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Improving Cotton Cool Test Result Consistency Within and Among Laboratories

November 22, 2023

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Introduction:

The cotton cool test is an established vigor test in the Seed Vigor Testing Handbook (Association of Official Seed Analysts, 2009) that has been used in seed testing for over 60 years. Typically, seeds are placed in an inner chamber (a plastic bag) enclosing vertically positioned rolled towels or acrylic "crisper" boxes with horizontally positioned rolled towels. If the temperature inside the inner chamber begins to exceed 18°C as set by the outer chamber, then the seeds accumulate more growing degree days (heat units) in the seven-day test duration. Standardization of an outer chamber that maintains 18°C ±0.5°C has been achieved; however, temperature control in the inner chamber has yet to be refined and could be a significant source of error among laboratories. Evaluation of seedlings states that a normal is considered any seedling having a combined hypocotyl and root length of 4 cm or greater, emphasizing the importance of temperature maintenance for consistent growth. Inconsistencies in cotton cool test results have been known for years, and this study's objectives are: 1) to better understand "seed" and "experimental" sources of variation; 2) to compare the "outer" and "inner" chamber model used for Accelerated Aging test for relevance to the Cool Test standardization; and 3) implement the measurement of Growing Degree Days (GDDs) as an additional tool to evaluate the uniformity of laboratory inner and outer chambers regimes during the seven day 18°C test period and compare accumulated GDDs to average germination result responses. The overall study goal is to minimize the experimental error contribution to test result variation among laboratories to provide the cotton seed end user more consistent quality data.

Materials and Methods:

Two cotton seed lots were obtained in the spring of 2023 for this study. Study 1 consisted of four shipments to three laboratories for testing. Each laboratory received four separate shipments of 16 letter-coded replicates consisting of 60 seed (eight replicates of two lots). These shipments were sent at time 0, time X2, time X3 and time X4, with the next subsequent shipment sent following completion of previous shipment. Once planted, a HarvestGuard™ (a data logger that measures temperature and accumulated heat units during the test, depicted in Figure 1) was reset to zero GDDs and placed inside the "inner" chamber with the replicates. After seven days at 18°C, each laboratory evaluated and recorded strong normal (≥4 cm), short sprouts (<4 and >2 cm), as well as abnormal, dead, firm seeds, and the accumulated GDDs on the data sheet. Once all data were received back from each laboratory, it was coded for analysis and statistics were analyzed through R Studio Version 4.0.3 using a split-split plot design. An ANOVA model was used to calculate means and variance with lab as the main plot, lot as the subplot and date as the sub-subplot. Significance was declared at $P < 0.05$ for each response variable. Least square means and co-efficient of variation are reported for each response variable in the tables below. A Spearman ranked correlation was used to determine if correlation was present between GDDs and response variables. All laboratories completed the four shipment sets within 6 months with one laboratory completing within 6 weeks.

Results:
The main objective of this study was to determine whether the cotton cool test method was repeatable and reproducible within and among three seed laboratories. An analysis of data showed that over all lots and dates, variation among replicates was not significant (F=0.65, P =0.7098) for ≥4 cm normals, indicating the test is repeatable within a laboratory (Table 1). Variation among laboratories (lab) was not significant for ≥4 cm normals (F=3.58, P=0.0555) but significant for <4 cm normals (F=14.63, P=0.0004) and total normals (F=5.82, P=0.0144), which indicates reproducibility for ≥4cm normals but variation in reproducibility for <4 cm normals and total normals among labs.

There was a significant difference between the two seed lots regarding ≥4 cm results (F=0.237.4, P =6.41E-13), as well as <4 cm results (F=221.01.4, P =1.28E-12), but not in total normals (F=2.15.4, P =0.1571). These results are expected as the two seed lots were selected based on different original vigor results. Additionally, these results highlight the importance of vigor testing along with germination testing as the differences between the two lots would not have been observed in a germination test with a total normal count. By completing a cool test, the lower vigor of lot 1 compared to lot 2 can be observed by the ≥4 cm and <4 cm count.

The effect of date was significant for all response variables except firm seed. As all four testing dates were completed within 6 months, this variation is another indication of a lack of reproducibility among laboratories. Lab by date interaction and date by lot interaction also showed significance, reinforcing the lack of reproducibility within laboratories testing the same seed lots at different dates.

This data supports a lack of reproducibility among seed laboratories performing the cotton cool test on the same seed lots at different dates, which has been a longstanding concern of the cool test (Tolliver, Savoy and Drummond, 1997.).

Table 1. F value and probablity for Cotton Cool Test germination responses for eight replicates of two seed lots averaged across four testing dates and three laboratories based on a split split plot analysis

	4cm+ Normal		< 4cm Normal		Total Normal		Abnormal		Dead		Firm	
Response Factors	F value	Pr(>F)[1]	F value	Pr(>F)[1]	F value	Pr(>F)[1]	F value	Pr(>F)[1]	F value	Pr(>F)[1]	F value	Pr(>F)[1]
Replicate	0.65	0.7098	0.69	0.6808	0.45	0.8539	0.83	0.5793	1.45	0.2621	0.57	0.7697
Lab	3.58	0.0555 .	14.63	0.0004 ***	5.82	0.0144 *	22.20	4.55E-05 ***	15.40	0.0003 ***	19.26	9.56E-05 ***
Lot	237.40	6.41E-13 ***	221.01	1.28E-12 ***	2.15	0.1571	1.97	0.1746	17.58	0.0004 ***	15.59	0.0007 ***
LAB:LOT	4.17	0.0298 *	2.95	0.0740 .	0.77	0.4775	1.13	0.3405	0.78	0.4715	2.23	0.1327
DATE	12.45	3.48E-07 ***	11.76	7.66E-07 ***	12.87	2.17E-07 ***	8.09	5.73E-05 ***	6.59	0.0004 ***	0.78	0.5099
DATE:LAB	5.13	9.54E-05 ***	2.44	0.0288 *	4.41	0.0004 ***	6.63	4.11E-06 ***	2.60	0.0207 *	0.82	0.5570
DATE:LOT	6.27	0.0005 ***	6.23	0.0006 ***	2.90	0.0376 *	1.93	0.1275	0.58	0.6325	2.78	0.0439 *
DATE:LAB:LOT	0.38	0.8930	1.23	0.2972	1.39	0.2251	0.73	0.6227	1.57	0.1625	0.80	0.5681

[1]Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Average cool test responses from the three laboratories across the two seed lots and four testing dates are reported in Table 2. Laboratory and date were coded using "Lab.Date" respectively, and ≥4 cm normal, <4 cm normal, total normal, abnormal seedlings, dead seed and firm seed were statistically analyzed. Growing degree days for each Lab.Date code was reported but was not statistically evaluated due to lack of degrees of freedom.

Mean ≥4 cm normal seedling percentages ranged from 54 to 73%, whereas responses of 73 and 71% significantly differed from the lowest responses, 62 and 54%. Much of the variation in ≥4 cm normals can be explained from the data with <4 cm normal (10 to 28%) across laboratories and dates. Considerable variation exists with the <4 cm normal with a CV% at 23.9%. By adding the ≥4 cm and <4 cm normals to obtain the total normals, considerable variation is eliminated (Table 2) with only the 93% total normal response being significantly different from the other responses (86-80%), with 80% as the lowest total normals.

Spearman rank correlation between cotton cool test ≥4cm normals and growing degree days was significant (r = 0.16986, P = 0.0185). This indicates that accumulating more GDD in the same 7-day period leads to more >4 cm normals.

Table 2. Average Cotton Cool Test germination responses for four replicates of two seed lots averaged across four testing dates and three laboratories based on a split split plot analysis.

Lab:Date	>4cm Normal		< 4cm Normal		Total Normal		Abnormal		Dead		Firm		Growing Degree Days
	%												
3:03	73	a	10	d	83	bc	13	a	4	bc	0	b	39
3:04	71	ab	12	cd	83	bc	14	a	2	c	0	b	36
1:01	70	abc	23	ab	93	a	3	c	4	bc	0	b	33
1:03	68	abc	18	bcd	86	b	6	bc	9	a	0	b	29
3:01	65	abc	18	bc	83	bc	14	a	2	c	0	b	19
3:02	65	abc	19	bc	83	bc	12	a	4	bc	0	ab	30
1:04	65	abc	21	ab	86	b	11	ab	4	bc	0	b	30
2:01	65	abc	21	ab	86	b	11	a	2	c	2	ab	24
2:03	65	abc	16	bcd	81	bc	14	a	4	bc	2	a	28
2:04	63	bc	20	bc	82	bc	13	a	4	bc	1	ab	21
2:02	62	cd	18	bcd	80	c	15	a	4	bc	1	ab	24
1:02	54	d	28	a	82	bc	12	a	6	ab	1	ab	34
CV (%)	7.4		23.9		4.0		31.5		38.6		118.2		
LSD	8.7		7.6		5.7		5.0		3.6		1.7		

Discussion:

This study revealed good repeatability among the three laboratories for ≥4 cm normal. Reproducibility issues were indicated by significant differences of <4 cm and total normals among labs and between date of test. “Outlier” results did occur, which may be due to experimental error related to temperature fluctuations. However, correlation between GDD and ≥4 cm results indicate that temperature differences impact vigor testing results within and among laboratories. Maintaining outer chamber temperature within ± 0.5C AOSA Vigor Testing Handbook guidelines is imperative and use of NIST temperature probes to calibrate and monitor outer chambers is required to assure all components of the chamber are functioning properly (one laboratory reported that one of the two circulation fans quit working during one 7-day period). Additional variation was noted with inner chamber temperature rising above outer chamber temperature due to temperature equilibration restrictions between the two chambers. Use of the HarvestGuard™ GDD monitors created some interest from laboratories in better understanding if GDD monitoring could be useful to the Cotton Cool Test. The hypothesis for usage of GDD monitoring is that variations in temperature in both

inner and outer chambers could be mediated if the laboratory could end the cool test using the theoretical GDDs of 30.8 [(64.4F, 18C) minus the cotton base growth temperature of 60F = 4.4 GDDs/24 hours times 7 days = 30.8 GDDs]. The inner chamber prototype shown in Figure 1 has a HarvestGuard™ recorder within the inner chamber regime. In theory, if laboratories could remove cool tests at 30 to 31 GDDs and evaluate for ≥4 cm normal seedlings, repeatability and reproducibility would improve among and within laboratories. Two of the three laboratories were using "TidbiT®" temperature monitoring sensors manufactured by Onset®. A comparison of the GDD values between the OnSet® "Pednant®" (newer version of TidbiT®) sensor and the HarvestGuard™ sensor is presented in Table 3. The two monitoring systems did not significantly differ (t-critical = 1.49, P = 0.17) for total GDDs.

Further studies on the cotton cool test will be expanded to at least five laboratories and incorporate accumulated GDD measurement (theoretical or calculated completion GDDs would be 30.8 GDDs) as a test termination criterion compared to the current termination criteria of seven days or 168 hours. Both HarvestGuard™ and Onset® dataloggers would be compared. The hypothesis is that 30.8 or 31 GDDs could replace the test termination criteria of 168 hours by either shortening the time or lengthening the test completion time caused by the variability of outer and inner chamber temperature.

Figure 1. From left to right: Inner chamber prototype with HarvestGuard™, close-up of HarvestGuard™ showing Low/High temperatures, GDUs and an Onset® Pendant®. (The abbreviation DDH use by Avatel™ means Degree Day Hours. Avatel™ uses the standard methods for calculating degree-days required by most current phenology models.)

Table 3. Comparison (t-test) of growing degree day calculations from two temperature monitor systems across 5 replications in 5 chambers in a corn (Zea mays) 50F cold test method.

Chambers	Growing Degree Days	
	HarvestGuard™	Onset® Pendant®
1	138	143
2	145	148
3	143	147
4	148	148
5	147	152

Literature Cited:

Association of Official Seed Analysts Seed Vigor Test Committee. *Seed Vigor Testing Handbook : Contribution No. 32 to the Handbook on Seed Testing*. Association of Official Seed Analysts 2002.

B.R. Savoy. Cool Germination Test: Principles and Applications in Cotton. https://www.jstor.org/stable/23433226.

J. Tolliver, B.R. Savoy and E.A. Drummond, 1997. Cool Germination Test on Cotton Variability Between Seed-Testing Laboratories. National Cotton Council, *Proceedings of the Beltwide Cotton Conference*, 1: 442-443.

K.A. Fiedler, A.L. Patin and T.J. Gutormson, 2007. "Cotton Cool Germination Study Group 2006-2007," *Seed Technology Newsletter*, 81.

K. Edmisten and G. Collins, 2022. Cotton Seed Quality and Planting Decisions. *NC State Extension Publications*, https://content.ces.ncsu.edu/cotton-information/cotton-seed-quality-and-planting-decisions.

M.I El Hawray and A.M. El Galfy, 2010. Identification of Low Temperature Requirements for Cool Germination Testing For Different Egyptian Cotton *Gossypium barbadense L.* Varieties. *Journal of Plant Production*, 1. https://jpp.journals.ekb.eg/article_86371_92942074fdfe9eae11c1fd0ce738b509.pdf.

Y. Bolek, 2006. Predicting Cotton Seedling Emergence for Cold Tolerance: *Gossypium barbadense*. *Journal of Agronomy*, 5: 461-465.

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