	6.2	6.7
Mean	*5.2 c*	*7.7 a*	*5.9 b*	
P rate * P quadrant (*P*=0.81)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. True positive indicates that an isolate has solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative indicates that an isolate has shown less $CaHPO_4$ solubilization than the mean of that particular medium and the formulation; false positive indicates that an isolate has good $CaHPO_4$ solubilization on solid medium but poor in liquid; and false negative is indicative of poor $CaHPO_4$ solubilization on solid medium. Control means no isolate addition.

canola plant dry mass, tissue P content or total P uptake. Overall, growth and tissue P content in canola were highest ($p < 0.05$) on the soils fertilized with superphosphate (Tables 4.3, 4.4 and 4.5).

4.3.2 Evaluation of *R. leguminosarum* isolates using a P solubilization binary model

The quadrant model failed to positively correlate isolates able to solubilize $CaHPO_4$ in laboratory screening to isolates able to solubilize P in the growth chamber experiments. A new $CaHPO_4$ solubilization mode–a binary model was then developed. Binary models are formulation-based models. Unlike the quadrant model in which isolates were classified by their ability to solubilize $CaHPO_4$ from both solid and liquid formulations within a medium, the binary model separates isolates as either positive or negative within a formulation. Therefore, the same eight isolates, previously selected from the quadrant model (Table 4.1) were re-grouped as either positive or negative groups within a formulation. Positive indicates that an isolate solubilized $CaHPO_4$ in an amount exceeding the mean of that particular formulation; negative indicates that an isolate solubilized $CaHPO_4$ in an amount less than the mean of that particular formulation. False positive and false negative group were eliminated.

In the case of the liquid binary model where the isolates were selected based on their ability to solubilize $CaHPO_4$ in the liquid formulation, none of the *R. leguminosarum* isolate groups from any of the three tested liquid formulations increased canola plant dry mass, tissue P content nor total P uptake compared to the control (Tables 4.6, 4.7 and 4.8). Furthermore, there was a significantly lower canola biomass obtained from the plants that were inoculated with the positive selection from the PVK than the control, and lower canola biomass obtained from the plants that were inoculated with the positive selection than the negative selection of YEM liquid formulations (Table 4.6). Overall, growth, tissue P content and total P uptake in canola were highest on the soils fertilized with superphosphate (Tables 4.6, 4.7 and 4.8).

In the case of the solid media binary model, where the isolates were selected based on their ability to solubilize $CaHPO_4$ on the solid formulation, none of *R. leguminosarum* isolate groups from any of the three tested liquid formulations increased

Table 4. 6 Dry matter production by canola grown in three P treatments inoculated with *R. leguminosarum* isolates grown in broth media.

Liquid	Dry mass (g pot $^{-1}$)			
Binary	0 P	Super P	Rock P	Mean
YEM				
Positive	2.57	2.99	2.48	2.68 B
Negative	2.68	3.17	2.65	2.83 A
Control	2.48	2.96	2.7	2.72 AB
Mean	*2.58 b*	*3.04 a*	*2.61 b*	
P rate * P binary (*p*=0.68)				
PVK				
Positive	2.87	3.2	2.98	3.02 B
Negative	2.93	3.41	3	3.12 AB
Control	3.12	3.21	3.44	3.26 A
Mean	*2.97 b*	*3.27 a*	*3.14 ab*	
P rate * P binary (*p*=0.08)				
MNBRI				
Positive	2.29	3.09	2.5	2.62
Negative	2.29	3.06	2.55	2.63
Control	2.26	3.13	2.57	2.65
	2.28 c	*3.09 a*	*2.54 b*	
P rate * P binary (*p*=0.98)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. Eight isolates were tested based on their ability to solubilize $CaHPO_4$ and grouped into binary groups according to more $CaHPO_4$ solubilized than the mean of all isolates (positive) or less $CaHPO_4$ solubilized than the mean of all isolates (negative). Controls are uninoculated canola plants.

Table 4. 7 Tissue P content of canola grown in three P treatments inoculated with *R. leguminosarum* isolates grown in broth media.

Liquid	Tissue P (mg g^{-1})			
Binary	0 P	Super P	Rock P	Mean
YEM				
Positive	1.6 c	2.6a	1.8 bc	2.0
Negative	1.7 c	2.7a	1.7 bc	2.0
Control	2.0 b	2.6a	1.8 bc	2.1
Mean	*1.8*	*2.6*	*1.8*	
P rate * P binary (*p*=0.009)				
PVK				
Positive	2.0	2.6	2.2	2.3
Negative	2.0	2.5	2.0	2.2
Control	2.0	2.4	2.1	2.2
Mean	*2.0 b*	*2.5 a*	*2.1 b*	
P rate * P binary (*p*=0.51)				
MNBRI				
Positive	2.3	2.4	2.3	2.3
Negative	2.2	2.5	2.2	2.3
Control	2.5	2.6	2.4	2.5
Mean	*2.3 a*	*2.5 a*	*2.3 a*	
P rate * P binary (*p*=0.66)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Values are the mean of four replicates. Eight isolates were tested based on their ability to solubilize $CaHPO_4$ and grouped into binary groups according to more $CaHPO_4$ solubilized than the mean of all isolates (positive) or less $CaHPO_4$ solubilized than the mean of all isolates (negative). Controls are uninoculated canola plants.

Table 4. 8 Total P uptake by canola grown in three P treatments inoculated with *R. leguminosarum* isolates grown in broth media.

Liquid	Total P uptake (mg pot $^{-1}$)			
Binary	0 P	Super P	Rock P	Mean
YEM				
Positive	4.3	7.8	4.4	5.3 B
Negative	4.6	8.5	4.7	5.7 A
Control	5.0	7.7	4.8	5.7 AB
Mean	*4.6 b*	*8.0 a*	*4.6 b*	
P rate * P binary (*p*=0.51)				
PVK				
Positive	5.9	8.3	6.7	7.0
Negative	6.0	8.7	6.1	6.9
Control	6.4	7.9	7.3	7.2
Mean	*6.1 b*	*8.3 a*	*6.7 b*	
P rate * P binary (*p*=0.08)				
MNBRI				
Positive	5.1	7.5	5.8	6.0
Negative	5.0	7.7	5.7	6.0
Control	5.6	8.0	6.2	6.5
Mean	*5.2 c*	*7.7 a*	*5.9 b*	
P rate * P binary (*p*=0.91)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. Eight isolates were tested based on their ability to solubilize $CaHPO_4$ and grouped into binary groups according to more $CaHPO_4$ solubilized than the mean of all isolates (positive) or less $CaHPO_4$ solubilized than the mean of all isolates (negative). Controls are uninoculated canola plants.

increased canola plant dry mass, tissue P content nor total P uptake compared to the control (Tables 4.9, 4.10 and 4.11).

Table 4. 9 Dry matter production by canola grown in three P treatments inoculated with *R. leguminosarum* isolates grown on solid media.

Solid	Dry mass mean (g pot $^{-1}$)			
Binary	0 P	Super P	Rock P	Mean
YEM				
Positive	2.54	3.13	2.58	2.57
Negative	2.7	3.02	2.55	2.76
Control	2.48	2.96	2.7	2.72
Mean	*2.58 b*	*3.04 a*	*2.61 b*	
P rate * P quadrant (*p*=0.28)				
PVK				
Positive	2.89 bc	3.34 a	3.16 ab	3.13
Negative	2.91 bc	3.26 a	2.82 c	3.00
Control	3.12 abc	3.21 abc	3.44 a	3.26
Mean	*2.97*	*3.27*	*3.14*	
P rate * P quadrant (*p*=0.008)				
MNBRI				
Positive	2.32	2.99	2.38	2.57
Negative	2.25	3.16	2.67	2.69
Control	2.26	3.13	2.57	2.65
Mean	*2.28 c*	*3.09 a*	*2.54 b*	
P rate * P quadrant (*p*=0.14)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Values are the mean of four replicates. Eight isolates were tested based on their ability to solubilize $CaHPO_4$ and grouped into binary groups according to more $CaHPO_4$ solubilized than the mean of all isolates (positive) or less $CaHPO_4$ solubilized than the mean of all isolates (negative). Controls are uninoculated canola plants.

Table 4. 10 Tissue P content of canola grown in three P treatments inoculated with *R. leguminosarum* isolates grown on solid media.

Solid	Tissue P mean (mg g^{-1})			
Binary	P 0	Super P	Rock P	Mean
YEM				
Positive	1.6	2.6	1.8	2.0 B
Negative	1.7	2.7	1.8	2.0 AB
Control	2.0	2.6	1.8	2.1 A
Mean	*1.8 b*	*2.6 a*	*1.8 b*	
P rate * P binary (*p*=0.06)				
PVK				
Positive	2.0	2.5	2.1	2.2
Negative	2.0	2.5	2.1	2.2
Control	2.0	2.4	2.1	2.2
Mean	*2.0 b*	*2.5 a*	*2.1 b*	
P rate * P binary (*p*=0.89)				
MNBRI				
Positive	2.3	2.5	2.4	2.4
Negative	2.1	2.4	2.2	2.2
Control	2.5	2.6	2.4	2.5
	2.3 a	*2.5 a*	*2.3 a*	
P rate * P binary (*p*=0.83)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. Eight isolates were tested based on their ability to solubilize $CaHPO_4$ and grouped into binary groups according to more $CaHPO_4$ solubilized than the mean of all isolates (positive) or less $CaHPO_4$ solubilized than the mean of all isolates (negative). Controls are uninoculated canola plants.

Table 4. 11 Total P uptake by canola grown in three P treatments inoculated with *R. leguminosarum* isolates grown in broth media.

Solid Binary	Total P uptake (mg pot $^{-1}$) 0 P	Super P	Rock P	Mean
YEM				
Positive	4.1	8.1	4.5	5.3
Negative	4.7	8.1	4.5	5.6
Control	5.0	7.7	4.8	5.7
Mean	*4.6 b*	*8.0 a*	*4.6 b*	
P rate * P binary (*p*=0.06)				
PVK				
Positive	6.0 c	8.6 a	6.9 bc	7.1
Negative	6.0 c	8.4 a	5.9 c	6.8
Control	6.4 bc	7.9 ab	7.3 abc	7.2
Mean	*6.1 b*	*8.3 a*	*6.7 b*	
P rate * P binary (*p*=0.04)				
MNBRI				
Positive	5.3	7.5	5.7	6.1
Negative	4.8	7.7	5.8	6.0
Control	5.6	8.0	6.2	6.5
Mean	*5.2 c*	*7.7 a*	*5.9 b*	
P rate * P binary (*p*=0.41)				

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Values are the mean of four replicates. Eight isolates were tested based on their ability to solubilize $CaHPO_4$ and grouped into binary groups according to more $CaHPO_4$ solubilized than the mean of all isolates (positive) or less $CaHPO_4$ solubilized than the mean of all isolates (negative). Controls are uninoculated canola plants.

Overall, growth, tissue P content and total P uptake in canola were higher on the soils fertilized with superphosphate (Tables 4.9, 4.10 and 4.11).

4.3.3 Effect of *R. leguminosarum* isolates

Because neither model (quadrant or binary) was able to relate the ability of the *R. leguminosarum* isolates to solubilize $CaHPO_4$ in the laboratory to P-uptake efficacy in the growth chamber, the effect of individual isolates was examined. The effect of *R. leguminosarum* on canola dry mass, tissue P content or total P uptake varied from one isolate to another, but none of the isolates consistently increased these parameters relative to the uninoculated control (Tables 4.12, 4.13 and 4.14). Overall, growth, tissue P content and total P uptake in canola were highest on the soils fertilized with triple superphosphate compared to the control (Tables 4.12, 4.13 and 4.14). One of the false positive selections (S003a-4) from the PVK medium showed a tendency to increase total P uptake by canola from all P treatments (Table 4.14).

4.4 Discussion

Canola plant dry mass, tissue P content or total P uptake did not increase with the inoculation of the *R. leguminosarum* true positive group that showed high $CaHPO_4$ solubilizing ability in bench-top laboratory screening (Tables 4.3, 4.4 and 4.5). Furthermore, canola plant dry mass, tissue P content and total P uptake were not affected by any of the P solubilizing *R. leguminosarum* groups selected from any of the three tested media. The quadrant model failed to relate *R. leguminosarum* isolates selected for their relative abilities to solubilize $CaHPO_4$ liquid and solid media, to performance in soil amended with different P sources. *R. leguminosarum* selection based on the ability to solubilize $CaHPO_4$ from the two formulations within a medium was not effective.

There are a few reasons that might contribute to the failure of the *R. leguminosarum* quadrant model: firstly, only two isolates were used from each quadrant within a medium. The results may have been different if all isolates in the quadrant were tested to increase sample population size. Secondly, the differences in $CaHPO_4$ solubilization among quadrants within a medium may be too small to be distinguishable in soil conditions.

Table 4. 12 Effect of *R. leguminosarum* isolates on canola dry matter production under different P fertilized treatments

Isolate	Quadrant	Dry mass (g pot $^{-1}$)			
		0 P	Super P	Rock P	Mean
YEM					
S003a-4	FP	2.42	2.90	2.55	2.63 BC
S008a-4	TP	2.44	3.07	2.48	2.66 BC
S010a-4	FN	2.84	2.98	2.68	2.83 ABC
S013a-2	FP	2.90	3.44	2.76	3.03A
S013b-3	TN	2.88	3.10	2.69	2.89 AB
S016b-1	FN	2.56	2.75	2.37	2.52 C
S016b-2	TN	2.52	3.27	2.59	2.80 ABC
S027a-1	TP	2.42	3.11	2.54	2.69 ABC
Control		2.49	2.96	2.71	2.72 ABC
Mean		*2.61 b*	*3.06 a*	*2.58 b*	
		P rate * Isolate (*p*=0.43)			
PVK					
S003a-4	FP	3.26 abc	3.40 ab	3.30 abc	3.31
S008b-2	FP	2.72 cdef	3.63 a	3.00 abcdef	3.12
S010a-4	TP	2.61 ef	3.26 abc	3.17 abcde	3.01
S011a-2	TN	2.76 cdef	3.33 abc	2.51 f	2.87
S014a-1	TN	3.00 abcdef	3.39 ab	3.22 abcd	3.20
S014b-3	FN	3.04 abcdef	3.11 abcde	2.94 abcdef	3.03
S015b-1	FN	2.88 bcdef	3.21 abcde	2.65 def	2.91
S020a-1	TP	2.95 abcdef	3.23 abcd	3.17 abcde	3.12
Control		3.12 abcde	3.21 abcde	3.44 ab	3.26
		2.92	*3.31*	*3.04*	
		P rate * Isolate (*p*=0.0003)			

Table 4.12 (continued)

Isolate	Quadrant	Canola Dry mass (g pot $^{-1}$)			
		0 P	Super P	Rock P	Mean
MNBRI					
S002a-3	TN	2.09	2.61	2.24	2.31 B
S006a-2	FN	2.46	3.31	2.52	2.76 A
S012a-2	TN	2.32	2.87	2.36	2.51 AB
S013b-3	TP	2.19	3.37	2.66	2.74 A
S014a-1	FP	2.43	2.98	2.77	2.72 A
S014b-3	FP	2.27	3.17	2.74	2.72 A
S019b-5	TP	2.14	3.14	2.51	2.59 AB
S022a-2	FN	2.44	3.19	2.40	2.67 A
Control		2.26	3.13	2.57	2.65 A
Mean		*2.28 c*	*3.08 a*	*2.53 b*	

P rate * Isolate (*p*=0.21)

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. True positive (TP) indicates that an isolate has solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative (TN) indicates that an isolate has shown less $CaHPO_4$ solubilization than the mean of that particular medium and formulation; false positive (FP) indicates that an isolate has good $CaHPO_4$ solubilization on solid medium but poor in liquid; and false negative (FN) is indicative of poor $CaHPO_4$ solubilization on solid medium. Control means no isolate addition.

Table 4. 13 Effect of *R. leguminosarum* isolates on canola tissue P content under different P fertilized treatments

Isolate	Quadrant	Tissue P (mg g $^{-1}$)			
		0 P	Super P	Rock P	Mean
YEM					
S003a-4	FP	1.6 cd	2.8 a	1.9 bc	2.1
S008a-4	TP	1.5 d	2.6 a	1.7 bcd	1.9
S010a-4	FN	1.8 bcd	2.6 a	1.9 bc	2.1
S013a-2	FP	1.7 bcd	2.6 a	1.7 bcd	2.0
S013b-3	TN	1.8 bcd	2.7 a	1.7 bcd	2.0
S016b-1	FN	1.7 bcd	2.7 a	1.8 bcd	2.0
S016b-2	TN	1.7 bcd	2.7 a	1.8 bcd	2.0
S027a-1	TP	1.6 cd	2.4 a	1.8 bcd	1.9
Control		2.0 b	2.6 a	1.8 bcd	2.1
		1.7	*2.6*	*1.8*	
	P rate * Isolate (p=0.009)				
PVK					
S003a-4	FP	2.2	2.5	2.2	2.3 A
S008b-2	FP	1.8	2.3	1.8	2.0 B
S010a-4	TP	2.2	2.7	2.4	2.4 A
S011a-2	TN	1.9	2.6	2.1	2.2 AB
S014a-1	TN	2.2	2.6	2.0	2.3 AB
S014b-3	FN	2.2	2.5	2.1	2.3 AB
S015b-1	FN	1.9	2.5	2.2	2.2 AB
S020a-1	TP	2.0	2.6	2.1	2.2 AB
Control		2.0	2.4	2.1	2.2 AB
		2.0 b	*2.5 a*	*2.1 b*	
	P rate * Isolate (p=0.62)				

Table 4.13 (continued)

Isolate	Quadrant	Canola Tissue P (mg g $^{-1}$)			
		0 P	Super P	Rock P	Mean
MNBRI					
S002a-3	TN	2.4	2.8	2.4	2.6
S006a-2	FN	2.2	2.4	2.3	2.3
S012a-2	TN	2.2	2.4	2.4	2.3
S013b-3	TP	2.1	2.3	2.1	2.2
S014a-1	FP	2.0	2.6	2.2	2.3
S014b-3	FP	2.2	2.5	1.9	2.2
S019b-5	TP	2.3	2.3	2.5	2.4
S022a-2	FN	2.4	2.5	2.5	2.4
Control		2.5	2.6	2.4	2.5
Mean		*2.2 c*	*2.5 a*	*2.3 ab*	
	P rate * Isolate (*p*=0.82)				

Within a medium, values followed by the same letter are not significantly different (p=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. True positive (TP) indicates that an isolate has solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative (TN) indicates that an isolate has shown less $CaHPO_4$ solubilization than the mean of that particular medium and formulation; false positive (FP) indicates that an isolate has good $CaHPO_4$ solubilization on solid medium but poor in liquid; and false negative (FN) is indicative of poor $CaHPO_4$ solubilization on solid medium. Control means no isolate addition.

Table 4. 14 Effect of *R. leguminosarum* isolates on canola total P uptake under different P fertilized treatments

Isolate	Quadrant	Total P uptake (mg pot $^{-1}$)			
		0 P	Super P	Rock P	Mean
YEM					
S003a-4	FP	3.9 bc	8.0 a	4.9 bc	5.3
S008a-4	TP	3.7 c	7.9 a	4.3 bc	5.0
S010a-4	FN	5.1 b	7.8 a	5.1 bc	5.9
S013a-2	FP	5.1 bc	9.0 a	4.6 bc	6.0
S013b-3	TN	5.1 bc	8.3 a	4.6 bc	5.8
S016b-1	FN	4.4 bc	7.4 a	4.0 bc	5.1
S016b-2	TN	4.3 bc	8.8 a	4.6 bc	5.6
S027a-1	TP	3.9 bc	7.7 a	4.5 bc	5.1
Control		5.0 bc	7.7 a	4.8 bc	5.7
Mean		*4.5*	*7.7*	*4.6*	
		P rate * Isolate (*p*=0.02)			
PVK					
S003a-4	FP	7.3 abcdef	8.5 ab	7.3 abcdef	7.7
S008b-2	FP	5.1 g	8.5 abc	5.6 fg	6.4
S010a-4	TP	5.7 fg	8.9 a	7.8 abcde	7.5
S011a-2	TN	5.3 g	8.6 ab	5.2 g	6.4
S014a-1	TN	6.7 bcdefg	9.0 a	6.5 cdefg	7.4
S014b-3	FN	6.7 bcdefg	7.9 abcd	6.3 defg	6.9
S015b-1	FN	5.5 fg	8.1 abcd	5.8 fg	6.4
S020a-1	TP	5.9 efg	8.4 abc	6.8 bcdefg	7.0
Control		6.4 defg	7.9 abcd	7.3 abcdef	7.2
Mean		*6.0*	*8.4*	*6.5*	
		P rate * Isolate (*p*=0.001)			

Table 4.14 (continued)

Isolate	Quadrant	Canola Total P uptake (mg pot $^{-1}$)			
		0 P	Super P	Rock P	Mean
MNBRI					
S002a-3	TN	5.1	7.4	5.5	5.9
S006a-2	FN	5.4	8.0	5.8	6.3
S012a-2	TN	5.1	6.8	5.6	5.8
S013b-3	TP	4.6	7.8	5.6	5.9
S014a-1	FP	5.0	7.7	5.9	6.1
S014b-3	FP	4.9	7.9	5.3	5.9
S019b-5	TP	4.8	7.3	6.2	6.0
S022a-2	FN	5.7	7.9	5.9	6.4
Control		5.6	8.0	6.2	6.5
Mean		*5.1 c*	*7.6 a*	*5.8 b*	
		P rate * Isolate (*p*=0.93)			

Within a medium, values followed by the same letter are not significantly different (*p*=0.05), according to Tukey's Honestly Significant Difference Test. Lower case non-italicized letters compare means of P treatments for the isolates. Lower case italic letters compare means of P treatments. Upper case letters compare means of different $CaHPO_4$ solubilizing *R. leguminosarum* groups. Values are the mean of four replicates. True positive (TP) indicates that an isolate has solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative (TN) indicates that an isolate has shown less $CaHPO_4$ solubilization than the mean of that particular medium and formulation; false positive (FP) indicates that an isolate has good $CaHPO_4$ solubilization on solid medium but poor in liquid; and false negative (FN) is indicative of poor $CaHPO_4$ solubilization on solid medium. Control means no isolate addition.

Overall, the *R. leguminosarum* isolates classified within the quadrants did not increase canola plant dry mass, tissue P content or total P uptake relative to the controls.

The binary models examined and compared P solubilizing *R. leguminosarum* selected from solid-based and liquid-based media separately. Canola dry mass, tissue P content and total P uptake were not affected by the *R. leguminosarum* positive nor negative groups. In some cases, plants not inoculated with *R. leguminosarum* had a higher dry mass and tissue P content than those inoculated with the *R. leguminosarum* positive group.

The effect of *R. leguminosarum* on canola dry mass, tissue P content and total P uptake varied from one isolate to another, but was not different from the controls. None of the tested *R. leguminosarum* isolates significantly impacted canola dry mass, tissue P content and total P uptake ($p = 0.05$).

According to Gyaneshwar et al. (2002) it is common to identify P solubilizing microorganisms under laboratory conditions, but field performances of the P solubilizing microbes are highly variable. These authors further indicate that the variability in field performance by the P solubilizing microorganism is due to the laboratory conditions employed in the screening process for the isolates not reflecting soil conditions (Gyaneshwar et al., 2002).

The $CaHPO_4$ solubilization efficacies of *R. leguminosarum* isolates from the three tested media, ranged from 93 to 372 mg L^{-1} in liquid cultures might not be high enough to have any meaningful impact on the growth of canola plants under growth chamber conditions. *R. leguminosarum* among the isolates tested does not have an effective P solubilization ability. The fungus *Penicillium bilaiae*, an active ingredient in the commercial P solubilizing inoculant JumpStart®, solubilized 690 to 2492 mg L^{-1} $CaHPO_4$ in the same three tested media. Hence, perhaps only the most efficient P solubilizers may perform in soils.

Another possibility for negligible affects of the *R. leguminosarum* isolates could be due to the low inoculation rate onto the seed. According to Jjemba and Alexander (1999), the effectiveness of a microorganism depends on the initial inoculum density. Furthermore, the ability to survive and multiply is the main factor influencing the competitiveness of microorganisms in soils. Twenty-five radish (*Raphanus sativus* L**.**)

seeds were treated with 1 mL of 10^8 cfu mL^{-1} of either *R. leguminosarum* or *Bradyrhizobium japonicum* cultures and radish dry matter yield varied. Only 25% of the 266 tested strains increased dried matter, with one *B. japonicum* increasing dry matter by 60% comparing to uninoculated control (Antoun et al., 1998). Soybean growth was enhanced by the P solubilizing bacteria *Burkholderia* sp., *Enterobacter* sp., and *Bradyrhizobum* sp. when 25 seeds were inoculated with 4 mL of 10^8 cfu mL^{-1} of bacterial cultures (Ferandez et al., 2007). In our growth chamber experiments, 2,500 canola seeds were treated only with 1 mL containing approximately 10^8 cfu mL^{-1} of *R. leguminosarum* culture. *R. leguminosarum* inoculum density in this study clearly was lower than levels used by other researchers. In general, the population size of the introduced microorganism declines rapidly upon inoculation in soils (Ho and Ko, 1984). Survival and growth of *R. leguminosarum* subsequently might have been affected leading to an undesirable result.

Soils used in the experiments were highly buffered calcareous soils. Highly buffered alkaline soils have an inhibitory effect on the secretions of organic acids by some bacteria (Gyanneshar et al., 2002). *Citrobacter koseri* only solubilized rock P on unbuffered solid medium, but not on medium buffered with 100 mM Tris-HCl at pH 8.0. In contrast, *Enterbacter asburiae* solubilized rock P on both buffered and unbuffered medium (Gyanneshar et al., 2002). According to Fernandez et al. (2007), the amount of P released in a buffered medium by a group of bacteria ranged from 0.07 % to 4.82%, in comparison to 3 to 24% of total P in a medium without a buffer. However, *P. bilaiae* produced a tenfold increase in soluble P from rock P in a buffered medium, compared to an unbuffered medium (Takeda and Knight, 2006). There were larger amounts of citric and oxalic acids produced, but the concentration of other organic acids such as malonic and succinic acids were higher in the non-buffered medium (Takeda and Knight, 2006). The type of organism and organic acid produced by the organism may be affected differently by the buffering capacity of the substrate. Organisms such as *P. bilaiae* or *Enterbacter asburiae* might be less sensitive to pH changes, and their P solubilization efficacy remain functional or even enhanced under highly buffered conditions. Organisms like *R. leguminosarum* and *Citrobacter koseri* are more sensitive to pH changes under growth conditions and subsequently their P solubilization is more dramatically influenced.

5.0 SUMMARY AND CONCLUSIONS

Phosphate solubilizing microorganisms have become more important and relevant in P fertilizer management and application. Total soil P has accumulated from P fertilization and mismanagement; whereas soluble P has become less available with the increase in P fertilization in soil. The efficacy of different PSM has been difficult to compare because of the diverse results achieved with different methods, media or even formulations. The study was set out to assess the ability of *R. leguminosarum* isolated from the Canadian Prairies to solubilize $CaHPO_{4,}$ to determine the relationship between solubilization ability for $CaHPO_4$ on solid and liquid formulation of same composition by the isolates, to determine the effect of C and N on the growth of *R. leguminosarum* isolates and their abilities to solubilize $CaHPO_{4.}$

The variation in the ability of 30 *R. leguminosarum* isolates to solubilize $CaHPO_4$ observed in the study indicated that the isolate, medium components, medium formulation, and nutrient availability greatly influenced the outcome. The effect of medium formulation was clearly demonstrated in this study. The liquid method was more sensitive in detecting P in solution; more isolates responded to the three liquid formulations than the solid formulations of the same media. Fewer *R. leguminosarum* isolates showed visible P solubilization on the solid media. The highest number of *R. leguminosarum* isolates showing P solubilization ability in liquid than solid media may be because of nutrient availability. Nutrients in the liquid method are readily available to the isolates, as opposed to the solid media where the nutrients might be limited by water availability. It is possible that only the most efficient $CaHPO_4$ solubilizing *R. leguminosarum* isolates produce visible results on the solid media. Overall, $CaHPO_4$ solubilization by *R. leguminosarum* obtained from the solid method did not correspond to that of the liquid method. The results obtained from *R. leguminosarum* isolates grown on solid media containing $CaHPO_{4,}$ therefore, cannot be used to predict isolates ability to solubilize $CaHPO_4$ in liquid methods. Additionally, liquid methods were labor intensive and time consuming. On the other hand, the solid medium methods were easy to perform and less time constricted.

Medium components, especially C and N concentrations, probably affected *R. leguminosarum* isolates to solubilize $CaHPO_4$. *R. leguminosarum* isolates solubilized $CaHPO_4$ differently depending on the tested medium and its C and N concentration. The highest average P concentration solubilized by the thirty *R. leguminosarum* isolates was obtained from PVK cultured broth.

$CaHPO_4$ solubilization by *R. leguminosarum* isolates in liquid broth is associated with a decrease in the pH. Among the three tested media, the lowest pH by the thirty *R. leguminosarum* isolates was obtained from PVK. Although no relationship was detected between P concentration and pH in PVK incubated with *R. leguminosarum*, the highest P concentration was probably due to the composition in the PVK medium. These results suggest that PVK is the most suitable of the three tested media (YEM, PVK and MNBRI) for screening $CaHPO_4$ solubilizing *R. leguminosarum*. The modified Pikovskaya's phosphate medium is a complex medium with high ammonium N content. Ammonium N reduced medium pH and promotion of P solubilization (Cunningham and Kuiack 1992; Zhao et al., 2002; Pradhan and Sukia 2005;). However, despite its promotion of P solubilization, ammonium N at the higher concentration tested inhibited the growth of *R. leguminosarum* isolates in this study. At a concentration of 0.5 g $(NH_4)_2SO_4$ L^{-1}, it promoted the solubilization of $CaHPO_4$ but inhibited the propagation of *R. leguminosarum*. A concentration of N at 0.1 g $(NH_4)_2SO_4$ L^{-1} optimized P solubilization and growth of *R. leguminosarum*. High ammonium N stimulates *R. leguminosarum* to solubilize $CaHPO_4$ on a per cfu basis. Ammonium N can be beneficial or toxic depending on its concentration and the evaluated microorganism. The solubilization of $CaHPO_4$ is perhaps a function of the stressful survival of *R. leguminosarum*. Ammonium N at a concentration of 2.5 g ammonium L^{-1} inhibited P released by *Pseudomonas* sp. in liquid medium (Nautiyal, 1999). Thus, the solubilization of P is influenced not only by the concentration of ammonium N, but equally regulated by the threshold of an isolate to ammonium N. If an organism tolerates ammonium N, an increase in P solubilization would likely be observed. Otherwise, a negative impact of ammonium N would be likely concluded.

Testing microorganisms for P solubilization under soil conditions is an important step for the confirmation of laboratory results, and necessary for any meaningful implications. In this study, three sets of each eight *R. leguminosarum* isolates were selected separately based on their ability to solubilize $CaHPO_4$ from the three screening media. A quadrant model was developed based on the ability of thirty *R. leguminosarum* isolates to solubilize $CaHPO_4$ on both solid and liquid formulations within a medium. The quadrant model demonstrated the relationship of an isolate in its ability to solubilize $CaHPO_4$ in two formulations within a medium, although the results on solid did not correlate to the results of liquid. Isolates were selected from the quadrant model for testing in soils under growth chamber conditions. Efficacy of P solubilization by each isolate fell in one of the four quadrants based on its solubilization on solid: true positive, true negative, false positive and false negative. Lines separating the quadrants were the mean of P solubilization by the thirty isolates from each of the solid or liquid formulations of the same medium. True positive indicated that an isolate had solubilized $CaHPO_4$ to an extent exceeding the mean of that particular medium and formulation; true negative indicated that an isolate had shown less than mean ability in solubilizing $CaHPO_4$ for that particular medium and formulation; false positive indicated that an isolate had an above mean ability in P solubilization on solid medium, but poor in liquid broth; and false negative was indicative of poor ability in solubilizing $CaHPO_4$ on solid medium but greater than mean ability in liquid broth. Selected isolates presented different solubilization efficacies for $CaHPO_4$.

The *R. leguminosarum* isolates were tested for their effect on canola dry mass and tissue P content in soil with various P fertilizer sources. No isolate from any of the quadrants showed any significant effect on canola dry mass and tissue P content compared to the controls ($p \geq 0.05$) regardless of their ability to solubilize $CaHPO_4$ in the various tested formulations and media under laboratory conditions. Based on these findings, the quadrant model from the laboratory screening of three media (YEM, PVK and MNBRI) failed to predict the performances of *R. leguminosarum* in soil. Even though $CaHPO_4$ solubilizing *R. leguminosarum* can be successfully identified from the three screening media YEM, PVK and MNBRI, but the P solubilization ability of these isolates have not yet been confirmed in soil under growth chamber conditions.

Variations have been found in the effectiveness of PSM under soil conditions (Kucey et al., 1989; Nautiyal et al., 1999). *Bacillus* sp. released P from organic P, but was not capable of solubilizing P from mineral P (Gyaneshwar et al., 2002). According to Gyaneshwar et al. (2002), it is common to identify PSM under laboratory conditions, but rare to find a microorganism that consistently performs under field conditions. These authors further concluded that the variability in field performances by the PSM is due to the laboratory screening process in which the parameters employed for the isolates do not reflect soil conditions. Laboratory screening for PSM with selected media is conducted under controlled environmental conditions, whereas conditions in soil are uncertain. The performance of microbes is strongly influenced by soil conditions such as pH and temperature (Nautiyal et al., 1999; Leggett et al., 2001).

A few possibilities might have contributed to the insignificant impact of *R. leguminosarum* on canola dry mass and tissue P content. *R. leguminosarum* isolates might not be the most efficient P solubilizers in the two tested soils. *R. leguminosarum* isolates solubilized $CaHPO_4$ in the three tested media ranging from 93 to 372 mg L^{-1} and they might not be efficient enough to cause any meaningful impact on the growth of canola plants under growth chamber conditions. *P. bilaiae*, active in JumpStart ® have shown variable field results from positive to no response or even a negative response (Jakobsen et al., 2005). In this study, P concentration in solution was solubilized by *P. bilaiae* ranging from 690 to 2491 mg L^{-1}.

The low inoculation rate used could be the second reason for the ineffectiveness of *R. leguminosarum* isolates. Canola seeds of 100 g (2,500 seed) were treated with 1 mL inoculum containing about 10^8 cfu mL^{-1} of *R. leguminosarum* culture in these growth chamber experiments. Other researchers have applied rates up to 100-time higher (Antoun et al., 1998; Ferandez et al., 2007). According to Jjemba and Alexander (1999), the effectiveness of a microorganism depends on the initial inoculum density. With a high inoculation rate, sufficient survival and population of the organisms would probably result, subsequently leading to better competition with other microbes in the soils. A higher inoculum density should be used for testing P solubilizing microorganisms in soil because higher inoculum density compensates for the loss of microorganisms due to an unprotected delivering system.

The pH buffering capacity of the soil heavily influences P solubilization. Soils used in the study were highly buffered calcareous soils. The highly buffered alkaline soils were found to have an inhibitory effect to secretions of high concentrations of organic acids from some bacteria (Gyanneshar et al., 2002). Takeda and Knight (2007) found that *P. bilaiae* produced soluble P tenfold higher from a rock P in a buffered medium compared to an unbuffered medium, concurrently with large amounts of citric and oxalic acids production (Takeda and Knight, 2007). The type of organism appears to be affected differently by the substrate buffering capacity. Organisms such as *P. bilaiae* might be more tolerant to pH changes, and thus its P solubilization efficacy would be maintained under a wide range of pH conditions. Organisms such as *R. leguminosarum* that might be more sensitive to pH changes are subsequently affected more for their P solubilization.

This study has focused on screening the ability of *R. leguminosarum* to directly solubilize P from an inorganic P source in media with the hope of solubilizing P in soil for plant uptake. Although *R. leguminosarum* isolates solubilized $CaHPO_4$ in liquid and solid formulations in the media YEM, PVK and MNBRI, they failed to show any significant impact on the canola dry mass and tissue P content under the growth chamber conditions. As Richardson (2003) states, the poor understanding of the interaction between physical and chemical characteristics of soil and P solubilization is a major limitation to the application of PSM. Phosphate solubilization is a complex process involving both organism and soil. Understanding the relationship between P solubilization and survival in soil conditions of a particular microorganism is necessary for efficient P solubilization to occur. According to Rajput et al. (2007), PSM works effectively on residual rock P and on higher grades of rock P. In future soil-based testing of PSM, a higher inoculum density should be considered. Soils with different P sources and status should also be used in the testing.

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