Aphanomyces euteiches Root Rot of Alfalfa: Improving Storage of Cultures in the Lab for Long-Term Experimental Success

Olivia Steinmetz, Douglas Rouse, Samantha Rude, Alex Bilgri
University of Wisconsin - Madison

Introduction

Aphanomyces euteiches is the subject of continued study, both for plant breeders and research pathologists. Current methods of storage include sterile water tubes and/or sterile media plates (Figure 1), though both result in limited viability up to only a few months between transfers. There is also concern that continual sub-culturing may lead to a loss or change in virulence of that isolate. A straightforward alternative is to keep infected plant material and vermiculite in 50ml plastic centrifuge tubes at -20 degrees Celsius, for over one year at a time (Figure 2).

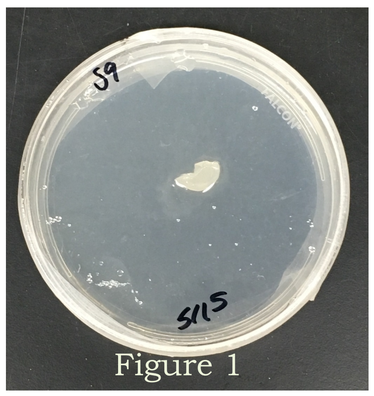

Figure 1

Figure 2

Figure 1: A typical potato dextrose agar (PDA) plate with *A. euteiches* growing on it, kept on the counter for one – two months.

Figure 2: A 50ml plastic centrifuge tube filled with vermiculite and *A. euteiches* infected plant roots from an inoculate flat. We have successfully kept these in the freezer for as little as one week and as long as 14 months so far.

First Steps

Flats of vermiculite were planted with 25 Vernal seeds per growing cell, and inoculated with a mycelial suspension of an isolate of interest: "S9." One to five weeks later, presence of oospores in plant roots was microscopically confirmed (Figure 3), and the material was scooped out of the growing cells and into 50ml plastic centrifuge tubes, which were then placed into a -20 degree Celsius freezer.

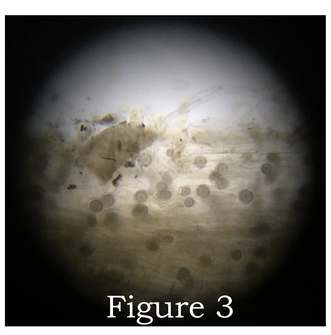
Figure 3

Figure 3: *A. euteiches* oospores in an infected alfalfa root two weeks post-inoculation, observed prior to freezing. Oospores are the survival spores, which survive winter freezing in nature.

Second Steps

The frozen tubes were removed between one week and 14 months later, and left at room temperature overnight before testing for viability. The thawed materials were scooped into a flat and 25 seeds of Vernal were planted in each growing cell. Seedlings were checked every few days, and symptomatic plants' roots were checked for oospores. With presence of oospores confirmed (Figure 4), roots were surface sterilized and placed on MBV. Once enough growth occurred, hyphal tips were then transferred onto PDA with streptomycin, and then finally pure cultures were obtained on normal PDA.

Figure 4: Oospores found in a two-week old alfalfa seedling grown in material that had been frozen for one week.

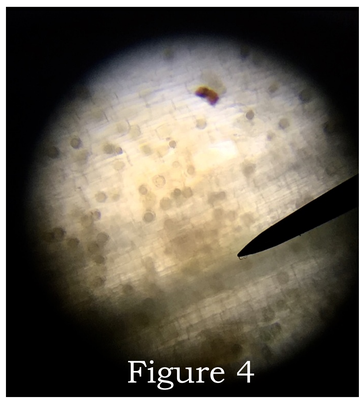
Figure 4

Third Steps

Once pure cultures of S9 were obtained from material that had been frozen for varying lengths, these plates were used to inoculate flats with a mycelial suspension to see if freezing had changed the S9 isolate in any way (Figure 5). We used two different industry cultivars "A" and "B," as well as WAHP-5, WAHP-1, and Vernal in this comparison.

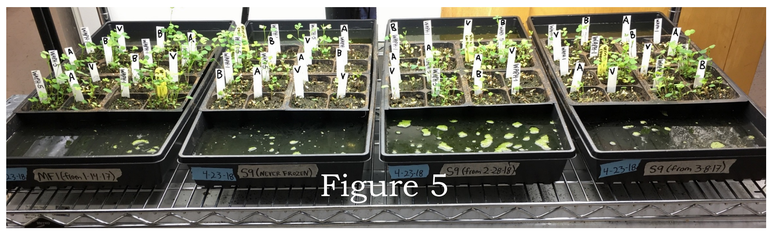

Figure 5

Figure 5: This is the setup for the S9 comparison, two weeks after planting. Flats left to right: inoculated with MFI that had been frozen for 14 months, inoculated with S9 from the counter that had never been frozen, inoculated with S9 frozen for one week, and inoculated with S9 frozen for one year.

Results

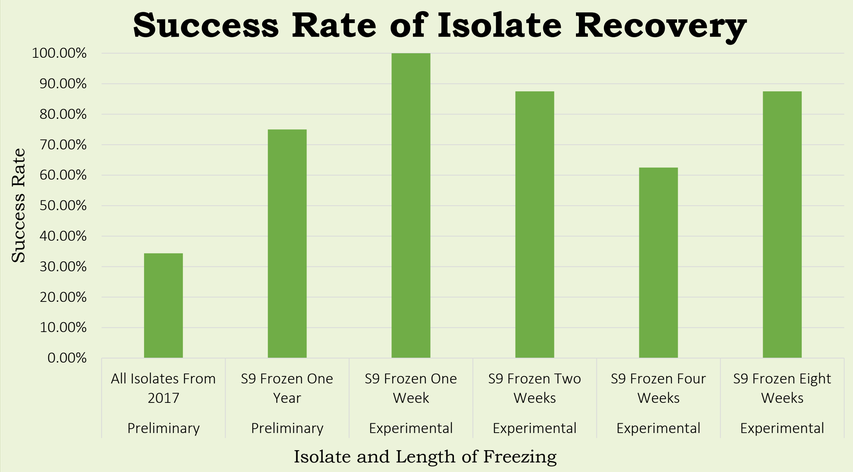

Figure 6: When the tubes from 2017 were taken out of the freezer, 11 of the 32 isolates were successfully baited onto a pure PDA culture. Four of those 32 were S9, and three of those were successfully recovered. Once new tubes were made and experimentation with freezing length began, eight out of eight from one week, seven out of eight from two weeks, five out of eight from four weeks, and seven out of eight from eight weeks were recovered.

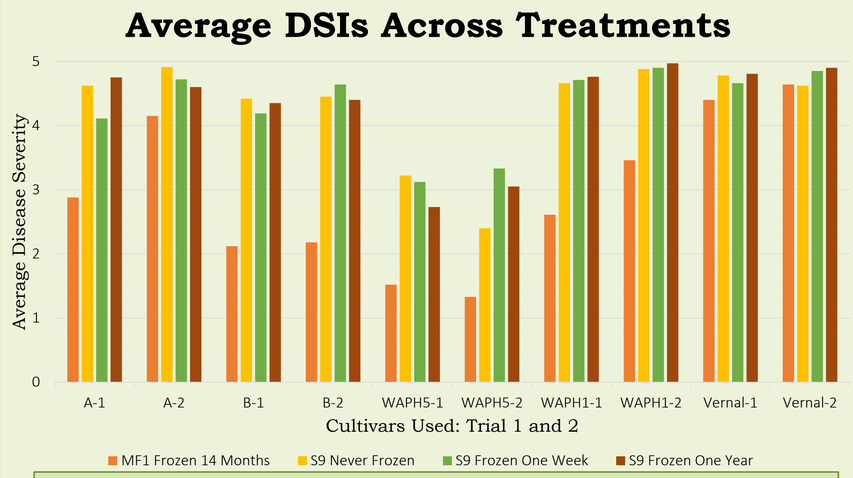

Figure 7: As expected, MF1 showed less severe disease than the three S9 isolates. Across the ten cultivars in both trials, S9 that had been frozen versus never frozen did not lead to a significant difference in disease severity.

Conclusions and Next Steps

Successfully recovering frozen isolates became easier once the protocol for making tubes was standardized (Figure 6); exactly how tubes were made in 2017 is unknown, as are any potential differences in preliminary isolate quality. Results of a one-way ANOVA test confirmed that there was not a statistically significant difference in the mean disease severities from the different S9 treatments within each cultivar (Figure 7). This provides confidence that storing isolates in a freezer is a viable long-term option.
Looking ahead, it would be wise to repeat this process with any remaining tubes from 2017 and 2014. The material used in the S9 comparisons was refrozen, and the possible effects of repeated freezing are also of interest.