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VIABILITY AND GERMINATION OF SCOTS PINE SEEDS AFTER FREEZING OF HARVESTED CONES IN VITRO

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7 VIABILITY AND GERMINATION OF SCOTS PINE SEEDS AFTER FREEZING OF
8 HARVESTED CONES *IN VITRO*

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21 ABSTRACT

22 Scots pine (*Pinus silvestris* L.) cone and seed water content were analyzed in two consecutive
23 seasons during maturation stage in the autumn and in January and March before seed
24 dispersal. Cones with different water contents were subjected to 2 h freezing at -30°C and
25 seed viability and laboratory germination of seeds from individual cones after treatment were
26 analyzed. Seed water content could be well predicted with cone water content measurement
27 and the general relationship between these two could be described with a generalized logistic
28 function. On average, cones had 5–10% higher water content than the seeds inside them.
29 The higher the cone water content at the onset of freezing treatment, the higher was the
30 proportion of seeds with apparent damage (based on visual inspection of seeds using x-ray
31 images) in that particular cone. High water content in cones also resulted in decreased
32 germination after freezing treatment. The critical cone water content for 50 % germination
33 after freezing at -30°C was approximately 31.3 % (fresh weight basis). This corresponds to
34 21.6 % water content in seeds.

35 **Keywords:** freezing, maturation, cone moisture, x-ray, cone storage

1. INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is characterized a three-year reproductive cycle typical to many other pine species (Owens and Blake 1985). In the first year, reproductive buds are initiated and formed. Pollination takes place in early summer of the second year. After pollination, pollen tube and ovule development is initiated, but it stops, usually in mid-summer. The development resumes the following spring and, after syngamy, a period of seed growth and development follows. Seeds mature in the fall of the third growing season after flower-bud initiation. In boreal zone of Fennoscandia, this phase occurs in late September – October, approximately 17 months after pollination and four months after syngamy (Sarvas 1962). When mature, cones remain intact on trees over winter and seeds are released next spring, typically in May – June.

Anatomically mature Scots pine seeds have fully developed embryo and megagametophyte tissues. In conifers, this stage is generally used as an indicator of when cones can be picked and successfully after-ripened (Edwards 1980). Based on Simak’s (1980) embryo and megagametophyte classification analysis of these tissues has become a routine analysis to estimate the germination potential of conifer seeds in forest tree seed centers in Northern Europe.

Besides anatomical maturation, conifer seeds exhibit biochemical (Kardell et al. 1973) and physiological (Edwards 1980) maturation. These are general terms referring to biochemical and physiological changes in both the embryo and megagametophyte during late stages of maturation and include the change from mobile to storage forms of reserves, seed dehydration and decline in respiratory activity. Physiological and biochemical activity in

61 Scots pine seeds continues after the accumulation of reserves is complete and the embryo is
62 fully elongated. Håkansson (1956), for example, found mitotic activity and formation of
63 starch in Scots pine embryos in late October in seed samples collected in southern Sweden. In
64 this case, the seeds were anatomically fully developed in the middle of August. Recently,
65 Tillman-Sutela & Kauppi (2014) have shown that maturation of Scots pine seed surface
66 structures – nucellar and megaspore membranes and the three – layer seed coat – play an
67 important role in the maturation process as a whole. The physiological maturation of the
68 above – named structures appears to take place independently of the anatomical development
69 of the embryo and megagametophyte, continuing several weeks after the embryo has reached
70 its final size.

71
72 Fundamental to physiological changes in life cycle of orthodox seeds is their dehydration at
73 the final stage of development (Kermode et al. 1986). In a dry state, seeds can tolerate a
74 number of diverse environmental conditions, including extremes in temperature. For Scots
75 pine in Fennoscandia, where temperatures below -30°C are common during winter months,
76 this is critical as seeds must simultaneously mature and become freeze tolerance in order to
77 survive harsh winter conditions.

78
79 During seed ripening and the overwintering phase, Scots pine seed development can be
80 disturbed or interrupted by negative temperatures, leading to seed damage and low
81 germinability. Simak (1972) found that the early stages of embryogenesis were more
82 sensitive to cold treatment than were the later ones. In material collected from northern
83 Sweden, Kardell (1974) demonstrated that in natural environments the proportion of Scots
84 pine seed with damaged embryos – as determined with a tetrazolium test – increased from
85 zero in September to 53% at maximum in February. In a later study, however, he found that

86 during the period from the beginning of September to the middle of October, freezing of
87 collected cones at temperatures of -5°C , -10°C and -15°C for two, four and eight hours
88 resulted in an increased germination rate. In the following year, the same combinations of
89 time and temperature caused both embryo damage and decreased germination (Kardell,
90 1976).

91 Many studies report large annual variation in moisture content of Scots pine cones and seeds
92 during the late stages of maturation at the onset of winter (Sahlen & Abbing 1995, Tillman-
93 Sutela & Kauppi 2014). Nygren & Pulkkinen (1994) for example, noticed that in northern
94 and southern Scots pine clones growing in a common garden in southern Finland, seed water
95 content varied from approximately 40 to 20 percent (fresh weight basis) at the end of the
96 maturation phase, depending on year and clone, respectively. Thus it may be hypothesized
97 that, depending on the environmental conditions prevailing during maturation, cone and seed
98 dehydration could be incomplete, resulting in seed damage during winter months.

99
100 With contradictory results on the role of sub-zero temperatures on Scots pine seed
101 maturation, optimizing the timing of cone collections is problematic. In Fennoscandia, it is
102 often a necessity to start the collections as early as possible before the harsh winter conditions
103 and snow make the operations difficult and more costly. Planning collection timing is further
104 complicated by the fact that clonal sequence and approximate dates of ripening vary (Bonner
105 1991). Starting collections too early usually results in low germination and short storage life
106 of seeds, while postponing the collection to early winter will increase the risk of frost
107 damage. These risks can be avoided by timely collection and proper post-collection handling
108 of cones and seeds, ie. cone storage and possible artificial ripening of the seeds (Edwards
109 1980, Sahlen & Abbing 1995). Nevertheless, when increasing amounts of genetically
110 improved Scots pine seed is used in nurseries and regeneration areas, it is of utmost

111 importance to optimize the collection timing, so that valuable seed is not lost due to improper
112 timing of collections.

113

114 The objectives of the current study were to evaluate if cone water content could be used to
115 predict the water content of the seeds inside them. A further objective included testing cold
116 hardness of harvested Scots pine seeds while still in cones, to determine how their
117 subsequent germination and possible freezing injury are affected by cone water content.

118

Draft

119 2. MATERIAL AND METHODS

120

121 *Cone collections*

122 Open-pollinated Scots pine cones were collected from Suhola seed orchard no. 404 (62°15'N,
123 27°42'E, 88 m a.s.l.) in southern Finland. This 1.5 generation orchard was established in
124 1997, with an area of 26.2 ha and with 35 clones. The number of grafts per clone varies from
125 104 to 208. Seeds collected represented two crops, maturing in two consecutive growing
126 seasons in 2011 and 2012. Collections for the first crop were done on Nov 21st, 2011, Feb
127 30th 2012 and March 26th 2012. The seed collection date for the second crop was Nov 5th,
128 2012.

129 In the first season, in November 2011, cones were collected from three clones (E1881, K689
130 and K393) and from three grafts per clone. The clones and individual grafts were randomly
131 chosen at the beginning of the study, and were used in every collection. During November
132 2012 collection, one more clone (K942), also with three grafts, was included in the study.
133 From each graft, 25 – 30 cones were picked on each occasion throughout the crown, and
134 transported in sealed, separate plastic bags in an insulated box to the seed laboratory at the
135 Finnish Forest Research Institute (now Natural Resources Institute Finland) in Suonenjoki
136 (approximately a one – hour drive from the seed orchard).

137

138 *Cone and seed water content*

139 In the laboratory, 3-5 cones from each graft were randomly chosen for the analysis of cone
140 and seed water content. For every collection, each clone was thus represented by 9 – 15
141 cones.

Each cone was cut in four quarters (lengthwise). One of these was used for cone water content measurement by weighing it fresh and after drying at +103 °C for 17±1 hours. From the other three quarters, a bulk sample of 15 – 20 seeds was picked manually, and the sample water content was measured gravimetrically on a fresh weight basis using the constant temperature oven method (103 °C, 17±1 h) (International Rules...2005). Cones damaged by pine cone weevil, *Pissodes validirostris* Gyll., were excluded from the study.

148

Cone freezing treatment and seed extraction

Further, in each collection, 24 cones per clone, 8 from each graft were randomly divided into control and freezing treatment groups, both including 12 cones per clone. Cones from different clones and grafts were kept separate, so that individual cone water content at the onset of freezing could be measured (except first collection, see below). Cones were treated as follows: (1) freezing treatment: starting at +5 °C and temperature lowered at a rate of 5 °C h⁻¹ until -30 °C was reached. After 2 h at -30 °C, temperature was raised again up to +5 °C at a similar rate. The freezing treatment as a whole took 16 hours to complete and was done using a controlled freezing cabinet (Weiss WT-600/70). In the control treatment, the cones were stored in a cold room at +5 °C for the same amount of time. The cones were not exposed to light during the treatments.

The first collection in Nov 2011 was a pilot study in which seeds extracted from a group of four cones from each clone-graft-treatment combination were treated as a combined sample. Thus, no data on individual cone water content at the onset of the freezing treatment was collected.

164 In later collections, individual cones were kept separate during the treatment. Similarly,
165 seeds extracted from them were also analyzed cone-wise. Number of filled seeds per cone
166 varied from 11 to 49. As each clone was represented by four cones from each of three grafts,
167 the total number of seeds from each clone in the germination tests in different collections
168 varied from 330 to 518. Individual cone water content (fresh weight basis) at the onset of
169 freezing was measured gravimetrically by weighing cones (accuracy 0.01 g) before the
170 freezing treatment. After seed extraction (+38 °C, ventilated oven, 48 h) cones were further
171 dried at +103 °C to a constant weight.

172 Seed wings were removed manually and seeds were stored in airtight pouches at +5 °C
173 (typically 2 – 3 weeks) before germination tests.

174

175 *Germination and seed x-raying*

176 Seeds were germinated, cone-wise in a 90 mm Petri dish, each with two layers of germination
177 paper (Munktell 1701) moistened with 5 ml of tap water. Dishes were placed in a
178 germination cabinet (FLOHR Instruments GC 10/11; 20 °C, 16 h day, RH 98 %, 1000–1500
179 lux) and germination was monitored for 14 days. Seed was considered as germinated when
180 the primary root and hypocotyl together exceeded four times the length of an individual seed
181 (International rules... 2005).

182 Prior to germination tests, seeds from each cone were x-rayed (Faxitron MX-20, IL, USA;
183 Fuji medical x-ray film AD Mammography, exposure time 18 kV, 10 s). Based on the x-ray
184 image, filled seeds were classified as follows; (1) seeds with visible differentiation between
185 embryo, megagametophyte and seed coat (= 'normal seeds') and (2) seeds without visible
186 differentiation and showing even optical density throughout the image (= 'grey seeds', Figure

1., cf. Simak 1980, Sahlen et al. 1995). In the following, these two types will be referred to as category 1 and category 2 seeds. The former were found to be viable and capable of germination. Category 2 'grey seeds', as based on our earlier experience do not germinate in a germination test, and are somehow deteriorated. In esterase activity measurements (data not shown) they have lower enzyme activity than viable, germinable seeds. Therefore, statistical analysis of germination data was based only on the number of filled, viable (category 1) seeds per cone. However, the proportion of these 'grey seeds' in each cone was used as an independent variable when modelling the cone-wise germination response of category 1 seeds.

Weather data

The weather data for the two growing seasons 2011 and 2012 were obtained using a set of climate data from the Finnish Meteorological Institute (Venäläinen et al. 2005). This data set consists of daily mean temperature and precipitation data interpolated onto a 10*10 km grid. The growing season temperature sum, monthly mean temperature and precipitation during the seed maturation period (September – December) in the Suhola orchard, as well as their long time averages (1981 – 2010) were calculated for the nearest grid, the center of which lies approximately 4 km north of the study site.

Statistical analysis

In order to describe the relationship between cone and seed water content, a non-linear logistic function was used. As this model was only used for general description, a common line was fitted to the data, ignoring the possible curvature differences according to clones and

210 years. Further, we made the assumption that the continuous explanatory variable, cone water
211 content, was measured without error.

212 The cone and seed water content data were further analyzed with a linear mixed model to
213 estimate and partition the total random variability between and within individual clones,
214 grafts and cones. In this analysis, the collection date was taken as a fixed effect and clone,
215 graft and cone effects were considered random. The variance component model was:

216 (1)
$$y_{ijkl} = \mu + a_i + b_{ij} + c_{ijk} + e_{ijkl}$$

217

218 where y_{ijkl} = observation on cone or seed sample from cone l in graft k in clone j in collection
219 i ; μ is the intercept; a_i is the fixed collection date effect, b_{ij} is the random clone effect, c_{ijk} is
220 the random graft effect and e_{ijkl} is the random cone effect.

221 Statistical analysis of germination and seed deterioration data vs. cone water content and
222 freezing treatment could only be done with material from three collections (January and
223 March 2012, November 2012) as we had no values for individual cone water contents from
224 the first collection in Nov 2011.

225 The effect of cone water content on the presence of deteriorated, category 2 seeds (as
226 determined from the x-ray images) after cone freezing at -30°C was analyzed using a
227 generalized linear mixed model. This data was binary, as the response variable was
228 categorical with two classes: the presence of at least one deteriorated seed (yes/no) in a given
229 cone. Cone water content prior to freezing treatment (W), was used as a fixed term and
230 *collection x clone* interaction as a random term. The random term imposes a correlation on
231 all observations from the same collection – clone combination.

232 The basic model was as follows:

233 (2) $\text{logit}(p) = b_0 + b_1 W + u_{\text{collection} \times \text{clone}}$

234 where $\text{logit}(p)$ = the probability that at least one seed per cone is deteriorated. For binary
235 data models in general the residuals provide no information about the adequacy of the fitted
236 model (Collet 2003) and thus, we used ROC-curve analysis (Eng, 2014) as a checking
237 diagnostic for this model.

238 The germination data was grouped binary, where the proportion of germinated seeds (out of
239 the number of category 1 seeds per each cone) after 14 days of incubation was the response
240 variable. Fixed terms in the model were: Cone freezing treatment (F), cone water content
241 prior to freezing treatment (W), and the proportion of category 2 seeds (P) found in each
242 individual cone (on x-ray image) after cone freezing. As with the seed deterioration model,
243 we used *collection x clone* interaction as a random term in order to take account of correlation
244 among observations from the same collection and clone.

245 The basic form of the model was:

246 (3) $\text{logit}(p) = b_0 + b_1 F + b_2 W + b_3 P + u_{\text{collection} \times \text{clone}}$

247 where $\text{logit}(p)$ = germination percent of category 1 seeds after 14 days incubation.

248 Overall fit of the Model 3 was examined by looking at graphical summaries of the
249 standardized residuals vs. fitted values and covariates. Individual model terms were assessed
250 by comparing the log-likelihood statistics of two nested, candidate models and Wald –
251 significance tests for parameter coefficients. Fixed model terms were considered statistically
252 significant at $p \leq 0.05$. For all analyses, logistic regression procedures of Genstat 17 (VSN
253 International...2014) were used.

254

255

3. RESULTS

Cone and seed water content

For each collection, seed and cone water content were closely correlated (Fig. 2.). The general relationship could be described with a generalized logistic regression:

$$\text{Seed water content, \%} = A + C / (1 + \exp(-B * (\text{Cone water content, \%} - M)))$$

with the following parameters (SE's in brackets):

$$A = 11.156 (0.552)$$

$$B = 0.225 (0.0213)$$

$$C = 21.86 (1.08)$$

$$M = 31.715 (0.378).$$

The regression accounted for 95.6% of the variance of the data; estimated standard error of observations was 1.37 %.

On average, cones had 5 – 10% higher water content than the seeds inside them. Clonal, seasonal and annual variation in both seed and cone water content was evident. In general, the two consecutive crops were different: in November 2011, average water content of the cones was 27.7% and 17.0% in seeds. The following year's crop had much higher average values: 40.7% in cones and 29.8% in seeds, respectively (Table 1.).

In autumn 2011, clone E1881 had the highest cone water content (29.2% fwt basis), whereas next year, clone K393 had the highest values (46.9%). Winter desiccation of cones and seeds was obvious: during the period from November 2011 to March 2012 cones and seeds lost approximately 7% and 4% of their fresh weight, respectively. Further studies by the authors

indicate (data not shown) that during the winter 2013 – 2014 the cones and seeds dried down to approximately 12 and 8% water content (fresh weight basis) respectively, before seed dispersal.

Weather conditions

In both seasons, temperature climate during late stages of maturation in September–November was milder than the long – term average, but the growing season 2011 as a whole was considerably warmer with final temperature sum (+5 °C threshold) reaching 1546.8 degree days. In 2012 the temperature sum was close to the average value on the study area. In both years, cone collections were started approximately two weeks after the accumulation of the temperature sum ended, ie. daily mean temperatures were constantly below +5 °C. In terms of precipitation, September was exceptional in both years as monthly rain fall was 99.6 mm in 2011 and 111.6 mm in 2012. These values are approximately twice and three times the long – term average value, respectively (Table 2.).

Seed viability and germination after cone freezing in vitro

The probability of category 2 seeds in cones (as determined from x-ray images) and germination of category 1 seeds depended on cone water content at the onset of freezing treatment. The higher the cone water content, the higher was the probability of having at least one of category 2 seeds in a cone (Figure 3.). For Model 2, cone water content (Wald statistics 28.44/1 df) was statistically significant ($p < 0.001$, Table 4.). ROC-statistics for this model were 0.972 (SE 0.017) indicating good fit (cf. supplementary Figure 1.)

299 For germination, the situation was similar: the higher the cone water content at the onset of
300 freezing, the lower was the germination of filled, category 1 seeds from that particular cone
301 after treatment. For germination model, cone water content interaction with freezing
302 treatment (Wald statistics 205.8 / 1 df) and the proportion of category 2 seeds per cone (15.2
303 /1 df) were statistically significant factors affecting germination (Figure 4., Table 5., $p < 0.001$
304 for both terms). For model 3, standardized residuals vs. fitted values did not indicate any
305 problems, so we considered model fit to be satisfactory (cf. supplementary Figure 2.).

306

307 DISCUSSION

308

309 Cone water content and specific gravity have been used as an index of seed ripening for
310 many *Pinus* species (see Edwards 1980 for a comprehensive review), but only a few studies
311 have reported data on seed water content inside maturing Scots pine cones (Messer 1958,
312 Matyas 1971, Nygren & Pulkkinen 1994, Tillman-Sutela & Kauppi 2014). The results of this
313 study suggest, that seed water content can be predicted with a reasonable accuracy from cone
314 water content measurements. On average, cones had 5 – 10% -units higher water content
315 than the seeds inside them.

316 Annual and clonal differences in cone and seed water content were obvious. High cone water
317 content values found in November 2012 may have resulted from high precipitation in the
318 study area during the month of September that year, as it was nearly three times the annual
319 long – term average value (Table 2.). In the previous year, precipitation in September was
320 also high (approximately twice the annual average), but due to a high temperature sum during
321 that season, cones and seeds may have dried prematurely and were not, perhaps, affected by
322 the high precipitation period in September.

In a three – year study, Nygren & Pulkkinen (1994) found that at the late maturation phase at the end of October, Scots pine seed water content was approximately 20 % -units lower in a warm growing season (1035 degree days, +5 °C threshold) than in a cool season (869 degree days). Tillman-Sutela & Kauppi (2014) found that during two consecutive seasons in 1996 and 1997 Scots pine seed water content at the beginning of October was approximately 5 – 9 % -units lower in a warm growing season than in a cool season. The result was consistent over three different geographical regions in Finland. The results of this study may indicate that in a given clone, the rate of maturation drying is not the same every year. This was also found by Remröd & Alfjorden (1972) for a Scots pine seed orchard: ‘early clones’ in a given year could be ‘the late ones’ in the next year. As suggested by Bonner (1991) orchard managers should be familiar with the sequence and approximate dates of ripening of all their clones. Due to limited number of clones in our study, our result needs to be confirmed with more data so that the whole range of seed maturation within an orchard in different years could be covered.

As revealed by variance component analyses, for each collection, the largest variation in both seed and cone water content was at the cone level (Table 3.). This result has implications when sampling cones for monitoring water content in the field, stressing the importance of taking samples throughout different parts of the crown in each clone or tree. The estimated zero variance component at the graft level is logical as grafts from the same clone are genetically identical and thus, do not account for any of the random variation. The small part of the variation explained by clonal level may be merely due to the fact that the number of clones in our study was very limited. Thus, further research is needed to confirm these results.

347 Most studies on freezing tolerance of conifer seeds have been done in relation to the
348 germination process or seed storage conditions (von Schönborn 1964, Cremer & Mucha
349 1985, Coursolle et al. 1998, Hawkins et al. 2003) but much less information is available on
350 their freezing tolerance while still intact in cones. Simak (1972), when studying the influence
351 of low temperatures (approximately -3°C , 2 – 3 h.) on embryogenesis of Scots pine *in vivo*,
352 found that even in anatomically fully developed seeds, germination declined 15% -units after
353 treatment. Further, he observed differences in response between cones of a single tree as well
354 as visual damage between individual seeds in a single cone. Our results indicate similar
355 variation because after freezing treatment, an individual cone could include filled seeds of
356 two categories: germinating, category 1 seeds, and presumably deteriorated, non-germinating
357 ‘grey seeds’(category 2). This classification is speculative, however, as we have only indirect
358 and subjective evidence of seed deterioration, based on interpretation of x-ray image data.

359

360 Whether freezing seeds in intact cones or after extraction, all studies indicate decreasing
361 freezing tolerance of conifer seeds during the transition from a dry to imbibed condition. In
362 general, our results are in agreement with those on freezing extracted Scots pine seeds in
363 imbibed condition. According to Model 4 critical cone water content for 50 % germination
364 after freezing at -30°C was approximately 31.3% (fwt basis). This corresponds to
365 approximately 21.6% water content in seeds.

366

367 A study by von Schönborn (1964) found that Scots pine seeds at 30% water content (fresh
368 weight basis) could withstand freezing to -10°C , whereas at 10% water content they could
369 survive at -40°C . Hawkins et al. (2003) found that after freezing of imbibed seeds of
370 lodgepole pine (with water contents varying from 25–45 %) to -25°C , their survival ranged
371 from 0 to 11%. Pamuk et al. (2003) consider 15–20% water content to be the critical value

for Scots pine seed freezing damage. This value is in agreement with that found in this study but however, freezing duration in their experiment was much longer (2 and 5 days) than in ours. Our results are also in line with the results from noble fir (*Abies procera* Rehd.) cone and seed freezing tests (Tanaka 1980). Using his model, estimated germination of noble fir seeds after cone freezing to -30°C at 35 % cone water content is 7.9% and according to Model 4 in our study it is 4.7% for Scots pine.

Tanaka (1980) observed that, with noble fir, the lethal temperatures were slightly lower in cone freezing tests than in the seed-freezing tests and attributed this difference to the insulating effect of cones scales when cones are frozen. Insulating effect is also important in another aspect: Keefe and Moore (1982) could demonstrate that in Scots pine seeds, the three – layer seed coat acts as nucleation barrier and retards ice growth towards inner seed structures, the megagametophyte and the embryo. Therefore, surface dry although fully imbibed (53.9% water content dry weight basis) Scots pine seeds could stand sub-zero temperatures better than those embedded in a moist substrate.

In both cone storage and in natural environments seeds are often exposed to freezing temperatures more than once and repeated freezing can cause greater embryo damage than a single frost. Further, prolonged freezing periods during natural frosts could result in greater damage than that observed in this study (Tanaka 1980).

In our study, germination of normal seeds (category 1, seeds without visible symptoms of damage in an x-ray image) was consistently lower if they came from the cones that had category 2 ‘grey seeds’ than from those that had none (Figure 4.). According to the Model 4 one percent increase in the proportion of category 2 seeds in a given cone, corresponds to an

approximately 6.5% decrease in the odds of germination of other, healthy looking seeds in that particular cone, also. We conclude that presence of 'grey seeds' in a cone indicates possible damage to other seeds in that cone which, however, cannot be detected in radiography. However, evidence of seed deterioration in our study is only indirect and has to be interpreted with caution. Kardell (1974, 1976) hypothesized that, besides damage (as determined with tetrazolium test) during overwintering, negative temperatures may induce dormancy in part of the Scots pine seeds, and that this dormancy could be broken by chilling. Our results cannot confirm this hypothesis, as chilling treatment of filled, but non-germinating seeds was not included in our study.

Conclusions

The timing of cone collections according to seed maturation is one of the key questions in the management of Scots pine seed orchards. Much of the earlier work has been concentrated on monitoring anatomical seed development and especially embryo size, when predicting the quality of the seed crop. The results obtained in this study stress the importance of maturation drying in order for seeds to attain hardiness and remain viable throughout the winter months. It is suggested that monitoring cone water content should become a routine analysis in seed orchards and used as an indicator of both seed ripening and the development of Scots pine seed frost tolerance.

Our results also have implications for storing cones during the post-harvest period. In practical seed procurement, harvested cones are often stored outdoors for long periods. In these conditions, cones are subjected to freezing temperatures in late fall and early winter. Emphasis should be put on protecting the cones from rain and allowing them to dry, exposed to free movement of air, in an interim storage shed or similar.

422

423 Our results may have implications with respect to effects of climatic warming on
424 reproductive success of Scots pine. One of the most important ways by which northern
425 forests will respond to predicted climate change is through variations in seed maturation
426 (Meunier et al. 2007). Generally, growing season temperature conditions at the northern
427 latitudinal and upper altitudinal limits of Scots pine in Fennoscandia, are frequently too low
428 to permit normal seed development. At the polar timber line in northern Finland the
429 probability of achieving a mature, germinating Scots pine seed crop is very low,
430 approximately 0.02 – 0.03 (Henttonen et al. 1986). According to Almqvist et al. (1998) 975
431 degree days (+5 °C threshold, accumulated heat sum from start of the growing season until
432 August 31) are needed for 95% germination of Scots pine seed. Current scenarios (Jylhä et
433 al. 2009) predict a significantly warmer future climate in Finland. This could mean improved
434 climatic conditions for Scots pine seed development and maturation in general. However, the
435 projected increase in precipitation – especially during the winter months and in northern
436 Finland - could disturb the maturation drying of seeds and their natural hardening in late fall
437 prior to severe winter conditions.

438

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TABLES

Table 1. Cone and seed water content (% , fresh weight basis; mean±SE) by clone in different collections. For every collection, each clone is represented by 9 – 15 cones from three randomly selected grafts; from each cone a bulk sample of 15 – 20 seeds was analyzed.

	Clone							
	<u>K393</u>		<u>K689</u>		<u>E1181</u>		<u>K942</u>	
	<u>Cone</u>	<u>Seed</u>	<u>Cone</u>	<u>Seed</u>	<u>Cone</u>	<u>Seed</u>	<u>Cone</u>	<u>Seed</u>
Crop 2011								
Nov 2011	27.7 (0.4)	17.4 (0.2)	26.3 (0.4)	16.7 (0.4)	29.2 (0.4)	16.7 (0.3)	..	..
Jan 2012	27.3 (0.6)	16.9 (0.2)	26.4 (0.6)	17.1 (0.2)	28.4 (0.6)	17.0 (0.2)	..	..
March 2012	21.5 (0.5)	13.6 (0.3)	19.9 (0.5)	12.5 (0.3)	21.0 (0.5)	13.2 (0.3)	..	..
Crop 2012								
Nov 2012	46.9 (2.1)	35.1 (1.8)	37.6 (1.1)	27.3 (1.1)	43.3 (0.4)	30.1 (0.3)	35.1 (0.8)	26.5 (0.9)

Table 2. Annual temperature sum (+5 °C threshold), monthly precipitation and mean temperature in the study area during the seed maturation period.

Year	Temperature sum	Precipitation, mm			Mean temperature, °C		
		Sep	Oct	Nov	Sep	Oct	Nov
2011	1546	99.6	48.9	30.4	11.1	5.6	1.9
2012	1302	111.6	60.5	40.6	10.4	4.1	1.1
Average (1981–2010)	1258	48.2	54.7	47.6	9.0	3.8	-2.0

Table 3. Components of variance for cone and seed water content (fresh weight basis) based on mixed linear Model 1.

Term	Variance estimate	SE	Proportion, %
<i>Cone water content</i>			
Clone level	2.202	1.589	24.3
Graft level	-0.139	0.125	0.0
Cone level	6.864	0.672	75.7
<i>Seed water content</i>			
Clone level	1.184	0.941	19.4
Graft level	-0.061	0.108	0.0
Cone level	4.934	0.484	80.6

Table 4. Parameter estimates (with SE) of the Model 2.

<i>Fixed parameters</i>	estimate	SE	t-value	appr. p-value
Constant	-19.73	4.04	-4.884	<.001
Cone water content	0.641	0.12	5.342	<.001
<i>Random parameters</i>				
Heterogeneity factor	-0.887	0.14	-6.336	<.001
Collection x Clone	-2.39	1.04	-2.291	0.112

Table 5. Parameter estimates (with SE) of the Model 3.

<i>Fixed parameters</i>	estimate...	SE.....	t-value.....	appr. p-value
Constant	3.578	0.550	6.505	<.001
Cone water content	0.0021	0.0102	0.2059	0.413
Freezing treatment ^a	12.86	1.090	11.798	<.001
Cone water x Freezing treatment ^a	-0.5559	0.0388	14.327	<.001
Proportion of deteriorated seeds/cone	-0.0669	0.0171	3.912	<.001
<i>Random parameters</i>				
Collection x Clone	-2.252	0.520	4.331	<.001

^a as compared with control at +5 °C

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FIGURE CAPTIONS

Figure 1. Example of an x-ray image of a fully developed Scots pine seed (category 1, middle) and two seeds without visible differentiation of embryo and megagametophyte and showing even optical density throughout the image (= 'grey seeds', category 2).

Figure 2. Scatterplot showing the general relationship between water content (fwt basis) of Scots pine cones and the seeds inside them in different collections and the predicted value according to generalized logistic function.

Figure 3. Probability of presence of at least one category 2 seed ('grey seeds', as determined from x-ray image) in an individual cone after 2 h cone freezing treatment at -30°C , as a function of cone water content (fresh weight basis) at the onset of freezing (Model 2).

Figure 4. Model 3 predictions of germination of category 1 Scots pine seeds from individual cones after 2 h cone freezing treatment at -30°C as a function of cone water content (fresh weight basis) at the onset of freezing. Open circles: Control seeds after storage of cones at $+5^{\circ}\text{C}$; triangles: seeds from cones that had no deteriorated (category 2) seeds after freezing; squares: seeds from cones that had 5 % deteriorated (category 2) seeds after freezing. The standard errors are approximate as the model is not linear.

Figure 1.

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Figure 3.

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SUPPLEMENTARY MATERIAL

Supplementary Figure 1. ROC-curve ($\pm 95\%$ confidence limits) for Model 2.

iti14 vs. freeze*cone water + grey%+u

Supplementary Figure 2. Residual diagnostics of the Model 3.