Effect of Temperature and Prechilling treatment on Hemp (*Cannabis sativa*) Germination

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INTRODUCTION

Since the 2018 Farm Bill legislation, hemp has become an important cash crop, which opened the door for conducting a wide range of scientific research and crop improvement (1). The total licensed hemp acres across 34 states in 2019 was 511,442. However, this number dropped by 9% in 2020 (2). In November 2020, there were 27,434 acres of outdoor hemp registered in Oregon (3).

Using high quality seeds is the corner stone of any successful production program (4). Oregon State University has seed certification program for hemp in place, which requires inspecting the crop at different stages of development in the greenhouse and in the field. In addition, seed quality is tested after harvest, for purity, viability, and PCR gender testing before issuing the blue tags (5).

New hemp varieties have been continuously developed since the Farm Bill Legislation in 2018. The current germination recommendation need to be validated and/or updated to make sure that it achieves maximum potential germination for a wide range of varieties. Furthermore, measuring the repeatability of the germination test results among labs is important to attain uniformity in seed testing.

There are two main problems in germinating hemp seeds, firstly, hemp seeds have various levels of dormancy, some are short-lived (1), and others are deeper. Recently, several new varieties have been developed by some seed companies and were sent to OSU Seed Lab for testing. It was observed that some of which possess deep dormancy, not only in freshly harvested seeds, but also in stored seeds. Newly developed triploid varieties that has been stored for 3-6 months or more were found to possess dormancy ranging from 26 to 41% at the end of the standard germination test (Personal communication). Such seeds were found to be viable when tested by the TZ test. The current AOSA Rules for germinating hemp seeds do not require prechilling treatment (AOSA Rules vol. 1, Table 6A) for breaking dormancy. Secondly, during germination at 20-30°C, medium to low quality hemp samples develop extensive fungal infection. In some cases, many seedlings were dead at the final count (7 days). It was therefore hypothesized that germination temperature at 20-30°C provides an optimal warm environment for fungal growth; and at 15-25°C, the growth of fungus may slow down.

The overall objective of the study was to evaluate and establish conditions that achieve maximum germination potential in hemp seeds. The specific objectives were to: 1) Measure the effectiveness of prechilling treatment on breaking the dormancy of six hemp varieties and the possibility of adding it to the AOSA Rules, if proven effective; 2) Compare germination temperature of 15-25°C to the current AOSA 20-30°C with and without prechilling, and the possibility of adding 15-25°C as an alternating temperature regime to the AOSA Rules; and 3) Measure the variability among six labs when the same study protocol was followed.

MATERIALS AND METHODS

Seed Materials

Six hemp samples that represent different qualities and levels of dormancy were used in the study. Samples were produced in summer of 2020.

National Referee

A national referee was conducted to measure the effectiveness of prechilling treatment on breaking the dormancy and the alternating 15-25°C effect on germination compared to 20-30°C. **S**ix private and state seed labs from USA and Canada that have experiences in testing hemp seeds were selected to participate in the national referee to ensure the reliability of the results and the objectivity of the conclusions attained.

Study Protocol

Six blind samples including 1600 seeds each were sent to the participating labs. Four replicates of 100 seeds each were planted according to the following four germination methods:

1. **Control method:** The **current AOSA standard germination method** (AOSA Rules Table 6A) was used as a baseline for comparison. No prechill treatment, seeds were germinated at 20-30°C. The 1st count was in day 3 and the final count in day 7.
2. **Current AOSA method with 7d prechill at 10°C**. The objective was to determine whether the prechilling treatment might overcome any dormancy that may be exist.
3. **Germination at 15-25°C without prechill**.
4. **Germinated at 15-25°C with 7d prechill at 10°C**.

Media: Paper toweling (T), moistened with water, either as folded or rolled towel test in horizontal or vertical position was used.

Tetrazolium Test: In all methods, at the end of the germination tests, the ungerminated seeds were tested by TZ to determine whether the seeds were dead or dormant.

Summary of study protocol: Six varieties, six participating labs, and four combinations of temperatures and prechill treatments.

Temperature	Pre-chill (10°C, 7d)
20-30°C (AOSA)	No (baseline method/control)
	Yes
15-25°C	No
	Yes

Germination Procedures

- Four-100 seed replications were planted from each of the six samples for each of the above four treatments.
- The first count was done at day 3 to minimize the secondary infection, if any, and were discarded. Final count at day 7. Normal seedlings was counted and recorded.
- Data sheets were prepared for each lab for uniformity in recording.

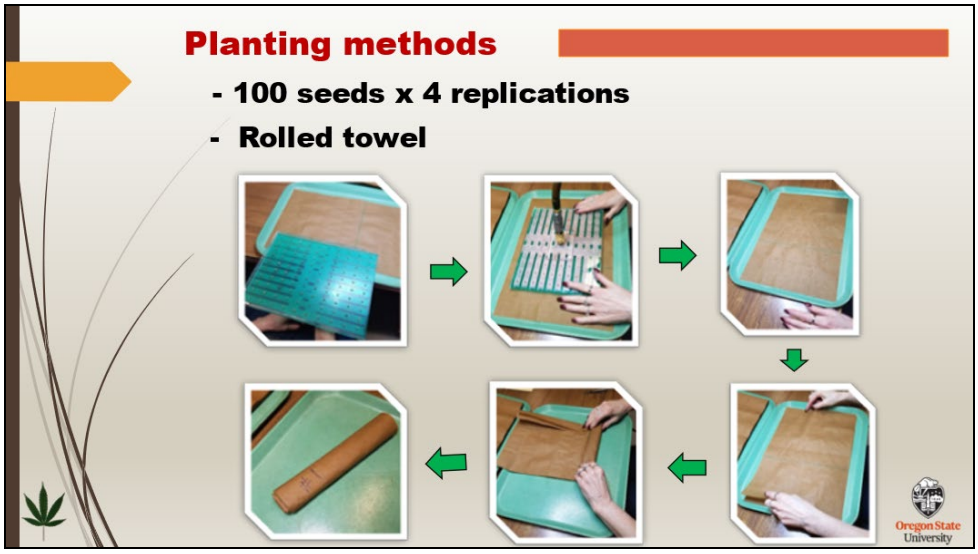

Figure 1. Planting method of hemp seeds. Four replications of 100 seeds each on moistened germination paper using rolled towels.

- At the end of the germination test, TZ tests were conducted on all firm ungerminated seeds. Seeds were soaked overnight (16 hours) in water, and then were cut longitudinally so that the TZ solution stains the internal tissues of seeds. Seeds were then soaked in 1% tetrazolium solution at 30°C overnight, and then evaluated and classified into viable or nonviable seeds. Viable seeds were evenly stained red or ¼ or less of the tip of the radicle unstained. The embryo may need to be separated from the seed coat to see the staining pattern clearly.

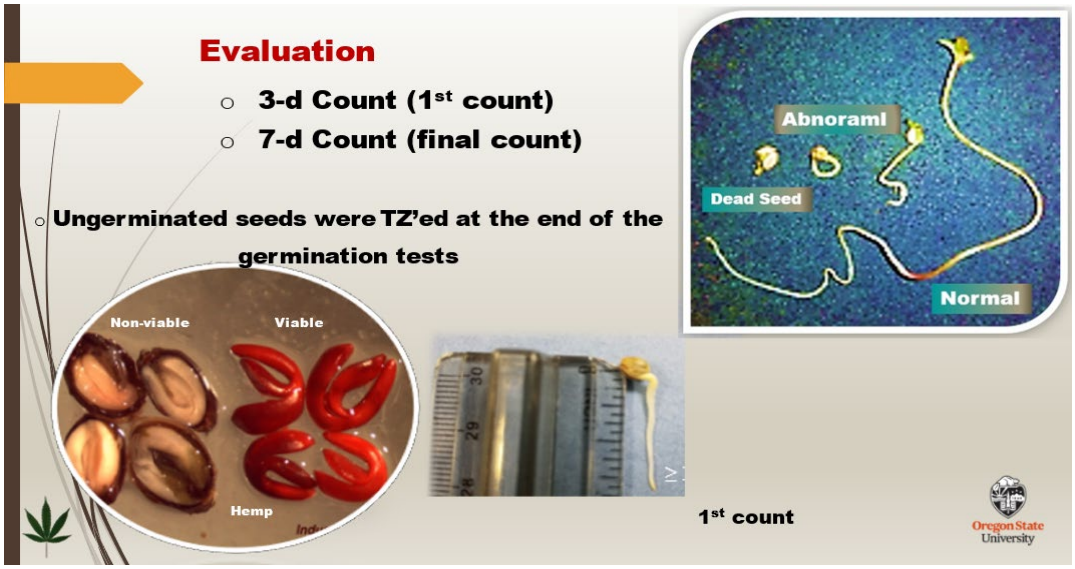

Figure 2. Evaluation of germination and TZ tests. At 3-day count, seedling developed a normal root system approximately 1.5 cm with root hair. Normal seedlings have well developed shoot and root systems. Abnormal seedlings have deformed shoot and/or root systems. For TZ test, seeds with embryonic axes uniformly stained red were viable; unstained, non-viable.

Experimental Design and Data Analysis

The experimental design used was three-factor completely randomized design (CRD), with four replications of each treatment. The factors were varieties, method of germination (temperature/prechilling), and participating labs. The data were collected and were subjected to analysis of variance (ANOVA) to measure the effect of the different treatments on the final germination/viability and dormancy results. Mean separation test (LSD) performed at 0.05 probability level whenever the effect of the treatments was significant. Standard error was calculated to estimate the variability across samples and treatments. The statistical package MSTAT was used in the statistical analysis.

RESULTS AND DISCUSSION

The ANOVA indicated that varieties, method of germinations (germination and prechilling treatments), and laboratories affected the germination and dormancy of the six hemp varieties significantly at $P \leq 0.05$ (Table 1). Some hemp varieties had higher germination (var. 5 and 6) than others (var. 2 and 3); some varieties had deeper dormancy (var 2 and 3) than others (5 and 6); some varieties responded to the prechilling better than others, and different patterns of germination at 20°C-30°C and 15-25°C among varieties were recorded (Figs. 3 and 6). In addition, the interactions among these factors were significantly different (at $P \leq 0.001$), indicating that the responses of varieties to the germination regimes were not similar among labs (Table 1).

Table 1. Analysis of variance (ANOVA) for the effects of four temperature and prechilling combinations on germination of six hemp varieties tested in six different laboratories.

Source of variation	df	3-d count	7-d count	Dormant seeds	Viable by TZ
		Mean Square (Prob0.05)			
Varieties (V)	5	37480.2***	17084.1***	24441.4***	22933.1***
Method of germination (M)	3	76240.9***	9515.2***	9935.7***	9974.0***
Labs (L)	5	18883.0***	3411.7***	1291.5***	142.1***
Interaction					
V x M	15	3932.9***	2644.8***	2649.1***	196.3***
V x L	25	1146.3***	302.6***	399.7***	30.9***
M x L	15	2176.6***	794.5***	844.9***	53.1***
V x M x L	75	805.1***	232.1***	210.9***	16.0***

*** Highly significant at 0.001 level of probability.

First Germination Count at Day 3

Prechilling treatment improved the germination of first count at day 3 in both 20-30°C and 15-25°C at 60 and 56%, respectively, compared to non-chilled seeds at 39% and 10%, respectively (Fig. 3A). The prechilling at 10°C for 7 days affected seed germination in two ways, 1) break dormancy, and 2) work as hydropriming, which activate the germination enzymes, so that once moved to the warmer temperature, seedlings grow faster and more uniform than non-chilled seeds. The non-chilled seeds germinated better at 20-30°C than 15-25°C (Fig. 3A), probably because the warmer temperature helped seedlings to grow faster.

Varieties 2 and 3 have deeper dormancy than varieties 1, 4, 5, and 6 (Fig. 3B). Varieties 4, 5 and 6 had the least dormant seeds and the highest first germination count at 53%, 55% and 62%, respectively, whereas varieties 2 and 3 had only 17% (Fig. 3B). The average first count of germination over varieties, germination regimes and labs was 41%, with LSD $_{(0.05)} = 7.6$.

Significant variation in the first germination count among laboratories at $P \leq 0.05$ were reported. Lab two was conservative in evaluating first count seedlings, whereas labs 5 and 6 were generous (Fig. 3C). This is probably because the criteria of classifying young seedlings into normal and abnormal in the first count, based on the morphological evaluation of the roots, was not consistent in all labs. Roots that are not stubby or deformed, with length of approximately 1.5 cm and root hairs developed are classified as normal. Such seedlings were removed at 1st count to reduce the secondary infection. This is particularly important in medium to low quality seeds, where the fungal infection can be high, and the seedling growth is slow. In a preliminary study by the authors, it was found that young seedlings with root length of approximately 1.5 cm and root hairs developed (without shoot system developed) produced normal seedlings.

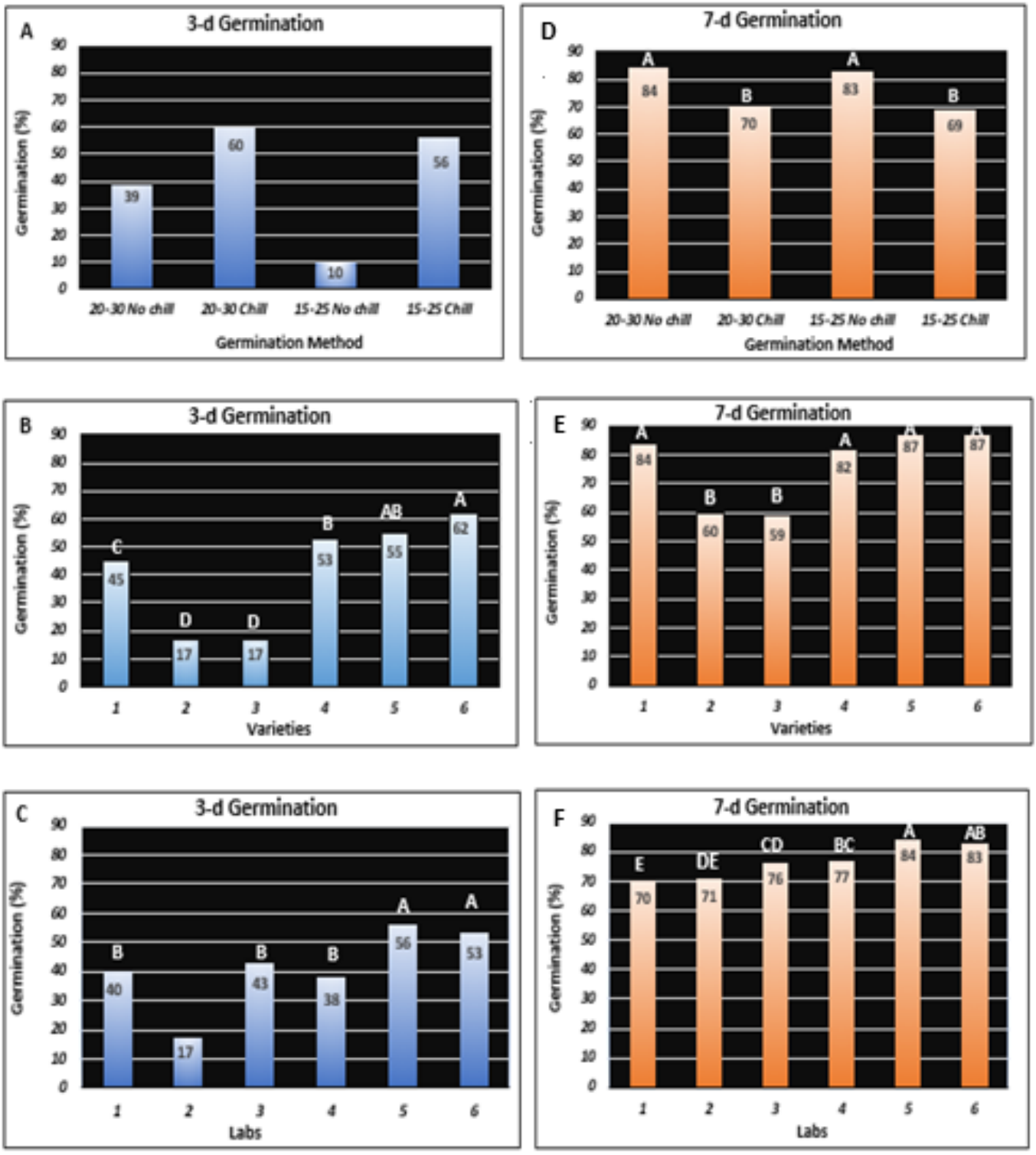

Figure 3. Average 3- day germination (first count) and 7- day germination (final count) of six hemp varieties tested using four combinations of temperature and prechilling treatments at six different laboratories. Means with different letters are significantly different from each other at $P \leq 0.05\%$.

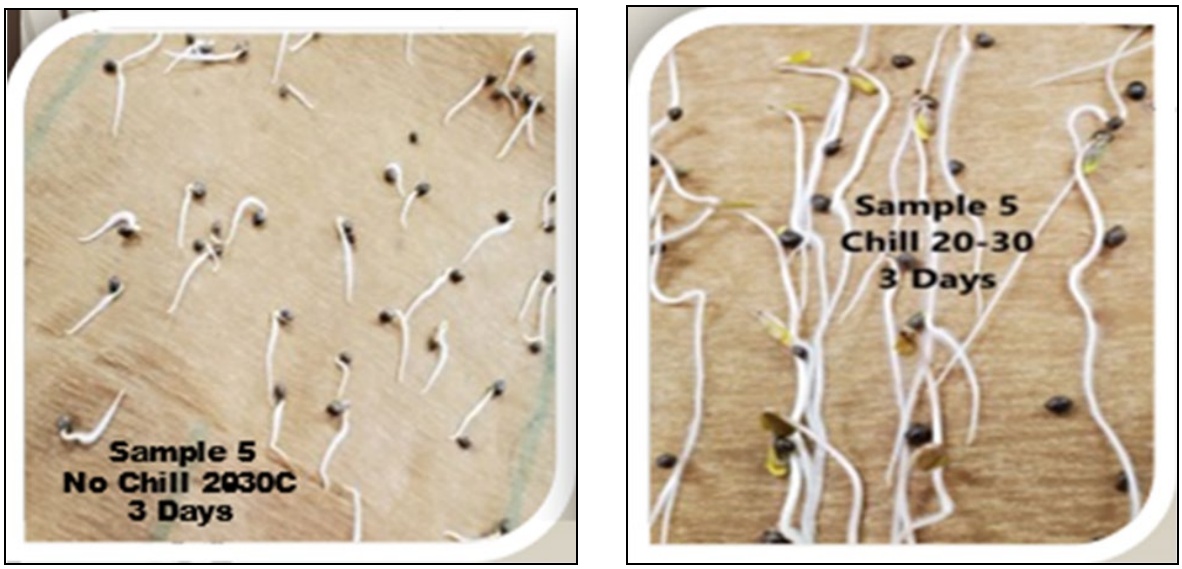

Figure 4. Example of first count (day 3) germination with and without prechilling treatment of hemp variety No. 5 (*low dormancy*) at 20-30°C.

Figure 5. Example of first count (day 7) germination with and without prechilling treatment of hemp variety No. 2 (*deep dormancy*) at 15-25°C.

Final Germination Count at Day 7

Final germination counts at day 7 increased significantly compared to the first count at day 3 in both chilled and non-chilled seeds of all varieties (Figs. 3D & 3E). Four out of the six varieties (1, 4, 5, and 6) had slight or no dormancy, and therefore had significantly higher final germination percentage than varieties 2 and 3, which had deep dormancy and less final germination counts. This is probably because the prechilling treatment did not completely break the dormancy of varieties 2 and 3. Both treatments 20-30°C

and 15-25°C with no prechill had similar average final germination of 84% and 83%, respectively. Likewise, both treatments 20-30°C and 15-25°C with chill had similar average final germination of 70% and 69%, respectively (Fig. 3D). This suggested that the prechilling treatment, along with the varieties, not the temperatures, made the difference in the final germination count (Figs. 3D and 3E).

Varieties 2 and 3 had deeper dormancy than varieties 1 & 4 & 5 & 6, which have light dormancy. Prechilling treatment was not completely effective in breaking the dormancy of varieties 2 and 3. The final germination of varieties 2 and 3 was 60% and 59%, respectively. The final germination of varieties 1, 4, 5 and 6 was 84%, 82%, 87%, and 87%, respectively (Fig. 3E). The average final germination count over varieties, germination regimes and labs was 77%, with $LSD_{(0.05)} = 5.9$.

Significant variation among **laboratories** at $P \leq 0.05$ were reported. Laboratories 1 and 2 were conservative in evaluation and Labs 5 and 6 were generous (Lab 3F).

Sample 1. The average final germination of non-chilled seeds ranged between 94% for 20-30°C to 91% for 15-25°C; whereas ranged between 79% for 20-30°C to 63% for 15-25°C in chilled seeds (Fig. 6). The variation in the final germination count among the six labs ranged between 73% and 92%. Clearly, sample 1 has some level of dormancy that was not broken by the prechilling treatment used in this study. That may indicated that the dormancy may be due to the seed coat or combination between physical and physiological dormancy.

Samples 2 and 3 had deep dormancy. The average final germination of non-chilled seeds of sample 2 ranged between 77% for 20-30°C to 81% for 15-25°C; whereas ranged between 40% for 20-30°C to 42% for 15-25°C in chilled seeds (Fig. 6). Similarly, the average final germination of non-chilled seeds of sample 3 ranged between 79% for 20-30°C to 71% for 15-25°C; whereas ranged between 46% for 20-30°C to 41% for 15-25°C in chilled seeds (Fig. 6). The variation in the final germination count among the six labs ranged between 46% and 75% in sample 2; and 45% and 72% in sample 3.

Sample 4 and 5 had low level of dormancy. The average final germination of non-chilled seeds of sample 4 ranged between 84% for 20-30°C to 83% for 15-25°C; whereas ranged between 81% for 20-30°C to 82% for 15-25°C in chilled seeds (Fig. 6). Similarly, the average final germination of non-chilled seeds of sample 5 ranged between 86% for 20-30°C to 88% for 15-25°C; whereas it was 88% in both 20-30°C and 15-25°C in chilled seeds (Fig. 6). The variation in the final germination count among the six labs ranged between 71% and 88% in sample 4; and 83% and 91% in sample 5.

Sample 6 does not have dormancy, the average final germination of non-chilled seeds of sample 6 ranged between 84% for 20-30°C to 87% for 15-25°C; whereas it was 88% in both 20-30°C and 15-25°C in chilled seeds (Fig. 6). The variation in the final germination count among the six labs ranged between 78% and 92%.

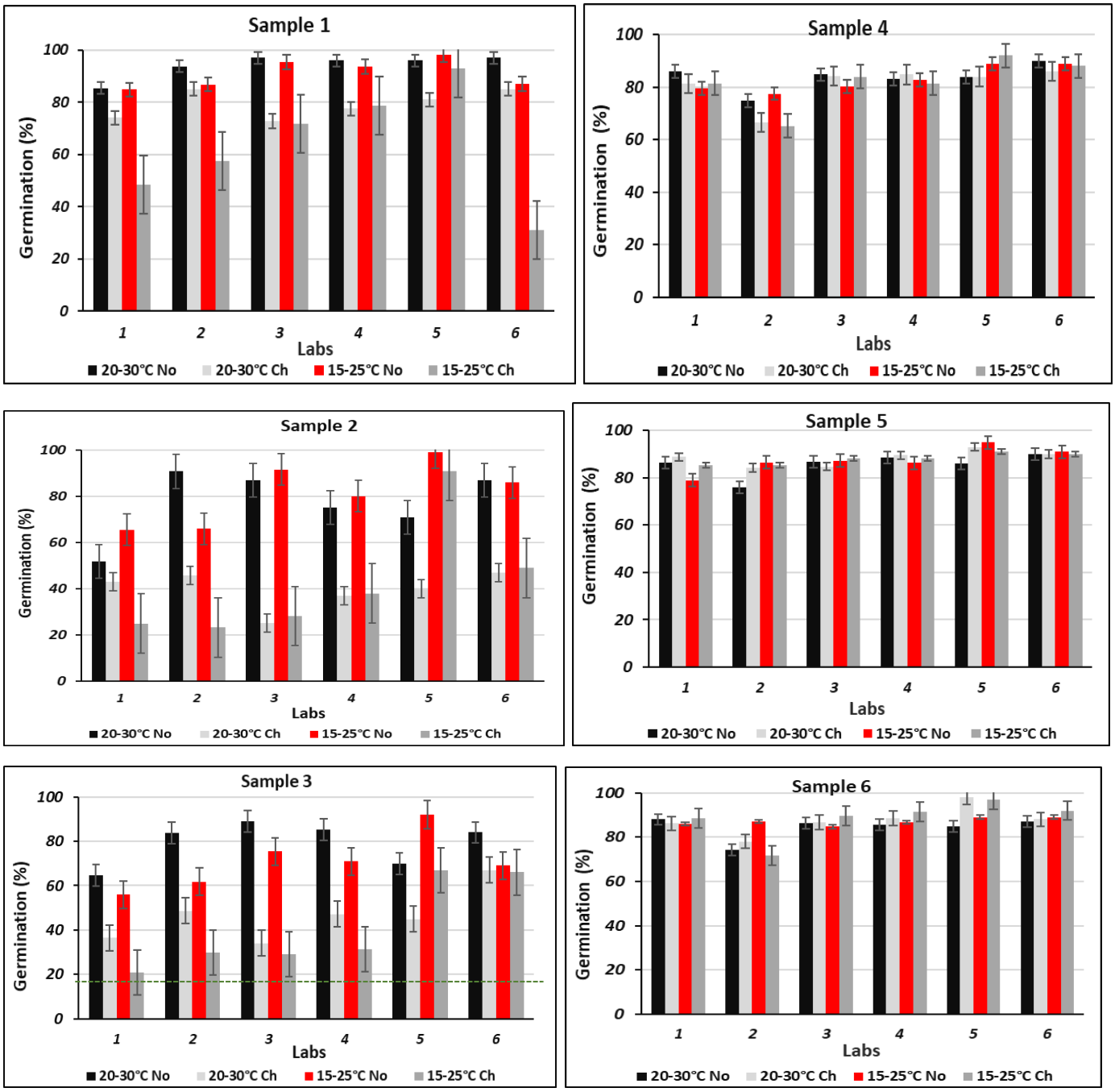

Figure 6. Final count (7 days) of six hemp varieties tested using four combinations of temperature and prechilling treatments at six different laboratories. Means with different letters are significantly different from each other at $P \leq 0.05\%$.

Number of Dormant Seeds

More dormant seeds were found in varieties 2 and 3 (average, 38 and 37 seeds, respectively), compared to varieties1, 4, 5, and 6 which had averages of 14, 8, 7, and 1 seeds, respectively (Fig. 7). These results showed that the prechilling treatments did not break the dormancy in varieties 2 and 3 completely, however, it may help in breaking the dormancy in the other varieties. The overall average of the dormant seeds over varieties, germination regimes, and labs was 17%, with $LSD_{(0.05)} = 4.92$.

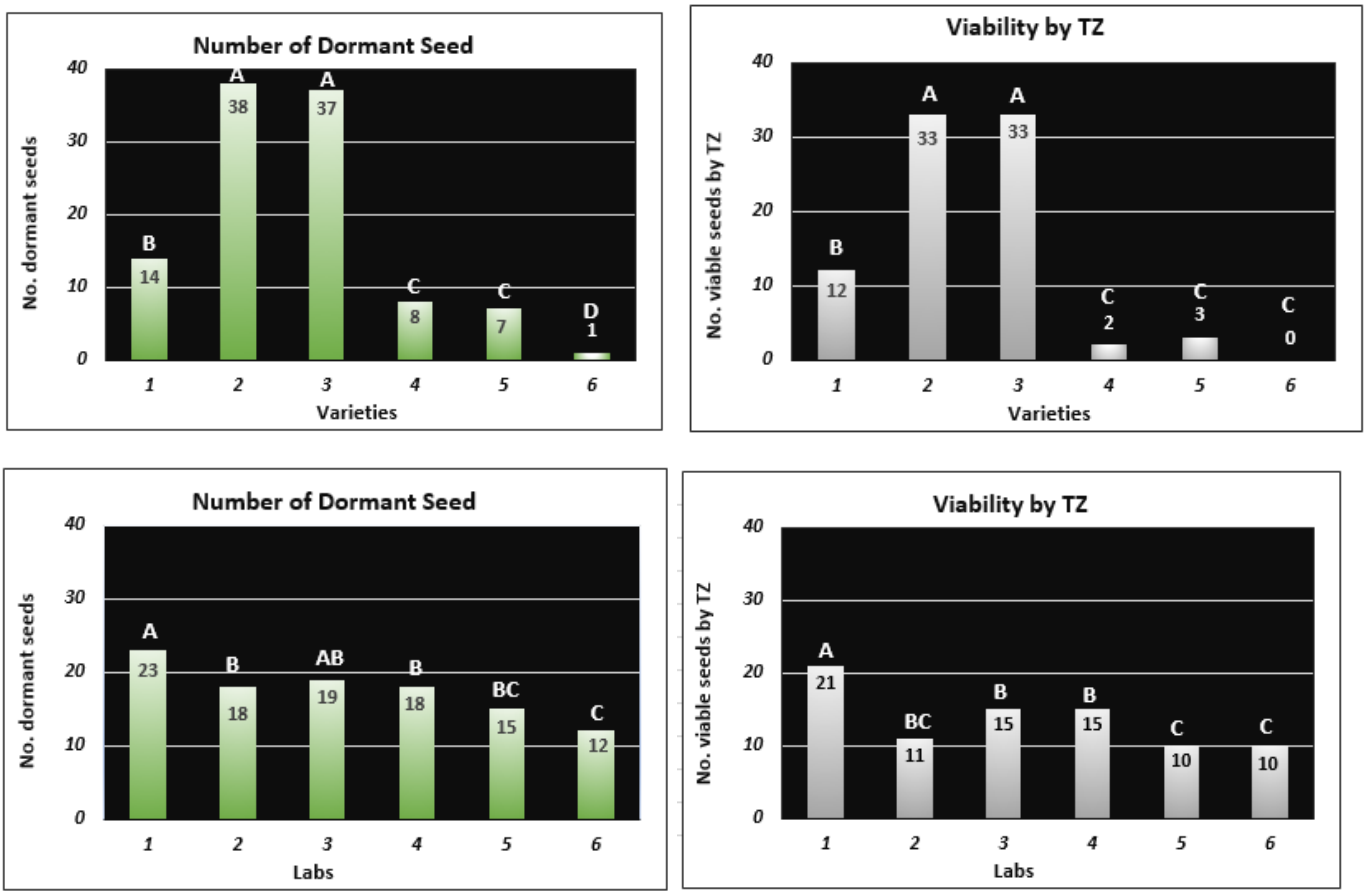

Figure 7. Average number of dormant seeds and number of viable seeds by TZ test of ungerminated seeds at the end of germination tests of six hemp varieties tested using four combinations of temperature and prechilling treatments at six different laboratories. Means with different letters are significantly different from each other at $P \leq 0.05\%$.

Significant variation among labs in number of dormant seeds (Fig. 7), with average ranging from 12 to 23 seed, with an average of 14 seeds over all other treatments, with LSD $_{(0.05)} = 4.85$.

Number of Viable Seed by TZ for Ungerminated Seeds after the Standard Germination Test

Most of the dormant seed were found to be viable when tested by TZ. This shows the importance of testing non-germinated seeds at the end of the standard germination test. In order to confirm whether ungerminated seeds were dead or dormant, it is crucial to conduct TZ test on ungerminated seeds to reflect the actual viability of the sample. This is particularly essential for the seeds that possess deep dormancy that is difficult to break with prechilling treatment. Varieties 2 and 3 had the deepest dormancy followed by variety 1. Varieties 4 and 5 had shallow dormancy, whereas variety 6 had no dormancy (Fig. 7 and 8).

These results indicated that further studies are needed to develop more effective dormancy breaking methods for hemp varieties with deep dormancy issue, such as varieties 2 and 3 used in this study (Fig. 7 and 8). However, the prechilling method has value in breaking shallower dormancy and producing fast uniform seedlings as it acts as hydropriming treatment.

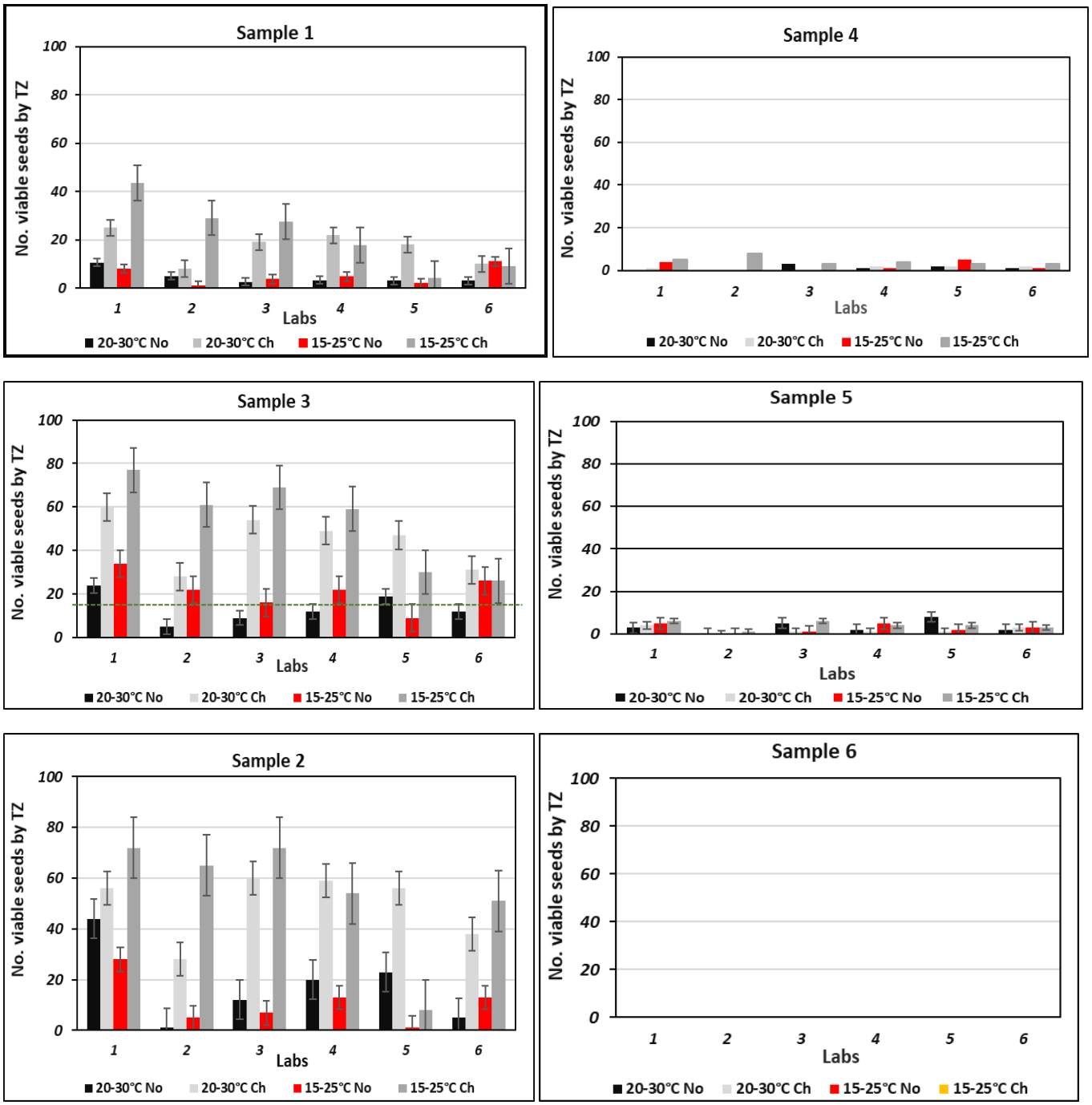

Figure 8. Average number of viable seeds by TZ test of ungerminated seeds at the end of germination tests of six hemp varieties tested using four combinations of temperature and prechilling treatments at six different laboratories. Means with different letters are significantly different from each other at $P \leq 0.05\%$. (Sample 6 did not have dormancy).

Sample 1. The average viable seed by TZ test of ungerminated seeds at the end of germination tests of non-chilled seeds was 5% for both 20-30°C and 15-25°C; whereas ranged between 17% for 20-30°C to 22% for 15-25°C in chilled seeds (Fig. 8). The variation in the viability by TZ among the six labs ranged between 7% and 22% in sample 1.

Samples 2 and 3 had deep dormancy. The average viable seed by TZ test of ungerminated seeds at the end of germination tests of non-chilled seeds of sample 2 ranged between 18% for 20-30°C to 11% for 15-25°C; whereas ranged between 50% for 20-30°C and 54% for 15-25°C in chilled seeds (Fig. 8). Similarly, the average viability of non-chilled seeds of sample 3 ranged between 14% for 20-30°C to 22% for 15-25°C; whereas ranged between 45% for 20-30°C to 54% for 15-25°C in chilled seeds (Fig. 8). The variation in the viability by TZ among of the six labs ranged between 22% and 50% in sample 2; and 24% and 49% in sample 3.

Sample 4 and 5 had low level of dormancy. The average viable seed by TZ test of ungerminated seeds at the end of germination tests of non-chilled seeds of sample 4 ranged between 1% for 20-30°C to 2% for 15-25°C; whereas ranged between 1% for 20-30°C to 4% for 15-25°C in chilled seeds (Fig. 8). Similarly, the average viability of non-chilled seeds of sample 5 was 3% for both 20-30°C and 15-25°C; whereas it was 2% and 4% for 20-30°C and 15-25°C, respectively in chilled seeds (Fig. 8). The variation in the viability by TZ among of the six labs ranged between 2% and 3% in sample 4; and 0% to 5% in sample 5.

Sample 6 did not have dormancy, no viable seeds by TZ of chilled or non-chilled seeds for both 20-30°C and 15-25°C (Fig. 8). The variation in viability by TZ among the six labs is 0%.

Total Viable Seeds

Sample 1. The average total viability (final germination count + viability by TZ of ungerminated seeds at the end of germination test) of non-chilled seeds ranged between 84% for 20-30°C to 96% for 15-25°C; whereas ranged between 96% for 20-30°C to 85% for 15-25°C in chilled seeds (Fig. 9). The variation in the total viability among the six labs ranged between 80% and 95%.

Samples 2 and 3. They had deep dormancy. The average total viability of non-chilled seeds of sample 2 ranged between 95% for 20-30°C to 92% for 15-25°C; whereas ranged between 89% for 20-30°C to 97% for 15-25°C in chilled seeds (Fig. 9). Similarly, the average total viability of non-chilled seeds of sample 3 ranged between 93% for 20-30°C to 92% for 15-25°C; whereas ranged between 91% for 20-30°C to 94% for 15-25°C in chilled seeds (Fig. 9). The variation in the total viability among the six labs ranged between 81% and 97% in sample 2; and 85% and 95% in sample 3.

Sample 4 and 5. They had low level of dormancy. The average total viability of non-chilled seeds of sample 4 was 85% for both 20-30°C and 15-25°C; whereas ranged between 82% for 20-30°C to 86% for 15-25°C in chilled seeds (Fig. 9). Similarly, the average final germination of non-chilled seeds of sample 5 ranged between 89% for 20-30°C to 90% for 15-25°C; whereas it was 90% to 92% in 20-30°C and 15-25°C, respectively in chilled seeds (Fig. 9). The variation in the total viability among the six labs ranged between 73% and 90% in sample 4; and 83% and 95% in sample 5.

Sample 6 does not have dormancy, the average total viability of non-chilled seeds of sample 6 ranged between 84% for 20-30°C to 87% for 15-25°C; whereas it was 88% in both 20-30°C and 15-25°C in chilled seeds (Fig. 9). The variation in the total viability among the six labs ranged between 78% and 92%.

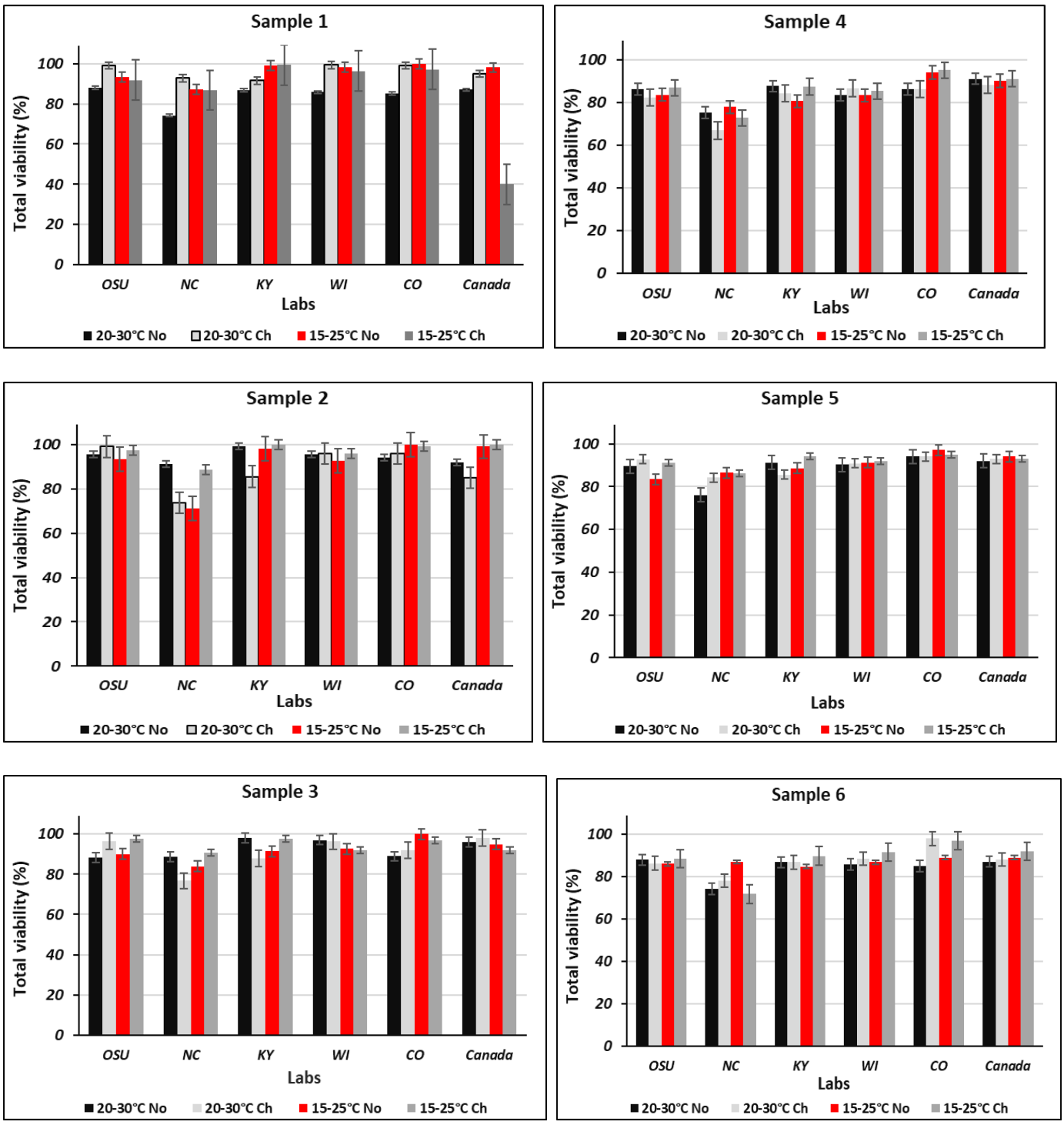

Figure 9. Total viable (normal seedlings after 7 days + viable seeds by TZ test of ungerminated seeds at the end of germination tests) of six hemp varieties tested using four combinations of temperature and prechilling treatments at six different laboratories. Means with different letters are significantly different from each other at $P \leq 0.05\%$.

CONCLUSIONS

First Count (3-day)

- Prechilling treatment helped achieving better 3-day count (average 62%) than non-chilled seeds (average 17 %) but did not break deep dormant seeds (var. 2 & 3).
- First count ranged from 62% to 44% for samples 6, 5, 4 & 1; the lowest was in samples 2 & 3 at 17%.
- Germinating at 20-30°C gave similar results to 15-25°C for chilled seeds.
- Variability among labs in 3-day count ranged from 17% to 56%. This may be due to subjectivity of criteria in the 3-day evaluations (1.5 cm with root hair).
- First count is important to control secondary infection.

Final Count (7-day) – Prechilling Treatment

- Prechilling seeds at 10°C for 7d were not enough to break deep dormant seed.
- Final germination ranged from 87 to 82% for samples 6, 5, 4 & 1; the lowest germination was in samples 2 & 3 at 59-60% for deep dormant varieties.
- Germination at 15-25°C gave similar results to 20-30°C for chilled or non-chilled seeds.
- Average germination of non-chilled samples was 84% and for chill was 70%. The two dormant samples may cause the average chilled seeds to drop.
- Variability among labs ranged from 84 to 70%.
- TZ of the ungerminated seeds at the end of the germination test is essential to determine the total viability of deep dormant hemp varieties.

RECOMMENDATIONS

- First count is important to control secondary infection.
- Prechilling achieve better 1st count and help in breaking short-lived light dormancy.
- TZ of the ungerminated seeds, at the end of the germination test, is essential to determine the total viability of deep dormant hemp varieties.
- Germination at 15-25°C gave similar results to 20-30°C for chilled or non-chilled seeds.
- Low-viable seed is needed in order to investigate potential advantage of 15-25°C by reducing the mold growth.

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REFERENCES

1. Sabry G. Elias, Yeaching Wu, and David C. Stimpson. 2020. Seed quality and dormancy of hemp (*Cannabis sativa* L.). J. Ag. Hemp Res. Vol. 2 (1): 1-15.
2. https://hempindustrydaily.com/2020-outlook-licensed-u-s-hemp-acreage-falls-9-from-2019-but-grower-numbers-increase-27/
3. https://extension.oregonstate.edu/crop-production/hemp/map-registered-outdoor-hemp-acreage-november-2020.
4. Elias, S.G**.** 2018. The Importance of Using High Quality Seeds in Agriculture Systems. Ag. Res. & Technol. Open Access J. Vol. 15 (4).
5. https://seedcert.oregonstate.edu/crop-information/hemp
6. AOSA Rules. Vol. 1. 2020.