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# PENICILLIUM BILAII: INTERACTIONS WITH BARLEY OR CANOLA, GROWTH IN RHIZOSPHERE SOIL, AND OVERWINTER SURVIVAL

BY

DOUGLAS OLIVER KEYES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PENICILLIUM BILAII: INTERACTIONS WITH BARLEY OR CANOLA, GROWTH IN RHIZOSPHERE SOIL, AND OVERWINTER SURVIVAL submitted by DOUGLAS OLIVER KEYES in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in SOIL BIOCHEMISTRY.

W.B. McGill (Supervisor)

J.A. Robertson

J.P. Tewari

Date October 11, 1990

*This thesis is dedicated to*

*Valerie*

## ABSTRACT

Dry matter and P yield of canola and wheat grown on alkaline soils are reportedly greater following inoculation with Penicillium bilaii, and P. bilaii cultures solubilize rock phosphate, suggesting that P availability to plants is greater in the presence of this fungus. Phosphate solubilization may, however, differ between alkaline and acidic soils because they contain different phosphate compounds, and the stability of cation-chelate complexes varies with pH. The effect of this fungus will also depend on its survival and distribution in soil.

In the present study, barley and canola were grown on a P deficient Malmo SiCL (pH 6.0) with or without i) P. bilaii and ii) monoammonium phosphate, added to the seed-row. Fungal (P. bilaii and native fungi) colony forming units (CFU), plant growth and seed yield parameters, and P and micronutrient concentrations were determined at various times during the growing season. In a separate field experiment, soil was uniformly inoculated to 5 cm and planted to canola. Fungi in rhizosphere and bulk soil were enumerated in the 2-4 cm, 6-8 cm, and 10-12 cm depths six weeks later and in bulk soil (2-4 cm) 1 year later. Canola was also grown in pots containing soil samples from Ap horizons (pH 5.5) having varying residual P, with or without P. bilaii. Fungi were enumerated, plant dry matter yield determined, and shoot P and micronutrient concentrations measured after 7 weeks.

Dry matter yield of canola was consistently greater with P. bilaii but P quantity was not affected. Under field conditions, Fe and Mn concentrations in canola were greater with P. bilaii. There was a 100 fold increase or a slight decrease in P. bilaii CFU following, respectively, uniform or seed-row inoculation; and P. bilaii survived overwinter. A restriction fragment length polymorphisms (RFLP) analysis indicated that two phenotypes of this fungus belong to the same species.

We concluded that P. bilaii grows and survives in the Ap horizon of a Malmo SiCL in central Alberta, and is associated with greater canola dry matter yield but does not affect barley growth. This work provides evidence that P. bilaii influences canola grown on acidic soils by mechanisms other than increased P availability.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## CHAPTER 1 INTRODUCTION

Biological activity has long been known to be greater in soil surrounding plant roots than in bulk soil (Hiltner 1904, as cited in Rovira 1956). Rovira (1956) attributed this activity to root exudates which are readily metabolized by many soil bacteria and fungi. Benefits to plant growth from microorganisms in the rhizosphere include: i) suppression of root pathogens (Wright 1956; Lumsden and Lewis 1989), ii) degradation of xenobiotics (Ghosal et al. 1985), iii) production of plant growth regulating substances (Mishra et al. 1987; Fallik and Okon 1989; Nieto and Frankenberger 1989), iv) production of siderophores (Page 1988), v) dinitrogen fixation (Lynch 1983; Hussain et al. 1987), and vi) mineralization of nutrients contained in organic matter (Lynch 1983). Another mechanism by which soil microorganisms may benefit plants is solubilization of minerals in soil resulting in release of plant nutrients including phosphorus (Katznelson and Bose 1959). This thesis focuses on phosphorus and micronutrient uptake, and plant growth following inoculation with the phosphate-solubilizing fungus, Penicillium bilaii, and on the growth and survival of this organism following inoculation into a Malmo SiCL.

Organic acids produced by microorganisms increase the rate of solubilization of Ca- Fe- and Al-phosphates (Johnston 1959a, 1959b), and reduce the extent of phosphate fixation by Fe and Al compounds (Struthers and Seiling 1950) and clays (Goring and Bartholomew 1952). Phosphate-solubilizing microorganisms solubilize minerals in solution culture apparently by decreasing the pH of the medium and/or by production of chelating agents, depending on the isolate and composition of the medium (Louw and Webley 1959; Duff et al. 1963; Banik and Dey 1982; Asea et al. 1988). The amount of phosphate solubilized in solution culture varies with the organism and the phosphate compound. Kucey (1983) found that fungi from Alberta soils generally solubilized more rock phosphate than did bacteria. The fungi with the greatest

phosphate solubilizing ability are generally considered to belong to the Penicillium and Aspergillus genera (Banik and Dey 1982; Kucey 1983). Fungi from forest tree seedbeds, in glucose amended medium for 20 days, solubilized up to 87.7% of the phosphate added; $Ca_3(PO_4)_2$ was most readily solubilized followed by fluorapatite and hydroxyapatite (Agnihotri 1970). Banik and Dey (1982) found in sucrose amended media that phosphate-solubilizing fungi and bacteria, on average, solubilized 19% and 10% as much $AlPO_4$ and $FePO_4$, respectively, as they did $Ca_3(PO_4)_2$.

Penicillium bilaii solubilizes rock phosphate in solution culture (Asea et al. 1989) and increased the $NaHCO_3$-extractable P in a Lethbridge SiCL (pH 7.7) under greenhouse conditions (Kucey 1988). Addition of this fungus to alkaline soils, alone or in combination with rock phosphate or monoammonium phosphate (MAP) has been associated with greater dry matter and P yields of wheat (Kucey 1987, 1988; Asea et al. 1988) and canola (Kucey and Leggett 1989). These observations suggest that P. bilaii benefits plant growth by increasing the availability of sparingly soluble phosphates in soil. Research regarding the effect of P. bilaii on plant growth and P uptake has only been reported for alkaline soils, however. In such soils Ca- and Mg-phosphate are the predominant forms of available P (Racz and Soper 1967). The relation of P. bilaii to P uptake by crops grown on acidic soils, in which Fe- and Al-phosphates may predominate (Alexander and Robertson 1968), has not been reported. Furthermore, the effect of this organism on acid sensitive crops has not been reported. Chapter 2 of this thesis deals with dry matter and nutrient yield of canola and barley following seed-row inoculation with P. bilaii, in an acidic field soil.

There have been no reports of the fate of P. bilaii following its inoculation into soil. Rhizosphere competence is required for a soil microorganism to affect plant growth. Penicillium species rapidly colonize fresh substrates and are generally more concentrated in the rhizosphere compared to bulk soil (Domsch et al. 1980), however, the microbial

community in the rhizosphere varies with plant species (Parkinson 1963). Hence, species-specific results may be obtained from P. bilaii inoculation. The potential for P. bilaii to influence other soil biota and subsequent crops depends on its overwinter survival. Penicillium bilaii was isolated from a southern Alberta soil; its survival ability in soils to which it is not endemic has not been determined. Chapter 3 reports on the distribution of P. bilaii following its introduction into a Malmo SiCL in central Alberta and on its growth and survival during the growing season and overwinter.

Penicillium bilaii and native soil fungi are enumerated by dilution plating and P. bilaii is normally identified by its colony morphology, pigmentation and ability to solubilize precipitated Ca-phosphate in the medium. Identification of imperfect fungi is problematic because many of the diagnostic characteristics used, including colony morphology, are often unstable (Raper and Thom 1949). Recent advances in molecular biology have provided techniques for identifying microorganisms that may be more reliable than traditional methods because they do not depend on gene expression. Because of the appearance of two phenotypes of P. bilaii, a DNA probe was developed to aid in its identification (Chapter 4).

Penicillium bilaii may benefit crops by solubilizing sparingly soluble fertilizer P reaction products, thereby increasing their availability to plants and, hence, the utilization efficiency of fertilizer P. Fertilizer P added to soil generally does not move out of the Ap horizon (Tisdale et al. 1985). By using samples of Ap horizon soil in pots to grow greenhouse crops, plant roots are confined to soil containing residual P. This may provide a better indication of residual P availability than growing the same plants on the same soil in the field. Calcareous or alkaline soils to which P has been added are likely to contain dicalcium phosphate dihydrite and octocalcium phosphate whereas in low pH soils added P is more likely to be associated with Fe and Al (Lindsay and Stephenson 1959; Soper and Racz 1980). There has been no report on the use of an acidic soil to

investigate dry matter and P yield of plants following inoculation with P. bilaii under greenhouse conditions. Furthermore, there has been no report that directly examines the relation of P. bilaii to residual fertilizer P availability. In chapter 5 we report dry matter yield and nutrient concentrations of canola plants grown in pots, under greenhouse conditions, using two acidic soils each having varying levels of residual P, with and without P. bilaii addition.

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# CHAPTER 2 DRY MATTER YIELD AND QUANTITIES OF ABOVE-GROUND PHOSPHORUS AND MICRONUTRIENTS IN BARLEY AND CANOLA WITH AND WITHOUT *PENICILLIUM BILAII* ADDITION TO AN ACIDIC FIELD SOIL[1]

## 2.1 Introduction

Field crops grown in western Canada take up about 10 kg $ha^{-1}$ of P in the form of $H_2PO_4^-$ or $HPO_4^{2-}$. Although soils on which these crops are grown contain large amounts of P, a very small portion of it is available to plants. Phosphate fertilizers are added to increase the amount of available P and thereby increase crop growth. Subsequent to dissolution, however, the phosphate in these fertilizers reacts with soil constituents to form sparingly soluble compounds such as dicalcium phosphate dihydrate in soils having neutral or higher pH, and iron and aluminum phosphates in soils having a low pH (Lindsay and Stephenson 1959; Soper and Racz 1980).

Organic acids such as citric, malic, tartaric, oxalic and lactic are produced by microorganisms and have been shown to increase the rate of dissolution of Ca- Fe- and Al-phosphates (Johnston 1959a, 1959b), and to reduce the extent of phosphate fixation by Fe and Al (Struthers and Sieling 1950) and by clays (Goring and Bartholomew 1952). The ability of an organic acid to dissolve Fe, Al and Ca compounds is proportional to the stability of the complexes formed, which in turn depends upon the structure of the acid and the solution pH (Struthers and Sieling 1950; Norval 1972). Organic ligands in soil also reduce Al toxicity (Barlett and Riego 1972) and are required to supply micronutrients to plants (Lindsay 1971).

Studies concerning phosphate-solubilization by microorganisms have been done within a narrow range of conditions generally using simple sugar substrates; it is not certain how closely these conditions resemble those of the rhizosphere. It is well established, however, that biological activity is greater in the rhizosphere than in bulk soil

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[1]A version of this Chapter will be submitted for publication (D.O. Keyes and W.B. McGill; Can. J. Soil Sci.).

(Rovira and Davey 1974) and the microbial community of the rhizosphere has a higher proportion of phosphate-dissolving microorganisms than bulk soil (Khan and Bhatnagar 1977). Solution cultures of some microorganisms increase the rate of dissolution of low solubility compounds, including phosphates, by reducing the solution pH and producing chelating agents including organic acids (Louw and Webley 1959; Duff et al. 1963; Asea et al. 1988). The amount and type of acids produced varies among isolates and with nutritional and environmental factors, which may in turn influence the mechanism and amount of phosphate solubilization in rhizosphere soil. For example, in the rhizosphere of wheat 2-ketogluconate is the predominant organic acid which, apparently, is produced by undetermined microorganisms utilizing glucose as a C source (Moghimi et al. 1978); however, $Ca^{++}$ chelation by this compound is negligible (Moghimi and Tate 1978).

Penicillium bilaii i) solubilizes rock phosphate in solution culture (Asea et al. 1988); ii) increases the $NaHCO_3$-extractable P in alkaline soil under greenhouse conditions (Kucey 1988); and iii) increases yield of P and dry matter of wheat (Kucey 1987, 1988; Asea et al. 1988) and canola (Kucey and Leggett 1989) grown in alkaline soils having low levels of extractable P, with or without addition of rock phosphate or monoammonium phosphate (MAP), under greenhouse or field conditions. These findings indicate that this fungus may increase P availability to plants. In addition, P. bilaii inoculation has been associated with greater zinc concentrations in wheat shoots (Kucey 1988). Research involving this fungus has been on western Canadian soils, generally having pH values greater than 7.0. However, approximately one third of the agricultural land in western Canada has a pH of less than 6.5 (P.F.R.A. 1983). Furthermore, the effects of P. bilaii inoculation on acid sensitive plant species has not been reported.

To extend our understanding of the range of conditions and crops over which inoculation with this fungus might be expected to benefit plant growth requires knowledge of its effects on plants grown on acidic soils and on acid sensitive crops

because i) phosphate solubilization by P. bilaii relies partly on acidification (Asea et al. 1988), ii) the chelating ability of organic acids is highly pH dependent (Norval 1972), and iii) there is a greater proportion of Fe- and Al-phosphates in acidic soils (Alexander and Robertson 1968; Soper and Racz 1980). Hence, the objective of this research was to test, under field conditions, the hypotheses i) that P. bilaii increases barley and canola dry matter yield on a slightly acid soil, ii) that P. bilaii increases above ground yield of P in these crops, with or without added MAP, and iii) that P. bilaii affects the concentration of selected cations in plants.

## 2.2 Materials and Methods

### *2.2.1 Site and Soil Description*

This study was conducted during 1988 and 1989 at the University of Alberta, Ellerslie Research Station (53° 26'N, 113° 22'W) approximately 10 km SW of Edmonton. The soil is a Malmo SiCL (Black Chernozemic). The N and C contents of the Ap horizon (0-10 cm) of this series are 5 and 52 g $kg^{-1}$, respectively, with a Db of 1.2 Mg $m^{-3}$ and a CEC of 40 cmol $kg^{-1}$ (Crown and Greenlee, 1978). The Ap horizon had a pH of 6.0 and contained 4 $\mu g$ $g^{-1}$ of extractable P by the method of Miller and Axley (1956). At field capacity this soil contains 38% $H_2O$ (gravimetric) (Maule and Chanasyk 1987). The area on which the plots were established had been cropped to barley in 1987 and was cultivated in the fall.

### *2.2.2 Agronomic Procedures*

Barley and canola were planted on adjacent (8 m apart) sets of plots. Ammonium nitrate (260 kg $ha^{-1}$) and ammonium sulfate (42 kg $ha^{-1}$) were broadcast in 1988 and ammonium nitrate (286 kg $ha^{-1}$) was broadcast in 1989. Ethalfluralin (1.4 kg $ha^{-1}$) was applied to the canola plots for weed control and was incorporated along with the fertilizer by rototilling to a depth of 14 cm prior to planting. Weed control in the barley was accomplished with an early (2 leaf stage) foliar application of metsulfuron methyl (4.45 g

$ha^{-1}$). Weeds that were not controlled chemically were removed manually.

Crop varieties, and seeding dates varied between years. The barley (Hordeum vulgare) varieties used were Sampson in 1988, and Winchester in 1989. The same canola variety, Brassica campestris v. Tobin, was used both years. In 1988 both crops were seeded on June 1; in 1989 both crops were seeded on May 18, however, the canola was re-seeded on June 19 without re-applying the treatments. The seeding rates were 80 and 6 kg $ha^{-1}$ for barley and canola, respectively.

*2.2.3 Inoculant*

The inoculants used contained P. bilaii and were prepared by growing this fungus on moistened wheat bran. The inoculant used in 1988 mistakenly contained a deviant phenotype of P. bilaii, type 'a' the source of which is not known. The inoculant used in 1989 was prepared from an isolate of P. bilaii that conforms to published descriptions of this species and is designated type 'b'. Although the same protocol was used both years to prepare the inoculants, the concentration of P. bilaii in the 1988 inoculant was approximately $10^7$ CFU $g^{-1}$ whereas the inoculant used in 1989 contained about $10^7$ CFU $g^{-1}$. The reason for this difference is not known. The inoculant preparation procedure and a description of the two P. bilaii phenotypes are given in Chapter 3.

*2.2.4 Experimental Design and Treatments*

For each crop, a complete factorial randomized complete block (4 blocks) design was employed with treatments consisting of two levels (+ and --) of P fertilization and two levels (+ and --) of inoculation. Crops were planted and treatments applied to 13 m long plots by one pass with a six row (15 cm row spacing), double disk seeder equipped with separate delivery systems for seed, fertilizers, and inoculum, which were all placed together at 5 cm for barley and 3 cm for canola. Crops were alternated between the two sets of plots the second year; the same treatment(s) were applied to each plot to avoid residual treatment effects.

The +MAP treatment provided P at 146 mg $m^{-1}$ $row^{-1}$ (9.6 kg $ha^{-1}$), except for barley in 1989 for which P was provided at a rate of 183 mg $m^{-1}$ $row^{-1}$ (12 kg $ha^{-1}$ ). Ammonium nitrate was applied to the -MAP treatments to balance the N content of the +MAP treatments. The +P. bilaii treatment consisted of 1.00 g $m^{-1}$ $row^{-1}$ of inoculant in 1988 and 0.25 g $m^{-1}$ $row^{-1}$ in 1989 because the results from work in 1988 indicated fewer P. bilaii propagules were likely required.

*2.2.5 Determination of Crop Growth Parameters*

Barley was harvested at three dates and canola at two dates each year. Barley plots were divided into 3 subplots, each 3 m long which in 1988 were harvested on July 4 (6 leaf; 1-2 tillers), July 31 (7-8 leaf; 2-3 tillers), and September 22 (mature); and in 989 were harvested on July 1 (7 leaf; no tillers), July 21 (7 leaf 1-2 tillers), and September 7 (mature). Each canola plot was divided into 2 subplots, each 5 m long which in 1988 were harvested on July 20 (early pod filling) and September 13 (mature); and in 1989 were harvested on July 21 (flowering) and September 12 (late pod filling). The following were determined for a 10 plant subsample from each plot and averaged: height, leaf area, number of tillers or branches, stem and leaf mass, head or pod mass, and number of heads or pods. Plants were removed from 5 randomly selected distances along each of the two center rows within the subplots and were dried at 76 C for 72 hours prior to weighing. Following removal of these plants, the remaining plants of the two center rows were cut at the soil surface, counted, dried and weighed. In addition, barley heads $m^{-1}$ $row^{-1}$ were counted at the onset of heading to determine the effect of treatments on crop maturity. For the final harvests, plants were threshed to determine seed yield, and 1000 grain mass.

*2.2.6 Plant Nutrient Concentrations and Quantity of P*

Plant material from the first barley harvest of both years, and from the first 1988, and the first and second 1989, canola harvests, was analyzed for P, Ca, Mg, Fe, Cu, Zn,

Mn and Al concentrations. Plants were stored dry at room temperature prior to analysis. The material was ground using a high speed mill with a stainless steel (0.5 mm) sieve and digested with nitric perchloric acid. Elemental concentrations were then measured by ICP. Quantity of these elements in plant shoots was calculated as the product of concentration times the respective total plot DM yields.

*2.2.7 Statistical Analysis*

Analysis of variance was performed on all parameters measured using the SAS (SAS Institute Inc. 1986) GLM procedure. The probabilities of differences between means were calculated using the PDIFF option of the LSMEANS procedure.

**2.3 Results**

*2.3.1 Barley Growth and Nutrient Uptake*

Analysis of variance indicates that dry matter yield of barley was, over all dates, significantly greater for +MAP than --MAP treatments (Figures 2.1 and 2.2; Table 2.1). Plant height, leaf area index (LAI), and number of tillers were also greater, overall, with this treatment although no difference was detected at some dates (Table 2.2). In 1988, seed yield was greater with than without MAP because there were more heads; in 1989, no response in seed yield components to MAP addition was observed (Figure 2.3 and Table 2.3). In both years, however, MAP appeared to hasten barley maturation (Table 2.4).

Quantity of crop P was significantly greater for the +MAP treatments prior to heading due both to a greater P concentration (1988), and to greater DM yield (Figures 2.4 and 2.5; Table 2.1). The concentrations of Ca and Mg were significantly greater but the concentration of Cu was significantly less for the +MAP treatments (Table 2.5). Aluminum and Fe concentrations were extremely high for plants of the first 1988 harvest and did not vary with MAP treatment (Table 2.5).

Penicillium bilaii did not affect barley DM yield, height, LAI, tillering, maturity, seed yield or any of the seed yield components measured (Figures 2.1, 2.2 and 2.3; Tables 2.1, 2.2, 2.3 and 2.4). Phosphorus concentration, but not above ground quantity, was significantly lower for +P. bilaii treatments (Figures 2.4 and 2.5; Table 2.1). Penicillium bilaii inoculation was associated with lower Ca and Mg concentrations in 1988 but higher concentrations of these elements in 1989; it was not observed to affect the concentrations of any other element (Table 2.5).

*2.3.2 Canola Growth and Nutrient Uptake*

Phosphate fertilization approximately doubled DM production of canola in this study (Figures 2.6 and 2.7; Table 2.6). Analysis of variance indicates that height, LAI, [illegible] branching were, overall, significantly greater for the MAP fertilized plants as well (Table 2.7). Seed yield was also approximately doubled with MAP: in 1988 largely due to more pods per plant, and in 1989 because of more plants and larger seed size (Figure 2.8; Table 2.8). The larger seed size associated with the +MAP treatments likely indicates hastened maturity in 1989 because plants were harvested before they had fully matured.

Phosphorus quantity was significantly greater with MAP fertilization primarily due to greater DM yield but also because of a significantly greater P concentration in plants grown with MAP (Figures 2.9 and 2.10; Table 2.6). The concentrations of Mg and Mn were, overall, greater for the +MAP than the --MAP treatments but the concentration of Zn was lower with MAP (Table 2.9, Analyis of Variance). Iron concentration varied significantly with the MAP treatment but the effect was inconsistent among sampling [illegible]ble 2.9) The concentrations of Ca, Mg, Cu, and Al did not vary with the MAP [illegible].

Analysis of variance indicates that, over all dates, P. bilaii addition was associated with greater canola DM yield (Figure 2.6; Table 2.6). The greatest and

statistically most significant increases occurred at the final harvests: in 1988 on the -MAP treatments, and in 1989 on the +MAP treatments. Plant height, LAI, and branching also tended to be greater in canola following addition of P. bilaii, however, there was considerable variability in these parameters (Table 2.7). Seed yield was, overall, greater for the +P. bilaii treatments, although none of the measured yield components was individually responsible for this difference (Table 2.8).

Quantity of P was, overall, not significantly greater in canola following P. bilaii addition, however, at the final harvest of 1989 P quantity in plants of the +MAP treatments was greater with P. bilaii addition due to a greater P concentration and greater DM yield (Figures 2.7 and 2.10; Table 2.6). Iron and Mn concentrations were significantly greater in plants inoculated with P. bilaii (Table 2.9), whereas the concentrations of Ca, Mg, Zn, Cu, and Al did not vary with P. bilaii inoculation.

## 2.4 Discussion

Differences in results between years in this study may be related to i) the use of a different P. bilaii phenotype having different phosphate solubilizing ability, ii) an approximately 400 fold greater inoculation rate in 1988 than in 1989; iii) differences in temperature and soil water distribution; iv) differences in planting date, plant age and growth stage; and iv) for the Fe and Al concentrations, differences in the amount of contamination with soil. In addition, differences in barley results between years may have been caused by the use of different barley variety (Sampson in 1988 and Winchester in 1989): barley varieties differ in their development, growth potential, pH tolerance, and nutritional requirements.

Canola DM yield and seed yield were greater following inoculation with P. bilaii. This is consistent with results previously reported for canola at this location and at Plum Coulee, Manitoba (Kucey and Leggett 1989), and for wheat at Lethbridge, Alberta (Kucey 1987, 1988). Barley growth, on the other hand, did not vary with the P. bilaii treatment

in this study. There are no previous reports concerning P. bilaii inoculation of barley, or of any monocotyledonous plant other than wheat (Kucey 1987, 1988; Asea et al. 1988). The reasons for the different results between crops following P. bilaii addition in the present study are not known. Fundamental differences between the two crops regarding nutrient acquisition include i) that barley has a fibrous root system whereas canola has a tap root, ii) that barley is mycorrhizal whereas canola is non-mycorrhizal (Hirrel et al. 1978), but is able to lower its rhizosphere pH in response to low phosphate availability (Mooreby et al. 1988), and iii) differences in nutritional requirements (Clark 1983). Distribution of roots in soil is controlled primarily by soil physical characteristics and soil water distribution, therefore, the different yield response to P. bilaii inoculation between the two crops was not likely due to differences in soil explored by the two root systems. Borlan et al. (1987) has shown that mycorrhizal plants can acquire phosphate from different sources than can non-mycorrhizal plants, however, Kucey (1987) found wheat yield and quantity of P was greater for P. bilaii inoculated plants regardless whether mycorrhizal fungi were present. This leaves differences in rhizosphere pH and acid tolerance, in addition to differences in nutritional requirements, as potential factors involved in determining the effect of P. bilaii on crop growth. Another hypothesis is that rhizosphere competence of P. bilaii differs between these two crops. This hypothesis is dealt with in Chapter 3.

There was a significant interaction between P. bilaii inoculation and P fertilization with respect to DM yield. This interaction differed between 1988 and 1989. In 1988 the greatest increase in canola growth associated with P. bilaii was observed in the -MAP treatment. This is consistent with the hypothesis that P. bilaii increases the availability of soil P but does not provide additional benefit to plants supplied with sufficient P by fertilization. In contrast, in 1989 increased growth associated with P. bilaii addition was greatest in the latter part of the growing season on the +MAP treatment. This result is

consistent with the hypothesis that P. bilaii increases the availability of fertilizer P reaction products formed during the growing season. Kucey and Leggett (1989) reported an increase in seed yield of Westar canola with P. bilaii addition at this location, however, they too were unable to find a meaningful interaction with added MAP or rock phosphate.

Greater quantity of P in plant tissue is required to conclude that there was an increase in available P. Overall, we did not find that P quantity in canola was significantly influenced by P. bilaii addition; although, for canola harvested on September 12, 1989, from the + MAP treatments, there was a higher concentration of P in the inoculated plants, thus providing further evidence that increased availability of fertilizer P reaction products may have been the cause of the greater yield with P. bilaii inoculation. This is in contrast to the results of Kucey and Leggett (1989) who found, on a Black Chernozemic soil (pH 7.7), that the greatest difference in the quantity of P in Westar canola seed between +P. bilaii and -P. bilaii treatments occurred without added P. The reasons for the different results concerning Phosphorus X P. bilaii interactions under field conditions is not known. The source of additional P taken up following P. bilaii inoculation of wheat under greenhouse conditions (pH 8.1) has been shown, using added $^{32}P$, to be primarily from soil rather than from added rock phosphate (Kucey 1988).

Calcium- and Mg-phosphates are the predominant form of extractable P in neutral or alkaline soils whereas Al- and Fe-phosphates comprise a greater portion of the extractable P in some low pH soils of western Canada (Racz and Soper 1967; Alexander and Robertson 1968); the quantity of P in plants following inoculation with P. bilaii was greater with soils having a pH above 7 but was generally not greater in the present study which was on soil having pH 6.0. This is consistent with the observations that many organic acids are more effective in solubilizing Ca- than Fe- or Al-phosphates (Johnston 1959a; 1959b) and, in solution culture, Aspergillus and Penicillium isolates solubilized

more $Ca_3(PO_4)_2$ than $AlPO_4$ or $FePO_4$ (Banik and Dey 1982). Other soil and climatic properties may also account for differences among experiments, however, the form of phosphates present in the soils can not be ignored.

An unexpected result of the present research was the reduction in P concentration of barley associated with the +P. bilaii treatments. Fungi, like plants, require P for growth and acquire this nutrient from the same sources as do plants but in a more efficient manner (Beever and Burns 1982). Furthermore, phosphate-dissolving bacteria may utilize for biomass production all the additional P made available by solubilization (Hmeidan 1982) and competition for P between plants and microorgnaisms may occur (Barber and Loughman 1967). In the present study, P. bilaii growth in the rhizosphere of barley was extensive (Chapter 3). Hence, the possibility that P. bilaii immobilized P, and thus reduced its availability to barley, deserves investigation.

The effect of P. bilaii on other soil organisms and its potential for producing phytohormones should also be investigated: Penicillium oxalicum is antagonistic to some plant pathogens (Kommedahl and Windels 1978), and some phosphate-solubilizing organisms, including a Penicillium species, produce auxins and gibberellins (Satter and Gaur 1987).

Soil microorganisms can affect the availability of nutrients additional to P. In the present study, N and S were added and their concentrations in plant tissue were not measured. Because Ca, Mg, Fe, Cu, Zn and Mn are subject to chelation, their concentrations were measured to determine the effect of both MAP fertilization and P. bilaii inoculation on uptake. Determination of plant nutrient concentrations is an effective means of assessing nutrient uptake rates (Jones 1972). In this study, P fertilization was associated with greater Ca and Mg concentrations in barley and greater Mg and Mn concentrations in canola, and with a lower Cu concentration in barley and a lower Zn concentration in canola. The reduction in Zn and Cu availability associated with

phosphate fertilization has been widely reported and is known to vary among crops (Marschner 1986). In the present study zinc concentration in both crops was above the suggested deficient limit of 20 mg $kg^{-1}$; Cu concentrations were bordering on deficient with values below the suggested 4 mg $kg^{-1}$ (Jones 1972). There was evidence of reduced Cu uptake by barley fertilized with MAP, particularly in 1989 (Table 2.5).

Penicillium bilaii inoculation was associated with greater concentrations of Fe and Mn in canola. Previous research on micronutrient uptake by wheat under field conditions found that Zn, but not Fe or Cu, concentration in seed was greater following inoculation with P. bilaii (Kucey 1988). In the present study, special precautions against soil contamination, such as leaf washing, were not taken and, therefore, plant samples undoubtedly contained some soil. Contamination would have affected Fe concentrations to a much greater extent than other micronutrients because of its high concentration in soil (average 3.8% [Tisdale et al. 1985]) compared to in plants. Aluminum concentrations also would have been greatly affected. Despite this limitation there were significant differences found among treatments that are not attributable to variation in contamination. The increase in Mn concentration in plants grown on P. bilaii inoculated soil was small but is consistent with the observation that P. bilaii reduces solution pH: because Mn solubility increases markedly with decreasing pH below 6.0, a reduction in rhizosphere pH would be expected to increase Mn availability. Iron concentration was significantly greater with P. bilaii inoculation. This is consistent with the findings of Kucey (1988) that P. bilaii is able to solubilize $Fe_3O_4$ in solution culture and also with many studies documenting plant uptake of Fe from siderophores (Page 1988). It has not been reported whether P. bilaii produces siderophores, however, this possibility is worthy of investigation.

Comparing barley and canola, it is not known why the concentration of different cations were affected by inoculation. Again, methods of nutrient acquisition and nutrient

requirements of the two crops differ which may have had an effect on ion uptake. The reason that, following P. bilaii inoculation, Fe concentration was greater in canola but was not greater in barley (Table 2.5) or in wheat (Kucey 1988) may be related to Fe chelation: grasses have an iron stress response that includes phytosiderophore production (Bienfait 1988), hence additional Fe chelates associated with P. bilaii may not increase Fe uptake.

## 2.5 Conclusion

Conclusions pertaining to field grown barley and canola on an acidic Chernozemic soil that follow from these results are:

1. P. bilaii was associated with greater dry matter and seed yield of canola but not of barley,
2. there was little evidence to support the hypothesis that P. bilaii increased canola yield through increased P availability, and;
3. P. bilaii inoculation was associated with greater Fe and Mn concentrations in canola.

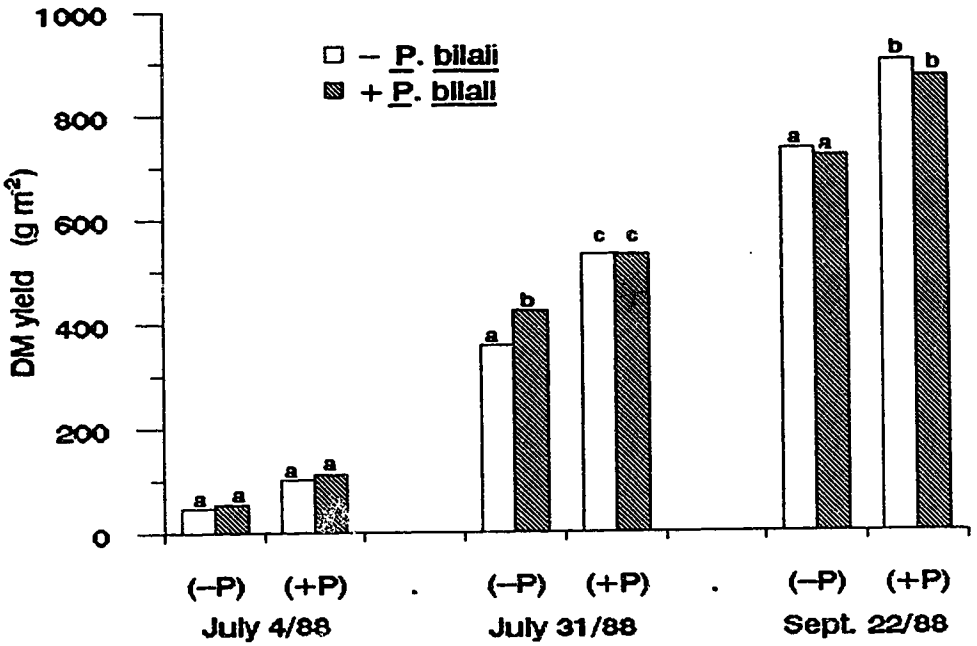


Figure 2.1. Dry matter yield of barley (planted June 1, 1988) at three dates with and without seed-row applied MAP (+P; –P), and with and without added P. bilaii. Bars having the same letter are not significantly different for a given date (P<0.05).

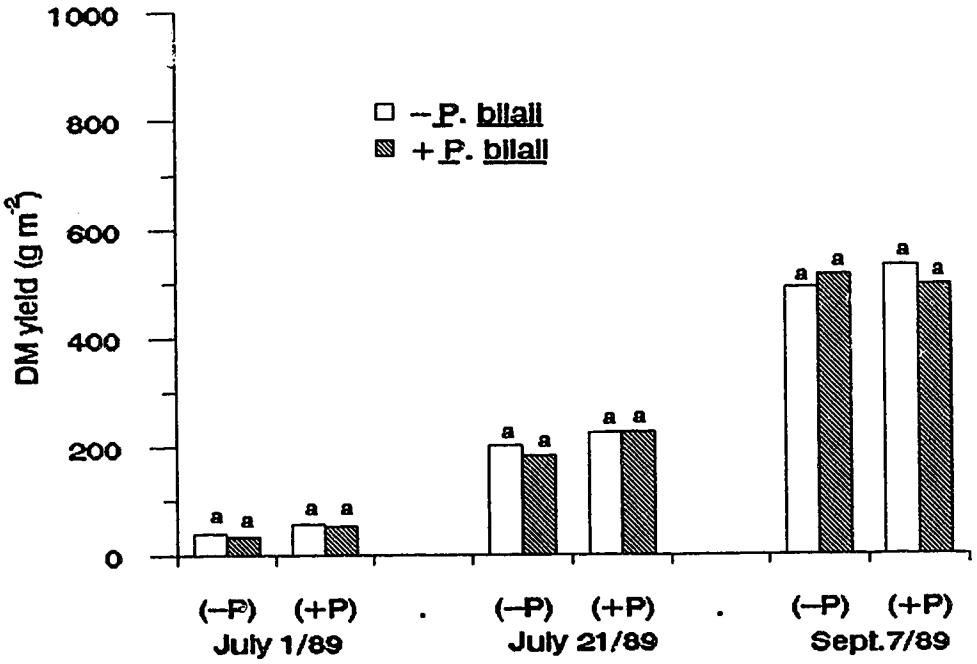


Figure 2.2. Dry matter yield of barley (planted May 18, 1989) at three dates with and without seed-row applied MAP (+P; –P), and with and without added P. bilaii. Bars having the same letter are not significantly different for a given date (P<0.05).

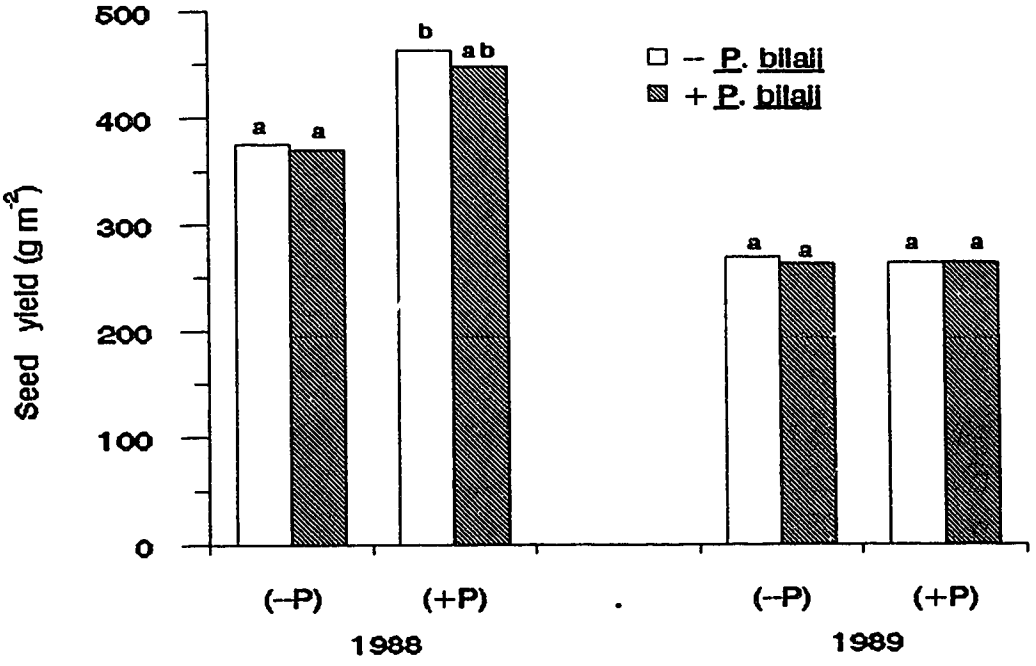


Figure 2.3. Seed yield of barley in 1988 and 1989, with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given year ($P<0.05$).

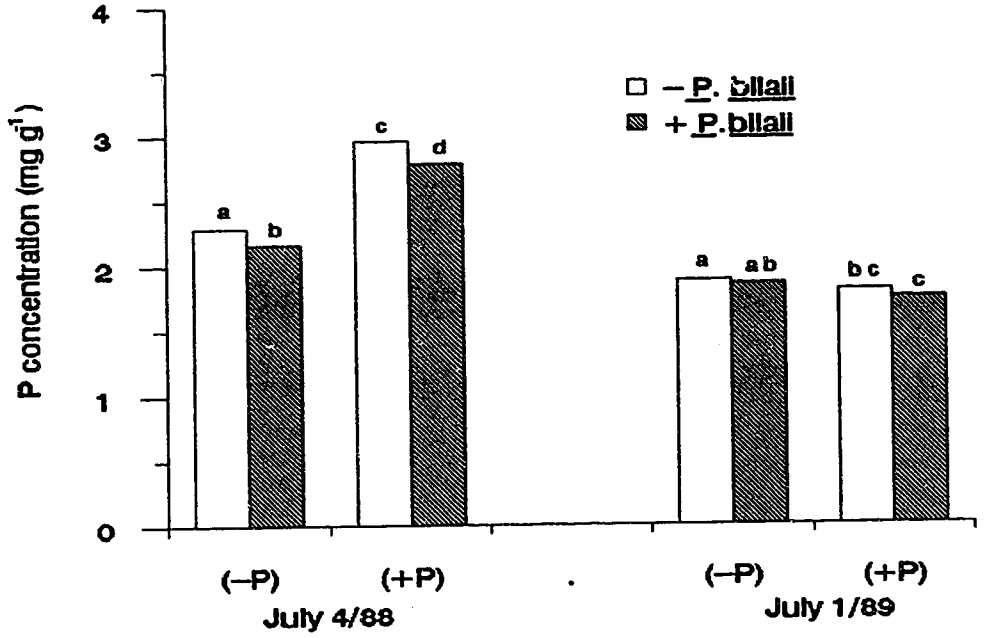


Figure 2.4. P. concentration in barley shoots prior to heading in 1988 and 1989, with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given year ($P<0.05$).

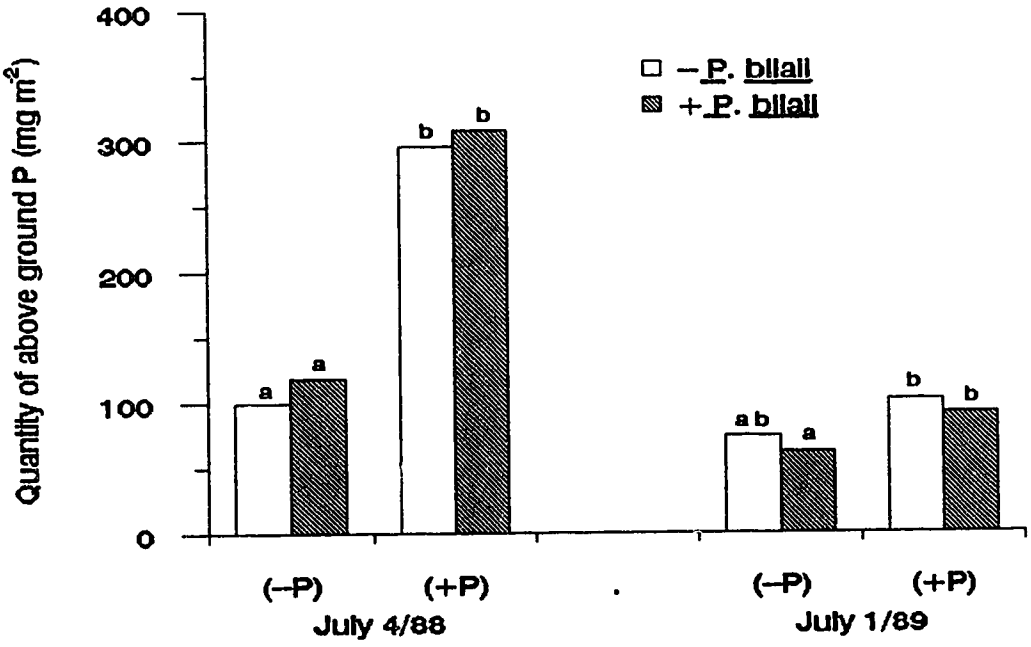


Figure 2.5. Quantity of P in barley prior to heading in 1988 and 1989, with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given year ($P<0.05$).

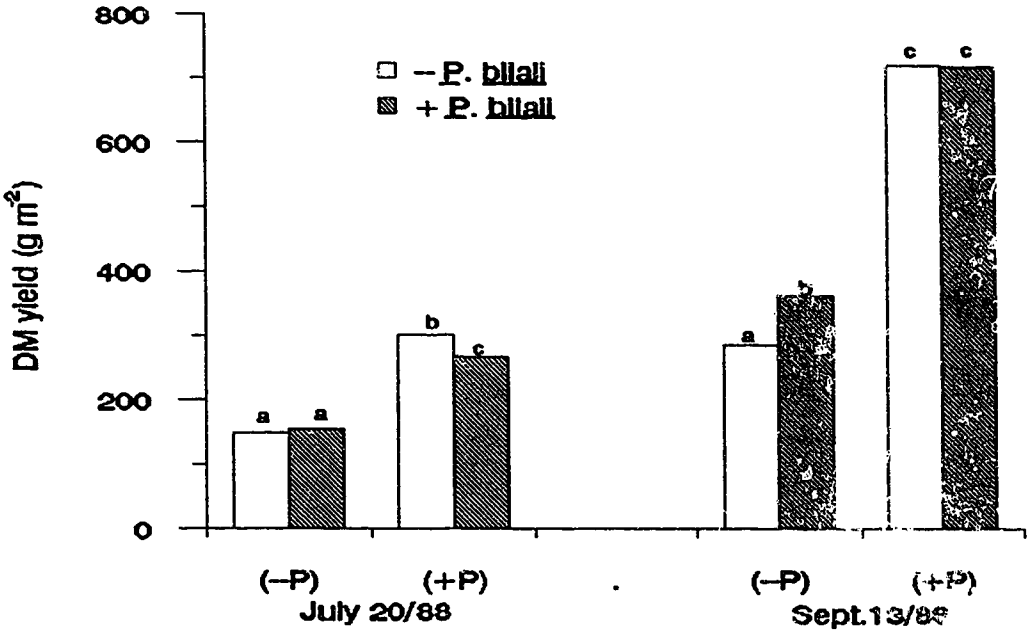


Figure 2.6. Dry matter yield of canola (planted June 1, 1988) at two dates with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given date ($p<0.05$).

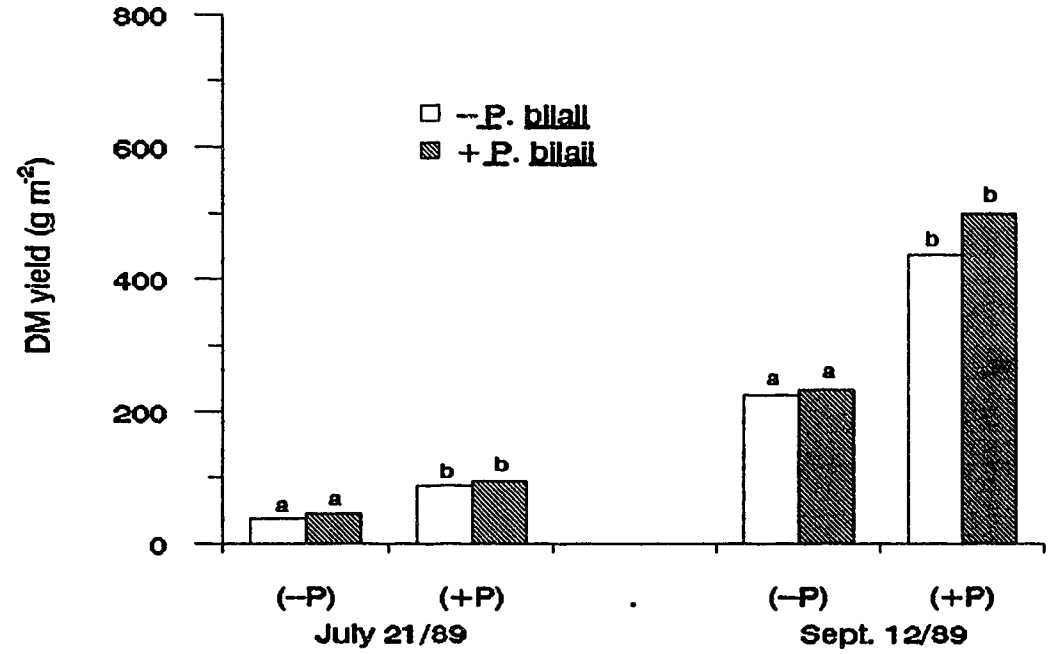


Figure 2.7. Dry matter yield of canola (planted June 19, 1989) at two dates, with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given date ($P<0.05$).

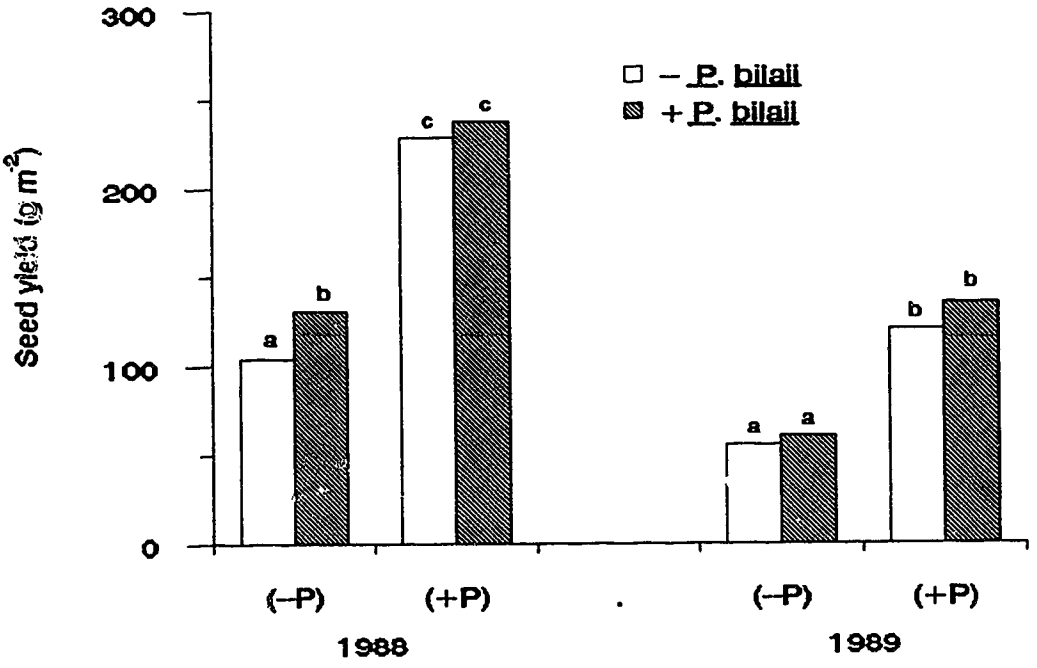


Figure 2.8. Seed yield of canola in 1988 and 1989, with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given year ($P < 0.05$).

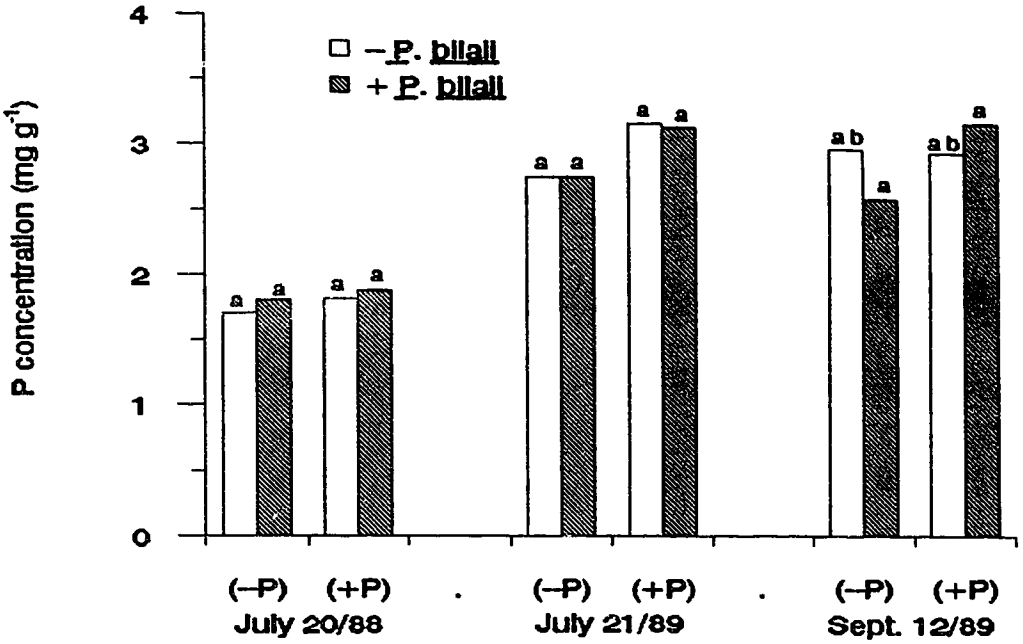


Figure 2.9. P concentration in the shoots of canola of varying ages grown with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given date (P<0.05).

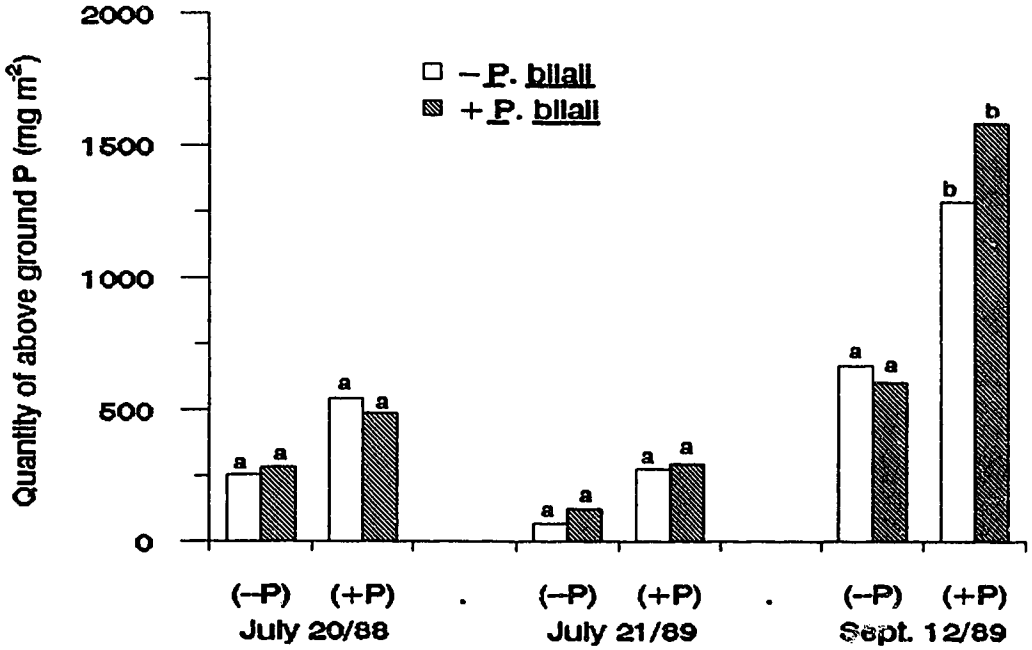


Fig. 2.10. Quantity of P in canola in 1988 and 1989, with or without seed-row applied MAP (+P; –P) and with or without added P. bilaii. Bars having the same letter are not significantly different for a given year (P<0.05).

Table 2.1 Analysis of variance for barley DM yield, P concentration and P quantity data (1988 and 1989).

| Source[1] | df | DM yield[a] | P conc.[b] | P quantity[b] |
|---|---|---|---|---|
| | | ---------- P* ---------- | | |
| Phosphorus (A) | 2 | 0.022 | 0.001 | 0.001 |
| P. bilaii (B) | 2 | 0.754 | 0.001 | 0.181 |
| A X B | 2 | 0.777 | 0.365 | 0.931 |

[a]Three dates per year. [b]One date per year.
[1]All sources listed were analyzed within year.
*Probability of no difference.

Table 2.2 Barley height, LAI, and number of tillers for plants grown with or without MAP and with or without added P. bilaii in 1988 and 1989.

| | MAP | | | |
|---|---|---|---|---|
| | ( - ) | | ( + ) | |
| | P. bilaii | | P. bilaii | |
| | ( - ) | ( + ) | ( - ) | ( + ) |
| **Height** | ---------- (mm) ---------- | | | |
| 1988 | | | | |
| July 4 | 217[a] | 233[a] | 315[b] | 314[b] |
| July 31 | 448[a] | 475[a] | 604[b] | 622[b] |
| Sept.22 | 702[a] | 702[a] | 717[a] | 719[a] |
| 1989 | | | | |
| July 1 | 195[a] | 189[a] | 234[b] | 231[b] |
| July 21 | 440[ab] | 431[a] | 451[ab] | 468[b] |
| Sept. 7 | 493[a] | 504[a] | 486[ab] | 457[b] |
| **LAI** | ---------- $cm^2$ $cm^{-2}$ ---------- | | | |
| 1988 | | | | |
| July 4 | 0.72[a] | 0.96[a] | 1.67[a] | 1.59[a] |
| July 31 | 1.81[a] | 1.72[a] | 2.05[a] | 2.30[a] |
| 1989 | | | | |
| July 1 | 0.51[a] | 0.46[a] | 0.65[a] | 0.61[a] |
| **Tillers** | ---------- tillers $plant^{-1}$ ---------- | | | |
| 1988 | | | | |
| July 4 | 1.57[a] | 2.00[a] | 3.00[b] | 2.90[b] |
| July 31 | 1.73[a] | 2.05[a] | 2.68[b] | 3.18[c] |
| Sept.22 | 2.15[a] | 2.13[a] | 2.68[b] | 2.70[b] |
| 1989 | | | | |
| July 1 | 0.03[a] | 0.03[a] | 0.25[a] | 0.38[a] |
| July 21 | 1.53[a] | 1.43[a] | 1.46[a] | 1.63[a] |
| Sept. 7 | 0.60[a] | 1.00[ab] | 1.30[b] | 1.20[b] |

Analysis of variance

| Source[1] | df | Height | LAI | Tillers |
|---|---|---|---|---|
| | | ---------- p* ---------- | | |
| Phosphorus (A) | 2 | 0.001 | 0.007 | 0.007 |
| P. bilaii (B) | 2 | 0.204 | 0.552 | 0.949 |
| A X B | 2 | 0.694 | 0.942 | 0.931 |

Within rows, values followed by the same letter are not significantly different (P<0.05).
[1]All sources listed were analyzed within year.
*P=probability of no difference.

Table 2.3 Seed yield components for barley grown with or without MAP and with or without added P. bilaii in 1988 and 1989.

| Date | MAP ( - ) P. bilaii ( - ) | MAP ( - ) P. bilaii ( + ) | MAP ( + ) P. bilaii ( - ) | MAP ( + ) P. bilaii ( + ) |
|---|---|---|---|---|
| 1988 | | | | |
| Plants $m^{-1}$ $row^{-1}$ | 166[a] | 172[a] | 135[b] | 140[b] |
| Heads $plant^{-1}$ | 2.3[a] | 2.2[a] | 2.9[ab] | 3.3[b] |
| 1000 seed mass(g) | 31.5 | 31.5 | 30.4 | 30.3 |
| Seed yield(g $m^{-2}$) | 375[a] | 369[a] | 463[b] | 447[ab] |
| 1989 | | | | |
| Plants $m^{-1}$ $row^{-1}$ | 164[a] | 158[a] | 167[a] | 165[a] |
| Heads $plant^{-1}$ | 1.5[a] | 1.9[a] | 2.1[a] | 2.0[a] |
| 1000 seed mass(g) | 37.6 | 35.7 | 36.0 | 36.9 |
| Seed yield(g $m^{-2}$) | 268[a] | 263[a] | 264[a] | 264[a] |

Analysis of variance

| Source[1] | df | Plants $m^{-1}$ $row^{-1}$ | Heads $plant^{-1}$ | 1000 seed mass | Seed yiels |
|---|---|---|---|---|---|
| | | p* | | | |
| Phosphorus (A) | 2 | 0.005 | 0.040 | 0.074 | 0.035 |
| P. bilaii (B) | 2 | 0.554 | 0.746 | 0.541 | 0.904 |
| A X B | 2 | 0.923 | 0.439 | 0.038 | 0.971 |

Within rows, values followed by the same letter are not significantly different (P<0.05).
[1]All sources listed were analyzed within year.
*Probability of no difference.

Table 2.4 Barley heading at selected dates with or without MAP and with or without added P. bilaii.

| Date | MAP ( - ) P. bilaii ( - ) | MAP ( - ) P. bilaii ( + ) | MAP ( + ) P. bilaii ( - ) | MAP ( + ) P. bilaii ( + ) |
|---|---|---|---|---|
| | ---------- heads $m^{-1}$ $row^{-1}$ ------- | | | |
| July 25, 1988 | 16 | 14 | 40 | 45 |
| July 14, 1989 | 10 | 6 | 157 | 161 |

n=4 blocks; counts of 6 m of row per block.

Table 2.5 Concentrations of Ca, Mg, Fe, Cu, Zn, Mn, and Al in barley shoots prior to heading in 1988 and 1989.

| Element | MAP ( - ) P. bilaii ( - ) | MAP ( - ) P. bilaii ( + ) | MAP ( + ) P. bilaii ( - ) | MAP ( + ) P. bilaii ( + ) |
|---|---|---|---|---|
| | % | | | |
| Ca | | | | |
| 1988[1] | 0.964[a] | 0.932[a] | 1.400[b] | 1.335[c] |
| 1989[2] | 0.737[a] | 0.786[a] | 0.822[b] | 0.875[b] |
| Mg | | | | |
| 1988[1] | 0.229[a] | 0.218[a] | 0.316[b] | 0.299[c] |
| 1989[2] | 0.236[a] | 0.252[b] | 0.262[b] | 0.282[c] |
| | $\mu g^{-1}$ | | | |
| Fe | | | | |
| 1988[1] | 1232 | 1333 | 1188 | 1078 |
| 1989[2] | 650 | 503 | 389 | 592 |
| Cu | | | | |
| 1988 | 8.28[a] | 8.30[a] | 5.30[b] | 5.75[b] |
| 1989 | 5.05[a] | 4.80[a] | 3.38[b] | 3.53[b] |
| Zn | | | | |
| 1988 | 38.3 | 40.3 | 37.6 | 37.8 |
| 1989 | 37.3 | 37.6 | 37.9 | 40.0 |
| Mn | | | | |
| 1988 | 48.5 | 50.7 | 50.5 | 52.5 |
| 1989 | 36.2 | 33.4 | 32.2 | 33.1 |
| Al | | | | |
| 1988 | 1653 | 1863 | 1236 | 1397 |
| 1989 | 699 | 527 | 375 | 441 |

Analysis of variance

| Source | df | Ca | Mg | Fe | Cu | Zn | Mn | Al |
|---|---|---|---|---|---|---|---|---|
| | | p* | | | | | | |
| A[1] | 2 | 0.001 | 0.001 | 0.215 | 0.001 | 0.284 | 0.520 | 0.145 |
| B[2] | 2 | 0.028 | 0.004 | 0.947 | 0.462 | 0.534 | 0.659 | 0.672 |
| A X B | 2 | 0.319 | 0.629 | 0.137 | 0.346 | 0.668 | 0.744 | 0.850 |

Values followed by the same letter are not significantly different (P<0.05).
[1]Phosphorus within year. [2]P. bilaii within year.
*Probability of no difference.

Table 2.6 Analysis of variance for canola dry matter yield, P concentration, and quantity of P (1988 and 1989).

| Source[1] | df | DM yield[a] | P concentration[b] | P quantity[b] |
|---|---|---|---|---|
| | | ---------- P* ---------- | | |
| Phosphorus (A) | 2 | 0.001 | 0.046 | 0.001 |
| P. bilaii (B) | 2 | 0.048 | 0.798 | 0.231 |
| A X B | 2 | 0.031 | 0.446 | 0.172 |

[a]Two dates per year. [b]One date in 1988; two dates 1989.
[1]All sources listed were analyzed within year.
*Probability of no difference.

Table 2.7 Canola height, LAI, and number of branches for plants grown with or without MAP and with or without added P. bilaii in 1988 and 1989.

| | MAP | | | |
|---|---|---|---|---|
| | ( - ) | | ( + ) | |
| | P. bilaii | | P. bilaii | |
| | ( - ) | ( + ) | ( - ) | ( + ) |
| Height | ---------- mm ---------- | | | |
| 1988 | | | | |
| July 20 | 554 [a] | 635[a] | 813[b] | 766[b] |
| Sept 13 | 689[a] | 733[a] | 893[b] | 875[b] |
| 1989 | | | | |
| July 21 | 188[a] | 194[a] | 307[b] | 271[ab] |
| Sept 12 | 592[a] | 554[a] | 692[b] | 651[b] |
| LAI | ---------- $cm^2$ $cm^{-2}$ ---------- | | | |
| 1988 | | | | |
| July 20 | 7.17[nd] | 8.79[nd] | 19.64[nd] | 17.71[nd] |
| 1989 | | | | |
| July 21 | 6.28[nd] | 7.72[nd] | 14.74[nd] | 14.20[nd] |
| Branches | ---------- branches $plant^{-1}$ ---------- | | | |
| 1988 | | | | |
| July 20 | 2.55[a] | 3.60[b] | 4.50[a] | 4.38[c] |
| Sept 13 | 3.25[a] | 3.38[a] | 4.10[b] | 3.94[b] |
| 1989 | | | | |
| July 21 | 2.47[a] | 2.42[a] | 4.28[b] | 3.85[b] |
| Sept 12 | 2.63[a] | 3.10[a] | 3.19[a] | 2.95[a] |

Analysis of variance

| Source | df | Height | LAI | Tillers |
|---|---|---|---|---|
| | | ---------- P* ---------- | | |
| Phosphorus (A) | 2 | 0.001 | 0.001 | 0.002 |
| P. bilaii (B) | 2 | 0.252 | 0.698 | 0.427 |
| A X B | 2 | 0.094 | 0.173 | 0.213 |

Within rows, values followed by the same letter are not significantly different (P<0.05). [nd]Not determined.
[1]All sources listed were analyzed within year.
*Probability of no difference.

Table 2.8 Seed yield components for canola grown with or without MAP and with or without P. bilaii inoculation in 1988 and 1989.

| Date | MAP ( - ) P. bilaii ( - ) | MAP ( - ) P. bilaii ( + ) | MAP ( + ) P. bilaii ( - ) | MAP ( + ) P. bilaii ( + ) |
|---|---|---|---|---|
| 1988 | | | | |
| Plants $m^{-1}$ $row^{-1}$ | $177^{a}$ | $113^{b}$ | $104^{b}$ | $95^{b}$ |
| Pods $plant^{-1}$ | $76^{a}$ | $89^{a}$ | $111^{b}$ | $94^{ab}$ |
| 1000 seed mass(g) | $2.5^{a}$ | $2.6^{a}$ | $2.7^{a}$ | $2.6^{a}$ |
| Seed yield(g $m^{-2}$) | $104^{a}$ | $131^{b}$ | $229^{c}$ | $237^{c}$ |
| 1989 | | | | |
| Plants $m^{-1}$ $row^{-1}$ | $154^{a}$ | $152^{a}$ | $168^{a}$ | $182^{a}$ |
| Pods $plant^{-1}$ | $47^{a}$ | $47^{a}$ | $51^{a}$ | $45^{a}$ |
| 1000 seed mass(g) | $1.7^{a}$ | $1.7^{b}$ | $2.1^{b}$ | $1.9^{ab}$ |
| Seed yield(g $m^{-2}$) | $55^{a}$ | $60^{a}$ | $120^{b}$ | $135^{b}$ |

Analysis of variance

| Source[1] | df | Plants $m^{-1}$ $row^{-1}$ | Pods $plant^{-1}$ | Seed mass | Seed yield |
|---|---|---|---|---|---|
| | | P* | | | |
| Phosphorus (A) | 2 | 0.047 | 0.030 | 0.021 | 0.001 |
| P. bilaii (B) | 2 | 0.133 | 0.795 | 0.337 | 0.055 |
| A X B | 2 | 0.256 | 0.083 | 0.338 | 0.351 |

Within rows, values followed by the same letter are not significantly different ($P<0.05$).
[1]All sources listed were analyzed within year.
*Probability of no difference.

Table 2.9 Concentrations of Ca, Mg, Fe, Cu, Zn, Mn, and Al in canola shoots in 1988 and 1989.

| | MAP | | | |
|---|---|---|---|---|
| | ( - ) | | ( + ) | |
| | P. bilaii | | P. bilaii | |
| Element | ( - ) | ( + ) | ( - ) | ( + ) |
| | ---------- % ---------- | | | |
| Ca | | | | |
| 1988[1] | 2.173 | 2.277 | 2.190 | 2.274 |
| 1989[2] | 3.482 | 3.628 | 3.530 | 3.517 |
| 1989[3] | nd | nd | 1.405 | 1.493 |
| Mg | | | | |
| 1988[1] | 0.211[a] | 0.201[a] | 0.218[b] | 0.234[b] |
| 1989[2] | 0.369[a] | 0.359[a] | 0.387[b] | 0.385[b] |
| 1989[3] | nd | nd | 0.204 | 0.219 |
| | ---------- mg kg$^{-1}$ ---------- | | | |
| Fe | | | | |
| 1988[1] | 191[a] | 290[ab] | 252[a] | 365[b] |
| 1989[2] | 369[a] | 508[b] | 365[a] | 408[a] |
| 1989[3] | 96[a] | 64[a] | 75[a] | 94[a] |
| Cu | | | | |
| 1988[1] | 3.05 | 3.20 | 3.05 | 3.25 |
| 1989[2] | 4.38 | 4.35 | 4.00 | 4.03 |
| 1989[3] | 3.00 | 2.85 | 4.03 | 4.45 |
| Zn | | | | |
| 1988[1] | 36.0[a] | 34.8[a] | 27.0[a] | 27.5[a] |
| 1989[2] | 55.4[a] | 57.4[a] | 38.9[b] | 38.4[b] |
| 1989[3] | 25.0[a] | 23.5[a] | 27.8[a] | 31.6[a] |
| Mn | | | | |
| 1988[1] | 15.3[a] | 19.1[a] | 20.3[a] | 21.3[a] |
| 1989[2] | 25.4[a] | 28.2[a] | 26.2[a] | 26.5[a] |
| 1989[3] | 14.5[a] | 14.3[a] | 14.3[a] | 17.0[a] |
| Al | | | | |
| 1988[1] | 195 | 328 | 272 | 294 |
| 1989[2] | 408 | 558 | 396 | 448 |
| 1989[3] | nd | nd | 65 | 82 |

(continued on next page)

Table 2.9 (continued)

Analysis of variance

| Source | df | Ca | Mg | Fe | Cu | Zn | Mn | Al |
|---|---|---|---|---|---|---|---|---|
| | | P* | | | | | | |
| A[4] | 2 | 0.933 | 0.046 | 0.037 | 0.209 | 0.007 | 0.010 | 0.566 |
| B[5] | 2 | 0.262 | 0.835 | 0.004 | 0.842 | 0.852 | 0.023 | 0.175 |
| A X B | 2 | 0.666 | 0.385 | 0.740 | 0.809 | 0.859 | 0.268 | 0.488 |

Values followed by the same letter are not significantly different (P<0.05).
[1]Harvested July 4, 1988 (early pod filling).
[2]Harvested July 1, 1989 (flowering).
[3]Harvested Sept. 12, 1989 (late pod filling).
*Probability of no difference.
[4]Phosphorus within year.
[5]P. bilaii within year.

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# CHAPTER 3 GROWTH, SURVIVAL AND RHIZOSPHERE COMPETENCE OF P. BILAII UNDER FIELD CONDITIONS[1]

## 3.1 Introduction

It has long been known that the rhizosphere is more biologically active than bulk soil (Hiltner 1904, as cited in Rovira 1956) and it has been established that this activity may benefit plants (Rovira and Davey 1974). Bacteria capable of solubilizing sparingly soluble phosphates in solution culture have been isolated from the rhizosphere of wheat, corn, barley, ryegrass and red clover (Katznelson et al. 1963) and inoculation of beans (Vicea faba) with Bacillus megatherium var. phosphaticum has been associated with a greater quantity of plant P and higher yield (Khalafallah et al. 1982). Fungi with phosphate solubilizing ability have been isolated from the rhizosphere of wheat, barley and red clover (Katznelson et al. 1963), and from legume nodules (Chhonkar and Subba-Rao 1965). Aspergillus and Penicillium isolates are the most effective in solubilizing phosphates in solution culture (Katznelson et al. 1962; Chhonkar and Subba-Rao 1965; Agnihotri 1970; Khan and Phatnagar 1977; Kucey 1983; Thomas et al. 1985). Recently, inoculation with the phosphate-solubilizing fungus P. bilaii has been associated with greater wheat (Kucey 1987; Asea et al. 1988) and canola (Kucey and Leggett 1989) P quantity and dry matter yield under field conditions on alkaline soils. In research related to the present study, P. bilaii was associated with an increase in canola dry matter yield, although P quantity was not affected; dry matter yield of barley was not affected (Chapter 2).

The extent to which this fungus influences crop growth and nutrient uptake is certain to depend on its survival in soils and its rhizosphere competence. The competitive saprophytic ability of some phosphate-solubilizing Aspergillus and Penicillium isolates has been assessed by determining the rate of colonization of straw in soil following inoculation (Thomas et al. 1985), and the rhizosphere competence of P. oxalicum has been documented

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[1]A version of this Chapter will be submitted for publication (D.O. Keyes and W.B. McGill; Can. J. Soil Sci.)

(Windels and Kommedahl 1982). The fate of P. bilaii following its introduction into a field soil has, however, not been reported. The present research was, therefore, undertaken to determine i) the survival time, ii) the rhizosphere effect, and iii) the extent of downward movement, of P. bilaii under field conditions.

## 3.2 Materials and Methods

### *3.2.1 Site and Soil Description*

As described in Chapter 2.

### *3.2.2 Inoculant*

Penicillium bilaii, the fungus contained in the inoculant, belongs to the P. adametzii series. It has a monverticulate penicillus and produces conidia 2.5-3.0 $\mu$m in diameter. Penicillium bilaii grown on PDA with precipitated Ca-P (Katznelson and Bose 1959) at 22° C produces 2 colony types. One, designated type 'b', is initially uncolored (up to 4 days) then forms a dark green basal felt of sporulating hyphae from which ropes of aerial hyphae ascend to produce a funiculose textured colony (7 days). Colony margins consist of white non-sporulating hyphae. The colony reverse and surrounding media turns golden brown between 4 and 7 days. Odor is lacking. After 7 days, a clear zone is produced around each colony that extends out from the colony about one half the colony diameter. This colony description is similar to that of isolates of P. terlikowskii Zaleski (Raper and Thom 1949), P. bilaii Chalabuda (Kulik 1968), and P. bilaii Chalabuda (Pitt 1979), all of which Pitt (1979) considers synonomous with P. bilaii.

The other colony type, designated type 'a', grows at a similar rate but differs from type 'b' in many respects: colonies are light green, appressed and have a velvety texture; the colony reverse is not colored, and; clearing of the media is limited to a narrow zone or is lacking, after 7 days. The relationship of these two phenotypes is the subject of Chapter 4.

The wheat bran inoculant was prepared using a constant protocol but contained different types and amount of P. bilaii each year. The inoculant used in 1988, provided by Philom Bios Inc., contained P. bilaii type 'a' at about $10^9$ CFU $g^{-1}$; the inoculant used in

1989 was prepared from a P. bilaii culture, provided by Philom Bios Inc., and contained P. bilaii type 'b' at about $10^7$ CFU $g^{-1}$. The inoculant was prepared by plating P. bilaii onto PDA agar. A lawn of sporulating fungi developed within 7 days at 22°C and was brushed from the surface of the agar into a pan containing 250 g of double autoclaved wheat bran moistened with an equal mass of sterilized $H_2O$. The bran was stirred daily to maintain uniform moisture and to distribute the fungus throughout. After 7 days incubation at 22°C the bran, containing fungal mycelium and spores, had air dried and was ready for use. The inoculant used each year was plated to determine the density of P. bilaii CFU and to check for fungal contaminants.

*3.2.3 Experimental: Seed-row Inoculation (1988)*

Samples were obtained from the field experiment described in Chapter 2. Random samples were taken from one block of each crop and from three of the four treatments within each block: inoculated (--MAP and +MAP), and noninoculated (--MAP). These treatments were imposed on May 18, 1988, using a double disk seeder (15 cm row spacing) equipped with separate delivery systems for phosphate fertilizer, inoculant, and seed. The furrow depth was 5 cm for barley and 3 cm for canola. Further agronomic details are provided in Chapter 2.

The dates of sampling were July 4 and July 31 for barley, and July 20 and September 13 for canola. At each date 4 soil cores (10 cm diameter) were taken from mid-row positions within each plot. For each crop, at the earlier date samples were taken from a depth of 2-10 cm, and at the later dates samples were taken from the 2-10 cm and 12-20 cm depths. Roots with adhering soil were manually removed from the samples to obtain rhizosphere soil in which fungi were enumerated. Enumerations were also performed on parallel bulk soil samples from the inoculated, --MAP treatments. On October 15, 1988, the plots were rototilled to a depth of 14 cm and on May 9, 1989, fungi in bulk soil samples (0-10 cm depth) were enumerated.

*3.2.4 Experimental: Uniform Soil Inoculation (1989)*

For this experiment we used small plots (30 cm X 100 cm) and inoculated the soil uniformly. Soil was excavated to a depth of 5 cm for six replicate plots and 5 g of inoculant was thoroughly mixed with the soil from each plot. The soil was replaced in 1 cm increments, packing it lightly between additions, and finally, the inoculated and adjacent area was planted to canola by hand (15 cm row spacing; 2 cm between seeds within rows).

Fungi from the inoculated plots were enumerated immediately following inoculation (June 21, 1989), six weeks later (July 29, 1989), and again the following spring (May 15, 1990). The July 29, 1989, samples were taken from the 2-4 cm, 6-8 cm, and 10-12 cm depths by excavating a pit beside each plot and using a blade to slice horizontally into the bank to remove successive 2 cm depth increments. The area sampled extended 5 cm either side of a planted row for a distance of 50 cm; hence each sample consisted of 1000 $cm^3$ of soil. Subsamples of bulk and rhizosphere soil were removed from each sample. The May 15, 1990, samples (bulk soil samples; 2-4 cm depth) were taken from within the plots and from the noninoculated areas between plots.

*3.2.5 Enumeration of Fungi and Identification of P. bilaii*

Standard plate count methods were used to enumerate fungal CFU in rhizosphere and bulk soil samples and in the inoculants. Samples were placed in bottles containing 100 ml of sterilized 0.1% agar solution and shaken for 45 min. at 200 rpm on a rotary shaker. For each sample ten fold dilutions ($10^{-1}$ to $10^{-4}$) were prepared using the same agar solution. The PDA medium used contained 30 mg $L^{-1}$ streptomycin to inhibit bacterial growth, and precipitated calcium phosphate (Katznelson and Bose 1959) for detecting the phosphate-solubilizing ability of fungal colonies. Usually the $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions were plated (5 replicate plates; 0.1 ml $plate^{-1}$). Colonies of P. bilaii and other fungi were counted after 4-5 days at 22° C and identification of P. bilaii colonies was confirmed after 6-7 days by comparing them to colonies produced by the inoculant.

*3.2.6 Statistical Analyses*

The data for CFU were transformed ($\log_{10}$) and tested for normality by calculating the Shapiro and Wilk (1965) W statistic using the SAS (SAS Institute Inc. 1986) Univariate procedure. Those data sets without significant ($P<0.05$) deviation from normality were subjected to ANOVA using the SAS GLM procedure. The probabilities of differences between means were calculated using the PDIFF option of the SAS LSMEANS procedure.

**3.3 Results**

*3.3.1 Seed-row Inoculation (1988)*

Penicillium bilaii was enumerated to determine its survival and movement following seed-row inoculation; a simple method of inoculating field crops. P. bilaii was recovered from rhizosphere and bulk soil of inoculated barley and canola plots during the growing season, and from bulk soil the following spring. The rhizosphere effect for P. bilaii was similar for both crops and averaged 8.5 (Table 3.1). P. bilaii was detected below 10 cm in only 3 out of 16 samples from inoculated plots, indicating limited downward movement (Table 3.2). It was not recovered from any noninoculated plots.

The population density of P. bilaii declined during the growing season (Fig 3.1), however, the number of CFU recovered depended on crop and phosphate fertilization. Compared to canola, barley had a greater density and a higher proportion of P. bilaii in its rhizosphere (Table 3.2). Monoammonium phosphate was associated with more P. bilaii CFU at the earlier sampling dates, but with fewer at later sampling dates. Hence, the decline from one date to the next was only significant for the -MAP treatments (Fig 3.1).

The survival of P. bilaii over the longer term, May 18, 1988 to May 9, 1989, averaged 20.7%. This value was calculated from the soil bulk density (0.90 Mg $m^{-3}$), and by assuming that P. bilaii propagules were evenly distributed in the upper 14 cm of soil by cultivation in the fall of 1988, and remained so until spring sampling. Phosphate fertilization did not significantly affect P. bilaii survival but survival tended to be greater in plots cropped to barley than in those cropped to canola (Table 3.3).

Native soil fungi were also enumerated during the course of this experiment but no attempt was made to characterize them further. Their density was greater above than below the 10 cm depth (Table 3.4), was greater in the rhizosphere than in bulk soil (Table 3.5), and was greater earlier in the growing season (Table 3.4). No difference was found between crops. Neither phosphate fertilization nor inoculation with P. bilaii affected the population of native fungi in rhizosphere soil during the growing season; the trend toward greater numbers of CFU of native fungi in inoculated than in noninoculated plots the following spring was not significant (Table 3.6).

*3.3.2 Uniform Soil Inoculation (1989)*

The purpose of this approach was to minimize P. bilaii CFU density variation within inoculated plots so that the CFU counts correspond to actual densities; variability in numbers of CFU $g^{-1}$ of soil was reduced by uniform soil inoculation compared to seed-row inoculation. The average rhizosphere effect within the upper 10 cm was 7.1 and, as in the preliminary experiment in 1988, there was little movement to below 10 cm (Fig. 3.2). P. bilaii was, however, found below 6 cm although its density was much less at this depth than within the 2-4 cm inoculation zone (Fig. 3.2). Despite the density decline, the rhizosphere effect did not change with depth (i.e. there was not a significant interaction between "depth" and "soil" effects (Table 3.7)).

The population density of P. bilaii increased by 23 fold in the 6 weeks following inoculation, in contrast to the decline that occurred with seed-row inoculation during a similar period. The initial P. bilaii density values, from which the population changes were calculated, are based on direct enumeration of soil fungi at the time of inoculation. The population density thus determined was 39% greater than that calculated from the theoretical inoculation rate. The density of P. bilaii in both rhizosphere and bulk soil by the latter part of July was approximately 10 fold greater than it had been in 1988, and 31% of the P. bilaii present in the inoculated soil layer on July 29 survived through the latter part of the growing season and overwinter (Fig. 3.3). Some P. bilaii CFU were recovered from

from interplot soil samples.

The recovery of P. bilaii type 'a' in 1989 was unexpected. Whereas in the previous experiment P. bilaii recovered from soil was almost exclusively type 'a', the same type that the inoculant contained; in this experiment both types 'a' and type 'b' were recovered, even though the inoculant used contained almost exclusively P. bilaii type 'b' (type'a'comprised less than 1% and was detected in only one plot initially [Table 3.7]). At subsequent sampling dates, however, approximately equal numbers of type 'a' and type 'b' CFU were recovered from all plots (Fig. 3.3). Furthermore, no difference was found in the distribution of the two types: both were recovered from rhizosphere soil and from soil below the inoculation zone (Table 3.7).

The native fungi enumerated, again exhibited a significant rhizosphere effect and a decline in density with depth but there was no interaction between these two variables (Table 3.7). Their population followed the same trend with time as P. bilaii: increasing from June to July of 1989, and then decreasing by May, 1990 (Table 3.7). Inoculation did not influence CFU counts of other fungi, including the native phosphate-solubilizing fungi which comprised about 2% of the native fungal CFU (Table 3.5).

### 3.4 Discussion

This study of the autecology of P. bilaii focussed on the rhizosphere effect, downward movement, and survival time of this organism. Penicillium spp. typically colonize new substrates rapidly, but their hyphae do not extend far into the surrounding soil; rather, these fungi invade new substrates via spore dispersal (Burges 1960).

Dispersal of P. bilaii propagules through the soil was, however, limited under the conditions of this study, as indicated by the limited extent of its downward movement. Penicillium bilaii recovered from the soil in both experiments was mostly from within the inoculated zones, and few CFU were recovered from below 10 cm. Penicillium bilaii, like all Penicillia, is a prolific spore producer (except in liquid culture) and in the 1989 experiment spores would have been abundant within the upper 5 cm of soil. However, despite heavy

rainfall during the experiment, there is no evidence that conidia were transmitted more than 10 cm down the soil profile. This lack of downward movement by water is consistent with the hydrophobic nature of the penicillia spore coat (Burges 1950). These small conidia are adapted for wind rather than water dispersal.

The significant rhizosphere effect for P. bilaii both years clearly indicates its affinity for plant roots. Although there are no reports of the nutrient requirements of P. bilaii specifically, Penicillium species generally are strong saprophytes capable of metabolizing many carbohydrates (Hall 1981) and are therefore, like most soil microorganisms, able to utilize root exudates. Furthermore, as roots grow and produce fresh exudates, this group of fungi rapidly colonizes the new substrate and, hence, is generally more concentrated in the rhizosphere than in bulk soil (Domsch et al. 1980; Parkinson et al. 1963).

Rovira (1956) established that root exudates are selective for particular microorganisms and Parkinson et al. (1963) found that Penicillium spp. were enriched in the rhizosphere of some plants. That the rhizosphere effect for P. bilaii was greater than for native fungi in general indicates that there was selection for this species. Parkinson et al. (1963) further demonstrated that the groups of fungi concentrated in the rhizosphere vary with plant species and root age. In the present study there was apparently greater selection for P. bilaii by barley. Although the age of the roots sampled in these experiments is not known, the rhizosphere effect tended to decline through the 1988 growing season (Table 3.1), perhaps indicating that P. bilaii has a greater affinity for younger roots. Despite being concentrated in the rhizosphere, there was only a slight indication that the downward movement of P. bilaii might be facilitated by roots. This result is consistent with the observation that fungal hyphae normally do not extend more than 1 mm along a root (Parkinson et al. 1963).

Changes in the propagule density of a fungus such as P. bilaii is due to growth and/or sporulation, and survival of its conidia. Our results clearly show that P. bilaii has a large capacity for growth within soil under field conditions. The two inoculation methods

and sampling procedures used here yielded different but complementary data on P. bilaii survival. In the case of the seed-row inoculation, P. bilaii density at the location of inoculation was greater than the density measured over a large volume of soil because the fungus was confined to a narrow band within the seed row. Hence, there would have been greater competition for accessible substrate and higher accumulations of inhibitory metabolites, such as $CO_2$, within the band. Such conditions can contribute to a lack of growth and an increase in senescence (Burges and Fenton 1953; Garraway and Evans 1984). When the inoculum was evenly distributed throughout the sampling zone, measured densities were similar to the volumetric densities. Under these conditions, substrate within the entire volume of inoculated soil would have been accessible, and competition for soil derived substrates correspondingly reduced.

In this study added phosphorus was associated with an increased density of P. bilaii in the long run. Sustained growth of saprophytic organisms requires a continuous supply of nutrients and a suitable environment. Carbohydrates contributed to soil by roots and crop residues provide a source of carbon and energy to soil organisms. In addition, fungi require several micro- and macro-nutrients including phosphorus (Garraway and Evans 1984). The relationship between phosphate addition and survival of P. bilaii may be either direct or indirect via an increase in plant root growth.

The main abiotic factors that affect fungal growth are moisture, temperature, pH, irradiation, aeration and $CO_2$ concentration (Garraway and Evans 1984). Biotic factors that may affect fungi include antagonism from other microorganisms (Parkinson 1981) and grazing by soil mites (Moore 1988). Further research is required to determine the relative importance of these factors on survival of P. bilaii in soil under field conditions.

We attempted to determine the effect of P. bilaii on other soil fungi because of concerns that a high inoculum density may disrupt soil ecosystems. The effect of adding P. bilaii on native soil fungi were always small and positive but frequently not statistically significant. In the first experiment, P. bilaii appeared to stimulate growth and/or sporulation

of native fungi. Although the cause of this increase is not known, it is consistent with a similar result, observed following inoculation with P. oxalicum (Windels and Kommedahl 1982). Further research is required to provide a comprehensive examination of the influence of P. bilaii inoculation on soil biota. Fungi enumerated by the method used in this study represent a select group, and fungi are only one of many groups of soil biota.

Penicillium bilaii may be expected to influence subsequent field crops if sufficient propagules survive, although no such effect has been reported. The minimum density of P. bilaii required to affect crop growth under field conditions is unknown, however, under greenhouse conditions a uniform density of $10^2$ CFU $cm^{-3}$ of P. bilaii was sufficient to increase canola growth (Chapter 5).

An unexpected result of the 1989 experiment was the recovery of P. bilaii type 'a'. It seems unlikely that the small amount of type 'a' in the inoculant would have grown to equal the amount of type 'b'. An alternative hypothesis is that the change to P. bilaii type 'a' occurred during growth within the soil. Penicillia growing on artificial media commonly undergo morphological changes (Raper and Thom 1949). We have observed sectoring within P. bilaii colonies growing on PDA that suggests a change from type 'a' to 'b'. Results of RFLP analysis concerning the relationship between type 'a' and type 'b' are presented in Chapter 4.

### 3.5 Conclusion

The results from these two field experiments lead to the conclusions that in soils such as the Malmo SiCL under central Alberta climatic conditions, P. bilaii

1. is capable of growth following inoculation into soil under field conditions and survives overwinter;
2. has a significant rhizosphere effect which is greater than that for native fungi in general;
3. in field soil does not move below 10 cm in any significant amount, and;
4. is recovered as two phenotypes following inoculation with one phenotype.

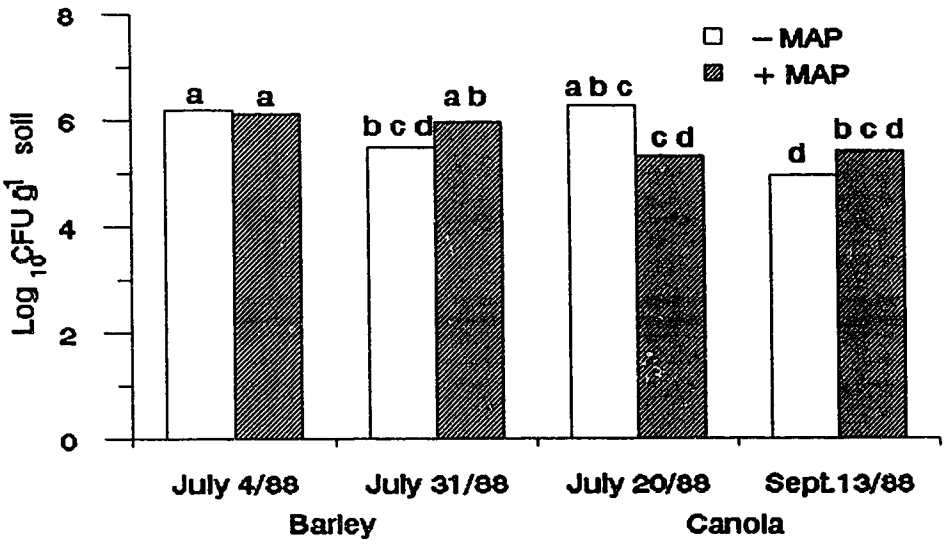


Figure 3.1. P. bilaii CFU density in the rhizosphere of barley and canola (2-10 cm depth) following seed-row inoculation on June1, 1988. Bars with the same letter are not significantly different ($P<0.05$).

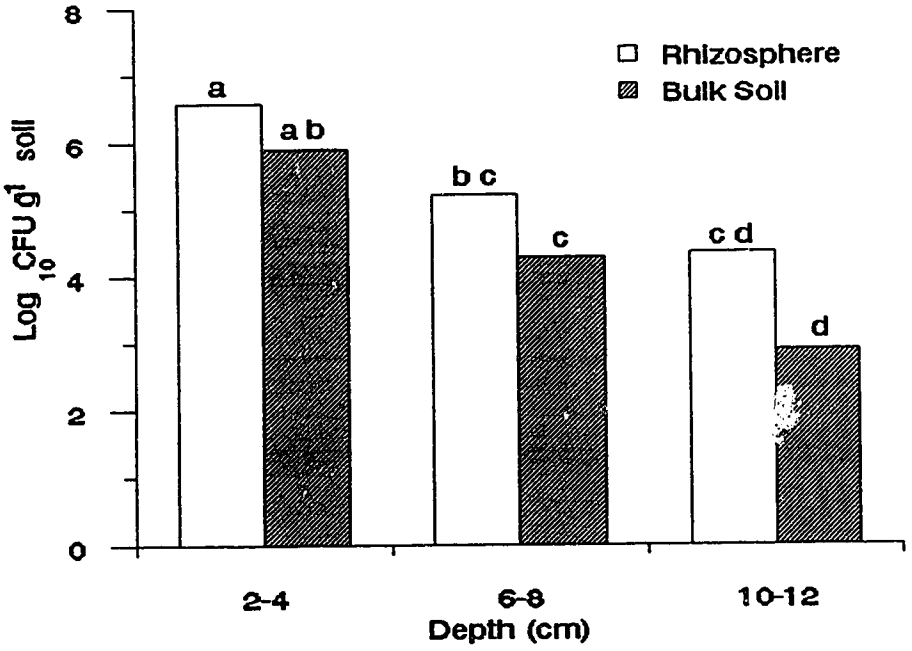


Figure 3.2. Depth distribution of P. bilaii in rhizosphere and bulk soil on July 29, 1989. Soil was uniformly inoculated to a depth of 5 cm on June 21, 1989. Bars having the same letter are not significantly different ($P<0.05$).

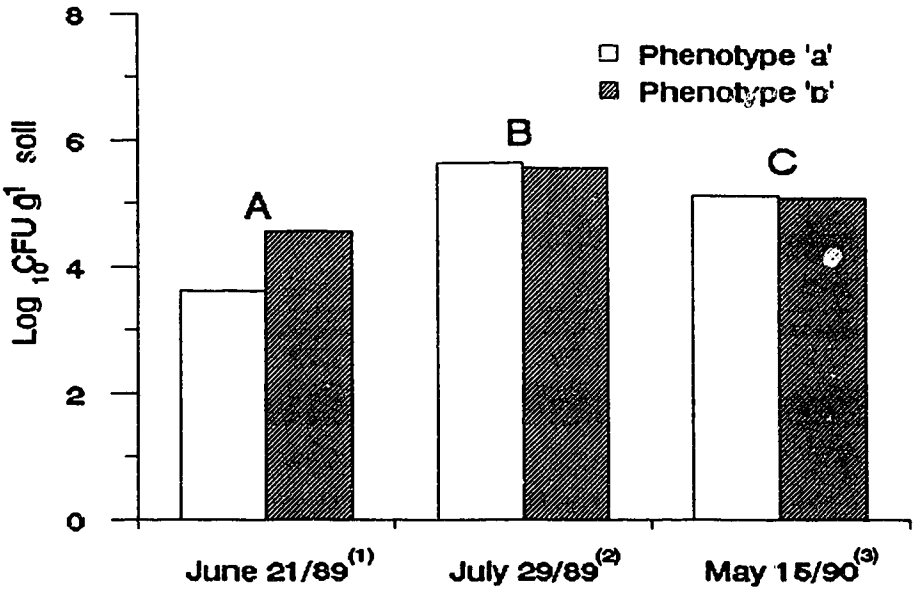


**Figure 3.3. Density (CFU) of two P. bilaii phenotypes in soil planted to canola (2-4 cm depth): at the time of inoculation (1), mid-way through the growing season (2), and the following spring (3). Dates having the same letter are not significantly different with respect to total P. bilaii density ($P<0.05$).**

Table 3.1 Penicillium bilaii rhizosphere effect and percent of total fungi within the 2-10 cm depth in seed-row inoculated barley and canola (1988).

| Crop (Date) | R:S[1] (P*) | Rhizo-sphere | Bulk Soil | Rhizo-sphere | Bulk Soil |
|---|---|---|---|---|---|
| | | - $\log_{10}$ CFU/g - | | ------ % ------ | |
| Barley (July 31) | 6.34 (0.013) | 5.486 | 4.684 | 62.9 | 34.4 |
| Canola (July 20) | 15.43 (0.043) | 6.268 | 5.079 | 63.8 | 45.1 |
| Canola (Sept. 13) | 3.81 (0.073) | 4.957 | 4.376 | 34.3 | 23.8 |

[1]Ratio of P. bilaii CFU in rhizosphere (R) versus bulk soil (S) *Probability of no difference between R and S. Overall Analysis for Canola: P<0.011 for R:S; P<0.007 for Date; P<0.836 for R:S X Date.

Table 3.2 Number of P. bilaii CFU recovered from the rhizosphere of seed-row inoculated barley and canola in 1988.

| Crop (Date) | Depth | | | |
|---|---|---|---|---|
| | 2-10 cm | | 12-20 cm | |
| | -MAP | +MAP | -MAP | +MAP |
| | ---------- $\log_{10}$ CFU $g^{-1}$ ---------- | | | |
| Barley (July 4) | 6.188 | 6.119 | | |
| Barley (July 31) | 5.485 | 5.964 | n/d | 4.000[1] |
| Canola (July 20) | 6.268 | 5.319 | | |
| Canola (Sept 13) | 4.955 | 5.415 | 3.301[1] | n/d |

Analysis of Variance for 2-10 cm depth

| Source | df | P* |
|---|---|---|
| Crop | 1 | 0.002 |
| Phosphorus within Crop | 2 | 0.498 |
| Date within Crop | 2 | 0.086 |
| Date X Phosphorus | 2 | 0.031 |

n/d none detected.
[1]Detected in one plot.
*Probability of no difference.

e 3.3 *Penicillium bilaii* CFU recovered from bulk soil on ), 1989, one year after seed-row inoculation of barley and canola, with or without added MAP.

| Crop | Treatment -MAP | Treatment +MAP | Mean |
|---|---|---|---|
| | ------- $\log_{10}$ CFU $g^{-1}$ -------- | | |
| Barley | 4.031 | 4.040 | 4.036 |
| Canola | 3.884 | 3.748 | 3.822 |

Analysis of Variance

| Source | df | P* |
|---|---|---|
| Crop | 1 | 0.098 |
| Phosphorus within Crop | 2 | 0.156 |

*Probability of no difference

Table 3.4 Native fungi CFU in the rhizosphere of seed-row inoculated barley and canola in 1988.

| Crop (Date) | Noninoculated 2-10 cm | Noninoculated 10-20 cm | Inoculated 2-10cm -MAP | Inoculated 2-10cm +MAP | Inoculated 10-20 cm -MAP | Inoculated 10-20 cm +MAP |
|---|---|---|---|---|---|---|
| | ---------- | | $\log_{10}$ CFU $g^{-1}$ | | ---------- | |
| Barley (July 4) | 5.577 | | 5.605 | 5.846 | | |
| Barley (July 31) | 5.264 | 5.070 | 5.202 | 5.140 | 4.734 | 4.515 |
| Canola (July 20) | 5.227 | | 5.443 | 5.225 | | |
| Canola (Sept 13) | 5.726 | 5.708 | 5.333 | 5.177 | 5.117 | 5.672 |

Analysis of Variance (for 2-10 cm)

| Source | df | P* |
|---|---|---|
| Crop | 1 | 0.104 |
| Phosphorus within Crop | 1 | 0.193 |
| *P. bilaii* within Crop | 2 | 0.451 |
| Date within Crop | 2 | 0.144 |
| Depth | 1 | 0.001 |

*Probability of no difference.
note: none detected in noninoculated plots.

Table 3.5 Rhizosphere effect of native fungi within the 2-10 cm depth of soil inoculated with P. bilaii and planted to barley or canola in 1988.

| Crop (Date) | R:S (P*) | Rhizosphere | Bulk Soil |
|---|---|---|---|
| | | ------ $\log_{10}$ CFU $g^{-1}$ ---- | |
| Barley (July 31) | 1.71 (0.364) | 5.202 | 4.969 |
| Canola (July 20) | 2.16 (0.024) | 5.443 | 5.108 |
| Canola (Sept. 13) | 2.38 (0.006) | 5.256 | 4.879 |

*Probability of no difference between rhizosphere and bulk soil. Overall Analysis for Canola: P=0.013 for R:S; P=0.032 for Date; P=0.610 for R:S X Date.

Table 3.6 Native fungi within the upper 10 cm of soil on May 9, 1989, one year following addition of P. bilaii and MAP.

| Crop | Noninoculated | Inoculated | |
|---|---|---|---|
| | | - P | + P |
| | -- ------- log10 CFU/g ---------- | | |
| Barley | 5.027 | 5.110 | 5.188 |
| Canola | 5.075 | 5.115 | 5.084 |

Analysis of Variance

| Source | df | P* |
|---|---|---|
| Crop | 1 | 0.236 |
| Phosphorus within crop | 1 | 0.494 |
| P. bilaii within crop | 1 | 0.149 |

*Probability of no difference.

Table 3.7 Penicillium bilaii and native fungal CFU depth distribution and survival in soil planted to canola following uniform inoculation with P. bilaii to a depth of 5 cm on June 21, 1989.

| | P. bilaii | | | | Native Fungi | |
|---|---|---|---|---|---|---|
| Enumeration | type 'b' | | type 'a' | | | |
| Date | S[a] | R[b] | S | R | S | R |
| | $\log_{10}$ CFU $g^{-1}$ soil | | | | | |
| June 21/89[c] | | | | | | |
| 0 - 5 cm | 4.559 | | 3.618* | | 5.001 | |
| July 29/89 | | | | | | |
| 2 - 4 cm | 5.566 | 6.258 | 5.634 | 6.305 | 5.472 | 5.753 |
| 6 - 8 cm | 3.886 | 4.759 | 4.058 | 5.020 | 5.150 | 5.495 |
| 10 - 12 cm | 0.000 | 4.343 | 2.881[1] | 0.000 | 5.152 | 5.639 |
| May 15/90 | | | | | | |
| 2 - 4 cm | 5.078 | | 5.118 | | 5.137 | |

Analysis of variance for July 21.

| Source | df | P. bilaii | Native Fungi |
|---|---|---|---|
| | | P* | |
| R versus S (A) | 1 | 0.028 | 0.000 |
| Depth (B) | 2 | 0.000 | 0.032 |
| A X B | 2 | 0.672 | 0.427 |

Analysis of variance for 2-4 cm depth at three dates.

| Source | df | P. bilaii | Native Fungi |
|---|---|---|---|
| | | P* | |
| Date | 2 | 0.000 | 0.078 |

[a]bulk soil samples [b]rhizosphere soil samples
[c]total P. bilaii applied based on enumeration of inoculant was 4.420 $\log_{10}$ CFU/g soil
[1]detected in 1 plot only
*Probability of no difference.

Table 3.8 Comparison of the number of P. bilaii and native fungi CFU between P. bilaii inoculated and noninoculated soil on May 15, 1990, one year after inoculation.

| Fungal Group | Source of Soil | |
|---|---|---|
| | Plot[a] | Interplot[b] |
| | --- $\log_{10}$ CFU $g^{-1}$ soil --- | |
| P. bilaii | 5.399 | 2.978[1] |
| Native fungi | 5.137 | 5.202 |
| Native P-solubilizing fungi | 3.465 | 3.450 |

[a]P. bilaii inoculated, [b]noninoculated.
[1]Detected in 2 out of 6 samples.

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# CHAPTER 4 A DNA PROBE FOR IDENTIFICATION OF PENICILLIUM BILAII[1]

## 4.1 Introduction

Molecular genetic techniques are being used increasingly to supplement traditional taxonomic methods. Their main advantage over traditional methods is that they do not require gene expression to detect differences in nucleic acid base sequences between organisms. They are particularly useful for identifying microorganism that often lack well-defined phenotypic variation. The identification of imperfect fungi, such as the Penicillia, is complicated by phenotypic instability (Raper and Thom 1949). Hence, molecular genetic techniques may provide more reliable information for identifying fungal species (Turner and Balance 1986).

Penicillium bilaii is a phosphate-solubilizing fungus that has been tested as an inoculant for field crops (Kucey 1987, 1988; Kucey and Leggett 1989; Chapter 2) and is being used commercially. Recent observations indicate two phenotypes in this species, and about one half the P. bilaii colony forming units (CFU) recovered from a soil were type 'a' following inoculation with nearly exclusively type 'b' (Chapter 3). Only type 'b', however, conforms with published descriptions of P. bilaii (Kulik 1968; Pitt 1979); hence, the relationship between the two types is uncertain.

One molecular genetic technique for identifying organisms uses a DNA probe to detect restriction fragment length polymorphisms (RFLP) following digestion of genetic material by a restriction endonuclease. This technique has been used to identify species of Aspergillus (Kozlowski et al. 1982), Sclerotinia (Kohn et al. 1988), and Armilaria (Smith and Anderson 1989); and strains of Phytophthora parasitica (Goodwin et al. 1989), Gaumannomyces graminis (Henson 1989), and Neurospora (Taylor and Natvig 1989).

The purpose of this study was to use RFLP analysis to test the hypothesis that the two P. bilaii phenotypes belong to the same species.

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[1]A version of this Chapter will be submitted for publication (D.O. Keyes, K.N. Egger and W.B. McGill; Can. J. Soil Sci.)

## 4.2 Methods and Materials

### *4.2.1 Fungal Isolates*

Sources of the fungal isolates used in this study are listed in Table 4.1. P. bilaii cultures and inoculant produced by growing the organism on moistened wheat bran, were provided by Philom Bios. Pure cultures of fungi recovered from soil were obtained by consecutively transferring part of colonies, 2 to 3 times, to fresh medium. Additional Penicillium species were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH).

The colony appearance of the two P. bilaii phenotypes differ considerably. (See Chapter 3 for a complete description of type 'a' and type 'b'.)

### *4.2.2 DNA Extraction*

Agar plugs from cultures of the fungi listed in Table 4.1 were grown in potato dextrose broth (100 ml) on a shaker for 7 days at 22° C. Mycelial pads were obtained by filtering each culture through Whatman #2 filter paper. Pads were lyophilized and ground to a powder in liquid nitrogen.

DNA was extracted using the mini-preparation method of Zolan and Pukkila (1986). Approximately 100 mg of mycelial powder was combined with 700 µl of extraction buffer (700 mM NaCl, 50 mM Tris-pH 8.0, 10 mM EDTA, 1% CTAB, 0.2% mercaptoethanol) in 1.5 ml microcentrifuge tubes and incubated at 60°C for 45 minutes. Chloroform:Octanol (700 µl) was then added, and the solution was mixed and centrifuged. DNA in the aqueous phase was precipitated with isopropanol and separated by centrifugation. The resulting pellet of crude DNA was dissolved in 300 µl of Tris-HCl:EDTA buffer (pH 8), aided by heating (60°C for 10 min). The mixture was again emulsified with chloroform:octanol and the aqueous phase transferred to fresh tubes. DNA was precipitated by sequential addition of 7.5 M ammonium acetate (50 µl) and cold ethanol (1 ml), and separated by centrifugation. The pellet containing DNA was then dissolved in 300 µl of 200 mM ammonium acetate, precipitated again by addition of cold ethanol (1 ml), and dried in a

vacuum oven. This purified DNA was dissolved in Tris-HCl:EDTA buffer (pH 8) and stored (-80°C) for later use.

*4.2.3 Preparation of DNA Probes*

Chromosomal and mitochondrial DNA of P. bilaii, isolate #1, were digested with EcoRI and the resulting fragments separated by gel electrophoresis. Many of these fragments were cloned in pUC18 plasmids. Plasmid DNA was purified using standard procedures (Maniatis et al. 1982). The plasmid vector pBR322 was used to clone pMF2 for use as a probe. The pMF2 sequence was ligated to PstI digested plasmids with which competent cells of Escherichia coli C600SF8 were transformed. pMF2 comprises a 5.8 kilobase repeat sequence from Neurospora crassa rDNA that carries the coding region of the 5.8S, 17S and 25S ribosomal subunits (Free et al. 1979). Probe DNA was nick-translated with dCT$^{32}$P (75 $\mu$Ci probe$^{-1}$) and purified using an Elutip column.

*4.2.4 Southern Blots*

DNA from the 13 isolates listed in Table 4.1 was digested with XbaI, EcoRI and ClaI endonucleases and the fragments separated by electrophoresis. The gel was 0.7% agarose, buffered to pH 8. Restricted DNA (1-5 $\mu$g) was placed at one end of the gel and a voltage (less than 5 V per cm) was applied. The gel was stained with ethidium bromide and DNA transferred to nylon membranes using the method of Southern (1975) as modified by Chomezynski and Qasba (1984).

*4.2.5 Hybridization Techniques*

Probe DNA was denatured by boiling (8 min) followed by chilling. Nylon membranes were placed in hybridization solution (263 mM sodium phosphate buffer [pH 7.4], 1 mM EDTA and 4% SDS) and denatured probe DNA was added. Hybridization was allowed to continue for 12-48 hours at 65° C. Membranes were then washed with elution solution (0.2 M Tris-HCl [pH 7.5] and 2% SDS) at 90° C to remove excess probe DNA. Autoradiographs were prepared from membranes containing hybridized DNA fragments from each of the three enzymes.

## 4.3 Results

### *4.3.1 DNA Extraction*

The quantity of DNA extracted differed among isolates so DNA from repeated extractions of the same isolate were combined where yields were low. There were no distinct bands produced by electrophoretic separation following digestion of fungal DNA with XbaI, EcoRI or ClaI indicating that a variety of fragment lengths were produced. This may indicate a large number of endonuclease recognition sites within the genome, or random DNA breakage during the extraction procedure.

### *4.3.2 Restriction Fragment Length Polymorphisms (RFLP)*

Probes produced from EcoRI fragments of P. bilaii DNA detected some of the same polymorphisms as the pMF2 probe but no additional variation was found and, therefore, the analysis of results obtained using these probes was not pursued.

The length of the fragments that hybridized with the pMF2 probe are listed in Table 4.2. The EcoRI and ClaI enzymes produced the most polymorphisms. Within the P. bilaii group no differences in RFLP patterns were observed between type 'a' and type 'b'. Although isolate #8 had different band positions than other P. bilaii isolates, the bands were shifted by a constant amount and so the pattern of banding was similar. Such shifts are commonly produced by pigments attached to the DNA which slows its movement in the gel. Adjustment for these shifts has been made to facilitate analysis.

The other Penicillium species and the unidentified fungi differed from the P. bilaii isolates by at least one band. Isolate #11, P. restrictum, and isolate #12, P. lividum, belong to the Monoverticulate group, as does P. bilaii, but are readily distinguished from P. bilaii on the basis of RFLP produced by each of the three enzymes (Table 4.2). Isolate #13, P. chrysogenum, although it belongs to the Asymetricata, had EcoRI and ClaI restriction fragment lengths identical to P. bilaii, but it was distinguishable by the presence of a 5.6 kilobase XbaI fragment. One unidentified soil fungus, isolate #9, exhibited weak bands with XbaI and ClaI, but with EcoRI the banding pattern was clear and was significantly different

from that of P. bilaii; the other unidentified fungus, isolate #10, showed different banding patterns with both EcoRI and XbaI.

### 4.4 Discussion

The RFLP analysis provides evidence that the two P. bilaii phenotypes are not distinguishable on the basis of the molecular genetic information provided here. They are, however, distinguishable from the other Penicillium species tested. Thus, although type 'a' does not conform to the published description of P. bilaii, it is not different from that taxa as determined using RFLP patterns of XbaI, EcoRI and ClaI fragments probed with pMF2. The number of isolates tested was small, however, and these data do not eliminate the possibility that Penicillium species, or even more distantly related species, share the P. bilaii banding pattern. Furthermore, other P. bilaii isolates may produce different banding patterns. Verification of these results would require that more P. bilaii and a wider variety of other fungal species be included in the RFLP analysis.

The isolates recovered from soil and identified as P. bilaii (isolates #5 - #8) had the same banding pattern as other P. bilaii isolates. This indicates that the criteria used to identify P. bilaii colonies were adequate (Chapter 3). The four isolates subjected to RFLP analysis, although typical, represent a small portion of the colonies identified as P. bilaii while enumerating fungi from inoculated field soils. Hence, these results do not exclude the posibility of incorrect identifications.

### 4.5 Conclusion

From these results, we conclude that there is no evidence that the two different phenotypes designated as P. bilaii are genetically different.

Table 4.1. Source and description of isolates used in RFLP analysis for P. bilaii identification.

| Isolate # | Species* | Source |
|---|---|---|
| *Laboratory Cultures* | | |
| 1 | P. bilaii 'b' | R.M.N. Kucey |
| 2 | P. bilaii 'b' | Philom Bios (Lethbridge) |
| 3 | P. bilaii 'b' | Philom Bios (Edmonton) |
| *Inoculum* | | |
| 4 | P. bilaii 'a' | Spring 1988 |
| *Recovered from soil* | | |
| 5 | P. bilaii 'a' | Barley rhizosphere |
| 6 | P. bilaii 'a' | Canola rhizosphere |
| 7 | P. bilaii 'a' | Spring 1989 |
| 8 | P. bilaii 'b' | Spring 1989 |
| 9 | Unknown | Spring 1989 |
| 10 | Unknown | Spring 1989 |
| *Related Fungi* | | |
| 11 | P. restrictum | UAMH # 5169 |
| 12 | P. lividum | UAMH # 5136 |
| 13 | P. chrysogenum | UAMH # 485 |

*'a' and 'b' refers to P. bilaii type 'a' and type 'b' described in Chapter 3.

Table 4.2 Restriction fragment lengths of P. bilaii and other fungal isolates produced by three restriction endonucleases and detected by pMF2 hybridization.

| Isolate number | Enzyme: XbaI | | EcoR1 | | | ClaI | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 3 | 1 | 2 | 3 |
| | Fragment Length (kilobases) | | | | | | | |
| 1 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 2 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 3 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 4 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 5 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 6 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 7 | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 8* | 4.9 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 9 | | 3.3 | 2.5 | 1.9 | 1.2 | | 2.0 | |
| 10 | 5.1 | 3.3 | 5.5 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |
| 11 | 4.6 | 3.3 | 2.5 | 1.9 | 1.2 | 6.5 | 2.0 | |
| 12 | 5.1 | 3.3 | 5.5 | 1.9 | 1.2 | 4.4 | 2.0 | 1.3 |
| 13 | 5.6 | 3.3 | 5.3 | 1.9 | 1.2 | 5.0 | 2.0 | 1.3 |

*Band positions adjusted by a constant proportion for all enzymes.

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# CHAPTER 5 INCREASED YIELD OF DRY MATTER BUT NOT OF P IN CANOLA FOLLOWING ADDITION OF PENICILLIUM BILAII UNDER GREENHOUSE CONDITIONS USING SOILS OF VARYING RESIDUAL P[1]

## 5.1 Introduction

The portion of fertilizer P recovered in above ground plant biomass in the year of application varies with the amount added, soil characteristics, crop species and timing of additions (Wild 1988), but is typically 25% or less. Most of the remaining fertilizer P is adsorbed to soil constituents, or precipitated as less soluble phosphate compounds (Lindsay and Stephenson 1959; Soper and Racz 1980).

Canola (Brassica napus var. Westar) dry matter (DM) yield, P concentration and quantity of P in plant tissue has been reported to be greater following inoculation with Penicillium bilaii under greenhouse and field conditions, with or without added monoammonium phosphate (MAP) or rock phosphate (Kucey 1987, 1988; Asea et al. 1988; Kucey and Leggett 1989). The above studies used soils deficient in available P with pH value greater than 7.0. In alkaline soils orthophosphate fertilizers progress toward dicalcium phosphate dihydrate and then octocalcium phosphate with a concomitant decrease in solubility (Soper and Racz 1980). Asea et al. (1988) found that P. bilaii, in solution culture containing dextrose as a C source, is able to increase the rate of rock phosphate solubilization. Rape root exudates contain high concentrations of sugars, primarily glucose and sucrose (Haggquist et al. 1984). These observations suggest that P. bilaii benefits canola plants by increasing the availability of sparingly soluble P compounds in rhizosphere soil. Hence, inoculation with P. bilaii may increase recovery of residual fertilizer P by plants.

Residual P is quantitatively significant in soils receiving regular applications of phosphate fertilizer. If P. bilaii increases the solubility of fertilizer P reaction products in soil, then inoculation with this fungus should increase both quantity of P and DM yield of crops grown on soils with a history of P fertilization; furthermore, this increase should be greater than that obtained for the same soils which have not had a history of P fertilization.

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[1] A version of this Chapter will be submitted for publication (D.O. Keyes, J.A. Robertson and W.B. McGill; Can. J. Soil Sci.).

In some acidic soils of western Canada, Fe- and Al-P comprise the greatest portion of extractable soil P (Alexander and Robertson 1968). Banik and Dey (1982) have reported that phosphate-solubilizing microorganisms, including some Penicillium species, are less effective in solubilizing $FePO_4$ and $AlPO_4$, than $Ca_3(PO_4)_2$. We have found that P. bilaii applied to a P deficient acidic (pH 6.0) field soil survived and grew in the Ap horizon and had a significant rhizosphere effect. It did not, however, affect P quantity in canola (Brassica campestris var. Tobin) either with or without added MAP, but was associated with greater dry matter yield. Hence, there is evidence that P. bilaii may not increase residual P uptake from low pH soils. Acidic agricultural soils are extensive in western Canada (P.F.R.A. 1983), therefore, information on the effectiveness of P. bilaii in increasing the availability of residual fertilizer P in such soils is needed. Because canola dry matter yield was greater following inoculation with P. bilaii whereas barley yield was not affected (Chapter 2), canola was chosen as the test crop for the present study.

The objective of the research reported here was to test, under controlled greenhouse conditions, the hypothesis that uptake of residual fertilizer P by canola (Brassica campestris var. Tobin) grown in acidic soils is increased following P. bilaii inoculation.

## 5.2 Methods and Materials

### *5.2.1 Description of Soils*

Soil samples were obtained from experimental field plots at two locations where P had been applied using various placements, rates, and application times over a 10 year period under both tilled and direct drill tillage practices (Robertson 1989). One location, 10 km SW of Edmonton, was on a Malmo SiCL (Black Chernozem) which contains 5 g $kg^{-1}$ N and 52 g $kg^{-1}$ C (Crown and Greenlee 1978). The other location, 110 km SW of Edmonton, was on a Breton L (Gray Luvisol) which contains 1.3 g $kg^{-1}$ N and 14 g $kg^{-1}$ C (McGill et al. 1986). The three categories of residual P listed in Table 5.1 were selected from among the 12 treatments of the tilled plots at each location. Soil cores (30) to a depth of 15 cm taken from each of the four replicate plots were combined, air dried and sieved (4 mm). The soil

samples from the Malmo series had a bulk density of 0.95 Mg $m^{-3}$ and a field capacity of 45.6% (gravimetric); the samples from the Breton series had a bulk density of 1.10 Mg $m^{-3}$ and a field capacity of 27.5% (gravimetric). Available phosphorus was determined using the Miller and Axley (1956) extraction procedure. The pH of both soils was 5.5 (1:2 $H_2O$).

*5.2.2. Inoculant Preparation*

A suspension of P. bilaii conidia was prepared by first plating P. bilaii type 'b' onto potato dextrose agar (PDA) and allowing it to grow for 10 days at 22°C to form a lawn of sporulating fungi. Sterilized water containing Agral 90 (0.125%), a non-ionic surfactant, was then added to the petri plate and a brush was used to free the conidia from the substrate and suspend them in the liquid. Finally, this suspension was poured off and the concentration of conidia determined by direct microscopic counting and later confirmed by dilution plating on PDA. The suspension was then diluted to a concentration of 9 X $10^3$ spores $ml^{-1}$ for use as an inoculant.

*5.2.3 Experimental*

Each of the 6 bulk soil samples was divided among 6 pots (2.1 L $pot^{-1}$). Of these, 3 pots were inoculated by spraying 10 ml of the diluted suspension onto soil while it was stirred, to obtain a density of 1 x $10^2$ conidia $cm^{-3}$ of soil within the 2-7 cm depth (900 $cm^3$). The 3 noninoculated pots had an equal amount of sterilized, deionized water added. Eight canola (Brassica campestris var. Tobin) seeds were placed in a circle on top of the moistened soil and covered with 2 cm of soil. All pots received $KHSO_4$ (77 mg $pot^{-1}$) and $NH_4NO_3$ (506 mg $pot^{-1}$) in solution. Plants were thinned to 3 per pot 10 days after seeding. Natural lighting (November and December) was supplemented by artificial lighting (12 h $d^{-1}$; 400 W Na lamp). Pots were watered to approximately 80% of field capacity, generally every second day. Plant shoots were harvested 49 days after seeding and dried (96 h at 76°C).

The following plant growth parameters were determined: i) emergence, ii) height/leaf area (visual rating) 22 days and 31 days after seeding, iii) days to onset of budding and

flowering, and iv) plant height and shoot DM yield 49 days after planting. Shoots were digested with a mixture of nitric and perchloric acid and analyzed by ICP (inductively coupled argon plasma) for P, Fe, Cu, Mn and Zn (Alberta Agriculture Plant and Animal Nutrition Testing Laboratory, Edmonton).

*5.2.4 Enumeration of Fungi*

In order to monitor growth and survival of P. bilaii in the soils, an additional 3 pots of each soil series (low residual P) were inoculated but were not planted. At harvest, soil samples (10 g) were removed from within the inoculated zone of planted (low residual P) and nonplanted pots. P. bilaii, other phosphate-solubilizing fungi, and total native fungi were enumerated on each sample according to the procedures in Chapter 3.

*5.2.5 Statistical Analysis*

Analysis of variance was performed on plant DM yields using the SAS (SAS Institute Inc. 1986) GLM procedure. If there was insufficient plant material from one pot for nutrient analyses, plant material from replicate pots were combined and treated as one observation. The number of fungal CFU were not normally distributed despite use of a $\log_{10}$ transformation, and therefore did not meet the criteria for ANOVA.

**5.3 Results**

*5.3.1 Canola Dry Matter Yield*

Canola DM yield was greater (Figure 5.1; Table 5.2) and time to maturity shorter (Table 5.3) with increasing levels of residual P. Overall, the low residual P soil produced 34% more dry mass than the soils without residual P; the high residual P soil produced an additional 62% increase. Plants grown on the Malmo soil produced greater DM yield than those on the Breton soil, for all residual P categories, and showed greater differences in DM yield associated with residual P (Fig. 5.1).

Dry matter yield was 14% greater overall in the P. bilaii inoculated treatments, and there was no statistically significant interaction with either the soil type or the residual P category (Table 5.2). Greater growth with more residual P and with inoculation was evident

from visual observations within 22 days.

*5.3.2 Canola Development*

The number of days to canola budding and flowering, decreased with increasing residual P, but no difference due to inoculation was found (Table 5.3). When compared to the soil with no residual P, canola budded and flowered 2 days earlier in the low residual P soils, and 4 days earlier in the high residual P soils. There was a significant interaction between soil series and residual P category: the greatest differences were with the Breton series.

*5.3.3 Nutrient Quantity and Concentration*

Increasing residual P was associated with a greater quantity and concentration of P in plant tissue. Residual fertilizer P did not affect the concentration of Fe and Mn in plant shoots (Table 5.4), but plants grown on soils with more extractable P tended to have lower Zn and Cu concentrations (Table 5.5).

Inoculated plants had a significantly lower P concentration. Thus, despite greater DM yield following inoculation, the quantity of plant P was not increased. Copper concentration in inoculated treatments tended to be lower than in noninoculated treatments and there was a significant interaction between P category and P. bilaii addition with regard to Zn and Mn concentrations: their concentrations were less following inoculation of soils with either no residual P or with high residual P, but were greater following inoculation of soils with low residual P (Table 5.4 and 5.5).

*5.3.4 Growth and Survival of P. bilaii*

Penicillium bilaii grew in both planted and nonplanted pots. The number of P. bilaii CFU in planted soils increased by 100 fold from the initial inoculation rate, accounting for 11.3% of the total fungi enumerated, with more in the Breton than in the Malmo series (Figures 5.4 and 5.5). For the Breton series, there were more P. bilaii in planted than in nonplanted pots (Figure 5.4). Native phosphate-solubilizing fungal CFU accounted for 4.3% of the native fungi enumerated in planted soils.

## 5.4 Discussion

Extractable soil P, canola DM yield, shoot P concentration and P quantity were greater with increasing residual P. This provides evidence that P availability to plants was greater in soil to which P had been applied. Furthermore, although there was about 50% more P added over a 10 year period to soils in the high residual P category compared to the low residual P soils, plants grown on the high residual P soils contained nearly double the P quantity (Figure 5.3). This indicates that P availability was related to time since P additions were made as well as to the total amount added. These results are consistent with reports of the benefit of residual fertilizer P to crops (Tisdale et al. 1985). Plants generally respond to P addition early in their growth (Wild 1988) and the difference associated with residual P were detected prior to canola budding in this study.

The minimum evidence required for increased plant P availability is a greater quantity of P in plant tissue. P. bilaii inoculation was associated with greater canola DM yield under the conditions of the present experiment, however, despite increasing the number of phosphate-solubilizing fungi present, inoculation was not found to increase plant P quantity. Hence, there was no evidence found to support the hypothesis that P. bilaii, as added, benefited canola growth by increasing P availability.

Our results differ from those of Kucey and Leggett (1989) who found, also under greenhouse conditions, significantly greater canola DM yield, P concentration and P quantity following inoculation with P. bilaii. The reason for the different results is not known. Although Kucey and Leggett (1989) used B. napus, whereas in the present study B campestris was used, this is an unlikely reason because an increase in P quantity in other species, wheat and peas, following inoculation has been reported (Kucey 1987). Kucey and Leggett (1989) placed pellets containing P. bilaii ($3.6 \times 10^6$ CFU pellet$^{-1}$) in a layer 1 cm below the seed whereas in the present study P. bilaii CFU were uniformly distributed throughout a 5 cm layer of soil. This difference also is an unlikely reason for the different results because we found extensive growth of P. bilaii in the inoculated soil, indicating that

our inoculation technique was effective. Kucey and Leggett (1989) used a Brown Chernozem with a pH of 7.2 for their study whereas in our study both soils had a pH of 5.5. The forms of P in soils of western Canada are known to vary with other soil properties including pH (Racz and Soper 1967; Alexander and Robertson 1968). Banik and Dey (1982) found that Penicillium and Aspergillus species were more effective in solubilizing $Ca_3(PO_4)_2$ than either $FePO_4$ or $AlPO_4$. The hypothesis that P. bilaii more effectively solubilizes Ca-phosphates than it does other forms of soil P is consistent with the observation that plant P quantity is only greater following inoculation of plants grown on alkaline soil. This hypothesis warrants further study.

Micronutrient interactions with P have been widely reported although the underlying mechanisms are poorly understood (Olson 1972). In the present study we found Cu and Zn concentrations in canola shoots were inversely proportional to residual P. Deficiency was indicated by Cu concentrations in plants grown on the high residual P soils (Jones 1972).

Organic ligands in soil are required to maintain an adequate supply of micronutrients to plants (Norval 1972) and P. bilaii may produce metabolites that affect micronutrient uptake by plants. P. bilaii increases the rate of solubilization of Cu, Fe, and Zn compounds in solution cultures and has been associated with a greater Zn concentration in wheat grown under greenhouse conditions (Kucey 1988). Under field conditions, we found that Fe and Mn concentrations in canola were greater following inoculation with P. bilaii (Chapter 2). In the present study, Zn and Mn concentrations were greater in canola shoots following inoculation of the low residual P soils but were less both for soil lacking residual P and for the high residual P soil. The cause of this interaction is not known but availability of these elements was unlikely to have affected plant growth because they are generally adequately supplied by acidic soils (Tisdale et al. 1985) and their concentrations in plant tissue did not indicate deficiency (Jones 1972). Copper concentration tended to be lower in plants following inoculation, regardless of the residual P category. Differences in nutrient uptake between greenhouse and field studies may result because in planted pots, roots are confined

to a limited volume of soil, generally soil from the Ap horizon, whereas under field conditions plant roots are free to extend down into lower horizons, thus exploring a greater volume of soil and soil of varying composition and characteristics.

Lower P and micronutrient concentrations in P. bilaii inoculated compared to noninoculated plants has not been reported previously. This result may be partially, but not completely, explained by dilution of nutrients in the plant tissue due to the greater growth associated with P. bilaii inoculation. An alternative hypothesis is that P. bilaii immobilized soil P and thus limited its availability to plants. P. bilaii growth was extensive in the present study and competition between plants and microorganisms for soil P has been reported (Barber 1973).

The direct cause of the increase in canola growth associated with P. bilaii inoculation in our research is not known. There are reports of root disease suppression by Penicillium species (Johnson 1952; Wright 1956; Liu and Vaughan 1965; Kommedahl and Windels 1978). Sattar and Gaur (1987) reported the production of auxins and gibberellins by many phosphate-solubilizing microorganisms including a Penicillium species. In this study, P. bilaii may have benefitted canola by means other than increasing nutrient availability. Research is needed to determine the effect of P. bilaii on root pathogens of canola and to determine whether this fungus produces plant growth regulators.

The enumeration of fungi in this study provides evidence that P. bilaii is an effective competitor in soil under greenhouse conditions and that it grows better in planted than in nonplanted soils. Hence, the potential for P. bilaii to benefit plant growth has been shown.

## 5.5 Conclusions

Results of this research suggest the following conclusions:

1. from the time of inoculation to 49 days after, there was a 100 fold increase in P. bilaii CFU in planted soils;
2. P. bilaii, at the densities obtained, did not increase P availability to canola, regardless of the P fertilization history of the soil, and;

3. canola DM yield was greater following P. bilaii inoculation, however, the cause was not found to be phosphorus-related, hence, further research into alternative mechanisms is required.

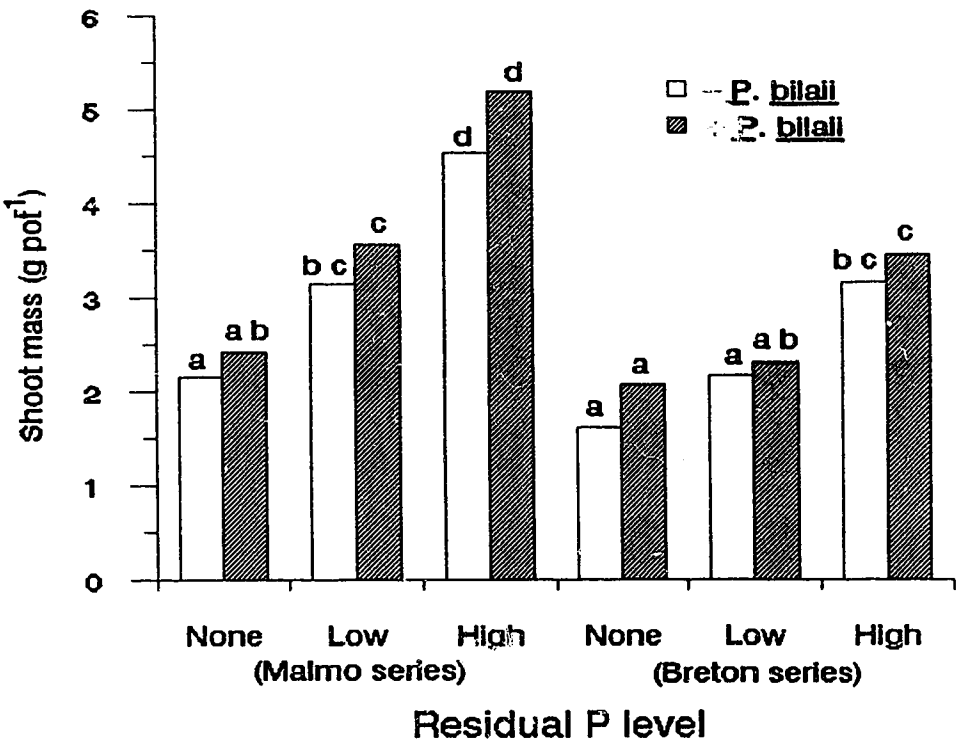


Figure 5.1. Dry matter yield of canola shoots grown in soil with varying residual P, with or without added P. bilaii. Bars having the same letter are not significantly different for a given soil ($P<0.05$).

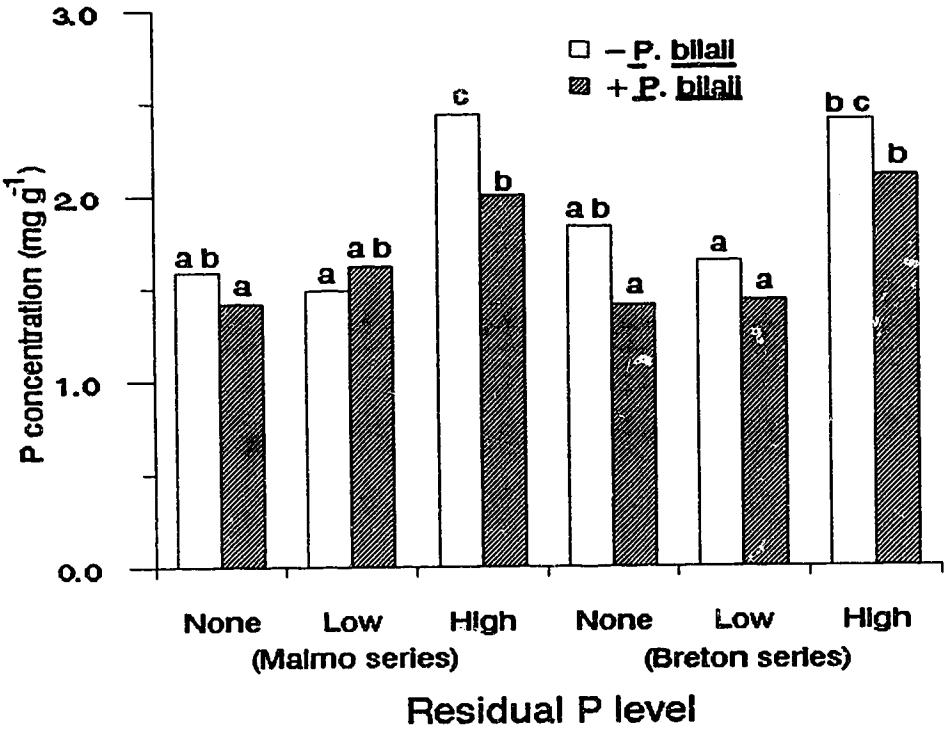


Figure 5.2. Shoot P concentration of canola grown in soil with varying residual P, with or without added P. bilaii. Bars having the same letter are not significantly different for a given soil ($P<0.05$).

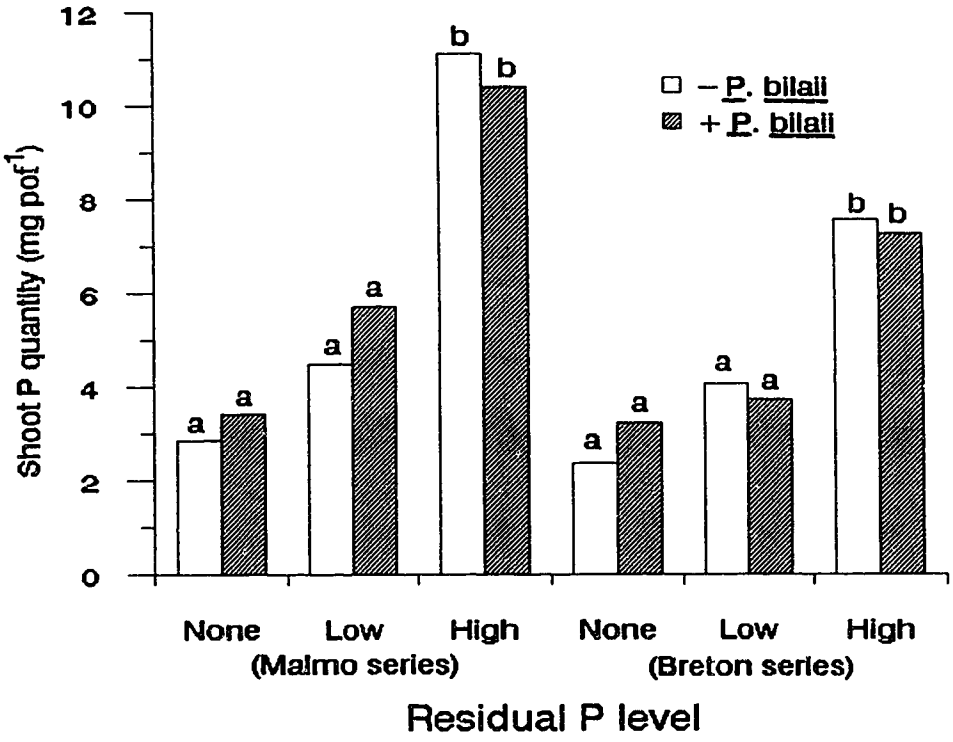


Figure 5.3. Shoot P quantity of canola grown in soil with varying residual P, with or without added P. bilaii. Bars having the same letter are not significantly different for a given soil ($P<0.05$).

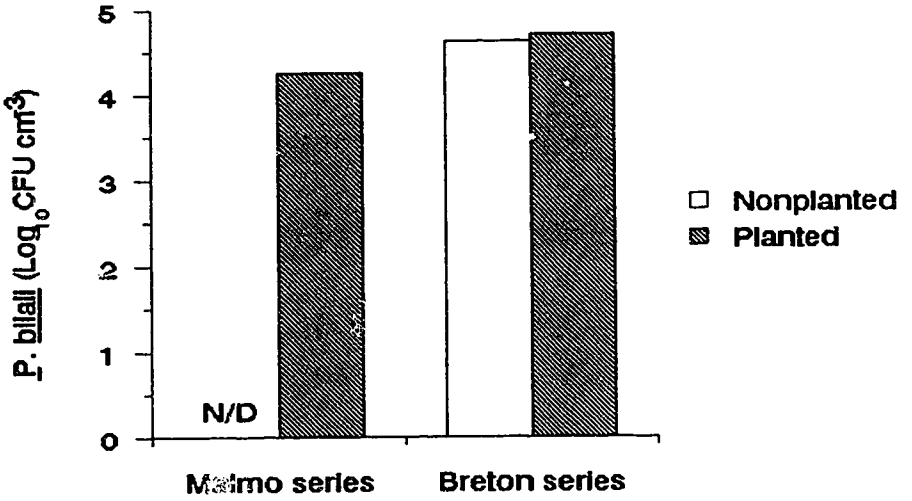


Figure 5.4. P. bilaii CFU recovered from nonplanted and planted soil. N/D=not determined

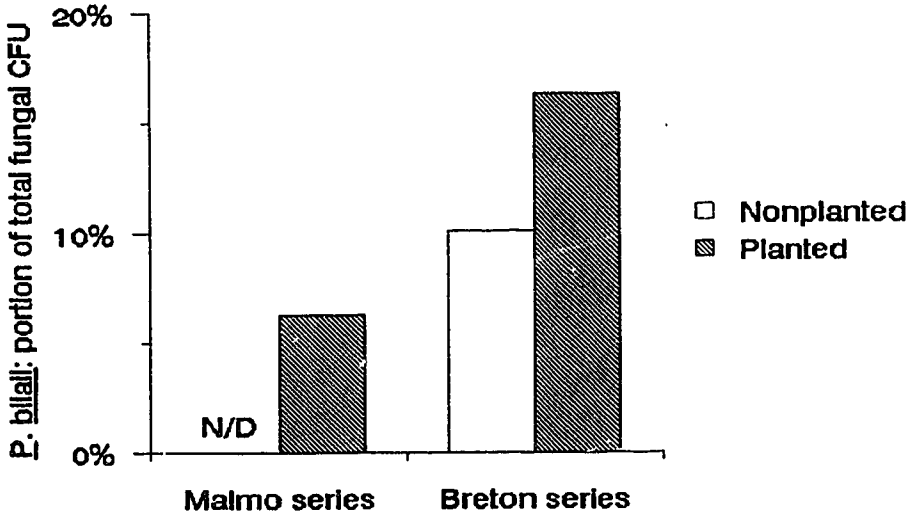


Figure 5.5. P. bilaii recovery from nonplanted and planted soil, as a portion of total fungal CFU. N/D=not determined.

Table 5.1 Fertilization history and extractable P of the Ap horizon of a Malmo and a Breton series soil used in a greenhouse experiment to evaluate the effects of P. bilaii addition on canola growth and nutrient uptake.

| Residual P Category | Fertilization History* | Extractable P ($ug\ g^{-1}$) | |
|---|---|---|---|
| None | No P fertilizer applied 1980-89 or previously. | Malmo<br>Breton | 7<br>8 |
| Low | P applied; 17 kg $ha^{-1}$ 1980-83, 20 kg $ha^{-1}$ 1984-86. | Malmo<br>Breton | 11<br>10 |
| High | P applied; 17 kg $ha^{-1}$ 1980-83, 20 kg $ha^{-1}$ 1984-89. | Malmo<br>Breton | 16<br>15 |

*N at 100 kg $ha^{-1}$. In some years a N fertilizer containing S was used.

Table 5.2 Analysis of variance for canola DM yield, P concentration and P quantity results shown in Figures 5.1, 5.2, and 5.3.

| Source | df | DM yield | P Conc. | P Quantity |
|---|---|---|---|---|
| | | ---------- P* ---------- | | |
| Soil Series (A) | 1 | 0.001 | 0.635 | 0.008 |
| P level (B) | 2 | 0.001 | 0.001 | 0.001 |
| P. bilaii C) | 1 | 0.049 | 0.022 | 0.688 |
| A X B | 2 | 0.064 | 0.862 | 0.077 |
| A X C | 1 | 0.921 | 0.401 | 0.797 |
| B X C | 2 | 0.789 | 0.270 | 0.602 |
| A X B X C | 2 | 0.903 | 0.459 | 0.672 |

*Probability of no difference.

Table 5.3 Number of days under greenhouse conditions to budding and flowering of canola grown on two soils with varying residual P, with or without added *P. bilaii*.

| Soil | Residual P Category | Time to Budding *P. bilaii* ( - ) | Time to Budding *P. bilaii* ( + ) | Time to Flowering *P. bilaii* ( - ) | Time to Flowering *P. bilaii* ( + ) |
|---|---|---|---|---|---|
| | | days | | days | |
| Malmo | None | 28.7[a] | 27.3[a] | 40.7[a] | 40.4[a] |
| | Low | 27.7[a] | 26.2[a] | 40.8[a] | 39.1[a] |
| | High | 27.6[a] | 28.6[a] | 38.9[a] | 40.6[a] |
| Breton | None | 34.8[d] | 31.4[bcd] | 46.7[b] | 45.6[b] |
| | Low | 33.0[cd] | 27.7[abcd] | 44.8[b] | 41.3[ab] |
| | High | 25.1[ab] | 24.6[a] | 37.1[a] | 36.6[a] |

Analysis of Variance

| Source | df | Budding | Flowering |
|---|---|---|---|
| | | P* | |
| Soil Series (A) | 1 | 0.217 | 0.095 |
| P level (B) | 2 | 0.065 | 0.003 |
| *P. bilaii* (C) | 1 | 0.194 | 0.423 |
| A X B | 2 | 0.047 | 0.012 |
| A X C | 1 | 0.381 | 0.471 |
| B X C | 2 | 0.550 | 0.521 |
| A X B X C | 2 | 0.941 | 0.968 |

Numbers followed by the same letter are not significantly different for a given soil ($P<0.05$).
*Probability of no difference

Table 5.4 Concentrations of Fe and Mn in shoots of canola grown on two soils with varying residual P, with or without added *P. bilaii*.

| Soil | Residual P Category | IRON *P. bilaii* ( - ) | IRON *P. bilaii* ( + ) | MANGANESE *P. bilaii* ( - ) | MANGANESE *P. bilaii* ( + ) |
|---|---|---|---|---|---|
| | | $\mu g\ g^{-1}$ | | | |
| Malmo | None | 107.0 | 125.3 | 42.6[a] | 36.3[a] |
| | Low | 184.7 | 121.0 | 36.5[a] | 42.5[a] |
| | High | 153.3 | 95.7 | 51.0[a] | 45.3[a] |
| Breton | None | 227.0 | 173.0 | 105.9[ab] | 97.1[ab] |
| | Low | 133.5 | 179.5 | 82.9[a] | 97.6[ab] |
| | High | 124.0 | 157.3 | 105.0[b] | 85.0[a] |

Analysis of Variance

| Source | df | Fe | Mn |
|---|---|---|---|
| | | p* | |
| Soil series (A) | 1 | 0.180 | 0.001 |
| P level (B) | 2 | 0.620 | 0.306 |
| *P. bilaii* (C) | 1 | 0.607 | 0.420 |
| A X B | 2 | 0.456 | 0.354 |
| A X C | 1 | 0.399 | 0.739 |
| B X C | 2 | 0.991 | 0.047 |
| A X B X C | 2 | 0.353 | 0.436 |

*Probability of no difference.

Table 5.5 Concentrations of Cu and Zn in shoots of canola grown on two soils with varying residual P, with or without added *P. bilaii*.

| Soil | Residual P Category | COPPER *P. bilaii* ( - ) | COPPER *P. bilaii* ( + ) | ZINC *P. bilaii* ( - ) | ZINC *P. bilaii* ( + ) |
|---|---|---|---|---|---|
| | | $\mu g\ g^{-1}$ | | | |
| Malmo | None | $5.20^{b}$ | $5.00^{b}$ | $99.5^{a}$ | $79.7^{cd}$ |
| | Low | $5.27^{b}$ | $4.87^{b}$ | $55.4^{ab}$ | $72.5^{bc}$ |
| | High | $2.67^{a}$ | $1.93^{a}$ | $59.3^{ab}$ | $51.3^{a}$ |
| Breton | None | $5.50^{bc}$ | $5.05^{bc}$ | $105.0^{b}$ | $75.5^{a}$ |
| | Low | $5.70^{c}$ | $4.95^{bc}$ | $62.3^{a}$ | $78.0^{ab}$ |
| | High | $4.07^{ab}$ | $3.23^{a}$ | $72.7^{a}$ | $64.0^{a}$ |

Analysis of Variance

| Source | df | Cu | Zn |
|---|---|---|---|
| | | P* | |
| Soil Series (A) | 1 | 0.041 | 0.151 |
| P level (B) | 2 | 0.001 | 0.001 |
| *P. bilaii* (C) | 1 | 0.052 | 0.226 |
| A X B | 2 | 0.123 | 0.526 |
| A X C | 1 | 0.670 | 0.661 |
| B X C | 2 | 0.795 | 0.006 |
| A X B X C | 2 | 0.976 | 0.915 |

Numbers followed by the same letter are not significantly different for a given soil ($P<0.05$).
*Probability of no difference.

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# CHAPTER 6 SYNTHESIS

## 6.1 Summary of Results

This study concerning interactions between the phosphate-solubilizing fungus P. bilaii, and barley or canola plants grown on acidic soil, has shown i) that P. bilaii can grow, and survives overwinter in these soil and ii) that it does not affect barley growth but does affect canola growth, but by mechanisms other than increased phosphate availability.

Research reported in Chapter 2 showed little evidence that P quantity in canola shoots was greater following P. bilaii addition, despite a greater dry matter yield. Evidence that P. bilaii influenced plant nutrient uptake under field conditions was provided by i) greater Fe and Mn concentrations in canola shoots, and ii) lower P concentration in barley shoots, following inoculation.

Results reported in Chapter 3 reveal the potential for extensive P. bilaii growth in a Malmo SiCL and a rhizosphere effect for P. bilaii of approximately 10; P. bilaii, however, made an insignificant contribution to rhizosphere fungi recovered from below 10 cm.

Two phenotypes of P. bilaii were used as inoculants: type 'a' and type 'b', and both types could be recovered from soil. In one experiment, however, type 'a' was recovered from soils to which P. bilaii of almost exclusively type 'b' had been added. A RFLP analysis, reported in Chapter 4, provides evidence that type 'a' and type 'b' belong to the same species.

The results of a green house study, Chapter 5, confirmed that P. bilaii can grow in Ap horizon soil. Again, there was greater canola dry matter yield following P. bilaii addition but, again, shoot P quantity was not affected.

## 6.2 Agronomic Implications

Research results reported in this thesis have implications for the use of P. bilaii as an inoculant for crops grown on acidic soils in central Alberta. That canola but not barley yields were greater following P. bilaii addition, provides evidence that the effect of P. bilaii

on crops is species-specific.

Downward movement of P. bilaii within inoculated soil is apparently limited. Hence, inoculum placement is likely to be critical in determining the extent of contact between P. bilaii and plant roots, and thus its potential to influence plant growth. High rates of inoculation were used in this study ($10^7$ to $10^9$ CFU $m^{-1}$ $row^{-1}$) and there were indications of competition for available soil P between the added organism and barley, as Barber and Loughman (1967) have shown. Inoculation rate may, therefore, be another critical factor determining the effect of P. bilaii on crop dry matter yield.

Because P. bilaii propagules can survive overwinter, a single inoculation may influence subsequent annual crops or provide a lasting benefit to perennial plants, provided P. bilaii is beneficial to that particular species.

Results of the present study indicate that inoculation with P. bilaii may increase canola yield but is unlikely either to increase the utilization efficiency of phosphate fertilizers or to reduce the fertilizer requirements of canola grown on acidic soils.

### 6.3 Scientific Implications

Recovery of P. bilaii from inoculated soil by dilution plating coupled with RFLP analyses of selected isolates was a successful method of tracing this organism in soil. This capability, along with the relatively high survival rate of P. bilaii, make this fungus well suited to either studies involving microorganism X plant interactions or investigations into the effects of introduced species on indigenous soil biota. Detailed studies are needed to discover the factors determining the survival, growth and movement of P. bilaii in soil.

Phenotypic instability under field conditions, which is suggested by the results of the present study, presents technical difficulties in tracing P. bilaii but also provides an opportunity for studying the factors controlling such changes, which may be related to its survival or activity in soil.

Research is also required to determine the mechanisms by which canola dry matter yield is increased following P. bilaii addition to acidic soils. Although previous work has

suggested that solubilization of sparingly soluble phosphates in soil by acidification, and perhaps cation chelation, is the primary mechanism by which plant growth is increased (Kucey 1987, 1988; Asea et al. 1988; Kucey and Leggett 1989), this mechanism does not provide an adequate explanation of the results obtained in the present study.

Penicillium bilaii may benefit plants grown on acidic soils by i) competitive exclusion of deleterious microorganisms including root pathogens or ii) production of metabolites directly beneficial to plants or active against deleterious microorganisms. Competition between pathogenic and nonpathogenic microorganisms is widespread (Domsch et al. 1980). Penicillium oxalicum suppresses pea seedling diseases (Kommedahl and Windels 1978) and P frequentens is antagonistic to Pythium species on sugarbeet and mustard roots (Wright 1956; Liu and Vaughan 1965). Included among the metabolites produced by P. bilaii may be i) antibiotics: many Penicillia produce antibiotics (Domsch et al. 1980); ii) phytohormones: Sattar and Gaur (1987) have found that gibberllins and auxins are produced by many phosphate solubilizing microorganisms, including a Penicillium species, or; iii) chelating agents, including siderophores (Page 1988) or organic acids (Louw and Webley 1959). All of these mechanisms may be species-specific and, therefore, could have produced the different results obtained for barley and canola.

Greater canola dry matter yield following inoculation with P. bilaii in this study appears likely to have resulted from suppression of deleterious microorganisms. Canola is susceptible to many soil borne plant pathogens (Martens et al. 1988) and, although seedling blight was not observed and emergence was not affected by P. bilaii addition, root rot of canola is common in Alberta and may have been reduced in the present study by growth of P. bilaii in the rhizosphere. Furthermore, greater Fe quantity in canola shoots following P. bilaii addition is not inconsistent with a disease suppression mechanism: pathogens may compete with plants for available Fe (Barash et al. 1988).

## 6.4 General Conclusions

In addition to identifying many areas for future study, the following conclusions are suggested by the research reported in this thesis:

1. P. bilaii was capable of growth and overwinter survival in the Ap horizon of a Malmo SiCL;
2. P. bilaii was associated with greater canola growth but, under field conditions, did not affect barley;
3. there was no evidence found to suggest P. bilaii increased plant P uptake from native soil phosphates, added MAP, or residual fertilizer P, and;
4. evidence was provided that a phenotype of P. bilaii not previously described belongs to this species.

## 6.2 References

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