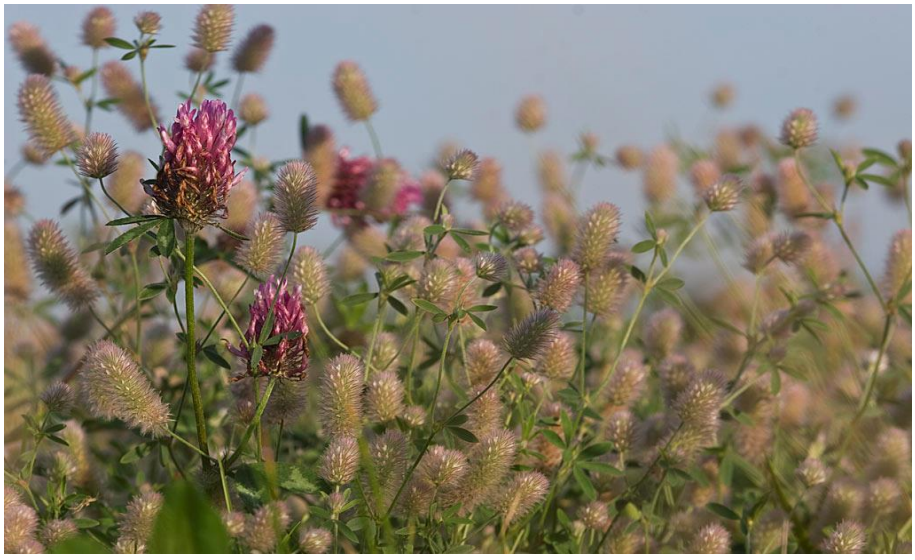
Thesis study on cold germination

A STUDY ON THE EFFECT OF TEMPERATURE AND DURATION OF COLD STRATIFICATION ON THE GERMINATION PROCESS OF TEN PRE-SELECTED WILDFLOWER SPECIES

RICHMOND, MADELON R.



Thesis study on cold stratification

A STUDY ON THE EFFECT OF TEMPERATURE AND DURATION OF COLD STRATIFICATION ON THE GERMINATION PROCESS OF TEN PRE-SELECTED WILDFLOWER SPECIES

Student: Madelon R. Richmond

Education: Applied Biology, Aeres University of Sciences, Almere

Graduation teacher: Leo van der Sluijs

4th of April 2022, Nijeberkoop

AERES UNIVERSITY OF APPLIED SCIENCES

Cruydt Hoeck WILDEBLOEMENZADEN

Preface

During this study, the germination process of ten pre-selected species is tested by cold stratification, where different temperatures and duration of stratification have been applied to test the effect of these two factors on the germination process. This thesis is written to satisfy the requirements of the study: Applied Biology at Aeres University of Sciences in Almere (Netherlands).

My interests are in (-native-) plant species and all that comes with. This brought me to Cruydt-Hoeck, a company that collects, grows, and sells native plants species of the Netherlands and Flanders. The study subject and research questions were developed together with Anouk Schalski, collection manager at Cruydt-Hoeck. During this study I was helped by Jojanneke Bijkerk (owner of Cruydt-Hoeck) and Karlijn Bokhorst (production advisor).

I would like to thank Jojanneke Bijkerk for the opportunity to perform my thesis at Cruydt-Hoeck. Anouk Boersma guided the process. I could ask her anything and I really appreciated that. Thanks to Karlijn Bokhorst for actively thinking along with the study.

I hope you enjoy reading.

Madelon R. Richmond

Nijeberkoop, 4th of April, 2022

Summary

Modernisation and intensification of the agricultural land in Europe has led to habitat fragmentation. Due to habitat fragmentation, wild plant populations became small and isolated. Plant populations need to be reinforced to prevent inbreeding depression and raising them ex situ is a generally considered as a valid way to accomplish this need. Therefore, it is necessary to gain knowledge about the germination process of wild plant species. For this study, the objective was to answer the following main question: "What effect does cold stratification have on the germination of ten pre-selected difficult germinators?".

Ten European plant species were evaluated on germination behaviour under influence of temperature and duration of cold stratification. For two, four- and six-weeks Petri dishes containing agar and twenty-five seeds per dish were placed in a cooling environment (2, 5 and 8 °C) resulting in nine experimental groups. After the stratification period, the dishes were placed in a germination box with a temperature of 19 – 26 °C and germination was noted every three days. Statistical analysis using general linear model and analysis of variance was performed to make a statement about the effectiveness of the applied germination methods.

Five out of ten species did not germinate in high numbers and therefore statistical analysis was not performed. Duration of stratification, temperature, and the interaction between these two factors showed a large or medium effect on *Betonica officinalis, Geranium pratense, Primula elatior, Sanguisorba officinalis* and *Trifolium arvense* based on the $p<0,05$ or PES-value.

Germination temperature, an uneven dispersal of light and fungi might have had a negative influence on the germination process of the ten species. Follow-up studies can apply lower temperatures in the germination box and make use of an even light distribution. To prevent formation of fungi, sterile seeds or a certain humidity can be applied. To confirm the suggested outcome of this study, further investigation is needed using larger sample size.

In case of *Consolida regalis, Knautia arvensis, Lycopus europaeus, Stachys sylvatica* and *Succisa pratensis* a period of cold stratification showed no effect on the establishment of seedlings. However, the control group did not germinate either. The 6 weeks/8 °C and 6/5 treatment had the highest germination rate for *Betonica officinalis, Geranium pratense* and *Primula elatior. Sanguisorba officinalis* responded best to the 6/8, 6/2, 2/2 and 2/5 treatment. The same applies for *Trifolium arvense* only without the 2/5 treatment.

Samenvatting

Modernisatie en intensivering van de agrarische sector in Europa heeft geleid tot habitatfragmentatie. Door habitatfragmentatie zijn wilde planten populaties kleiner geworden en geïsoleerd van elkaar komen te liggen. Plantpopulaties moeten worden versterkt om inteeltdepressie te voorkomen. Het opkweken van zaden ex situ is hiervoor over het algemeen een erkende manier van werken. Hierbij is het van belang om meer kennis te vergaren over het ontkiemingsproces van wilde planten. Voor deze studie is er gedoeld op het beantwoorden van de hoofdvraag: "Welk effect heeft koude stratificatie op de ontkieming van tien voor geselecteerde moeizame kiemers?".

Tien Europese planten zijn onderzocht op kiemgedrag onder invloed van temperatuur en duur van koude stratificatie. Voor twee, vier en zes weken zijn Petri schaaltjes met agar en 25 zaden geplaatst in een koelkast van 2, 5 en 8 °C resulterend in negen experimentele groepen. Vervolgens zijn de schaaltjes in een kiemkast geplaatst met een temperatuur van 19 – 26 °C en ontkieming werd om de drie dagen genoteerd. Statistische analyse middels algemeen lineair model en analyse van varianten is uitgevoerd om een uitspraak te doen over de effectiviteit van de toegepaste kiemmethoden.

Vijf van de tien soorten zijn niet in grote getalen ontkiemd, waardoor een statistische analyse voor deze soorten niet is uitgevoerd. Duur van stratificatie, temperatuur en de interactie tussen deze twee factoren hadden een gemiddeld of groot effect op de ontkieming van *Betonica officinalis*, *Geranium pratense*, *Primula elatior*, *Sanguisorba officinalis* en *Trifolium arvense* gebaseerd op de $p<0,05$ of PES-waarde.

Kiemtemperatuur, verdeling van licht en schimmelvorming heeft mogelijk een negatieve invloed gehad op het ontkiemingsproces van de tien soorten. Bij vervolgonderzoek kunnen lagere temperaturen toegepast worden in de kiemkast en gebruik gemaakt worden van een optimale gelijkmatige verdeling van licht. Om schimmelformatie te voorkomen kan er gewerkt worden met steriele zaden of kan de luchtvochtigheid aangepast worden. Vervolgonderzoek met een grotere steekproefomvang is nodig om de resultaten van dit onderzoek met grotere zekerheid te bevestigen.

Bij *Consolida regalis*, *Knautia arvensis*, *Lycopus europaeus*, *Stachys sylvatica* en *Succisa pratensis* lijkt de gekozen methode geen effect te hebben gehad op de ontkieming, daarentegen is ook de controlegroep niet ontkiemd. Voor de overige vijf soorten is de conclusie dat de 6/8 en 6/5 behandeling het hoogste ontkiemingsaantal oplevert voor *Betonica officinalis*, *Geranium pratense* en *Primula elatior*. *Sanguisorba officinalis* reageerde het beste op de 6/8, 6/2, 2/2 en 2/5 behandeling. Hetzelfde geldt voor *Trifolium arvense*, alleen zonder de 2/5 behandeling.

Table of content

Chapter 1 | Introduction

Modernisation and intensification of agricultural land use during the last half century in Europe has led to a major decline in biodiversity on the continent (Storkey et al., 2012). Habitat fragmentation is one of the main causes leading to this decline. Habitat fragmentation might lead to more endangered species which have a higher chance on going extinct (Wilcove et al., 1986). Habitat specialized vascular plants still decrease significantly due to habitat fragmentation, even though further fragmentation is not occurring. Landscape area loss is still occurring and the management strategies to maintain these habitats are insufficient due to time-delayed extinction (Krauss et al., 2010). This means that populations present in fragmented habitats will eventually go extinct (Tilman et al., 1994; Kuussaari et al, 2009). Habitat fragmentation causes important genetic consequences. Smaller and isolated populations experience a heavier effect of genetic drift, which results in loss of genetic diversity within these populations. Thereby, increasing the degree of kinship and the degree of inbreeding, may lead to inbreeding depression (inbreeding depression is mating between individuals that are related by ancestry (Possingham et al., 2001; Frankham et al., 2010). Therefore, solutions need to be found to provide the increment of native plant populations.

The raising of native plant populations ex situ is seen as a valid way of reducing the extinction of these populations (Bowes, 1999). The local ecotypes can be maximized by multiplying plants from seed and a higher genetic diversity can be reached (Fay and Muir, 1990; Fay, 1992). Therefore, knowledge of germination characteristics is crucial to ensure the success of the establishment of the plants (Leiblein et al., 2014). To germinate, all seeds need to absorb a species-specific minimum of water and oxygen. Water content that is too high or too low might inhibit germination (Baskin and Baskin, 1998). In this study, germination is defined as emergence of the radicle from the seed. A critical temperature and moisture supply are factors that are required for germination and seed dormancy can be defined as failure to germinate under preferable conditions, although the seed is viable (Bewley, 1997; Baskin and Baskin, 2005). Seed dormancy is an essential aspect for seeds to germinate at the right moment of the year to increase the chances of survival and growth (Taiz and Zeiger, 2010). A dormancy breaking treatment needs to be applied to a dormant seed, to become non-dormant and to make germination possible. To propagate native plants species ex situ more knowledge of the right germination conditions and types of dormancies is needed (Baskin and Baskin 1998; Hilhorst 2007).

Environmental factors influence the germination process and the break of dormancy, such as light, hormones, nitrate, temperature, oxygen, and carbon dioxide (Baskin and Baskin, 1998; Taiz and Zeiger, 2010). The degree of seed dormancy can vary among and within plant species (Ista, 1966; Ista, 1993), between individuals of the same population and even between inflorescences on the same individual (Baskin and Baskin, 2004). A different germination rate can be the consequence of the degrees and sensitivity of the mentioned germination factors (Baskin and Baskin, 1998). Dormancy and germination requirements in wild populations are adapted to local climate and conditions (Baskin and Baskin, 1998). According to Baskin and Baskin (2005) germination tests that obtain various responses to certain conditions are representative for the population. To be considered is the large intra-populations variation, due to differences in hormonal levels in some wild populations (FinchSavage and Leubner-Metzger, 2006), which also influence the germination process. Among herbaceous seed plants in temperate regions, temperature is the most important dormancy-breaking factor and for these species a winter period is required for the majority to germinate (Baskin and Baskin, 1988), where germination in autumn can be prevented (Baskin and Baskin, 2014; Porceddu et al., 2013). In these environments a sufficient biomass to resist the freezing winter is missing in small seedlings, whereby seedling survival is enhanced by timing of germination (Jaganathan et al., 2015). The seeds become non-dormant during winter and germination in spring or early summer can be accomplished (Mondoni et al., 2009; Schwienbacher et al.,2011; Wang et al., 2017).

The types of seed dormancy can be classified as physiological, morphological, morphophysiological, physical and combination dormancies (Baskin and Baskin, 2004). Germination in physiological dormancy is prevented by physiological mechanisms in the embryo, in the endosperm or in the seed testa (coat) (Amen 1968; Baskin and Baskin 1998). Low temperatures may break physical dormancy (Baskin and Baskin, 1988) and a dormancy breaking treatment (warm, cold or both) followed by a growth period (warm or cold) can break morphophysiological dormancy (Baskin and Baskin, 1998). To break seed dormancy, stratification is

the horticultural term for treating seeds with cold or warm temperatures where winter or summer can be simulated. The seed testa's become water permeable due to low temperatures, making them porous and inhibition possible (Baskin and Baskin, 1988). Propagation from seed is inexpensive and most of the times effective, but for many European plant species the germination strategy is not fully understood (J. Bijkerk, personal communication, 1st November 2021).

This study focusses on ten pre-selected European plant species known as *Betonica officinalis, Consolida regalis, Geranium pratense, Knautia arvensis, Lycopus europaeus, Primula elatior, Sanguisorba officinalis, Stachys sylvatica, Succisa pratensis* and *Trifolium arvense*. These species are native to Europe, and some are known to use cold stratification to germinate. Others are known to be more difficult to germinate. Their mechanism for germination is not fully understood (J. Bijkerk, personal communication, 1st November 2021). Six of these ten species are registered as endangered in the Netherlands (*Betonica officinalis, Consolida regalis, Geranium pratense, Knautia arvensis, Primula elatior* and *Sanguisorba officinalis), Succisa pratensis* and *Knautia arvensis* are stated as vulnerable. *Consolida regalis* is critically endangered in the Netherlands. The four other mentioned species (*Lycopus europaeus, Stachys sylvatica, Succisa pratensis* and *Trifolium arvense*) are stated as non-threatened species (NDFF & FLORON, 2021 a-j). These species are known as cold and/or difficult germinators, from which the ex-situ germination has been irregular (J. Bijkerk, personal communication, 1st November 2021). This irregular germination success has led to the aim of answering the following question: "What effect does cold stratification have on the germination of ten pre-selected difficult germinators?" To answer the main question, three sub questions have been formed:

1. What is the effect of duration of cold stratification on germination (success and rate)?
2. What is the effect of temperature on germination (success and rate)?
3. What is the effect of the interaction between temperature and duration of cold stratification on germination?

The objective of the present study is to determine if cold stratification can regulate the germination of the ten selected species. This study aims to determine which pre-treatments have the highest germination rate and which pre-treatment is most suitable for each of the ten pre-selected species. The hypothesis is that the cold stratification pre-treatment will regulate the germination process of the mentioned species.

Chapter 1 describes the introduction, the matter of this study and the main- and sub questions. Chapter 2 describes the materials and methods that will be used for this study. The results are presented in chapter 3, where different treatments are discussed separately. Chapter 4 contains the discussion, where a summary of the results is shown, and the methods and results are discussed. The conclusion and answers to the sub- and main question(s) is answered in chapter 5, Conclusion. Bibliographic information can be found in chapter 6. Rough data, species overviews, suggested registration form and germination results are shown in the appendix.

Chapter 2 | Materials and methods

Chapter two describes the way this study was carried out, where 2.1 describes the seed collection. Paragraph 2.2 describes the germination test and 2.3 describes the data analysis that was applied.

2.1 Seed collection

Seeds were collected from various nature reserves in the Netherlands and were gathered in 2020 with *Consolida regalis* as an exception, which was from a harvest of a seed production in 2019 (see Appendix I for an overview of the selected species and Appendix III for plant characteristics). After harvesting, the seeds were mechanically cleaned without any acids, to only have the seeds from the plants left. First, the plants were dried and cleaned from other seeds or plant material using sieves of varied sizes. Subsequently, these seeds were kept in a dark seed vault at Cruydt-Hoeck with a temperature of 5,5 °C and a relative humidity of around 30%. The mean storage period, i.e., the period from harvesting to germination was for nine species one year and for one species *(Consolida regalis)* it was two years. For this experiment, the seeds had been taken out of the vault and were placed in another vault with a temperature of 15 °C for storage and 8/16 hours diurnal cycles. Every two weeks the seeds were taken out of the second vault, counted, and sown into an agar medium (see 2.2 Germination test).

2.2 Germination test

The ten mentioned plant species were tested on germination success under influence of two factors: temperature (°C) and duration of cold stratification (in weeks) (see Appendix II for the registration form). The study included three treatments: two, four and six weeks of cold stratification, and three different temperatures: 2 °C, 5 °C and 8 °C measuring the germination of 50 seeds per temperature and duration of cold stratification. In total there were nine treatments with one control group receiving no stratification period. Every two weeks seeds were collected from the vault and were sown into agar medium.

First, a cold stratification period of six weeks was performed on the ten mentioned species. 50 seeds per species were used per temperature and duration of cold stratification (150 seeds per species per sowing moment). The seeds were sown per 25 seeds in one disinfected (ethanol) Petri dish of Soda-lime (20x100mm) and an agar powder CMN was used to make cold and moist stratification possible. The germination substrate does not influence germination, since germination is just a matter of water retention capacities and that seeds don't get drenched (Baskin and Baskin, 1998), which made Agar powder of CMN suitable. For the agar medium the ratio of Davies, Di Sacco and Newton (2015) were used. Once the agar was poured into the Petri dishes (30-33 cl per dish) the seeds were sown in the agar using forceps that were disinfected with ethanol and for every new dish the table and forceps were disinfected again. The dishes were sealed using Parafilm and a corresponding code was written on the Parafilm. Plated seeds were chilled at 2 °C, 5 °C and 8 °C to break dormancy for a period of six weeks. Per species six Petri dishes within total 150 seeds were prepared: two dishes to be placed in an environment of 2 °C, two dishes were placed in a 5 °C environment and two dishes were putted in an 8 °C cooling environment. This procedure was repeated for the four- and two-weeks period. An overview of seed counting, sowing, and screening of germination is shown in table 1 on the next page.

After six weeks a control group was sown (the seeds had been kept in the 15 °C vault) in the same way as the groups that had a cold stratification period. Once the control group was sown, the dishes in the cooling environment were collected and all the dishes were transferred to the growing environment which contains a germination box, and this box maintains a temperature between 20 °C to 26 °C and 8/16 hours diurnal cycles. Studies of Baskin and Baskin (1998) show that an alternating diurnal temperature rhythm is better for germination. To maximise germination for the studied species, the temperature regime according to ISTA (1996) was applied. The light in the germination box did not reach the end of the box, which made rotation of the dishes necessary. Every Wednesday the seeds were rotated, to provide the required light.

On the third day after sowing, the first screening took place. Every two days (Monday, Wednesday, and Friday) the germination status was evaluated up to 28 days. Screening and counting of the germinated seeds were done every morning on the mentioned days between 8.00 and 10.00. A seed was defined as germinated when 2 mm of the radicle had emerged (Bewley, 1997). Marker dots were placed on the Petri dish to

avoid double calculations. The emergence of fungi in the dishes and on the seeds, made counting more difficult, because some fungi have thin threads that look like an embryo or root, so a microscope had to be used to follow germination. Counting's were registered in a registration form (Appendix I).

Table 1

Overview of planned activities to execute the germination test

Performed activities										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
Sown + cold stratification	3-12-2021			19-11-2021			5-11-2021			
Germination box	17-12-2021			17-12-2021			17-12-2021			17-12-2021
Screening 1	17-12-2021			17-12-2021			17-12-2021			
Screening 2	20-12-2021			20-12-2021			20-12-2021			20-12-2021
Screening 3	22-12-2021			22-12-2021			22-12-2021			22-12-2021
Screening 4	24-12-2021			24-12-2021			24-12-2021			24-12-2021
Screening 5	27-12-2021			27-12-2021			27-12-2021			27-12-2021
Screening 6	29-12-2021			29-12-2021			29-12-2021			29-12-2021
Screening 7	31-12-2021			31-12-2021			31-12-2021			31-12-2021
Screening 8	3-1-2022			3-1-2022			3-1-2022			3-1-2022
Screening 9	5-1-2022			5-1-2022			5-1-2022			5-1-2022
Screening 10	7-1-2022			7-1-2022			7-1-2022			7-1-2022
Screening 11	10-1-2022			10-1-2022			10-1-2022			10-1-2022
Screening 12	12-1-2022			12-1-2022			12-1-2022			12-1-2022
Screening 13	14-1-2022			14-1-2022			14-1-2022			14-1-2022

2.3 Data analysis

Duration of cold stratification (2, 4 and 6 weeks) and influence of temperature during stratification (2, 5 and 8 °C) on the duration of germination and the percentage of established seedlings was analysed statistically. The methods that were used were General Linear Model, with 95 % significance and a Multi Analysis of Variance (ANOVA). N is the total number of seeds in one accession and X is the total of germinated seeds in that accession (Olsson et al., 2010). All statistical analysis was performed using IBM SPSS Statistics 28.0.1.0 (142).

With interpreting data in SPSS, the significance- and Partial Eta Squared level was noted. To interpret the values for Partial Eta Squared the following rules of Z. (2021) were used:

- .01: Small effect size
- .06: Medium effect size
- .14 or higher: Large effect size

Chapter 3 | Results

This chapter describes the results that were retrieved by screening establishment of radicals of seedlings, whereafter statistical analysis was performed. In the subsequent paragraphs, the results are treated separately for five species showing significant effect of stratification treatment. *Betonica officinalis* is being assessed in section 3.1, followed by *Geranium pratense* in section 3.2. 3.3 describes the results for *Primula elatior* and 3.4 for *Sanguisorba officinalis*. Results of *Trifolium arvense* are shown in section 3.5. In Appendix V the data from SPSS per species is shown. Five species did not respond to the cold stratification treatment and germination has virtually not occurred. Therefore, the right conditions for germination were not met or seeds were not viable. The species this applied to were *Consolida regalis, Knautia arvensis, Lycopus europaeus, Stachys sylvatica* and *Succisa pratensis*. See Appendix IV for the results.

The nine different treatments were coded according to temperature and duration of stratification. In table 2 these codes are listed as used during statistical analyses in SPSS.

Table 2

Description of used terms for treatment

In SPSS	Duration of cold stratification	Temperature
2/2	Two weeks	2 °C
2/5	Two weeks	5 °C
2/8	Two weeks	8 °C
4/2	Four weeks	2 °C
4/5	Four weeks	5 °C
4/8	Four weeks	8 °C
6/2	Six weeks	2 °C
6/5	Six weeks	5 °C
6/8	Six weeks	8 °C
Con	Control-group	Control-group

Table 3 shows data retrieved from SPSS using Anova to analyse the data. Per species the significance and the Partial eta squared (PES) is shown per sub-question (effect of temperature, effect of duration of cold stratification and the interaction between these two). While discussing the results per species, reference will be made to table 3.

Table 3

p-value and PES for duration of stratification, temperature, and the interaction between these two terms

Species	*Duration*		*Temperature*		*Interaction*	
	Sig.	PES*	Sig.	PES*	Sig.	PES*
Betonica officinalis	0,066	0,419	0,316	0,206	0,315	0,352
Geranium pratense	0,696	0,07	0,215	0,041	0,939	0,07
Primula elatior	<,001	0,953	<,001	0,807	0,464	0,280
Sanguisorba officinalis	<,001	0,767	0,076	0,402	0,435	0,293
Trifolium arvense	0,663	0,079	0,663	0,079	0,833	0,125

** PES stands for Partial eta squared*

3.1 Betonica officinalis

Table 3 shows that duration of stratification does not meet the required significance value of p<0,05, but it must be considered that this value is with p<0,066 almost significant. No significance is shown at the p-value for temperature (0,316) and the interaction between duration and temperature (0,315). However, for all three terms the PES-value raises above the 0.14 that is required to have an effect.

As for the different treatments that were applied, figure 1 gives a clear overview of the germination of seeds during the 28 days of screening. Treatment 6/8 stands out with the highest germination rate, followed by treatment 6/5. This figure confirms the significance and PES of *Betonica officinalis*. Figure 2 shows the germination mean where duration of stratification is placed together with temperature. Germination rate rises once the stratification period is longer, with the highest germination rate for 6/8 treatment.

Figure 1

Total germination per treatment for Betonica officinalis

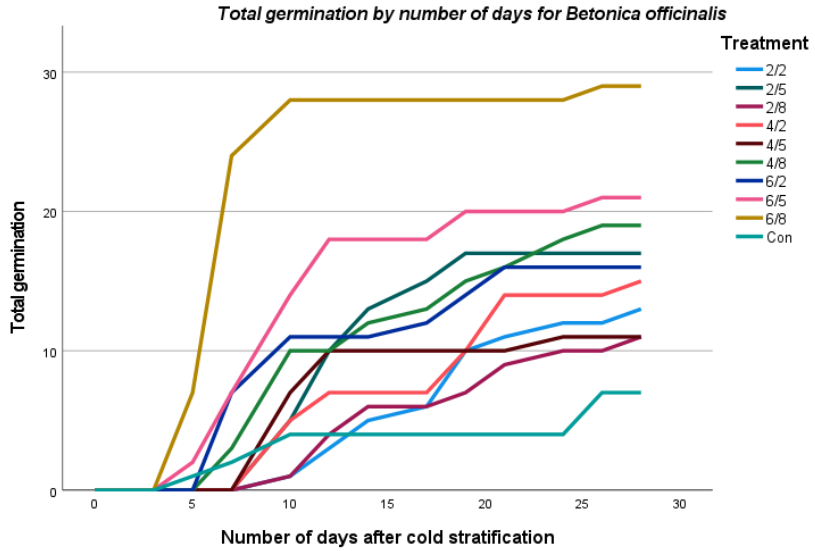
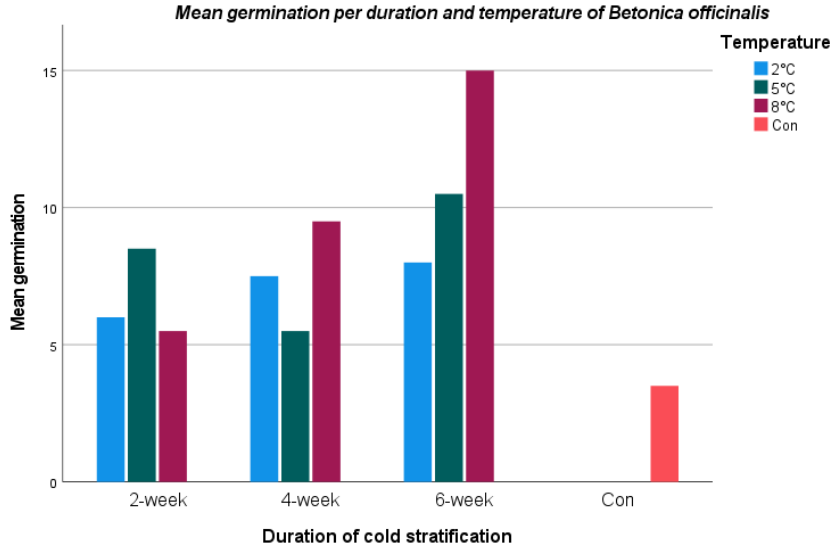


Figure 2

Mean germination per duration and temperature for Betonica officinalis



3.2 Geranium pratense

The results for *Geranium pratense*, as seen in table 3, show no significance at the p<0,05 for duration (0,696), temperature (0,215) nor the interaction between these terms (0,939). However, for duration and interaction a PES of 0,07 and a PES of 0,041 for temperature suggest that there is a medium effect.

Considering figure 3, most of the seeds germinated after the four-week stratification period with a temperature of 5 °C. Cold stratification for six weeks in an 8 °C cooling environment shows a quick germination after three days and then stabilizes. A two-week stratification period results in the lowest germination rate. Most germination occurred after 17 days. Figure 4 shows the germination mean during screening per treatment. Within the four- and six-week period, most of the seeds germinated after the 5 °C stratification. More seeds germinated during a longer stratification period, however, also without stratification, seeds germinated well.

Figure 3

Total germination per treatment for Geranium pratense

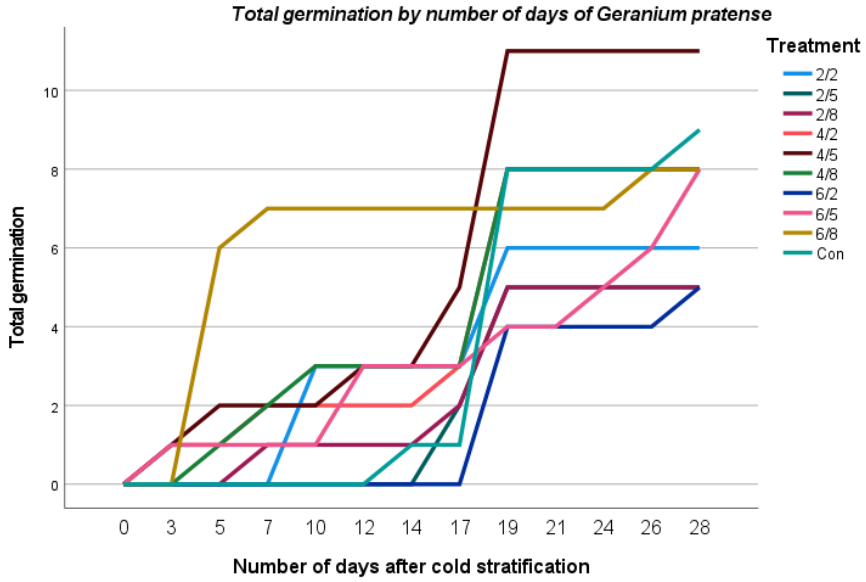
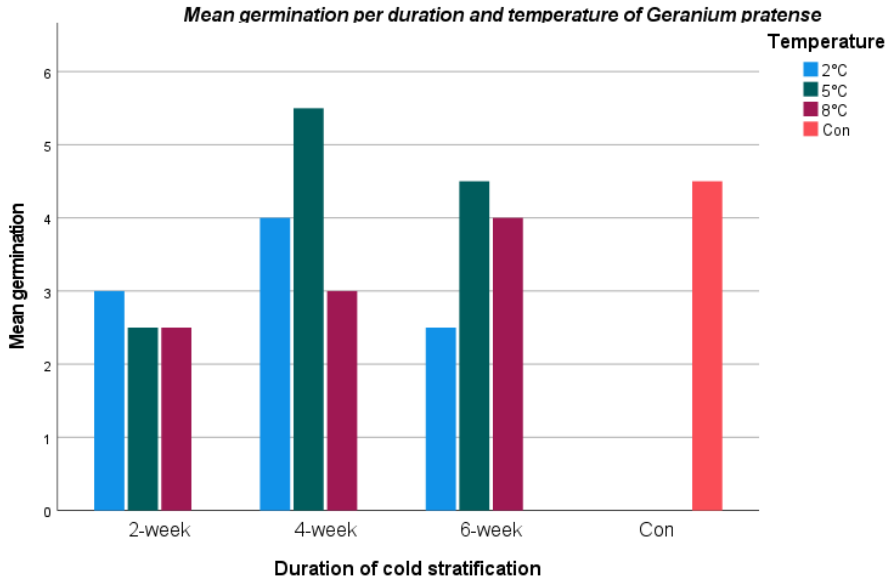


Figure 4

Mean germination per duration and temperature for Geranium pratense



3.3 Primula elatior

Even though not that many seedlings established of *Primula elatior* high significance levels and values could be retrieved. As shown in table 3 duration of stratification ($p<0,001$) and temperature ($p<0,001$) show a high significant effect on germination for this species. The high PES-value for both terms (duration: 0,953, temperature: 0,807) show that there is a large effect size. Duration of stratification and temperature during stratification have an important effect on germination. The interaction between these two terms seems to be less significant (0,464), but the PES for interaction (0,280) implies that there is a large effect size.

Figure 5 gives an overview of germinated seeds during the 28 days of screening per treatment. It shows that germination occurs mostly after the six-week stratification period and most seeds germinated at 5 °C. The second figure, figure 6 confirms figure 5 by showing mostly germination after six weeks of stratification.

Figure 5

Total germination per treatment for Primula elatior

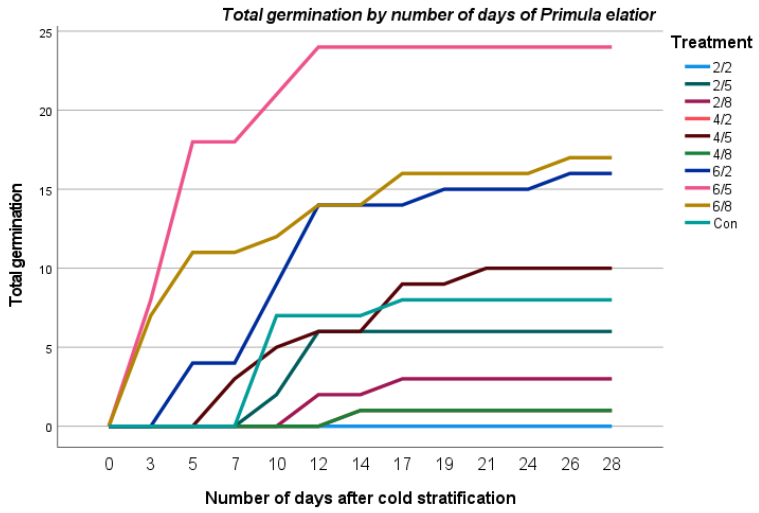
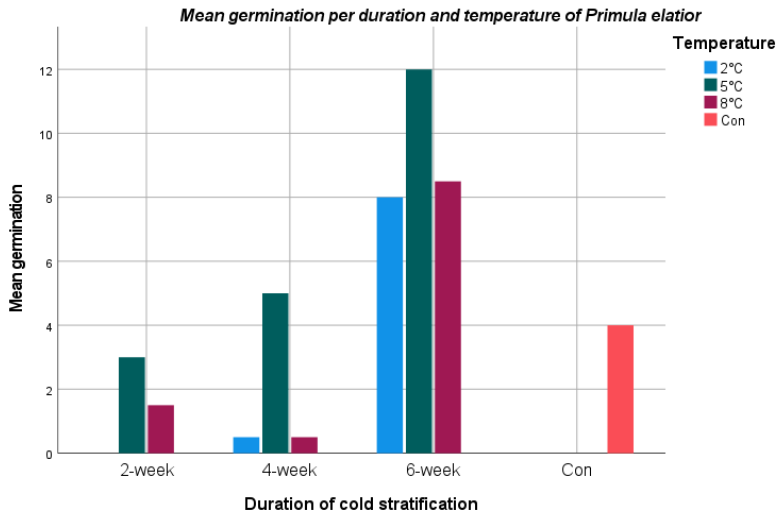


Figure 6

Mean germination per duration and temperature for Primula elatior



3.4 Sanguisorba officinalis

Sanguisorba officinalis germinated in relatively high numbers compared to the other species, which had a positive effect on the analysis. Considering the results for *Sanguisorba officinalis*, as seen in table 3, show no significance at the p<0.05 for interaction between duration (0,435) and stratification (0,076). However, the p-value for duration (<,001) shows this term has a considerable influence on germination. The p-value of temperature almost meets the required significance level. For all measured terms, the PES-value is higher than 0,14 and therefore shows a large effect on germination.

Figure 7 shows the total germination for *Sanguisorba officinalis* during screening days for every treatment. This species germinated in relatively high numbers, with most germination after the six weeks at 5 °C treatment. The six weeks at 2 °C treatment follows and the two weeks period at 2 °C and 5 °C make up the third and fourth place. This is confirmed by the second figure 8, which shows most germination within the two- and six-week stratification period.

Figure 7

Total germination per treatment for Sanguisorba officinalis

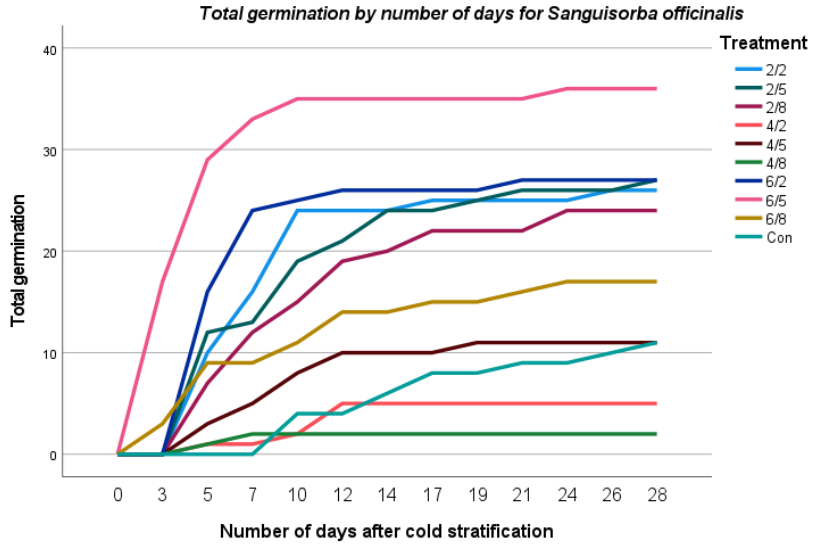
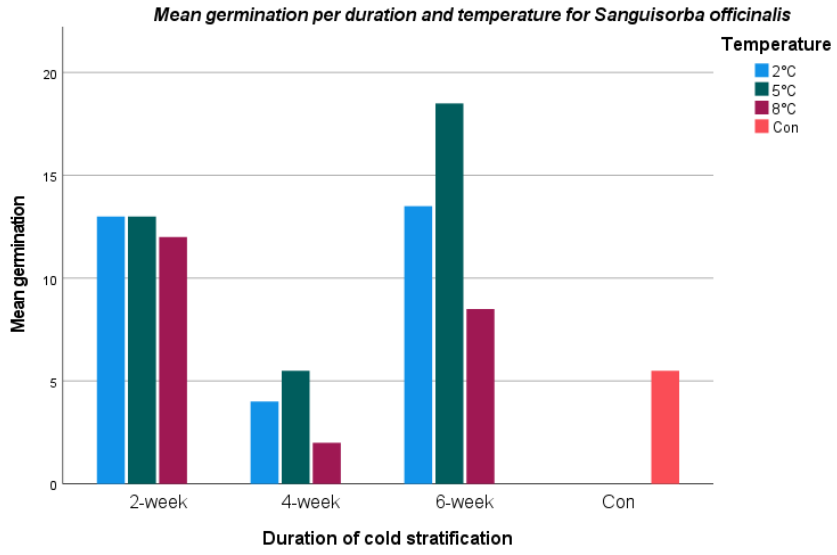


Figure 8

Mean germination per duration and temperature for Sanguisorba officinalis



3.5 Trifolium arvense

Trifolium arvense was the only species that mostly germinated during the cold stratification at all temperatures and during screening germination declined. This must be taken account since germination was already noted on day 0 (figure 9). However, germination did not occur on day 0 of screening. When germination in the cooling environment exactly has occurred is unknown. For all three terms the significance level is too high to meet the p<0,05 value (duration: 0,663; temperature: 0,663; interaction: 0,833). The same counts for the PES-value of duration (0,079) and temperature (0,079), although a medium effect for these terms can be recognized. The interaction between duration and temperature (0,125) has a higher PES-value than for those terms separately, but still counts for a medium effect size.

Figure 9 shows germination for *Trifolium arvense* on day 0, where most germination occurred for the six-week, 8 °C treatment, six-week, 2 °C period and the two-week, 5 °C treatment. After three days the four-week, 5 °C took over in seedling establishment. According to figure 10, high germination occurred within the two- and six-week stratification period for 2 °C. Germination declined for a temperature of 5 °C as the weeks for stratification increased. Highest and more consisted establishment of seedlings can be seen at a temperature of 2 and 8 °C.

Figure 9

Total germination per treatment for Trifolium arvense

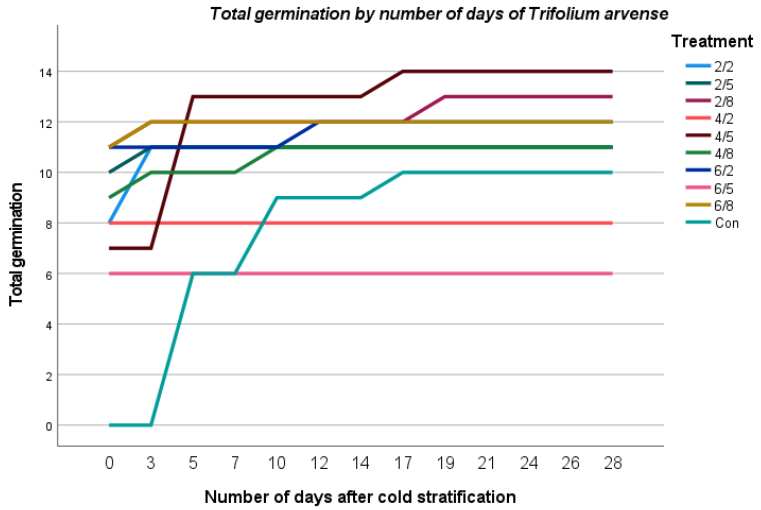
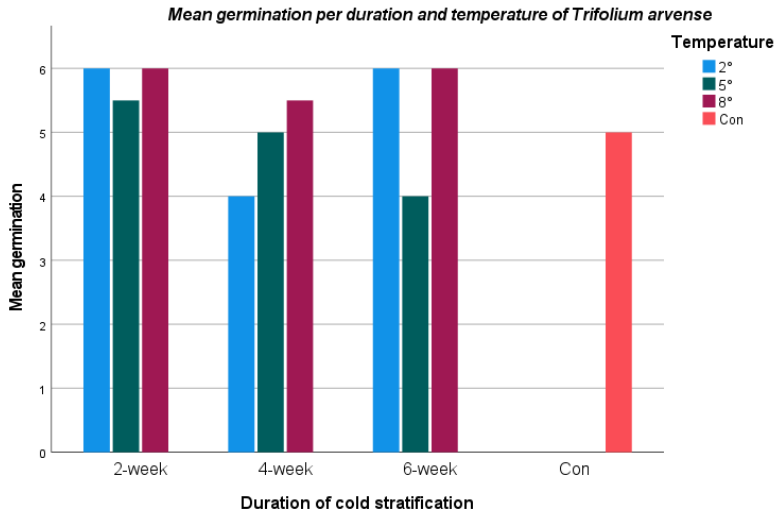


Figure 10

Mean germination per duration and temperature for Trifolium arvense



Chapter 4 | Discussion

Aim of this thesis was to establish which method of pre-treatment has the highest germination rate and which treatment is optimal for the establishment of seedlings for the ten selected species.

The expectation was for regulation in the germination process across all ten species, this turned out to be incorrect. Five out of ten did not respond with high germination rates and therefore statistical analysis was not performed. This applied for *Consolida regalis, Knautia arvensis, Lycopus europaeus, Stachys sylvatica* and *Succisa pratensis*. *Betonica officinalis, Geranium pratense, Primula elatior, Sanguisorba officinalis* and *Trifolium arvense* did respond well to the treatment.

Betonica officinalis germinated quite well under certain conditions, and there is small evidence that temperature has a significant effect on germination. The PES of temperature, duration of stratification and the interaction between these two was relatively high, and this suggests a large effect on establishment of seedlings. Treatment 6/8 and 6/5 showed the highest germination rate for this species. Establishment of seedlings was accomplished at Cruydt-Hoeck with earlier research with temperatures of 5 – 15 °C and this implies that temperatures in the germination box have been too high. Lower temperatures and more time for germination is worth investigating further.

Consolida regalis did not respond well to the applied treatments. However, this species showed high germination rates during sowing experiments from Cruydt-Hoeck, using temperatures of 20 °C during the day and 10 °C at night. According to Kew (-a, n.d.), mostly lower temperatures during the night result in higher germination rates. Otte (1996) described during a germination test on the Munich plain, this species had a strong preference for low germination temperatures and Lang et al. (2016) had the highest germination rates in a climate chamber where the seeds stayed for six weeks. For further studies at least six weeks of stratification period should be applied with lower temperatures during the night.

For *Geranium pratense* no significant p-value could be retrieved with the sample size, but the PES-value for the three terms suggests a medium effect size. Figures show that treatments 6/8, 6/5 and 4/5 results in the highest germination rate. Studies from Van Assche & Vandelook (2006) show cumulative germination percentages after an eight-week stratification period of 5 °C, whereafter the seeds got transferred to a 23 °C environment. Results within this study could be confirmed with a larger sample size and a longer stratification period, and this is worth investigating further.

Knautia arvensis hardly germinated during this study and a reason for this might be that this species did not need a cold stratification period, since it is known that this species has an irregular germination process. Earlier sowing experiments of Cruydt-Hoeck sowed an 80% germination rate after six weeks with temperatures between 15 – 19 °C. The experiment would show if a cold stratification period would regulate germination, instead it had a negative influence on the process. Possibly a larger effect can be retrieved with a larger sample group, no cold stratification, and higher temperatures. However, the control-group did not germinate either, which implies that the environment of the germination box or the seeds have not met the right conditions for germination.

Lycopus europaeus responded the least to the treatments, only three seeds germinated. According to Kew (-b, n.d.), *Lycopus europaeus* needs warm days and cold nights to germinate. The duration of cold stratification might have been too short and a larger variation in temperatures might be needed. Germination is possible with low night temperatures (10 °C) and high day temperatures (25 °C) (Kew -b, n.d.). This suggestion can be confirmed by Thompson (1969), who writes that *Lycopus europaeus* is absolutely depended on diurnal temperature fluctuations and light. The study did not show specific temperatures that are needed but did prove that a diurnal fluctuation of more than 7 °C must be applied for germination. A cumulative effect was retrieved and so, rise and fall of temperature is essential for *Lycopus europaeus*' germination.

The germination rate of *Primula elatior* showed high significance values in duration of stratification and temperature. The PES-value for these terms were high too, which suggests a large effect size. The conditions and length of stratification have a large effect for establishment of seedlings. *Primula elatior* responded best to the 6/5 treatment and on the six-week stratification period overall. The experience of Cruydt-Hoeck with *Primula elatior* is high germination rates in March and April when sowing in January and

February (J. Bijkerk, personal communication, 24th January 2022) and these results suggests that a shorter stratification period is also suitable for this species. Further germination of the species might have occurred with lower temperatures in the germination box, since this species germinates mostly in spring (Browne, 1995).

Results from *Sanguisorba officinalis* suggest that duration has a significant effect on the germination process of this species, and a significant effect of temperature was almost met. This could be met with a larger sample size. For all three terms a large effect size on germination came out of the results. Most of the seeds of *Sanguisorba officinalis* germinated during the 6/5 treatment, followed by 6/2, 2/2 and 2/5 treatments. The longest stratification period (six weeks) had the highest germination rate, and a longer stratification period could have a positive effect on germination. However, studies by Holloway & Matheke (2003) show germination of *Sanguisorba officinalis* with temperatures ranging from 5 °C to 30 °C, which imply rapid germination under controlled conditions and suggesting no special pre-treatments would be necessary for this species.

For *Stachys sylvatica* the same applies as for *Lycopus europaeus*. Sowing in December might give establishment of seedlings in March and April, due to lower temperatures at night. In addition, Taylor & Rowland (2010) studied germination of *Stachys sylvatica* and showed establishment of seedlings in light to moderate shade. The red/far red ratio was more critical than reduced irradiance on the plant's response to shading, which might have an influence on germination. Further studies could take this in account for germination success.

During sowing experiments at Cruydt-Hoeck *Succisa pratensis* germinated with a rate of 60% within three weeks at a temperature of 18,8 °C without stratification. As a slow germinator a short cold stratification period might have been enough to trigger germination for *Succisa pratensis*. Another explanation for the low germination rates is the light requirement of *Succisa pratensis*. Isselstein et al. (2002) showed a slight improved of germination for *Succisa pratensis* between existing vegetation, which implied that this species has a lower light requirement. Grime et al. (1981) confirmed this who has also found higher germination rate of the species in the dark or under a leaf canopy. An explanation for this could be that *Succisa pratensis* favours high moisture levels for germination. Follow-up research could experiment with various levels of light and moisture to find the optimal levels for this species.

Seeds of *Trifolium arvense* germinated during the cold stratification period, which implies that this species does not need warmth to germinate. In 1916 Harrington showed that *Trifolium* seeds were weakened by temperature fluctuations (below 10 °C and above 20 °C). Harrington concluded that *Trifolium* would germinate in spring, with high fluctuations in temperatures occur. Sowing experiments of Cruydt-Hoeck showed that light has a positive influence on germination compared to darkness (sowing deeper). Since this species started germinating in the cooling environment, no significant values could be retrieved. The PES-value for *Trifolium arvense* implies a medium effect size for the three terms, which could be further investigated. Based on the figures most germination occurred during the 6/8, 6/2 and 2/5 treatment and most of the seeds germinated at a temperature of 8 °C. Further research should apply temperature fluctuations and a longer stratification period.

Warmth even seemed to inhibited germination of *Trifolium arvense* and this may have been the case for *Betonica officinalis, Primula elatior* and *Stachys sylvatica*. This suggests that cold stratification is an effective technique for germination, and in fact placing the seeds in a 5 °C environment until full germination could be the most effective method.

Seeds were sown every two weeks, after which the plates were placed in a cooling environment. The advantage of this method was that all the Petri dishes could be placed in the germination box at the same time. The seeds were not sown at the same time and slight differences in the working method or environment may have influenced the germination process and can be seen as a limitation. There is a potential that different sowing times could have influenced the results.

The light in the germination box did not reach the end of the box, which made rotation of the Petri dishes necessary. Every week the dishes were rotated on the same day, but the variation in light availability will most likely have had an influence on seedling establishment.

Temperature was not as constant as one would aim for in a germination test. As with the reach of the light, the temperature on the edge of the germination box was lower than in the middle of the box. The temperature ranged between 22 °C and 26 °C. The temperature at night was not measured. An estimation of 20 °C was made, but this temperature might have been too high for night temperature. Temperatures at night should be between 5 °C and 10 °C. For follow-up research there must be an even distribution of light and temperature throughout the germination box and temperature at night must be lower.

Large densities of fungi developed in and on the seeds in the Petri dishes. These fungi had two ways of negative influence on the experiment. First, the fungi grew in some cases faster than the seeds would germinate. This made the screening of germination difficult and sometimes the seeds could not be seen. Screening with a microscope made it an easier to detect germination. Second, it is unknown what the influence of these fungi was on the seeds and the germination process, and if this influence is positive or negative for establishment of seedlings. A conscious choice was made to work with non-sterilized seeds, since the experiment is about wildflowers. However, to research purely the germination of the plant species, working with sterilized seeds needs to be considered for more trustworthy results.

A Tetrazolium test can be performed for follow-up research, to guarantee the vitality of the seeds.

Five out of ten plant species didn't respond to the stratification treatment and was not analysed with statistics. The conditions that were sued were incorrect and different conditions should be applied for follow-up research. For the other five species more data was collected, but a longer stratification period, screening period and a larger sample size could have led to a higher percentage of meaningful results.

Chapter 5 | Conclusion

The objective of this thesis was to retrieve a stratification method for establishment of seedlings for *Betonica officinalis, Consolida regalis, Geranium pratense, Knautia arvensis, Lycopus europaeus, Primula elatior, Sanguisorba officinalis, Stachys sylvatica* and *Trifolium arvense*. The seeds were sown into Petri dishes containing an agar medium and placed in a cooling environment. Three different temperatures and durations of stratification were tested and the results of the effect of temperature, the effect of duration of stratification and the interaction between these two was described. By reaching the objective, an answer to the sub- and main question was retrieved.

Most results for the effect of temperature, duration, and the interaction between these two were insignificant. However, there are some measured effect sizes, and as such this suggests that this study is of insufficient size to show effect.

Five out of ten species did not germinate and different methods in combination with a larger sample size should be applied to find the right temperature and duration of stratification and herewith the most suitable treatment. This applied for *Consolida regalis*, *Knautia arvensis, Lycopus europaeus, Stachys sylvatica* and *Succisa pratensis*. Therefore, no answer on the objective of the study was retrieved, but methods used in this study can be excluded in further research. However, for the other five species some results were retrieved and a larger sample size during further investigation could confirm results and suggestions from this study.

Results of duration of stratification for *Betonica officinalis* suggests that this term has a significance influence on the germination process and a larger sample size could confirm this suggestion. The effect of temperature nor the interaction between duration and temperature could be measured properly, however the PES-value suggests that for all three terms there could be a large size effect. *Betonica officinalis* responded the most to the 6/8 and 6/5 treatment.

No significant effect was found for temperature, duration, and interaction in between on the germination process of *Geranium pratense*. However, values of PES for the mentioned terms implies that the researched terms could have a medium effect which could be confirmed with a larger sample size. Treatment 6/8, 6/5 and 4/5 gained most of the established seedlings for this species.

Duration of stratification and temperature both showed a significant effect on the germination of *Primula elatior*. Also, the PES for interaction of the two terms presented a large effect size and thus is important for germination. Most of the seeds of *Primula elatior* germinated during the six weeks of stratification, with a highest germination rate at the 6/5 treatment.

For *Sanguisorba officinalis* no significant value was measured for interaction between duration and stratification. However, the p-value for duration of stratification shows this term has a considerable influence on germination. For all measured terms, the PES-value is higher than 0,14 and therefore shows a large effect on germination. Further studies with a larger sample size could confirm these values. This species germinated mostly during the 6/5, 6/2, 2/2 and 2/5 treatments, which implies that even a shorter stratification period can be applied for germination.

Trifolium arvense germinated mostly during the stratification period and this implies that this species does not need warmth or light to germinate. Establishment of seedlings during stratification might be the explanation why the p-value of significance for all three terms is too high to meet the $p<0,05$ value. For the PES-value a medium effect can be seen for duration, temperature, and the interaction between these two. Seeds of *Trifolium arvense* had the highest germination rate during the 6/8, 6/2 and 2/5 treatments.

Suggested is for follow-up to make use of a larger sample size and longer stratification period. A solution on the development of fungi needs to be found, to out rule this possible effect on germination.

Chapter 6 | Literature

Amen, R. D. (1968). A model of seed dormancy, The Botanical Review, vol. 34, pp. 1–31.

Baskin, C. C. & Baskin, J. M. (1988). Germination ecophysiology of herbaceous plant species in a temperate region, American Journal of Botany, vol. 75, pp. 286–305.1988

Baskin, C. C. & Baskin, J. M. (1998). Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination. San Diego: Academic Press.

Baskin, C. C. & Baskin, J. M. (2005). Seed dormancy in wildflowers. In: McDonald M.B. and Kwong F. Y. (eds.), Flower Seeds: Biology and Technology. Wallingford: CABI Publishing, pp. 163–185.

Baskin, C. C. & Baskin, J. M. (2014). Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination, second ed. Elsevier Academic Press Inc., San Diego.

Baskin, J. M. & Baskin, C. C. (2004). A classification for seed dormancy. Seed Sci. Res., 14 (1), 1-16.

Bewley, J. D. (1997). Seed Germination and Dormancy, The Plant Cell, vol. 9, pp. 1055–1066.

Bowes, B. G. (1999). A Colour Atlas of Plant Propagation and Conservation. Manson Publishing Ltd, London.

Browne, K. (1995) A study of the Oxlips of Shadwell Wood. B.Sc. dissertation, Anglia Polytechnic University (now Anglia Ruskin University), Cambridge, UK.

Davies, R., Di Sacco, A., & Newton, R. (2015). Germination testing: procedures and evaluation. Technical Information Sheet_13a. Royal Botanic Gardens, Kew.

Fay, M. F. & Muir, H. J. (1990). The role of micropropagation in the conservation of European plants. In: Hernández Bermejo, J. E., Clemente, M., Haywood, V. (Eds.), Conservation Techniques in Botanic Gardens. Koeltz Scientific Books, Koenigstein, pp. 27–32.

Fay, M. F. (1992). Conservation of rare and endangered plants using in vitro methods. In Vitro Cellular Developmental Biology-Plant 28, 1–4.

Frankham, R., Ballou, J. D. & Briscoe, D. A. (2010). Introduction to conservation genetics. Cambridge University Press, Cambridge, UK.

Grime, J. P., G. Mason, A. V. Curtis, J. Rodman, S. R. Band, M. A.G. Mowforth, A. M. Neal, and S. Shaw. (1981). A comparative study of germination characteristics in a local flora. Journal of Ecology 69:1017–1059.

Harrington, G. T. (1916). Agricultural value of impermeable seeds. Journal of Agricultural Research6: 761–796.

Hilhorst, H. W. M. (2007). Definitions and hypotheses of seed dormancy. In: Bradford K. & Nonogaki H. (eds.), Seed Development, Dormancy and Germination, Annual Plant Reviews, vol. 27. Sheffield: Blackwell Publishing. pp. 50–71.

Holloway, P. S., & Matheke, G. E. (2003). Seed germination of Burnet, Sanguisorba spp. Native Plants Journal, 4(2), 95-99.

Isselstein, J., Tallowin, J. R. B., & Smith, R. E. N. (2002). Factors affecting seed germination and seedling establishment of fen-meadow species. Restoration Ecology, 10(2), 173-184.

ISTA, International Seed Testing Association. (1966). Internationale Vorschriften fur die Prüfung von Saatgut, Vol. 31/4, Wageningen, Germany.

ISTA, International Seed Testing Association. (1993). Rules for testing seeds. Seed Sci. Technol., 21, 1-259.

ISTA, International Seed Testing Association. (1996). International rules for seed testing, rules 1996, Seed Science and Technology, Zürich, Switzerland

Jaganathan, G. K., Dalrymple, S. E., & Liu, B. (2015). Towards an understanding of factors controlling seed bank composition and longevity in the alpine environment. The Botanical Review, 81(1), 70-103.

KEW. (n.d.-a). Seed Information Database: Search Results. Kew Royal Botanic Gardens. Retrieved on 24 January 2022, from https://data.kew.org/sid/SidServlet?ID=6353&Num=snr

KEW. (n.d.-b). Seed Information Database: Search Results. Kew Royal Botanic Gardens. Retrieved on 24 January 2022, from https://data.kew.org/sid/SidServlet?ID=14611&Num=b7E

Krauss, J., Bommarco, R., Guardiola, M., Heikkinen, R. K., Helm, A., Kuussaari, M., Lindborg, R., Öckinger, E., Pärtel, M., Pino, J., Pöyry, J., Raatikainen, K. M., Sang, A., Stefanescu, C., Teder, T., Zobel, M. & Steffan-Dewenter, I. (2010). Habitat fragmentation causes immediate and time-delayed biodiversity loss at different trophic levels. Ecology letters, 13(5), 597-605.

Kuussaari, M., Bommarco, R., Heikkinen, R. K., Helm, A., Krauss, J., Lindborg, R., Ökinger, E., Pärtel, M., Pino, J., Rodà, F., Steganescu, C., Teder, T., Zobel, M. & Steffan-Dewenter, I. (2009). Extinction debt: a challenge for biodiversity conservation. Trends in ecology & evolution, 24(10), 564-571.

Lang, M., Prestele, J., Fischer, C., Kollmann, J., & Albrecht, H. (2016). Reintroduction of rare arable plants by seed transfer. What are the optimal sowing rates? Ecology and Evolution, 6(15), 5506-5516.

Leiblein-Wild, M. C., Kaviani, R., & Tackenberg, O. (2014). Germination and seedling frost tolerance differ between the native and invasive range in common ragweed. Oecologia, 174(3), 739-750.

Mondoni, A., Daws, M. I., Belotti, J., & Rossi, G. (2009). Germination requirements of the alpine endemic Silene elisabethae Jan: effects of cold stratification, light and GA3. Seed Science and technology, 37(1), 79-87.

NDFF & FLORON. (2021a). NDFF Verspreidingsatlas | Betonica officinalis - Betonie. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/1244

NDFF & FLORON. (2021b). NDFF Verspreidingsatlas | Consolida regalis - Wilde ridderspoor. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/0396

NDFF & FLORON. (2021c). NDFF Verspreidingsatlas | Geranium pratense - Beemdooievaarsbek. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/0573

NDFF & FLORON. (2021d). NDFF Verspreidingsatlas | Knautia arvensis - Beemdkroon. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/0692

NDFF & FLORON. (2021e). NDFF Verspreidingsatlas | Lycopus europaeus - Wolfspoot. www.verspreidingsatlas.nl. Retrieved on 16 november 2021, from https://www.verspreidingsatlas.nl/0780

NDFF & FLORON. (2021f). NDFF Verspreidingsatlas | Primula elatior - Slanke sleutelbloem. www.verspreidingsatlas.nl. Retrieved on 16 november 2021, from https://www.verspreidingsatlas.nl/1014

NDFF & FLORON. (2021g). NDFF Verspreidingsatlas | Sanguisorba officinalis - Grote pimpernel. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/1137

NDFF & FLORON. (2021h). NDFF Verspreidingsatlas | Stachys sylvatica - Bosandoorn. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/1246

NDFF & FLORON. (2021i). NDFF Verspreidingsatlas | Succisa pratensis - Blauwe knoop. www.verspreidingsatlas.nl. Retrieved on 16 november 2021, from https://www.verspreidingsatlas.nl/1258

NDFF & FLORON. (2021j). NDFF Verspreidingsatlas | Trifolium arvense - Hazenpootje. www.verspreidingsatlas.nl. Retrieved on 16 November 2021, from https://www.verspreidingsatlas.nl/1296

Olsson, U., Englund, J-E. & Engstrand, U. (2010). Biometri – Grundläggande Biologisk Statistik,41 Lund: Studentlitteratur.

Otte, A. (1996). Populationsbiologische Parameter zur Kennzeichnung von Ackerwildkräutern. Zeitschrift für Acker- und Pflanzenbau 15:45–60.

Porceddu, M., Mattana, E., Pritchard, H. W. & Bacchetta, G. (2013). Thermal niche for in situ seed germination by Mediterranean mountain streams: model prediction and validation for Rhamnus persicifolia seeds. Annals of Botany, 112(9), 1887-1897.

Possingham, H. P., Lindenmayer, D. B., & McCarthy, M. A. (2001). Population Viability Analysis. Encyclopedia of Biodiversity, 831–843. https://doi.org/10.1016/b0-12-226865-2/00356-4

Schwienbacher, E., Navarro-Cano, J. A., Neuner, G. & Erschbamer, B. (2011). Seed dormancy in alpine species. Flora-Morphology, Distribution, Functional Ecology of Plants, 206(10), 845-856.

Storkey, J., Meyer, S., Still, K. S. & Leuschner, C. (2012). The impact of agricultural intensification and land-use change on the European arable flora. Proceedings of the Royal Society B, 279, 1421–1429.

Taiz, L. & Zeiger, E. (2010). Topic 23.18, http://5e.plantphys.net/chapter.php?ch=23 [05-02-2013].

Taylor, K., & Rowland, P. (2010). Biological Flora of the British Isles*: Stachys sylvatica L. Journal of ecology, 98(6), 1476-1489.

Thompson, P. A. (1969). Germination of Lycopus europaeus L. in response to fluctuating temperatures and light. Journal of Experimental Botany, 20(1), 1-11.

Tilman, D., May, R. M., Lehman, C. L., & Nowak, M. A. (1994). Habitat destruction and the extinction debt. Nature, 371(6492), 65-66.

Van Assche, J. A., & Vandelook, F. E. (2006). Germination ecology of eleven species of Geraniaceae and Malvaceae, with special reference to the effects of drying seeds. Seed Science Research, 16(4), 283-290.

Wang, G., Baskin, C. C., Baskin, J. M., Yang, X., Liu, G., Zhang, X., Ye, X. & Huang, Z. (2017). Timing of seed germination in two alpine herbs on the south-eastern Tibetan plateau: the role of seed dormancy and annual dormancy cycling in soil. Plant and Soil, 421(1), 465-476.

Wilcove, D. S., McLellan, C. H. & Dobson, A. P. (1986). Habitat fragmentation in the temperate zone. In: Conservation Biology, pp. 237-56 (Soule, M.E., ed.). Sunderland, MA: Sinauer.

Z. (2021, 1st March). What is Partial Eta Squared? (Definition & Example). Statology. Consulted on 26 January 2022, from https://www.statology.org/partial-eta-squared/

Appendix

Appendix I | Overview of the selected species with ID and code of provenance

ID	Code	Species	Number of seeds (x2)	Temperature	Length in time
1	200491	Betonica officinalis	25	2	2
2	200491	Betonica officinalis	25	5	2
3	200491	Betonica officinalis	25	8	2
4	200491	Betonica officinalis	25	2	4
5	200491	Betonica officinalis	25	5	4
6	200491	Betonica officinalis	25	8	4
7	200491	Betonica officinalis	25	2	6
8	200491	Betonica officinalis	25	5	6
9	200491	Betonica officinalis	25	8	6
10	200491	Betonica officinalis	25	Control	
11	2108267	Consolida regalis	25	2	2
12	2108267	Consolida regalis	25	5	2
13	2108267	Consolida regalis	25	8	2
14	2108267	Consolida regalis	25	2	4
15	2108267	Consolida regalis	25	5	4
16	2108267	Consolida regalis	25	8	4
17	2108267	Consolida regalis	25	2	6
18	2108267	Consolida regalis	25	5	6
19	2108267	Consolida regalis	25	8	6
20	2108267	Consolida regalis	25	Control	
21	200346	Geranium pratense	25	2	2
22	200346	Geranium pratense	25	5	2
23	200346	Geranium pratense	25	8	2
24	200346	Geranium pratense	25	2	4
25	200346	Geranium pratense	25	5	4
26	200346	Geranium pratense	25	8	4
27	200346	Geranium pratense	25	2	6
28	200346	Geranium pratense	25	5	6
29	200346	Geranium pratense	25	8	6
30	200346	Geranium pratense	25	Control	
31	200233	Knautia arvensis	25	2	2
32	200233	Knautia arvensis	25	5	2
33	200233	Knautia arvensis	25	8	2
34	200233	Knautia arvensis	25	2	4
35	200233	Knautia arvensis	25	5	4
36	200233	Knautia arvensis	25	8	4
37	200233	Knautia arvensis	25	2	6
38	200233	Knautia arvensis	25	5	6
39	200233	Knautia arvensis	25	8	6

40	200233	Knautia arvensis	25	Control	
41	200533	Lycopus europaeus	25	2	2
42	200533	Lycopus europaeus	25	5	2
43	200533	Lycopus europaeus	25	8	2
44	200533	Lycopus europaeus	25	2	4
45	200533	Lycopus europaeus	25	5	4
46	200533	Lycopus europaeus	25	8	4
47	200533	Lycopus europaeus	25	2	6
48	200533	Lycopus europaeus	25	5	6
49	200533	Lycopus europaeus	25	8	6
50	200533	Lycopus europaeus	25	Control	
51	190029	Primula elatior	25	2	2
52	190029	Primula elatior	25	5	2
53	190029	Primula elatior	25	8	2
54	190029	Primula elatior	25	2	4
55	190029	Primula elatior	25	5	4
56	190029	Primula elatior	25	8	4
57	190029	Primula elatior	25	2	6
58	190029	Primula elatior	25	5	6
59	190029	Primula elatior	25	8	6
60	190029	Primula elatior	25	Control	
61	200425	Sanguisorba officinalis	25	2	2
62	200425	Sanguisorba officinalis	25	5	2
63	200425	Sanguisorba officinalis	25	8	2
64	200425	Sanguisorba officinalis	25	2	4
65	200425	Sanguisorba officinalis	25	5	4
66	200425	Sanguisorba officinalis	25	8	4
67	200425	Sanguisorba officinalis	25	2	6
68	200425	Sanguisorba officinalis	25	5	6
69	200425	Sanguisorba officinalis	25	8	6
70	200425	Sanguisorba officinalis	25	Control	
71	200484	Stachys sylvatica	25	2	2
72	200484	Stachys sylvatica	25	5	2
73	200484	Stachys sylvatica	25	8	2
74	200484	Stachys sylvatica	25	2	4
75	200484	Stachys sylvatica	25	5	4
76	200484	Stachys sylvatica	25	8	4
77	200484	Stachys sylvatica	25	2	6
78	200484	Stachys sylvatica	25	5	6
79	200484	Stachys sylvatica	25	8	6
80	200484	Stachys sylvatica	25	Control	
81	200500	Succisa pratensis	25	2	2

82	200500	Succisa pratensis	25	5	2
83	200500	Succisa pratensis	25	8	2
84	200500	Succisa pratensis	25	2	4
85	200500	Succisa pratensis	25	5	4
86	200500	Succisa pratensis	25	8	4
87	200500	Succisa pratensis	25	2	6
88	200500	Succisa pratensis	25	5	6
89	200500	Succisa pratensis	25	8	6
90	200500	Succisa pratensis	25	Control	
91	200396	Trifolium arvense	25	2	2
92	200396	Trifolium arvense	25	5	2
93	200396	Trifolium arvense	25	8	2
94	200396	Trifolium arvense	25	2	4
95	200396	Trifolium arvense	25	5	4
96	200396	Trifolium arvense	25	8	4
97	200396	Trifolium arvense	25	2	6
98	200396	Trifolium arvense	25	5	6
99	200396	Trifolium arvense	25	8	6
100	200396	Trifolium arvense	25	Control	

Appendix II | Registration form

Screening date												
Temperature (°C)	2	5	8		2	5	8		2	5	8	
Duration of cold stratification (weeks)	2	2	2		4	4	4		6	6	6	Control
Species												
Betonica officinalis												
Consolida regalis												
Geranium pratense												
Knautia arvensis												
Lycopus europaeus												
Primula elatior												
Sanguisorba officinalis												
Stachys sylvatica												
Succisa pratensis												
Trifolium arvense												

Appendix III | Characteristics of the ten selected plant species

Species	Family	Habitat	Soil	Rareness	Status	Year collected	Province	Country
Betonica officinalis	Lamiaceae	Forest edges, grassland, roadsides, hollow roads	Loam, sand, sludge, marl, stony places	Rare	Endangered	2020	Limburg	Netherlands
Consolida regalis	Ranunculaceae	Fields, ruderal places	Sludge, light clay	Exceed-ingly rare	Critically endangered	2019	-	Germany
Geranium pratense	Geraniaceae	Rough roadsides, grassland, thicket, railways, em-bankments, forests	Sand, loam, sludge, clay	Quite rare	Least concern	2020	Duursche Waarden, Overijssel	Netherlands
Knautia arvensis	Caprifoliaceae	Roadsides, grassland, hollow roads, river embank-ments, railways, thicket, forest edge, Sea dunes	Sand, loam, marl, loess, sludge	Quite rare	Vulnerable	2020	Brabant – Zeeland	Netherlands
Lycopus europaeus	Lamiaceae	Waterfronts, swamps, sea dunes, grassland, thicket, forests	Sand, loam, sludge, light clay	Common	Least concern	2020	North-Limburg	Netherlands
Primula elatior	Primulaceae	Forests, coppice, waterfronts, grassland	Sludge rich sand, loam, loess, marl	Quite rare	Least concern	2020	Limburg	Netherlands
Sanguisorba officinalis	Rosaceae	Waterfronts, grassland, roadsides, river embank-ments, railways	Mixed sand, clay, peat bogs, loam	Quite rare	Least concern	2020	Limburg	Netherlands
Stachys sylvatica	Lamiaceae	Forests, forest edge, fields, roadside	Loam, loamy sand, sludge, clay, lo-ess, marl	Common	Least concern	2020	Overijssel	Netherlands
Succisa pratensis	Caprifoliaceae	Grasslands, sea dunes, roadside, sand ridges in peat swamps and ancient sphagnum reed land	Sand, loam, peat bogs	Common	Vulnerable	2020	Friesland	Netherlands
Trifolium arvense	Fabaceae	Fields, grassland, roadside, sea dunes, excavations, heath, railways	Low in calcium, loess, sand, or pebble soil	Common	Least concern	2020	Overijssel	Netherlands

Appendix IV | Results of germinated seeds per species per treatment (50 seeds)

Betonica officinalis										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	0	0	0	0	0	0
22-dec	0	0	0	0	0	0	0	2	7	1
24-dec	0	0	0	0	0	3	7	5	17	1
27-dec	1	5	1	5	7	7	4	7	4	2
29-dec	2	5	3	2	3	0	0	4	0	0
31-dec	2	3	2	0	0	2	0	0	0	0
3-jan	1	2	0	0	0	1	1	0	0	0
5-jan	4	2	1	3	0	2	2	2	0	0
7-jan	1	0	2	4	0	1	2	0	0	0
10-jan	1	0	1	0	1	2	0	0	0	0
12-jan	0	0	0	0	0	1	0	1	0	2
14-jan	1	0	1	1	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**13**	**17**	**11**	**15**	**11**	**19**	**16**	**21**	**28**	**6**

Consolida regalis										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	1	0	0	1	1	0
22-dec	0	0	0	0	1	3	0	3	3	0
24-dec	0	0	0	0	0	4	1	0	0	0
27-dec	0	0	0	0	0	3	0	0	1	0
29-dec	0	0	1	0	0	1	0	0	0	0
31-dec	0	1	3	0	0	2	0	0	1	0
3-jan	0	0	0	0	0	1	0	0	0	0
5-jan	0	0	2	0	0	0	0	0	0	0
7-jan	0	0	1	0	0	0	0	0	0	0
10-jan	1	0	0	0	0	0	0	0	0	0
12-jan	1	0	0	0	0	0	0	0	1	0
14-jan	0	0	0	0	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**2**	**1**	**7**	**0**	**2**	**14**	**1**	**4**	**7**	**0**

Geranium pratense										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	1	1	0	0	1	0	0
22-dec	0	0	0	0	1	1	0	0	6	0
24-dec	0	0	1	1	0	1	0	0	1	0
27-dec	3	0	0	0	0	1	0	0	0	0
29-dec	0	0	0	0	1	0	0	2	0	0
31-dec	0	0	0	0	0	0	0	0	0	1
3-jan	0	2	1	1	2	0	0	0	0	0
5-jan	3	3	3	5	6	5	4	1	0	7
7-jan	0	0	0	0	0	0	0	0	0	0
10-jan	0	0	0	0	0	0	0	1	0	0
12-jan	0	0	0	0	0	0	0	1	1	0
14-jan	0	0	0	0	0	0	1	2	0	1
17-jan	0	0	0	0	0	0	0	0	0	0
18-jan	0	0	0	0	0	0	0	3	0	2
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**6**	**5**	**5**	**8**	**11**	**8**	**5**	**11**	**8**	**11**

Knautia arvensis										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	0	0	0	0	0	0
22-dec	0	0	0	0	0	0	0	0	1	0
24-dec	0	0	0	0	0	0	0	0	2	1
27-dec	0	0	0	0	0	0	0	0	1	0
29-dec	0	0	0	0	0	0	0	0	0	0
31-dec	0	0	0	0	1	0	0	0	0	0
3-jan	0	0	0	0	0	0	0	0	0	0
5-jan	0	0	0	0	0	0	0	0	0	0
7-jan	0	0	0	0	0	0	0	0	0	0
10-jan	0	1	0	0	0	0	0	0	0	0
12-jan	0	0	0	0	0	0	0	0	0	0
14-jan	0	0	0	0	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**4**	**1**

Lycopus europaeus										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	0	0	0	0	0	0
22-dec	0	0	0	0	0	0	0	0	0	0
24-dec	0	0	0	1	0	0	0	0	0	0
27-dec	0	0	0	0	0	0	0	0	0	0
29-dec	0	0	0	0	0	0	0	0	0	0
31-dec	0	0	0	0	0	0	0	0	0	0
3-jan	0	0	0	2	0	0	0	0	0	0
5-jan	0	0	0	0	0	0	0	0	0	0
7-jan	0	0	0	0	0	0	0	0	0	0
10-jan	0	0	0	0	0	0	0	0	0	0
12-jan	0	0	0	0	0	0	0	0	0	0
14-jan	0	0	0	0	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**0**	**0**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**

Primula elatior										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	0	0	0	8	7	0
22-dec	0	0	0	0	0	0	4	10	4	0
24-dec	0	0	0	0	3	0	0	0	0	0
27-dec	0	2	0	0	2	0	5	3	1	7
29-dec	0	4	2	0	1	0	5	3	2	0
31-dec	0	0	0	1	0	1	0	0	0	0
3-jan	0	0	1	0	3	0	0	0	2	1
5-jan	0	0	0	0	0	0	1	0	0	0
7-jan	0	0	0	0	1	0	0	0	0	0
10-jan	0	0	0	0	0	0	0	0	0	0
12-jan	0	0	0	0	0	0	1	0	1	0
14-jan	0	0	0	0	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**0**	**6**	**3**	**1**	**10**	**1**	**16**	**24**	**17**	**8**

Sanguisorba officinalis											
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	0	0	0	17	3	0
22-dec	10	12	7	1	3	1	16	12	6	0
24-dec	6	1	5	0	2	1	8	4	0	0
27-dec	8	6	3	1	3	0	1	2	2	4
29-dec	0	2	4	3	2	0	1	0	3	0
31-dec	0	3	1	0	0	0	0	0	0	2
3-jan	1	0	2	0	0	0	0	0	1	2
5-jan	0	1	0	0	1	0	0	0	0	0
7-jan	0	1	0	0	0	0	1	0	1	1
10-jan	0	0	2	0	0	0	0	1	1	0
12-jan	1	0	0	0	0	0	0	0	0	1
14-jan	0	1	0	0	0	0	0	0	0	1
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**26**	**27**	**24**	**5**	**11**	**2**	**27**	**36**	**17**	**11**

Stachys sylvatica										
Temperature (°C)	2	5	8	2	5	8	2	5	8	
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	Control
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	4	3	0	0	0	0	0	0
22-dec	0	0	0	0	0	0	0	0	0	0
24-dec	0	0	0	0	0	0	0	0	0	0
27-dec	2	0	1	0	0	1	0	0	0	0
29-dec	0	4	0	2	0	3	0	0	2	3
31-dec	0	0	0	0	1	0	0	0	0	0
3-jan	0	0	0	0	0	0	0	0	1	0
5-jan	1	0	0	0	1	0	0	0	0	0
7-jan	0	0	0	0	0	0	0	0	0	0
10-jan	0	0	0	0	0	0	0	1	0	0
12-jan	0	1	1	0	0	0	0	0	0	1
14-jan	0	0	1	0	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**3**	**5**	**7**	**5**	**2**	**4**	**0**	**1**	**3**	**4**

Succisa pratensis										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	0	0	0	0	0	0	0	0	0	0
20-dec	0	0	0	0	0	0	0	0	0	0
22-dec	0	0	0	0	0	0	0	0	0	0
24-dec	0	0	0	0	0	0	0	0	0	0
27-dec	0	0	0	0	1	0	0	0	0	0
29-dec	0	0	0	0	0	0	0	0	0	0
31-dec	0	0	0	1	0	0	0	0	0	0
3-jan	0	0	0	0	0	0	0	0	0	0
5-jan	0	0	0	0	0	1	1	0	0	0
7-jan	0	0	0	0	0	0	0	0	0	0
10-jan	0	0	0	0	1	0	0	1	0	0
12-jan	0	1	2	0	0	0	0	2	0	1
14-jan	0	0	0	0	0	0	0	3	1	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**0**	**1**	**2**	**1**	**2**	**1**	**1**	**6**	**1**	**1**

Trifolium arvense										
Temperature (°C)	2	5	8	2	5	8	2	5	8	Control
Duration of cold stratification (weeks)	2	2	2	4	4	4	6	6	6	
17-dec	8	10	11	8	7	9	11	6	11	0
20-dec	3	1	1	0	0	1	0	0	1	0
22-dec	0	0	0	0	6	0	0	0	0	6
24-dec	0	0	0	0	0	0	0	0	0	0
27-dec	0	0	0	0	0	1	0	0	0	3
29-dec	0	0	0	0	0	0	1	0	0	0
31-dec	0	0	0	0	0	0	0	0	0	0
3-jan	0	0	0	0	1	0	0	0	0	1
5-jan	0	0	1	0	0	0	0	0	0	0
7-jan	0	0	0	0	0	0	0	0	0	0
10-jan	0	0	0	0	0	0	0	0	0	0
12-jan	0	0	0	0	0	0	0	0	0	0
14-jan	0	0	0	0	0	0	0	0	0	0
17-jan	0	0	0	0	0	0	0	0	0	0
19-jan	0	0	0	0	0	0	0	0	0	0
21-jan	0	0	0	0	0	0	0	0	0	0
Total	**11**	**11**	**13**	**8**	**14**	**11**	**12**	**6**	**12**	**10**

Appendix V | Data SPSS per species

Betonica officinalis

Tests of Between-Subjects Effects

Dependent Variable: Mean germination

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	189,450[a]	9	21,050	2,204	,117	,665
Intercept	980,865	1	980,865	102,708	<,001	,911
Duration	68,778	2	34,389	3,601	,066	,419
Temperature	24,778	2	12,389	1,297	,316	,206
Duration * Temperature	51,889	4	12,972	1,358	,315	,352
Error	95,500	10	9,550			
Total	1549,000	20				
Corrected Total	284,950	19				

a. R Squared = ,665 (Adjusted R Squared = ,363)

Geranium pratense

Tests of Between-Subjects Effects

Dependent Variable: Mean germination

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	19,800[a]	9	2,200	,237	,980	,176
Intercept	244,246	1	244,246	26,263	<,001	,724
Temperature	4,000	2	2,000	,215	,810	,041
Duration	7,000	2	3,500	,376	,696	,070
Temperature * Duration	7,000	4	1,750	,188	,939	,070
Error	93,000	10	9,300			
Total	372,000	20				
Corrected Total	112,800	19				

a. R Squared = ,176 (Adjusted R Squared = -,566)

Primula elatior

Tests of Between-Subjects Effects

Dependent Variable: Mean germination

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	364,200[a]	9	40,467	884441280848891200000000000000000,000	,000	1,000
Intercept	305,862	1	305,862	66849234968534280000000000000000000,000	,000	1,000
Temperature	59,111	2	29,556	64596754724273830000000000000000000,000	,000	1,000
Duration	279,111	2	139,556	30501324787100700000000000000000000,000	,000	1,000
Temperature * Duration	22,222	4	5,556	12142247128622860000000000000000000,000	,000	1,000
Error	4,575E-29	10	4,575E-30			
Total	734,000	20				
Corrected Total	364,200	19				

a. R Squared = 1,000 (Adjusted R Squared = 1,000)

Sanguisorba officinalis

Tests of Between-Subjects Effects

Dependent Variable: Mean germination

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	494,450[a]	9	54,939	5,257	,008	,826
Intercept	1464,188	1	1464,188	140,114	<,001	,933
Temperature	70,333	2	35,167	3,365	,076	,402
Duration	344,333	2	172,167	16,475	<,001	,767
Temperature * Duration	43,333	4	10,833	1,037	,435	,293
Error	104,500	10	10,450			
Total	2423,000	20				
Corrected Total	598,950	19				

a. R Squared = ,826 (Adjusted R Squared = ,669)

Trifolium arvense

Tests of Between-Subjects Effects

Dependent Variable: Mean germination

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	11,200[a]	9	1,244	,356	,932	,242
Intercept	492,938	1	492,938	140,840	<,001	,934
Temperature	3,000	2	1,500	,429	,663	,079
Duration	3,000	2	1,500	,429	,663	,079
Temperature * Duration	5,000	4	1,250	,357	,833	,125
Error	35,000	10	3,500			
Total	608,000	20				
Corrected Total	46,200	19				

a. R Squared = ,242 (Adjusted R Squared = -,439)