**Phenotypic Changes and DNA Methylation Status in Cryopreserved Seeds of Rye**

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### Background
- **Conserving genetic diversity is one of the major tasks for seed banks all over the world.** The USDA National Laboratory for Genetic Resources Preservation (NLGRP) currently uses two long-term conservation methods for orthodox seeds, cold storage at -18°C (conventional storage) and in vapor phase of liquid nitrogen at -193°C (cryopreservation).
- **However, some concerns remain about the long-term effects of cryopreservation on seed viability and stability.**
- **Rye** (*Secale cereale*) was chosen for this experiment based on availability of 40 accessions stored for 25 years at NLGRP using both storage methods. In addition, results from this experiment should be applicable to other cereal crops.
- **This experiment measured germination, early seedling root growth, and field phenotypes on individuals from the 40 rye accessions to compare effects from both storage methods.** We also conducted an AFLP-based methylation analysis for two accessions from each storage treatment.

### Objectives
- **Determine phenotypic differences between seed stored by standard and cryopreservation methods.**
- **Determine if changes in methylation pattern are associated with preservation methods.**

### Methods

#### Field Experiment
- **Winter**
  - 2014 to 2015 in ADEIEC Fort Collins, CO
  - Two moisture levels within each growth habit
  - Two blocks in each moisture level
  - Two raps in each block
- **Spring**
  - 2014 to 2015 in ADEIEC Fort Collins, CO
  - Two moisture levels within each growth habit
  - Two blocks in each moisture level
  - Two raps in each block
- **Record heading date, earliness date, and plant height**
- **Measure five individual plants per plot**
- **DNA Methylation**
  - One spring accession (V108) and one winter accession (Orka) from each storage treatment was analyzed
  - Up to 10 individuals from each accession and each storage treatment were used
  - DNA extracted from 2-week old seedlings of leaf tissue grown in the greenhouse
  - Use methylation sensitive AFLP (RFLP/ AcclSB) method
  - Fifteen selective primer sets were used
  - Statistical analysis using Fisher's Exact Test with BH FDR correction in SAS

#### Seedlings
- **Seeds from both storage treatments of each accession were planted side-by-side**
- **Data collected from five randomly selected plants per plot**
- **Measure mature spike length and spike weight**
- **Fifteen heads in each plot**
- **Normal germination percentage, abnormal germination percentage, root and shoot dry weight, root length, root diameter, root volume and root projected area measured at NLGRP.**
- **Statistical analysis using mixed model in SAS.**

### Results and Discussion

#### Seeding Experiment

**Summary statistics**

**Fig. 1.** Mean values of accessions for seedling traits with significant differences between the two storage treatments. (a) Normal and abnormal germination percentage. (b) Root length and average root diameter of winter rye set. (c) Root length of spring rye set.

**Comparison between two storage treatments across all accessions**

**Fig. 2.** Field photos of abnormal rye plant. (a) Pink pigmented plant observed soon after emerging (b) Albino plant in spring rye field (c) Branched head at maturity (d) The spike with ergot on it (e) A winter plot after emerging in Oct. 2014 (f) The winter rye field looked like in the summer of 2015

**Comparison between two storage treatments**

**Table 1.** Comparison between two storage treatments for quantitative field traits.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cryopreservation</th>
<th>Conventional</th>
<th>Difference (cryo-standard)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter</td>
<td>154.37±0.35</td>
<td>153.36±0.35</td>
<td>-1.01±0.14</td>
<td>0.97</td>
</tr>
<tr>
<td>Spring</td>
<td>78.93±0.11</td>
<td>78.93±0.11</td>
<td>0.00±0.01</td>
<td>0.13</td>
</tr>
</tbody>
</table>

**Table 2.** Comparison between two storage treatments for seedling dry weight.

<table>
<thead>
<tr>
<th>Treats</th>
<th>BMI</th>
<th>Model</th>
<th>Difference (cryo-standard)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal germination</td>
<td>BMI</td>
<td>Standard method</td>
<td>Cryopreservation</td>
<td>Conventional</td>
</tr>
<tr>
<td>Abnormal germination</td>
<td>BMI</td>
<td>Standard method</td>
<td>Cryopreservation</td>
<td>Conventional</td>
</tr>
</tbody>
</table>

**Methylation Experiment**

**Fisher's Exact Test for differences in methylation status**

**Fig. 3.** Two rye accessions were analyzed separately for changes in methylation patterns between storage treatments. A total of 311 and 308 potentially methylated AFLP loci were scored among individuals from the spring and winter habit accessions, respectively.

The p-values for Fisher's Exact Test compare the proportion of differences in methylation status among individuals between the two storage treatments at each locus. The Benjamini-Hochberg False Discovery Rate procedure was used to correct for multiple testing. Panel (a) shows p-values for spring accession V108 and (b) shows p-values for winter accession Orka.

- Only a few loci have raw p-values significant at the p<0.05 level. However, after FDR adjustment, no loci were significantly different for methylation status between treatments.

### Summary
- The germination tests showed that, across accessions, the cryopreserved seeds maintained higher germination with less damage than conventionally stored seeds after 25 years. However, different accessions perform differently for germination.
- Root diameter and total root length for winter rye and root diameter for spring rye were statistically different between the two storage treatments. Seedlings from cryopreserved winter rye had greater total root length than seedlings from conventionally stored seeds. However, the differences in mean values for these traits were small.
- Among the traits measured in the field, spike length in the winter rye was statistically different before storage treatments. Again, the difference in mean values for this trait is small.
- The metaAFLP experiment found no evidence of change in methylation status attributable to storage treatment using FDR adjusted p-values. However, both the sample sizes for individuals within the heterogeneous accessions and the number of methylation sensitive loci surveyed were relatively small.
- We found no evidence of detrimental effects from cryopreservation compared to conventional storage.