Different Freezing Factors associated with Saskatchewan Winters in relation to the Viability of *Penicillium bilaiae*

E.M. Sanders[1], J.D. Knight[1], L.V. Gusta[2]

[1]Department of Soil Science, University of Saskatchewan, Saskatoon, SK, S7N 5A8
[2]Department of Plant Science, University of Saskatchewan, Saskatoon, SK, S7N 5A8

Key Words: *Penicillium bilaiae*, temperature, freezing, canola

Abstract

Survival of *Penicillium bilaiae* over the winter months is essential for use on fall seeded canola crops. Through a series of freeze experiments it is has been demonstrated that *P. bilaiae* can successfully survive low temperatures down to -196 °C when there is a controlled decrease in temperature. *Penicillium bilaiae* is still viable after being frozen at -20 °C for 100 days. Cycles of freezes to -8 °C and thaws at 2 °C had no effect on viability of *P. bilaiae*. *Penicillium bilaiae* on seed should be able to successfully survive freezing conditions associated with a Saskatchewan winter.

1. Introduction

Penicillium bilaiae is a phosphate solubilizing organism that is a natural inhabitant of southern Alberta soils (Kucey, 1983). When applying *P. bilaiae* to fall seeded canola, it is necessary for the organism to possess the ability to survive the winter. Saskatchewan winters are unpredictable and can include low temperatures, long winters and freeze-thaw cycles.

The objective of the study is to determine if *P. bilaiae* has the ability to survive over a Saskatchewan winter on canola seed.

2.0 Materials and Methods

2.1.1 *Penicillium bilaiae* seed treatment

Seeds were inoculated with a commercial product ('Jumpstart', Philom Bios Inc.), a wettable powder containing 3.3 x10^6 CFU of *P. bilaiae* per gram of seed. The recommended rate of water was added to the product to create a slurry that could evenly be applied to seed. The certified Roundup Ready canola seed, variety LG3235 was used for the experiment.

2.1.2 Low temperatures (Experiment 1)

Just enough distilled water was added to dampen a piece of Kim wipe in a glass tube. To ensure freezing an ice nucleator, Thermica was added to the tube followed by

two grams of inoculated canola seed. The tube was capped and placed in a refrigerated circulating bath (Neslab© LT50DD) that was held at -3 °C.

The temperature of the bath gradually decreased 2 °C every hour (Gusta et al., 2001). Tubes were removed from the bath at -3 °C, -20 °C, -30 °C and -40 °C once the temperature had been maintained for one hour for each treatment temperature. After the removal of a tube the temperature was allowed to continue decreasing until the next treatment temperature. After the -40 °C treatment the remaining tubes were rapidly cooled by placing them in a -60 °C freezer. The final treatment (-196 °C) was reached by adding liquid nitrogen to the tubes. After removal of the tubes at each designated temperature the tubes were then placed in a 2 °C cooler and allowed to thaw overnight.

2.1.3 Long term freezing (Experiment 2)

A large sample of seed inoculated with Jumpstart was placed in a -20 °C freezer where it remained over the course of the experiment. At times 0, 1, 2, 3, 5, 7, 10, 15, 20, 40, 60, 80 and 100 days two gram samples were removed from the sample bag, covered and allowed to thaw overnight in a 2 °C cooler.

2.1.4 Freeze-thaw cycles (Experiment 3)

Method used for freezing is similar to experiment 1. The temperature was lowered 2 °C every hour until the final temperature of -8 °C was reached. This is an approximate average temperature (-8 °C) of soil during the winter at the 5 cm depth while under snow cover in Saskatoon, Saskatchewan (Archibold et al., 1996). The temperature was held at the final temperature for one hour and then allowed to thaw overnight in a 2 °C cooler. Treatments included four repetitive freeze-thaw cycles. After the first thaw the remaining three treatments were placed back into the bath the following day and the procedure was repeated until the fourth cycle was completed.

2.1.5 Survival of *P. bilaiae*

Standard plate count methods were used to enumerate colony forming units (CFU) of *P. bilaiae*.

All experiments included a control treatment that was plated immediately after inoculation and thus not exposed to freezing temperatures.

3.0 Results

Death curves of microbes generally fit a linear slope. When the log of the survival of the organism is graphed a decrease by one log is the same as 90% loss in viability. Therefore a one log drop in survival was used as the guideline for a loss in viability.

3.1 Low temperature survival

In contrast to a standard death curve, the death of *P. bilaiae* with decreasing temperature best fits a sigmoidal curve, r^2=0.886. In food microbiology it is argued that there is a certain degree of curvature to the death curves and that a straight line through the data is usually wrongly used for mathematical convenience (Legan and Peleg, 2001).

With a gradual decrease in temperature *P. bilaiae* survival was stable until -40 °C (Figure 3.1). There was a decrease in survival between -40 °C and -60 °C and then *P. bilaiae* viability stabilized again as the temperature continued to decrease to -196 °C.

The one log drop marker was not reached over the course of the experiment (Figure 3.1).

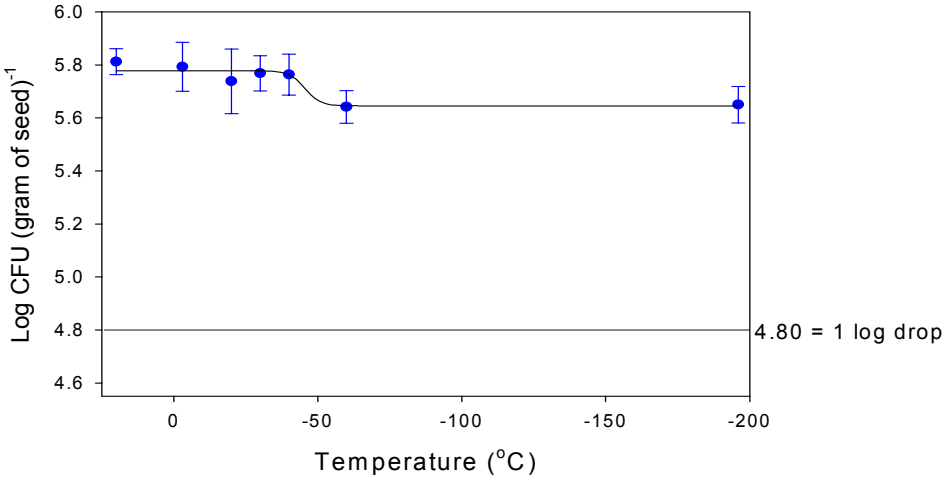

Figure 3.1. Survival of *P. bilaiae* with decreasing temperature

Even though Figure 3.1 shows a difference in *P. bilaiae* survival the statistical analysis indicates that there is no significant difference between any of the treatments and the control treatment. (Data not shown).

The graphs for percent recovery of *P. bilaiae* survival are not shown. At -60 °C there is still 69.8% survival of *P. bilaiae* and 65.7% at -196 °C.

3.2 Length of freeze

There is a steep decline in the death curve between time zero and one day (Figure 3.2). The exponential decay regression has an r^2 value of 0.805. The turning point is at three days, after when the slope begins a gradual decline. A one log drop in viability was not reached after 100 days when the temperature was held at -20 °C. A continuation of the slope of the line indicates that *P. bilaiae* could survive for 239 days (approximately 8 months) on seed until it is no longer considered viable or until a one log drop is reached.

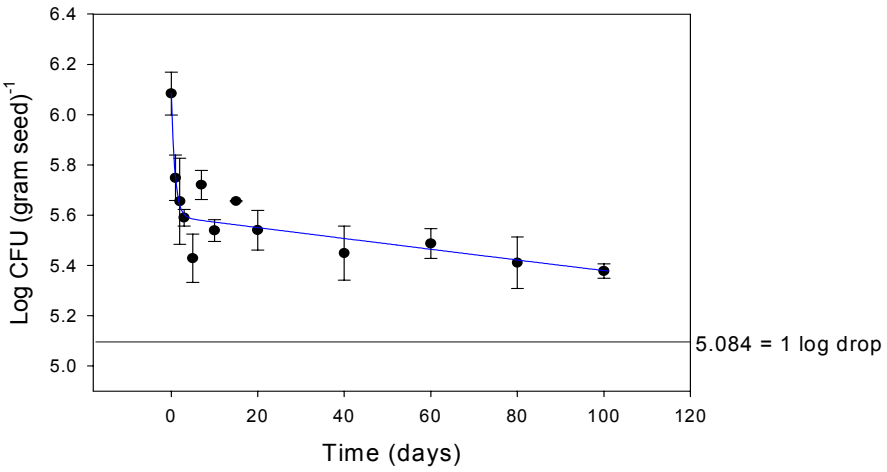

Figure 3.2 Survival of *P. bilaiae* with increasing time with temperature held at -20 °C

There is only a statistical difference between time zero and all other time points.

At day one only 33% of the *P. bilaiae* is viable and at day 100 there is only a slight further decline to 20%.

3.3 Freeze-thaw

Viability of *P. bilaiae* decreased between the control and the first freeze-thaw (Figure 3.3). Thereafter there was very little effect on *P. bilaiae* viability with increasing freeze-thaw cycles. The slope of the sigmoidal regression curve, r^2=0.929 does not reach the one log drop indicating that *P. bilaiae* is still viable after four freeze thaw cycles.

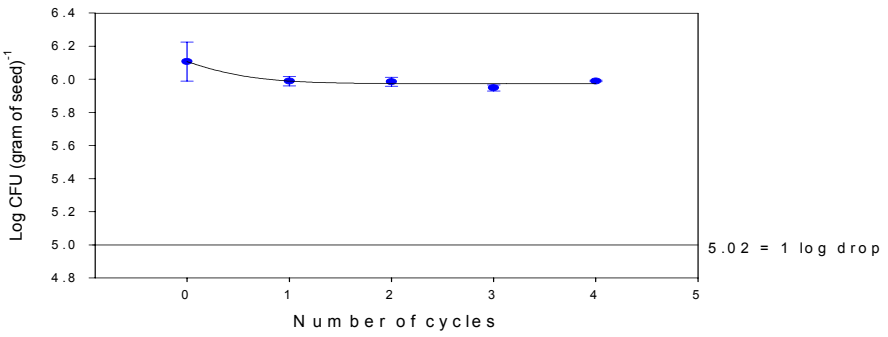

Figure 3.3. The effect of freeze-thaw cycles on survival of *P. bilaiae* when frozen to 8°C.

There is no statistical difference between any of the treatments and the control. The percent recovery of *P. bilaiae* drops to 81% after the first freeze-thaw cycle and increases again to 80% recovery with the fourth cycle.

4.0 Discussion

4.1 Survival of *P. bilaiae* at low temperatures

Fungal spores are very resistant to freezing temperatures where some have germinated successfully after prolonged exposure at -196 °C (Metting, 1993). Thus *P. bilaiae* would similarly be considered very resistant to freezing similarly; in relation to the control 65.7% of the *P. bilaiae* were still viable at -196 °C. More than 60% of the vegetative cells of *P. expansum* were able to grow after being frozen to -196 °C (Smith et al., 1986).

There is a critical point at -40 °C (Figure 3.1); any further decrease in temperature is associated with a drop in viability.

In relation to the low temperatures found in Saskatchewan *P. bilaiae* should be able to survive the winter and commence germination in the seasonally warmer spring temperatures.

4.2 Survival of *P. bilaiae* during exposure to an extended period of freezing temperatures

The significant amount of death found at the initiation of the experiment may be a response to cold shock with the sudden chilling of *P. bilaiae*. If *Escherichia coli* is growing in culture at 37 °C and is suddenly placed in a 5 °C environment 90% of the microbes will perish when otherwise the cold normally would not have the same effect (Ingraham and Ingraham, 1995). As well, rapid cooling of *Tetrahymena pyriformis* results in 99% loss of viability but when cooled slowly the organism can tolerate the freeze stress (Morris, 1987). It is probable that survival would have been greater between time zero and one day if the temperature had been gradually lowered since it was found in the previous experiment that *P. bilaiae* can withstand a temperature up to -40 °C with no large decrease in viability.

Loss of viability increases as time increases at freezing temperatures (Morris, 1987). Correspondingly, the viability of *P. bilaiae* continued to decrease as time increased.

These results indicate that *P. bilaiae* should survive the long, Saskatchewan winters with minimal loss.

4.3 Survival of *P. bilaiae* in response to freeze-thaw cycles.

There was no difference between *P. bilaiae* viability and increasing freeze-thaw cycles this may be due to the -8 °C temperature not being low enough to facilitate death. Figure 3.1 indicates that the temperature must reach -40 °C before there is noticeable death.

While in the stationary phase the filamentous fungi, *Geotrichum candidum* had drastic loss in viability with increasing freeze-thaw cycles (Thammavongs et al., 2000). After one cycle the *G. candidum* organism had a percent recovery just above 10 while with three cycles % survival was reduced to a value marginally above one. The high survival of *P. bilaiae* in relation to freeze-thaw cycles compared to the experiment by Thammovongs et al. (2000) is possibly due to the organisms being in different life stages. Park et al. (1997) determined that the yeast *Saccharomyces cerevisiae* had a higher freeze-thaw tolerance when in the lag phase (no growth period) compared to the stationary phase.

Penicillium bilaiae is able to survive the freeze-thaw cycles associated with Saskatchewan winters with no significant loss of viability.

5.0 Conclusion

All three experiments indicate that *P. bilaiae* should be able to successfully survive the winter. In nature, temperatures decrease slowly during a natural freeze stress (Ashworth and Kieft, 1989) thus the same drop in viability as was seen with the long term freeze experiment should not be an issue in the field.

6.0 References

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