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Effect of Short Periods of Cold Stratification on Seed Germination of Various Pine Species

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ABSTRACT

Pre-sowing seed treatments are crucial for improving the germination capacity of several tree species. This study aims to investigate the effect of cold stratification on the germination of five non-native pine species of the Libyan flora (*P.leiophylla*, *P.lawsonii*, *P.ayacahuite*, *P.oocarpa*, and *P.radiata*). These pine species have potential to be introduced into the climatic ecosystem of Al-Jabal Al-Akhdar region. Three treatments of no stratification (control), 15 days of cold stratification, and 30 days of cold stratification were applied to the selected pine species. The results revealed a significant positive effect of cold stratification on seed germination. Germination percentage increased from 46.7% when sowing without stratification (control treatment) to 70% when seeds were stratified for 15 and 30 days prior to sowing, respectively. No seed germinated during the first week in the first and second treatments while the germination percentage reached in the third treatment of 30 days of cold stratification to 24%. Germination for *P.leiophylla*, *P.lawsonii*, *P.oocarpa*, and *P.radiata* improved after applying different stratification treatments on both rate and time of germination while *P.ayacahuite* germination did not differ significantly with stratification compared to control treatment.

تأثير التعرض لفترات قصيرة من التنضيد البارد على إنبات بذور بعض أنواع جنس الصنوبر

عطية عبدالكريم موسى السنوسى

معاملات ما قبل البذر تعتبر حاسمة في تحسين قدرة الإنبات للعديد من الأنواع الشجرية. تهدف هذه الدراسة الى معرفة تأثير المعاملة بالتنضيد البارد على إنبات خمس أنواع صنوبرية غير محلية وغريبة على التركيبة النباتية الليبية وهي (*P.leiophylla*، *P.lawsonii*، *P.ayacahuite*، *P.oocarpa*، *P.radiata*). هذه الأنواع الصنوبرية لديها القابلية على التأقلم مع الظروف البيئية والمناخية لمنطقة الجبل الأخضر. تم تطبيق ثلاث معاملات وهي بدون تنضيد بارد (الشاهد)، و التنضيد البارد لمدة 15 يوم، والتنضيد البارد لمدة 30 يوم على الأنواع الصنوبرية المختارة. أظهرت النتائج تأثير إيجابي معنوي للتنضيد البارد على إنبات البذور. نسبة الإنبات إزدادت من 46.7 % بدون تطبيق معاملة التنضيد البارد الى 70% عند تطبيق التنضيد البارد لمدة 15 و30 يوم على التوالي. لم يحدث إنبات للبذور خلال الأسبوع الاول في المعاملتين الاولى والثانية بينما وصلت نسبة الإنبات خلال الأسبوع الأول من المعاملة الثالثة للتنضيد البارد لمدة 30 يوم الى 24%. تحسن الإنبات بالنسبة لبذور كل من *P.leiophylla* ، *P.lawsonii* ، *P.oocarpa* و *P.radiata* بعد تطبيق معاملات التنضيد البارد المختلفة بالنسبة لكل من معدل ووقت الإنبات، بينما إنبات بذور *P.ayacahuite* لم يحدث له تغيير معنوي عند المعاملة بالتنضيد البارد مقارنة بمعاملة الشاهد بدون تنضيد.

INTRODUCTION

Cold stratification is one of the most influential treatments applied in the germination processes of many cold and temperate tree species. It is described as a simulation process of dormancy conditions for most viable seeds in order to initiate the germination process. It can be simply defined as a pre-treatment of seeds by keeping them stored for a prolonged period of time at a certain cold temperature (usually at 50 C) (Piotto & Di Noi, 2003). Seed stratifications vary based on seed types and sizes, some seeds might be treated with different stratification methods such as warm stratification (Herranz, *et. al*., 1998., Bower, *et. al*., 2011) or any other pre-sowing seed treatments like mechanical scarification, chemical treatments with acids, gibberellin (Lim, *et. al*., 2015), insecticide and fungicide solutions. Furthermore, the purpose of all of these treatments was to enhance and accelerate seed germination (Barnett & McGilvray, 1971), and to achieve the complete development needed by the embryo to overcome physiological dormancy (Baskin & Baskin, 1998). The significant effect of stratification on seed germination is more evident especially when combined with scarification or using chemical growth regulators (Nawrot-Chorabik, *et. al*., 2021). It is indeed the most dormancy-breaker effective method used for the germination of various pine seeds (Cooke, *et. al*., 2002) (Ghildiyal, *et. al*., 2009) (Shen & Cho, 2021) even though some studies found no significant effect of stratification on pine seeds germination (Nelson, 2015). Applying cold stratification on seeds of some trees alters many chemo-physiological activities including activation of enzymes, hormones, and other metabolites which usually embryo needed for germination (Blanche, *et. al*., 1990).

Cold and temperate tree species, such as pines, have a relatively moderate to a high degree of dormancy which might prevent germination even under favourable growth conditions (Sharma, 2006., Jull & Blazich, 2000). Pine seeds vary in size and shape and therefore; in their response to different stratification periods. While some pine species require a short to no stratification period needed like P. halepinsis, P. contorta, P. palustris, other pine species might need a prolonged stratification period exceeding in several cases 60 days such as P.ponderosa, P. cembra, P. lambertiana (Krugman & Jenkinson, 1974). Seeds of pines as well as other Gymnospermae reserve much of nutrients in the megagametophyte the embryo unlike most Angiospermae seeds (Berlyn & Miksehe, 1972., Carpita, *et. al*., 1983). Several pine species are used in afforestation projects outside of their natural geographic habitats, they showed resilience and well adaption to various growth conditions in their new environment. In Libya, for instance, many exotic pine species were planted as ornamentals or shelterbelts trees around agricultural lands and adapted successfully to the local ecosystem. The current study aims to examine the effect of cold stratification on germination of several non-indigenous pine species by applying different short stratification periods.

MATERIALS AND METHODS

The study was carried out at the forestry laboratory, Faculty of natural resources and environmental sciences, Omar Al-Mukhtar University, Albaida, Libya. Seeds of the subjected pine species were obtained through The New Libya Association (TNL)(NGO). Seed source was Sheffield's Seed Company (https://sheffields.com/). In this study, seeds of five pine species were chosen for the experiment and were as follows:

1- ***P. leiophylla***: Chihuahua pine, and known also as the yellow pine. North American pine species distributed naturally in Mexico and southern the U.S, grow on elevations up to 3000m, and average precipitation range of 600-1000 mm.
2- ***P. lawsonii*:** Lawson's pine is a limited pine species only to certain places in Mexico on high elevations (1300 – 2600 m), and grows in warm temperate zones at average precipitation of 800mm.
3- ***P. ayacahuite*:** The Mexican white pine is native to mountainous areas in southern Mexico and western Central America at elevations ranging from 1800 to 3200 m.a.s.l. (Saborio, *et. al*., 1997). The species grows naturally in wet humid areas with raining summer season. It is a highly demanding species that require full sunlight and well-drained rich soils and temperature not exceeding 20^0 C.
4- ***P. oocarpa*:** Hazelnut pine, native to Central America and grows in environments of 15 – 24^0 C and precipitation range of 1000 – 1900 mm. It is extensively used in afforestation purposes around the world and produces high-quality wood compared to other pine trees.
5- ***P. radiata*:** known also as Monterey pine, native to Mexico and California. It is considered one of the fast-growing pine species and is favored in reforestation projects. It also has high-quality wood and is one of the pine species that has been previously introduced in Libya (Zunni & Biome, 2006).

The total number of seeds obtained were highly viable with 99% purity and over 95% germination rate according to the seed source (https://sheffields.com/). The total number of seeds (90 seeds) were soaked in water for 24 hours, all of which passed the floating test, and were divided into 3 different treatments as follows:

1- **Treatment 1**: without stratification; seeds were sowed immediately after the 24 hours soaking time.
2- **Treatment 2**: 15 days of cold stratification.
3- **Treatment 3**: 30 days of cold stratification.

Stratification treatments were performed by placing seeds in air-tight plastic bags containing damp sand as a medium and kept in the refrigerator at a temperature of 5^0 C. Control and stratified seeds were sowed on paper towels in Petri dishes and were checked for moisture every day to keep them damp, not over wet. Germination of seed was recorded on daily basis

Throughout the study period, data obtained were analyzed and graphs and tables were generated to illustrate the germination rate and time of each treatment. Univariate analysis of variance was applied to test the significance of stratification treatments on both rate and time of germination, and general linear model was applied to illustrate the effect of different treatments applied on the germination rate of the selected pine seeds in this experiment. All statistical analyses were performed using IBM SPSS statistics software 28.0.

RESULTS

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1. First treatment results (control):

The results indicated a significant effect of cold stratification on the germination of selected pine species (Figure 2). The effect was prominent on both time and rate of germination for all different stratification treatments. Table 1 illustrates rate and time of germination for all pine species in the first treatment (control). Total germination percentage of the control treatment was the lowest among other treatments with an overall percentage of 46.7%. The highest germination percentage was recorded during the third week of sowing (50%) followed by the fourth week (43%) and then the second week (7%). No germination occurred after a week of sowing (Figure 1).

Table 1: Time and percentage of germination for pine species in the first treatment (control treatment).

Species name	First treatment (control) germination percentage						
	1st week	2nd week	3rd week	4th week	Mean Avg.	Std.Deviation	Total percentage
P. leiophylla	0	0	16.7	33.3	1.10	1.51	50
P. lawsonii	0	0	16.7	33.3	1.10	1.51	50
P. ayacahuite	0	0	33.3	16.7	1.00	1.41	50
P. oocarpa	0	0	16.7	16.7	0.70	0.83	33.3
P.radiata	0	16.7	33.3	0	0.80	1.59	50
Average Percentage	0	3.3	23.3	20			46.7

2. Second treatment results (15 days of cold stratification):

Following the first stratification treatment (15 days of cold stratification), the results exhibited an obvious improvement in both germination percentage and time required for germination. Overall germination percentage in this treatment reached 70 % (Table 2). The highest germination percentage was achieved by *P. radiate* with (100%) germination percentage followed by *P. lawsonii* (83%), while the least germination percentages reached were for both species *P. ayacahuite* and *P. leiophylla* with only (50%) of seeds germinated. The highest germination rate in this treatment was recorded during the third week of sowing (43%), followed by second week (33%) then the fourth week (24%) while no germination occurred during the first week of this treatment similarly to control treatment (Figure 1).

Table 2: Time and percentage of germination for pine species in the second treatment (15 days of cold stratification).

Species name	Second treatment (15 days cold stratification) germination percentage						
	1st week	2nd week	3rd week	4th week	Mean Avg.	Std.Deviation	Total percentage
P. leiophylla	0	0	33.3	16.7	1.00	1.41	50
P. lawsonii	0	16.7	66.7	0	1.40	3.18	83.3
P. ayacahuite	0	16.7	0	33.3	1.00	1.63	50
P. oocarpa	0	16.7	16.7	33.3	1.30	1.16	66.7
P.radiata	0	66.7	33.3	0	1.40	2.85	100
Average Percentage	0	23.3	30	16.7			70

3.2 Third treatment results (30 days of cold stratification):

In the third treatment (30 days of cold stratification), enhancements were noted in the time required for germination compared to the previous 2 treatments. On the other hand, the total germination percentage was similar to that obtained from the second treatment of 15 days of cold stratification with a 70% germination percentage (Table 3). The highest germination rate in this treatment was recorded for *P. leiophylla*, *P. lawsonii*, and

P. radiata (83.3%), while *P. ayacahuite* had the least germination percentage of only (33.3%). The highest germination rate occurred in the second week (52%), followed by the first week (24%), the third week (19%), and finally the fourth week with the least germination occurrence (only 5%) (Figure 1).

Table 3: Time and percentage of germination for pine species in the third treatment (30 days of cold stratification).

Species name	Third treatment (30 days cold stratification) germination percentage						
	1st week	2nd week	3rd week	4th week	Mean Avg.	Std.Deviation	Total percentage
P. leiophylla	0	83.3	0	0	1.00	3.65	83.3
P. lawsonii	16.7	50	16.7	0	1.00	2.00	83.3
P. ayacahuite	0	0	16.7	16.7	0.83	0.83	33.3
P. oocarpa	0	33.3	33.3	0	1.00	1.82	66.7
P.radiata	66.7	16.7	0	0	0.60	2.19	83.3
Average Percentage	16.7	36.7	13.3	3.3			70

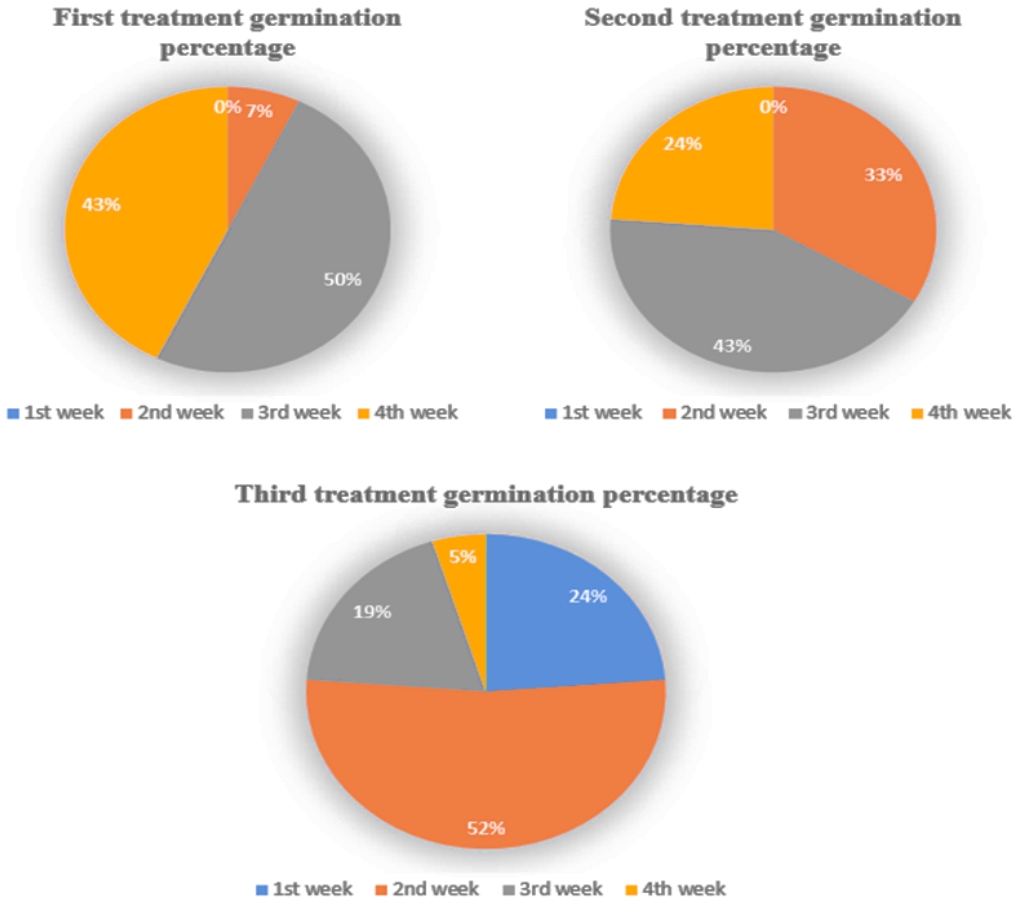

Figure 1: Germination percentage per week after sowing for all stratification and control treatments.

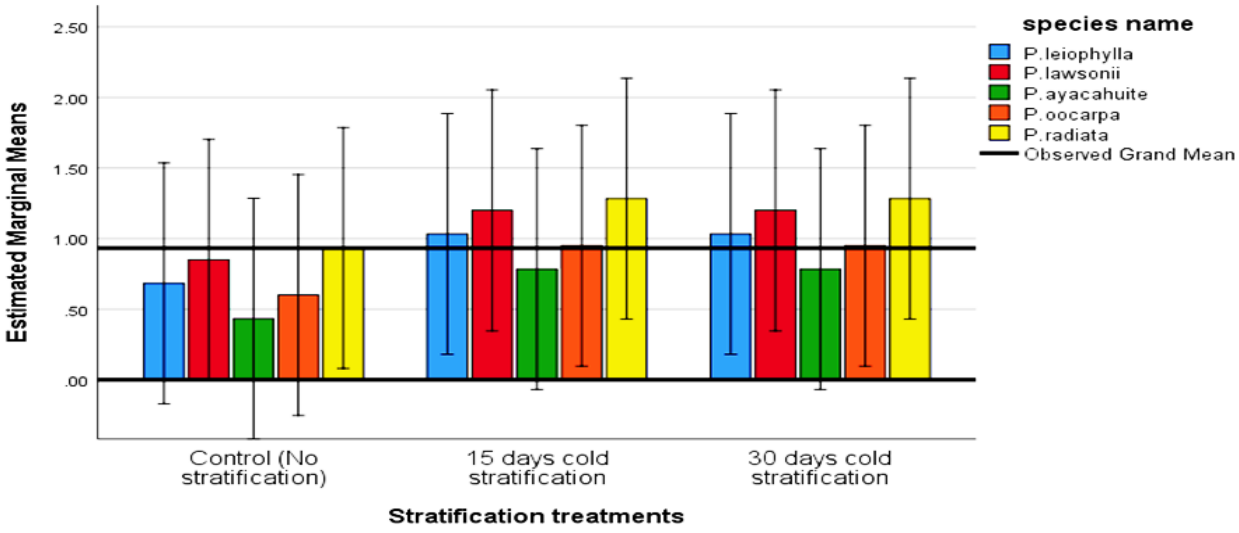

Figure 2: Estimated Marginal Means of Number of germinated pine seeds.

DISCUSSION

Treatment of seeds with cold stratification techniques by subjecting them to low temperature (4-6^0 C) for prolonged periods of time (up to 90 days in some species) has proven to be effective on enzymatic activity stimulation and helped promote germination in many tree seeds around the world (Nikolaeva, 1977., Nawrot-Chorabik, *et. al*., 2021). The current study confirmed as well the significance of cold stratification as a dormancy-breaking treatment for the germination of five different pine species. Cold stratification applications lead to an increase in the total germination percentage of pine seeds from 46.7% when no stratification was applied to 70% when stratification is applied for 15 and 30 days, respectively. These findings were in agreement with (Koyuncu, 2005) who reported an increase in germination rate from 33% in non-stratified seeds of Black Mulberry to up to 88% in seeds stratified for 100 days. Similar findings were reported by (Skordilis & Thanos, 1995) who demonstrated an increase from a 10% germination rate in untreated seeds to 74% and 98% in both *P. brutia* and *P. halepensis* seeds stratified for up to 3 months in seeds obtained from Thasos provenances in Greece.

The positive impact of cold stratification was more evident in the time required for germination. In general, stratified pine seeds germinate faster and require less time than non-stratified seeds. During the first week after sowing, no germination occurred in first and second treatments seeds while in the third treatment (30 days of stratification), germination occurred with a percentage of 24%. In the second week after sowing, the germination percentage gradually increased from 7% in the control treatment to 33% in the second treatment (15 days of cold stratification) and 52% in the third treatment of 30 days of cold stratification. These results indicated a positive relationship between the length of stratification period and the time required for germination for the studied pine species. These findings were in agreement with (Lim, *et. al*., 2015) who found similar results in their study of the effect of wet cold stratification and gibberellin treatments on the germination of dwarf stone pine seeds, and (Fowler & Dwight, 1964) who stated that any increase in the period of stratification will result in an increase in the percentage of white pine seeds germination rate. (Malik & Shamet, 2008., Biswas, *et. al*., 1972., Barnett & McGilvray, 1971) also reported similar trends in the impact of cold stratification on improving time and percentage of germination. Patterns of seeds germination were similar during the third and fourth weeks after sowing. In both times, the highest germination percentages were recorded in the first (control) treatment (50% and 43%), then in the second treatment (43% and 24%), and finally in the third treatment (19% and 5%), respectively.

Different pine species demonstrated different germination responses in terms of type and time of treatments applied. For instance, *P.radiata* had the highest germination rate compared to other pine species in this study. The best germination rate achieved was 100% following a stratification period of 15 days. Best recorded germination times for *P.radiata* were in the first week of the third treatment and the second week of the second treatment. *P.radiata* is one of the species which requires a short period of cold stratification (7 – 20 days) in order to achieve successful germination (Krugman & Jenkinson, 1974). *P.leiophylla*, on the other hand, had its best germination rate in the third treatment (83.5%) compared to only 50% in both first and second treatments. Concerning the time of germination, the highest germination percentage of *P.leiophylla* was during the second week of the third treatment (30 days of cold

stratification). *P.lawsonii* had the highest germination rate in both stratification treatments (second and third treatments) with a percentage of 83.5% compared to only 50% germination percentage in the control treatment. The third week of the second treatment exhibited the highest germination percentage of *P.lawsonii*. Contrary to other pine species, *P. ayacahuite* responded negatively to cold stratification treatments. The highest germination rate for *P.ayacahuite* was recorded in the first and second treatments (50%) while in the third treatment the percentage of germination was only 33.3%. The best germination time for *P.ayacahuite* was in both the third week of the first treatment and the fourth week of the second treatment. Finally, *P.oocarpa* had the highest germination rate in both the second and third treatments (66.7%) while had the least germination rate in the control treatment (33.3%). The fourth week of the second treatment and the second and third weeks of the third treatment were the times with best germination percentage of *P.oocarpa*.

CONCLUSION

Treating pine seeds with cold stratification prior to sowing might be of importance to achieve better germination results. The current study highlights the positive effect of applying short cold stratification periods on obtaining better germination rates. *P.radiata*, *P.leiophylla*, *P.lawsonii*, and *P.oocarpa* seeds had the highest germination percentages when stratified compared to the control treatment. On the contrary, *P.ayacahuite* was the only pine species in this experiment with no significant impact of cold stratification treatment. Reducing time until germination and breaking seed dormancy were the most influential impacts of cold stratification treatments on pine species.

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