

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.

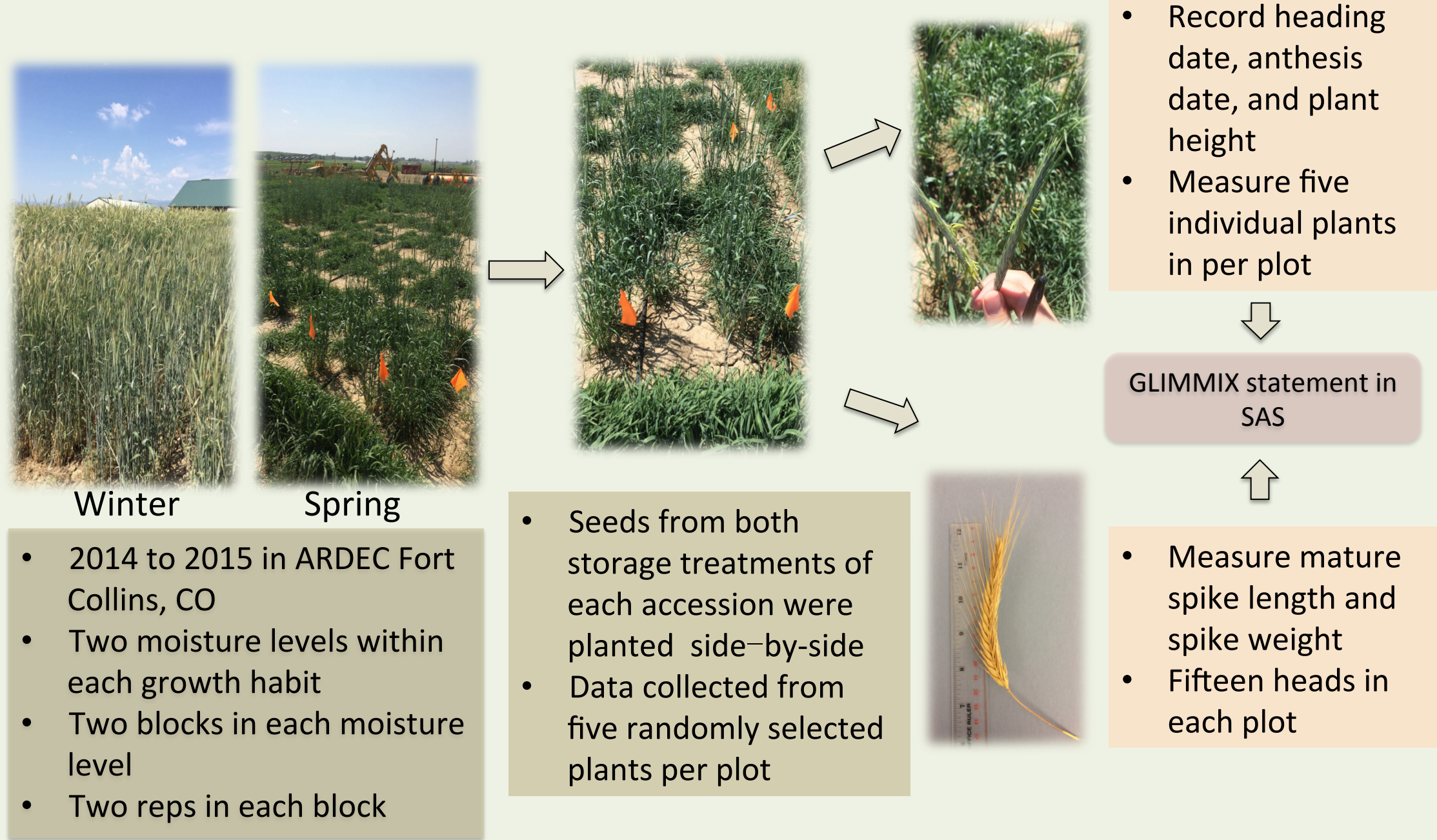
- This experiment measured germination, early seedling root growth, and field phenotypes on individuals from the 40 rye accessions to compare effects from both storage methods. We also conducted an AFLP-based methylation analysis for two accessions from each storage treatment.

Objectives

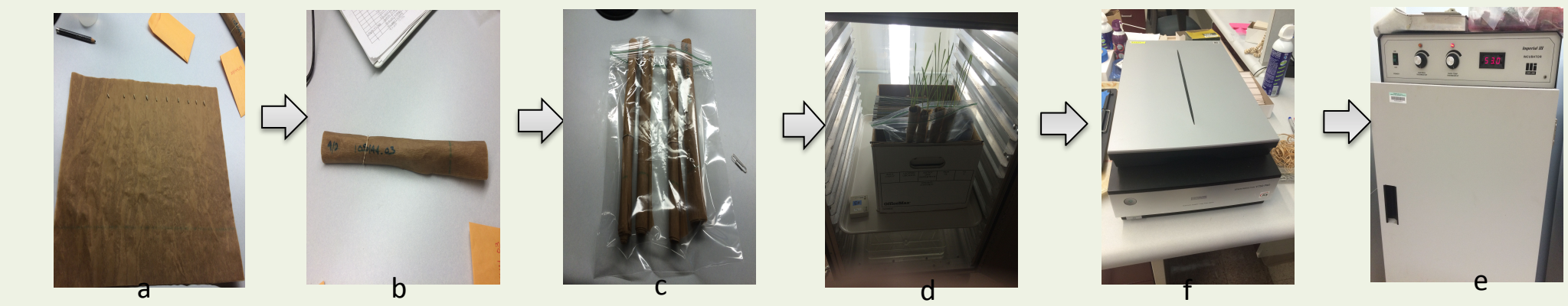
- Determine phenotypic differences between seed stored by standard and cryopreservation methods
- Determine if changes in methylation pattern are associated with preservation methods

Methods

Field Experiment

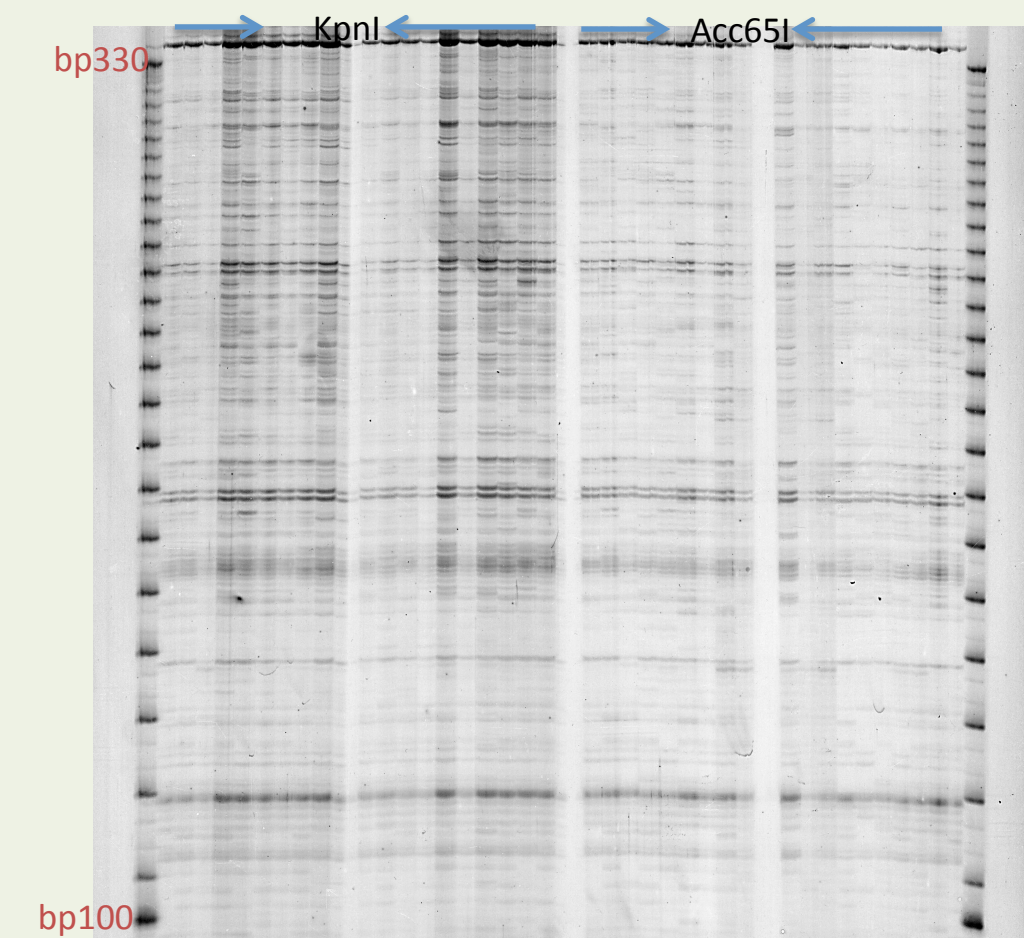


Seedlings



- 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 .

DNA Methylation



- One spring accession (V/108) and one winter accession (Omka) 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 seedling leaf tissue grown in the greenhouse
- Use methylation sensitive AFLP (KpnI/Acc65I) method
- Fifteen selective primer sets were used
- Statistical analysis using Fisher's Exact Test with BH FDR correction in SAS

Results and Discussion

Seedling Experiment

- Summary statistics

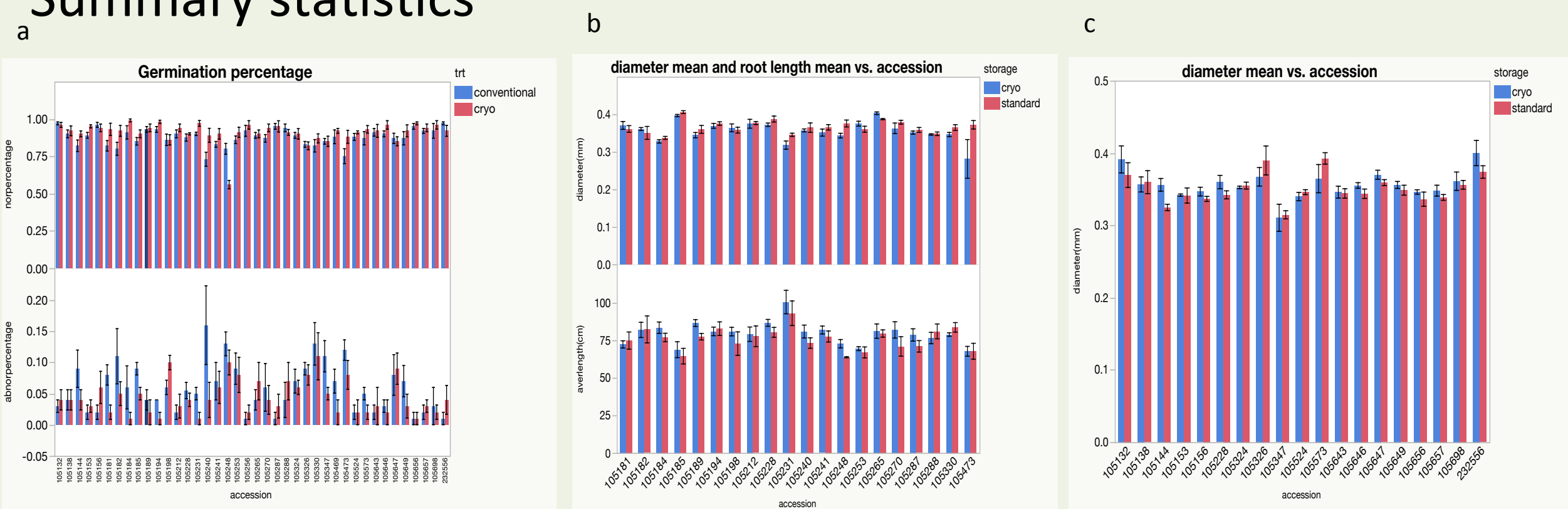


Fig. 1. Mean values of accessions for seedling traits with significant differences between the two storage treatments. (a) Normal and abnormal germination percentage. (b) Root length and average root diameter of winter rye set. (c) Average root diameter of spring rye set.

- Comparison between two storage treatment across all accessions

Table 1. Comparison between two storage treatments for germination and seedling dry weight

Traits	Cryopreservation	Standard method	Difference (cryo-standard)	P-value
Normal germination	0.91±0.0063	0.88±0.0063	0.031±0.0071	<.0001
Abnormal germination	0.044±0.0039	0.058±0.0039	-0.014±0.0044	0.0014
Seedling dry weight (g)	0.010±0.00020	0.011±0.00018	-0.00029±0.00025	0.30
Shoot dry weight (g)	0.0069±0.00016	0.0071±0.00014	-0.00025±0.00017	0.22

Table 2. Comparison between two storage treatments for seedling roots traits.

Traits	Habit	Cryopreservation	Standard Method	Difference (cryo-standard)	P-value
Root length (cm)	Spring	83.31±1.48	86.09±1.56	-2.77±1.82	0.13
	Winter	79.52±1.77	76±1.77	3.64±1.51	0.019
Project area (cm ²)	Spring	3.00±0.049	3.02±0.052	-0.038±0.063	0.55
	Winter	2.83±0.047	2.783±0.047	0.043±0.058	0.46
Surface area (cm ²)	Spring	9.38±0.15	9.5±0.16	-0.12±0.2	0.55
	Winter	8.88±0.2	8.74±0.2	0.14±0.18	0.46
Average diameter (mm)	Spring	0.36±0.0032	0.35±0.0033	0.006±0.003	0.05
	Winter	0.36±0.0035	0.37±0.0035	-0.011±0.004	0.007
Root volume (cm ³)	Spring	0.085±0.0016	0.085±0.0017	0.00059±0.002	0.77
	Winter	0.08±0.002	0.081±0.002	-0.00092±0.002	0.64

- Cryopreserved seeds had a higher normal germination percentage and a lower abnormal germination percentage than those from conventional storage (normal germination percentage $p < .0001$ and abnormal germination percentage $p = 0.0014$).
- Both spring and winter habit rye seedlings had significant differences for average root diameters. However, for spring rye, the average root diameter was larger in seedlings from cryopreserved seeds than from conventionally stored seeds while in winter habit accessions, seedlings from conventionally stored seeds had larger root diameter.
- There was a significant difference for total root length in seedlings of winter accessions. Seedlings from cryopreserved seeds had longer root lengths than those from conventional storage.

Field Experiment

- Field observations



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

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 between storage treatments. Again, the difference in mean values for this trait is small.
- The metAFLP 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.

- Comparison between two storage treatments

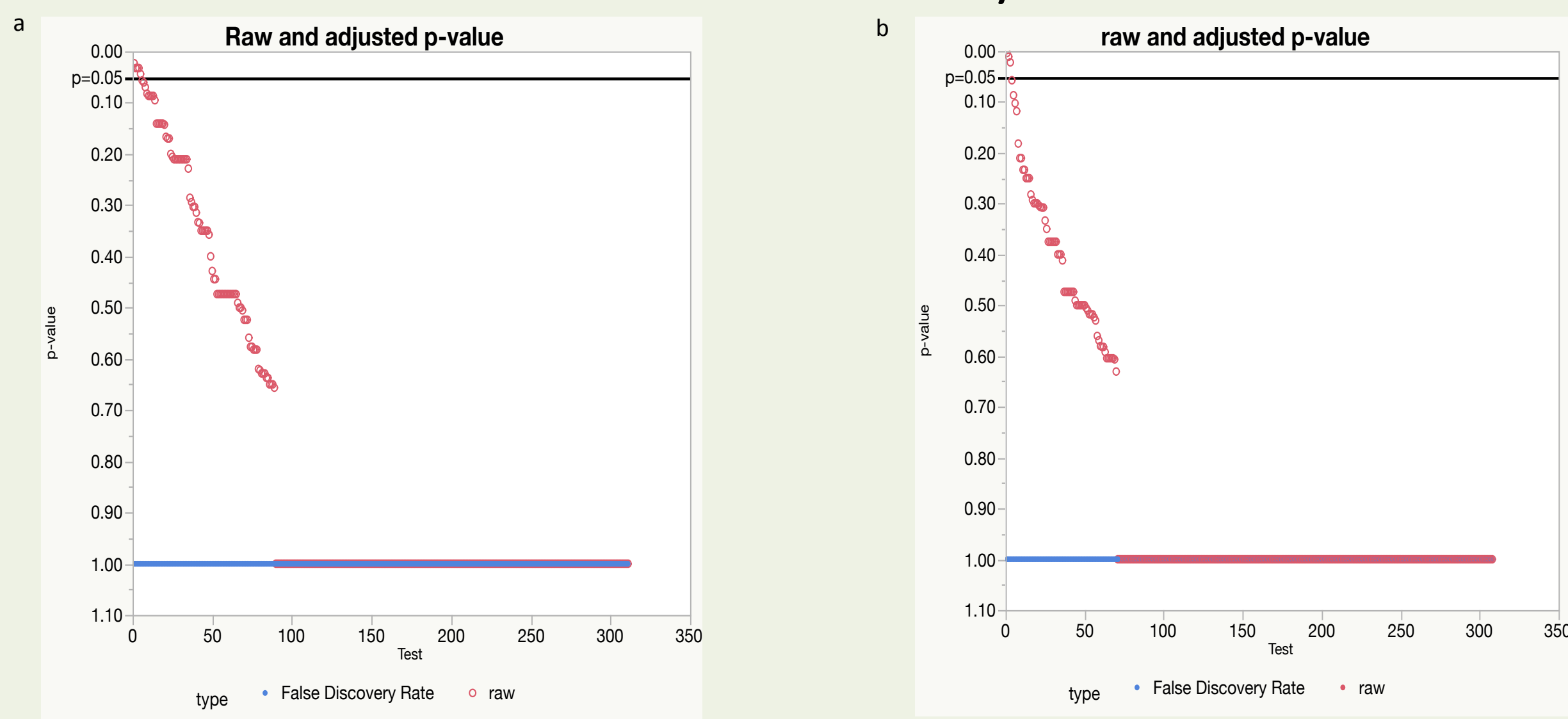
Table 3. Comparison between two storage treatments for quantitative field traits

Traits	Treatments	Cryopreservation	Conventional Method	Difference (cryo-standard)	P-value
Anthesis date	Winter	153.27±0.55	153.39±0.55	-0.13±0.14	0.37
	Spring irrigated	76.029±0.88	76.056±0.88	-0.027±0.45	0.95
	Spring rainfed	78.93±1.13	78.38±1.11	0.55±0.87	0.53
Plant height (cm)	Winter	172.22±0.54	172.73±0.54	-0.51±0.25	0.43
	Spring irrigated	111±3.13	112.35±3.16	-1.35±1.94	0.49
	Spring rainfed	104.96±2.20	103.85±2.20	1.11±1.42	0.44
Spike length (cm)	Winter	12.27±0.26	12.44±0.26	-0.18±0.087	0.045
	Spring irrigated	11.91±0.21	12.11±0.21	-0.20±0.19	0.29
	Spring rainfed	12.22±0.24	12.17±0.24	0.050±0.16	0.76
Spike weight (g)	Winter	1.76±0.058	1.78±0.058	-0.029±0.031	0.35
	Spring irrigated	1.14±0.060	1.19±0.060	-0.052±0.052	0.32
	Spring rainfed	1.15±0.061	1.13±0.061	0.016±0.054	0.78
Lodging	Winter	4.67±0.52	4.81±0.52	-0.14±0.10	0.18
	Spring irrigated	2.37±0.26	2.21±0.26	0.16±0.19	0.41
	Spring rainfed	2.067±0.17	1.91±0.17	0.15±0.13	0.25

- Of the five field phenotypes measured, only spike length in the the winter rye accessions was statistically different at $p = 0.05$ between the two storage treatments.
- No irrigated treatment was used in the winter rye experiment due to unusually high and frequent precipitation that occurred in the early spring through the grain filling period in 2015.
- Some accessions in the spring planted rye exhibited delayed and incomplete transition from vegetative growth to the reproductive phase suggesting they may have had an unmet vernalization requirement (and could be misclassified as strictly spring types). Therefore, the spring rye plants had larger standard error for both storage treatments for anthesis date and plant height.

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 V/108 and (b) shows p -values for winter accession Omka.
- 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.