Biocontrol of Damping-Off and Root-Rot Causing Pathogens by Novel Rhizobacteria Isolated in Saskatchewan

G.C.Y. Leung, R.K. Hynes, and L.M. Nelson.

Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, SK, S7N 5A8

Key Words: Rhizobium, rhizobacteria, plant growth promotion, fungal pathogens, biological control, inoculant, pulse crops (pea, lentil)

Abstract

In Saskatchewan, land area used to grow pulse crops is increasing, and maintenance of plant health is imperative to produce an economically viable resource. The objective of this study is to characterize novel rhizobacteria for plant growth promoting properties: enhancing the root and shoot growth of pea and lentil, and suppressing the growth and disease severity caused by fungal pathogens. From *in vitro* assays, isolates 5-6, 4-31, and 2-9 suppressed the growth of fungal pathogens. Mechanisms which suppress the growth of fungal pathogens may include the production of enzymes which degrade the cell wall of the fungi, and antifungal metabolites. In studies where rhizobacteria were inoculated in combination with commercial *Rhizobium* inoculants onto pea and lentil seeds, isolate 2-9 significantly increased the root dry weight of pea, and isolate 4-31 and 5-6 increased the ability of *Rhizobium* to fix nitrogen in pea and lentil, respectively. Fungicide compatibility studies using two commonly-used products (Apron®-FL, Crown®) showed that isolate 5-6 was compatible with both fungicides, whereas isolates 4-31 and 2-9 were compatible only with Apron®-FL. The three bacterial isolates (5-6, 4-31, and 2-9) chosen for possible development of a commercial inoculant show promise to be effective biological control agents against fungal pathogens.

Introduction

Fungal pathogens such as *Pythium*, *Rhizoctonia*, and *Fusarium* spp. cause significant economic losses to pulse producers. Currently, producers use chemical fungicides to reduce disease severity and incidence. However, concerns regarding pollution and effects on human health have prompted agriculturalist to decrease the use of chemicals (Agrios 1997). An alternative to chemical control of fungal pathogens are biological products. Plant-Growth-Promoting Rhizobacteria (PGPR) may effectively safeguard pulse crops from the above pathogens, while enhancing the growth of plants. Enhancement of crop yield is mediated by two methods, direct and indirect. Supplementation of nutrients, minerals, and other growth-promoting substances, are categorized under direct enhancement (Glick, 1995; De-Ming and Alexander 1988). The elimination of crop-deleterious agents, which in turn, increases crop production, are considered indirect enhancement (Elad and Chet 1987). Biological antifungal and plant-enhancing agents address society's concerns regarding the widespread use of chemical fertilizers and fungicides. Plant-growth-promoting rhizobacteria have been increasingly studied for direct and indirect plant enhancing properties. The objective of this study is the characterization of isolated rhizobacteria for direct and indirect plant growth promotion.

Materials and Methods

Rhizobacterial Isolates*:* Rhizobacterial strains, which were isolated by Hynes and Nelson (2001) from 6 fields in Saskatchewan, were chosen for further characterization. These pure-culture isolates were stored in 20% glycerol at -80°C, until needed. All subsequent assays utilized bacterial isolates inoculated from pure culture, and grown in one-half strength Tryptic Soy Broth (1/2 TSB) for 2 days on a gyratory shaker (148 rpm) at room temperature. *Pseudomonas chlororaphis* 63-28 (EcoSoil Inc., San Diego, CA) was included as a positive control. *Rhizobium leguminosarum* biovar *viciae* RGP2 (Becker Underwood, Saskatoon, SK) was used to inoculate pea, and ICAR 20 (Becker Underwood, Saskatoon, SK) was used to inoculate lentil. *Rhizobium* spp. were grown in Yeast Extract Mannitol (YEM : 0.5 g L^{-1} K_2HPO_4; 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$; 0.1 g L^{-1} NaCl; 0.5 g L^{-1} yeast extract; 10 g L^{-1} mannitol; 0.5 g L^{-1} $CaCO_3$) medium for 3 days on a gyratory shaker (148 rpm) at room temperature.

Fungal pathogens: Soil-borne pathogens of pulse crops included this study are *Pythium*, *Rhizoctonia*, and *Fusarium* spp. Fungal pathogens were provided by S.F. Huang (Alberta Research Council, Vegreville, AB).

Suppression of the growth of fungal pathogens by bacterial isolates: Assays were conducted on 1/5-strength Potato Dextrose Agar (PDA) plates, with a fungal plug placed in the centre, and bacteria streaked 3 cm perpendicular to the plug. Plates were incubated at room temperature for 2 days (*Pythium*), 3 days (*Rhizoctonia*), or >7 days (*Fusarium*). Fungal suppression was measured as the zone of inhibited growth.

Production of lytic enzymes: Protease was detected by the clearing of skim milk medium surrounding the rhizobacterial isolate. Chitinase was detected by a zone of clearing on minimal medium supplemented with colloidal chitin (Dunne et al., 1997).

Phosphate solubilization: The ability to solubilize inorganic phosphate was qualitatively measured by inoculating bacterial isolates onto Yeast Extract Diphosphate (YEDP) agar plates (Yang, personal communication), which contained yeast extract (5 g L^{-1}), dextrose (10 g L^{-1}), $Ca_3(PO)_2$ (2 g L^{-1}), and agar (12 g L^{-1}). Plates were incubated for 2 days at room temperature. Clearing of medium was indicative of inorganic phosphate solubilization.

Effect of bacterial isolates on plant growth and nodulation by *Rhizobium* in Leonard jars: Seeds were sown in sterile Leonard jars (Vincent 1970) and inoculated with bacterial isolates and *Rhizobium* spp. as described by Chanway et al. (1989). Plants were harvested after 5 weeks, and assayed for acetylene reduction ability, and shoot and root dry weight.

Fungicide compatibility: Seeds coated with fungicides Apron®-FL (Gustafson, Calgary, AB) and Crown® (Crompton, Elmira, ON) were inoculated with rhizobacterial isolates from either liquid culture or peat. Survival of the bacteria in the presence and absence of fungicide on the seed surface was determined at 0, 2, 6, 24 hours by serial dilution and plate count.

Results and Discussion

Suppression of the growth of fungal pathogens by bacterial isolates: Rhizobacterial isolates 2-9, 4-31, and 5-6 suppressed the growth of fungal pathogens (Fig. 1). This bio-fungicidal activity may enhance the competitiveness of the beneficial isolates *in situ*. The ability to strongly suppress one or more fungal pathogens is an attribute which may lead to the successful development of a commercial

inoculant. Isolate 4-31 showed the strongest suppression of growth of *Rhizoctonia* sp., 2-9 strongly suppressed the growth of *Pythium* and *Fusarium* spp., and 5-6 was able to strongly suppress the growth of all fungal pathogens tested. Rhizobacterial isolates 2-9, 4-31, and 5-6 show promise for the development of biocontrol inoculants.

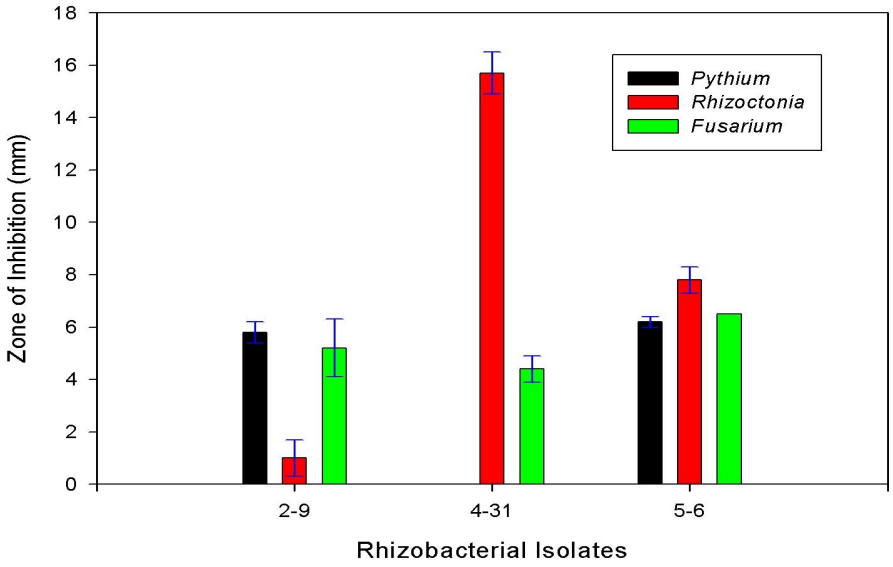

Figure 1. Suppression of the growth of fungal pathogens by rhizobacterial isolates *in vitro.*

Production of lytic enzymes: The ability to produce cell wall-degrading enzymes, such as, proteases and chitinases, (Table 1) may enhance the competitiveness of the bacterial isolates in the rhizosphere (Dunne et al. 1996).

Table 1. Ability of rhizobacterial isolates 2-9, 4-31, and 5-6 to produce lytic enzymes and solubilize inorganic phosphate.

	Production		
Isolate	Protease	Chitinase	Phosphate Solubilization
4-31	+	+	-
5-6	+	+	-
2-9	-	-	+

Values represent the mean of three replicates.

Phosphate solubilization: Isolate 2-9 was able to solubilize the phosphate in medium which contained pre-processed inorganic phosphate (YEDP) (Table 1). Clearing of the medium was observed after incubation overnight. The ability to solubilize inorganic phosphate may increase phosphate availability in the soil (Kucey 1983).

Effect of bacterial isolates on plant growth and nodulation by *Rhizobium* in Leonard jars: No isolate significantly increased shoot dry weight of pea cv Mozart compared to the control (*Rhizobium* RGP2 alone); however, isolate 2-9 increased the root dry weight (Fig. 2). This suggests that isolate 2-9 may be producing compounds which may increase root growth. Isolates 5-6 and 63-28 had a reduced root

dry weight compared to *Rhizobium* RGP2 only. Plants inoculated with isolate 4-31 had a significantly lower shoot dry weight compared to the control.

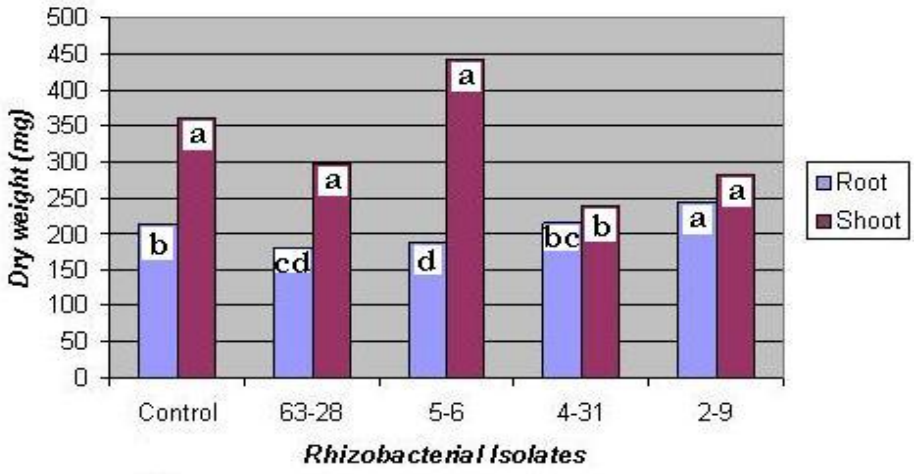

Figure 2. Effect of rhizobacterial isolates on the root and shoot growth of pea cv Mozart in Leonard jars. Values represent the mean of three replicates.

Pea plants inoculated with isolate 4-31 had a higher rate of acetylene reduction, whereas isolates 63-28, 5-6 and 2-9 had equal acetylene reduction compared to control plants (Figure 3). This suggests that isolate 4-31 may enhance the ability of *Rhizobium* RGP2 to fix atmospheric nitrogen.

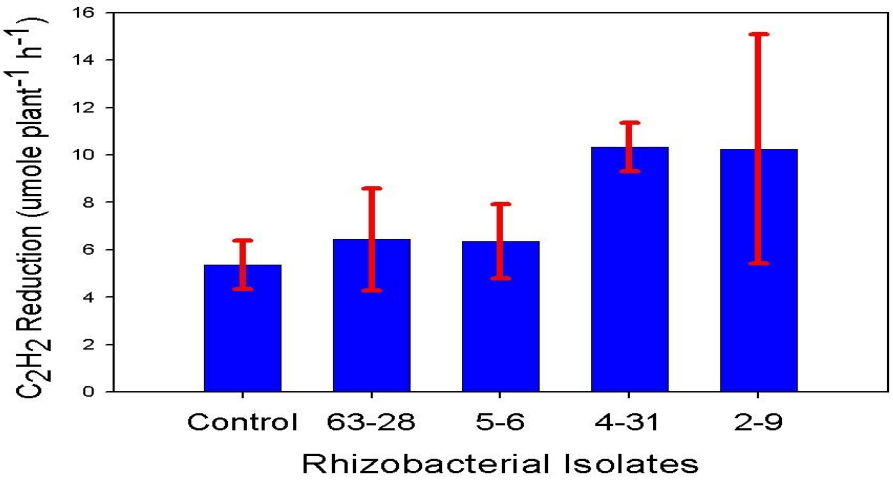

Figure 3. Effect of inoculation of rhizobacterial isolates on acetylene (C_2H_2) reduction activity of pea cv Mozart in Leonard jars. Values represent the mean of three replicates.

No isolate significantly (P<0.05) increased root dry weight, or shoot dry weight of lentil cv Milestone in Leonard jars compared to plants inoculated only with *Rhizobium* ICAR 20 (Fig. 4). Isolate 2-9 significantly reduced the dry weight of the shoot of lentil cv Milestone, whereas, this bacteria had no

significant effect on the shoot dry weight of pea cv Mozart. Thus, the effect isolate 2-9 had on the growth of plants was dependant on the pulse crop. Furthermore, 2-9 increased the root dry weight of pea, but had no effect on lentil.

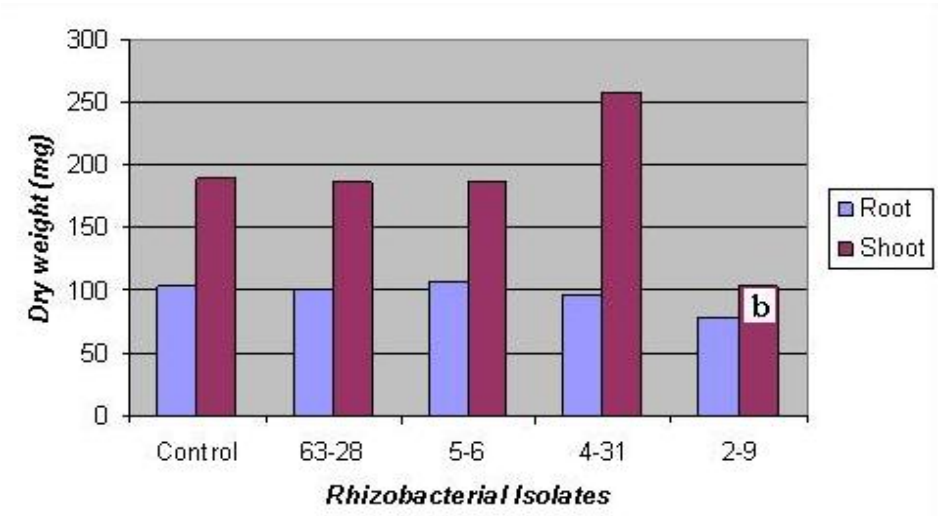

Figure 4. Effect of rhizobacterial isolates on the root and shoot growth of lentil cv Milestone in Leonard jars. Values represent the mean of three replicates.

Two rhizobacterial isolates increased the ability of lentil cv Milestone nodules to reduce acetylene—62-28 and 5-6 (Figure 5). Despite the observation that these two isolates had no significant effect on shoot and root dry weight, an increased ability to reduce acetylene implies that increased nitrogen fixation had no significant effect on the growth of the lentil plants. Possibly the effect may be seen in early plant growth, within the first 3 weeks. Pea plants inoculated with rhizobacterial isolate 4-31 had an increased reduction of acetylene compared to non-inoculated plants, yet no difference was seen with lentil plants. Therefore, the beneficial interaction with the rhizobacteria may be dependant on the *Rhizobium* species.

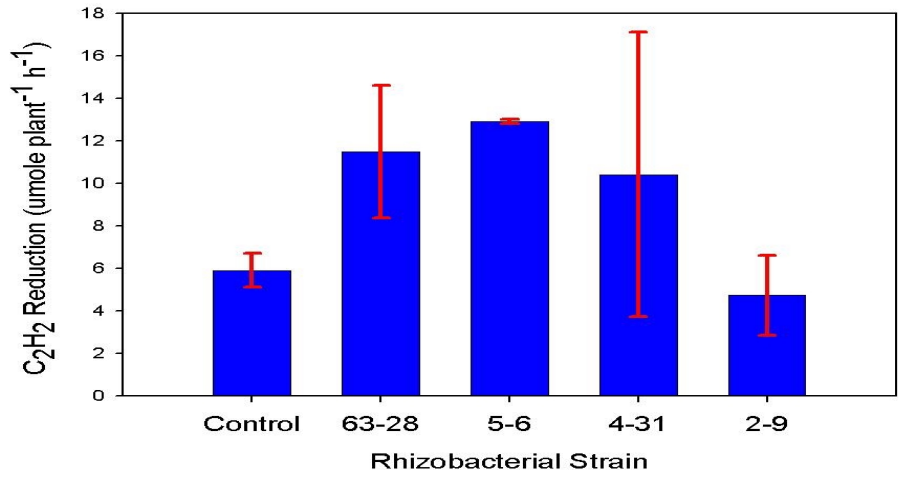

Figure 5. Effect of inoculation of rhizobacterial isolates on acetylene (C_2H_2) reduction activity of lentil cv Milestone in Leonard jars. Values represent the mean of three replicates.

Fungicide compatibility: Rhizobacterial isolate 2-9 in both peat and liquid formulations was observed to maintain the same CFU/seed on seeds not treated with fungicide and seeds treated with Apron®-FL; however, this isolate was unable to maintain the same CFU/seed on seeds coated with Crown® fungicide compared to seeds not treated with fungicide (Fig. 6).

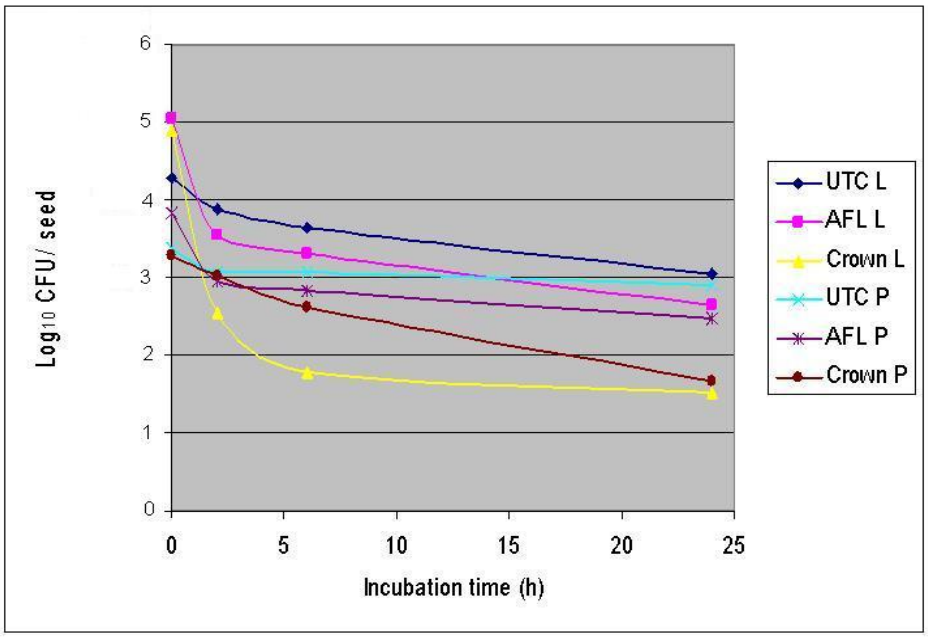

Figure 6. Survivability of rhizobacterial isolate 2-9 from liquid (L; ♦, ■, ▲) or peat culture (P; x, x, ●) on seeds coated with Apron®-FL (AFL) or Crown® fungicide. UTC, Untreated Control. Values represent the mean of three replicates.

Isolate 4-31 formulated in liquid culture was able to survive on seeds treated with Apron®-FL fungicide, however, could not survive on seeds treated with Crown® (Fig. 7). Peat inoculant was able to sustain the same CFU/seed on Apron®-FL-treated seeds compared to seeds not treated with fungicide, after long-term (24 h) incubation. Isolate 4-31 in peat formulation was not compatible with seeds treated with Crown® fungicide.

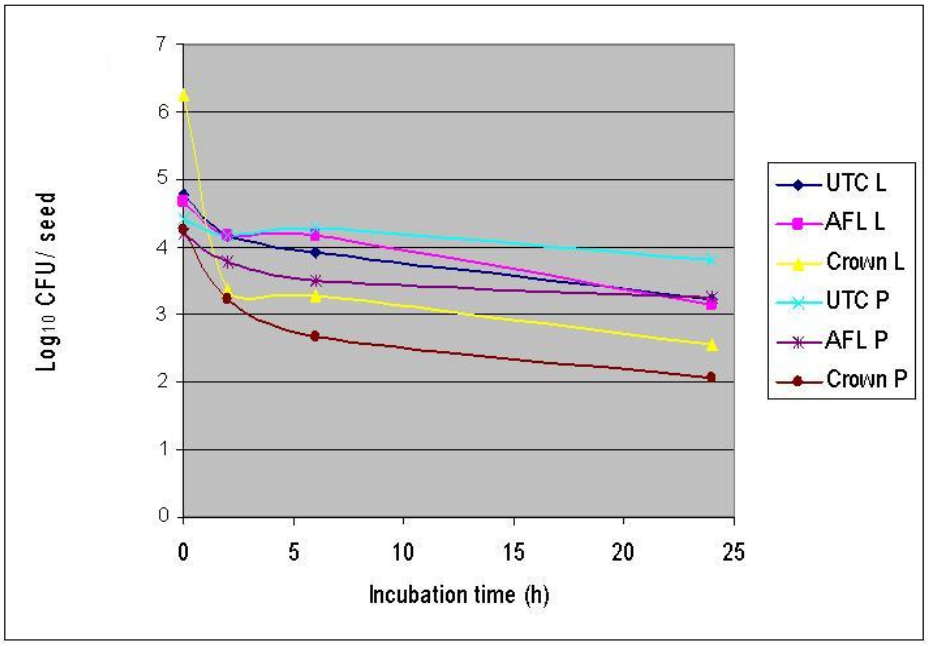

Figure 7. Survivability of rhizobacterial isolate 4-31 from liquid (L; ♦, ■, ▲) or peat culture (P; x, x, ●) on seeds coated with Apron®-FL (AFL) or Crown® fungicide. UTC, Untreated Control. Values represent the mean of three replicates.

Peat and liquid culture formulations with rhizobacterial isolate 5-6 showed no detrimental effects within the first 6 h of incubation when inoculated on seeds treated with fungicide (Fig. 8). After this, seeds treated with Crown® and peat inoculant had a reduced CFU/seed; and seeds treated with both fungicides had lower CFU than seeds not treated with fungicide and inoculated with liquid formulation.

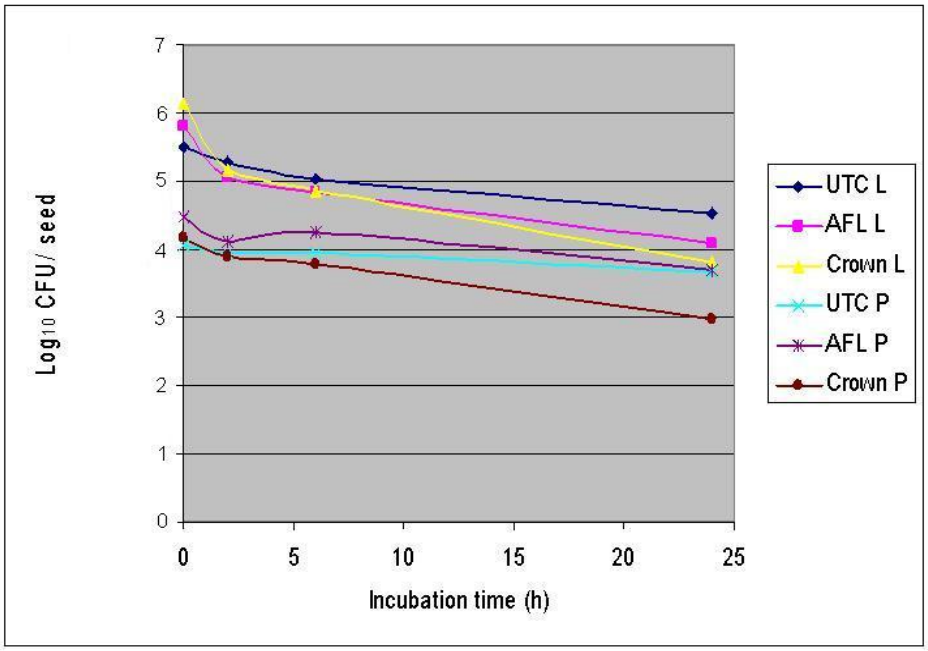

Figure 8. Survivability of rhizobacterial isolate 5-6 from liquid (L; ♦, ■, ▲) or peat culture (P; x, x, ●) on seeds coated with Apron®-FL (AFL) or Crown® fungicide. UTC, Untreated Control. Values represent the mean of three replicates.

Conclusions

The objective of this study is to characterize rhizobacterial isolates which demonstrate the ability to enhance the growth of pulse crops, pea and lentil. Rhizobacterial isolates 2-9, 4-31, and 5-6 suppressed the growth of fungal pathogens. Possible bio-fungicidal mechanisms may be the production of chitinases and proteases by isolate 5-6 and 4-31. Furthermore, these isolates have shown to be compatible with commercial *Rhizobium* inoculants. Isolates 2-9 and 4-31 are compatible with the fungicide Apron®-FL and not with Crown®; while, isolate 5-6 was compatible with both fungicides. Rhizobacterial isolate 2-9 was able to solubilize inorganic phosphate; this may increase the availability of this nutrient to pulse crops. Characterization of the differing crop-enhancing properties produced by the PGPR will aid in the successful development of a commercial inoculant.

Acknowledgements

We thank Marilyn Gould, Vindya Tennakoon, Karen Williams, Brent Chamberlain, and Guoping Yang for technical assistance. This study was supported by grants from the Natural Science and Engineering Research Council (NSERC), and the University of Saskatchewan President's NSERC fund.

References

Agrios, G.N. 1997. Plant Pathology, 4th ed. pp. 15-16.

Chanway, C.P., Hynes, R.K., and Nelson, L.M. 1989. Soil Biol. Biochem. **21:** 511-517.

De-Ming, L., and Alexander, M. 1988. Plant Soil **108**: 211-219.

Dunne, C., Delany, I., Fenton, A., Lohrke, S., Moenne-Loccoz, Y., and O'Gara, F. 1996. Biology of plant-microbe interactions. *Edited by* G. Stacey, B. Mullice, and P.M. Gresshoff. Int. Soc. for Molecular Plant-microbe Interactions, St. Paul, MN. pp. 441-448.

Dunne, C., Crowley, J.J., Moenne-Loccoz, Y., Dowling, D.N., de Brujin, F.J., and O'Gara, F. 1997. Microbiol **143**: 3921-3931.

Elad, Y., and Chet, I.1987. Phytopathology **77**: 190-195.

Glick, B.R. 1995. Can J Microbial **41**: 109-117.

Kucey, R.M.N. 1983. Can. J. Soil Sci. **68**: 261-270.

Hynes, R.K., and Nelson, L.M. 2001. Crops and Soils Proceedings 2001, pp. 237-242.

Vincent, J.M. 1970. *A Manual for the Practical Study of Root-Nodule Bacteria*, IBP Handbook No.15. Blackwell Scientific, Oxford.

Yang, G.P. 2002. Personal communication.