Canadian Journal of Forest Research

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Journal:	<i>Canadian Journal of Forest Research</i>
Manuscript ID	cjfr-2019-0049.R1
Manuscript Type:	Note
Date Submitted by the Author:	25-Feb-2019
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Keyword:	genetic load, inbreeding, inbreeding depression, Pinus sylvestris, tree breeding
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

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RESEARCH NOTE

Effects of inbreeding depression on seed production in Scots pine

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Abstract

Like other outcrossing species, Scots pine (*Pinus sylvestris*) is thought to carry a “genetic load” of deleterious recessive alleles. When these alleles occur as homozygotes in inbred progeny, their expression can give rise to so-called “inbreeding depression”. While this phenomenon has been studied in several conifer species through selfing, rather few studies have quantified inbreeding depression in crosses with lower levels of relatedness between parents. We report here on the generation of a set of F3 study materials in Scots pine, where 142 families arose from a mating design among 49 F2 parents, representing nine levels of expected inbreeding between 0.0 and 0.5, and repeated over two consecutive seasons. While the numbers of extractable seeds were unaffected, the proportion with fully developed embryos was strongly affected by inbreeding. This was expressed as inbreeding depression in the yield of full seeds per cone, but not in their average weight. Levels of germination of these full seeds were affected by inbreeding, but the depression was rather small and only weakly significant. The roles of pollen competition and polyembryony in mitigating the impact of inbreeding depression are discussed. The materials have been outplanted for future assessment of inbreeding depression on growth and survival.

Keywords: genetic load, inbreeding, inbreeding depression, Pinus sylvestris, tree breeding

Résumé

Comme plusieurs espèces à pollinisation croisée, le pin sylvestre (*Pinus sylvestris*) est porteur d'une « fardeau génétique » d'allèles récessifs délétères. Lorsque ces allèles se présentent sous la forme d'homozygotes chez la progéniture consanguine, leur expression peut donner lieu à ce que l'on appelle une « dépression consanguine ». Bien que ce phénomène ait été étudié chez plusieurs espèces de conifères par l'autoformation, peu d'études ont quantifié la dépression de consanguinité chez des croisements présentant des niveaux de parenté plus faibles entre les parents. Nous rapportons ici la génération d'un ensemble de matériaux d'étude F3 dans le pin sylvestre, où 142 familles issues d'un modèle d'accouplement chez 49 parents F2, représentant neuf niveaux de consanguinité attendue entre 0,0 et 0,5, ont été répétées pendant deux saisons consécutives. Bien que le nombre de graines extractibles n'ait pas été affecté, la consanguinité a fortement affecté la proportion d'embryons pleinement développés. Cela a été exprimé comme une dépression de consanguinité dans le rendement en graines complètes par cône, mais pas dans leur poids moyen. Les niveaux de germination de ces graines complètes ont été affectés par la consanguinité, mais la dépression était plutôt faible et seulement faiblement significative. Les rôles de la compétition du pollen et de la polyembryonie dans l'atténuation de l'impact en cas de dépression de la consanguinité sont discutés. Les matériaux ont été plantés pour l'évaluation future de la dépression de consanguinité sur la croissance et la survie. [Traduit par l'auteur]

*Mots-clés: fardeau génétique, consanguinité, dépression consanguine, Pinus sylvestris
amélioration des arbres*

1. Introduction

It is a well-known feature of outcrossing species that individuals typically carry a “genetic load” of recessive deleterious alleles. In natural outbreeding populations, the excess of heterozygotes over what would be expected under Hardy-Weinberg Equilibrium (HWE) “shields” these alleles from selection, as they are infrequently expressed in the homozygous form. When inbreeding does occur, for whatever reason, the recessive nature of these deleterious alleles causes them to be expressed most frequently when they occur as homozygotes, giving rise to “inbreeding depression” (Charlesworth and Charlesworth 1987; Williams and Savolainen 1996).

Forest tree breeders generally work under the assumption that natural populations of wind-pollinated species carry a significant genetic load and that its expression through inbreeding will result in problems, through reduction in genetic gain and/or problems with fecundity. Just as inbreeding occurs to some degree in natural populations, it is also expected to occur when seed orchard parents mate at random, where some fertilization events represent selfing, and others mating between relatives (Rosvall and Lindgren 2012).

Inbreeding has generally been regarded as something to be avoided in seed orchards, even when it results from mating between relatives, rather than through selfing. While it is thought that embryonic inbreeding depression from selfing will be so severe that seed set will usually not occur, the fear is that lesser relatedness between parents will result in successful seed set, with inbreeding depression expressed only later in the established plantation. Management of the breeding population with separate sublimes has often been recommended so that individual, unrelated seed orchard parents can be selected from each subline (Lowe and van Buijtenen 1986; McKeand and Bridgwater 1998). Alternatively, Lindgren et al. (2009) recommended penalizing

the relatedness of pairs of seed orchard parents for expected inbreeding depression while optimizing selection for gain under a constraint on overall gene diversity (or status number).

In many species, seed set from control-pollinations between relatives is lower than that for outcrosses, suggesting embryonic inbreeding depression occurs during seed formation. When self-pollination does occur in Scots pine through wind pollination, there may be up to three fertilization events per ovule, due to multiple archegonia and the ability of the micropyle to accommodate two to three pollen grains (Sarvas 1962). This can lead to so-called “simple” or “archegonial” polyembryony, producing embryos from multiple fertilization events. There is also the additional possibility that “cleavage” polyembryony will produce identical twins (Burdon and Zabkiewicz 1973). However many viable embryos might be present initially, intra-ovule competition among embryos almost always leaves a single embryo to germinate (Filonova et al. 2002; Sorensen 1982). Only when all embryos express lethal genes in the homozygous form are empty seeds guaranteed (Williams 2007, 2009).

While inbreeding depression affecting embryo development might mitigate the effects of inbreeding in the surviving embryos from self-pollinations, it is not known if this same mechanism is as effective in crosses with lesser amounts of inbreeding, i.e., inbreeding coefficients less than 0.5. This is of particular concern for seedling seed orchards composed of full-sib or half-sib families, and for breeding of related parents in breeding populations. Even if inbreeding depression does not result in embryo death, it may still be expressed during later stages of development, including seedling development and mortality, height growth and fecundity (Husband and Schemske 1996). Whether or not inbreeding depression has a significant impact on stand productivity depends on the dynamics of stand development and the degree of

self-thinning eliminating the inbred individuals from the plantation prior to harvest (Wang and Russell 2006).

Selfing studies suggest that inbreeding depression can be substantial, but these often base their calculations of inbreeding depression on a single level of rather severe inbreeding (e.g., Lundkvist et al. 1987 in Scots pine). Rather few field experiments have been established with material that represents various levels of inbreeding, resulting from crosses between other relatives (Durel et al. 1996; Ford et al. 2015; Matheson et al. 1995; Snieszko and Zobel 1988; Sorensen and Cress 1994; Woods and Heaman 1989; Wu et al. 1998a, b). To our knowledge, our crosses made under our current inbreeding project represent the first attempt to generate similar information for Scots pine (*Pinus sylvestris* L.).

We report here on the generation of a set of F3 study materials in Scots pine, where 142 families arose from a mating design among 49 F2 parents, representing nine levels of inbreeding between 0.0 and 0.5, and repeated over two consecutive seasons. The impact of inbreeding level on seed production and germination is evaluated.

2. Materials and methods

2.1 Controlled crossing

Controlled crosses for this study were made in an F2 Scots pine breeding archive near the Sävar field station (Latitude 63.88°N; Longitude 20.55°E) representing the T11 breeding population for northern Sweden. The F2s are non-inbred, but have a pedigree arising from two discrete cycles of controlled crossing, starting with F0 plus trees selected in unimproved natural stands.

72
73 From each of 24 full-sib families in the archive, we identified two individuals that were
74 producing pollen, and where at least one of those trees was also producing female strobili. Pollen
75 samples from all 48 trees were collected in 2012, extracted, dried and placed in 10 ml sealed vials
76 at -25°C. The inbreeding coefficient F for any given cross could be calculated as the coancestry
77 between the two F2 parents, under the assumption that the F0 founders are unrelated. One of the
78 two trees per family was used as a seed-producing parent, which was crossed with itself and with
79 its half sib, producing controlled crosses with inbreeding coefficients of 0.5 and 0.25,
80 respectively. The seed parent was also crossed with a tree from an unrelated family, producing a
81 cross with inbreeding coefficient 0.0, and with three additional related trees producing different
82 levels of inbreeding where $0.0 < F < 0.25$.

83
84 Isolation of receptive strobili from contaminating pollen was accomplished with either a 205 x
85 400 mm paper/plastic bag (STERIKING®, manufactured by Wipack Medical) or a plastic tube
86 (length 80 mm, diameter 15 mm), sealed with water-repellant foam in both ends. The pollen was
87 applied with a custom-made applicator, designed for small lots of pollen. The isolation material
88 was removed around three weeks after pollination and the strobili clusters tagged.

89
90 A total of 273 crosses were attempted over a two-year period, with 147 and 126 in 2013 and
91 2014, respectively. Crossing success (i.e., producing 1 or more harvestable cones) was 82% in
92 2013, and 90% in 2014. The crossing operation attempted to produce 154 families with unique
93 pedigree (Tables 1 and 2). Of these, 142 produced cones from at least one crossing attempt, and
94 92 were successful in both years. While the 12 unsuccessful families involved 11 different pollen

parents and a wide range of expected cross inbreeding, only four seed parents were involved (Table 1), although every seed parent produced at least one family.

2.2 Seed collection, extraction and testing

The crosses were collected and extracted in the fall of the second year after pollination. Opening of the cones was accomplished by two cycles of heat (52°C) and moisture, and seeds extracted from the cones in a vibrating mesh box. The extracted seeds are gently wet-dewinged and empty seeds were separated using a gravity separator, and embryo development of the remainder evaluated by X-ray analysis.

The following production and seed data were developed for each cross:

- Total seed set per cone (total number of full and empty seeds)
- Percentage full seed (confirmed by X-ray analysis of both full and empty seeds)
- Number of full seed per cone
- Weight per 1000 full seed

Total seed set and percentage full seed data were not available for eight of the crosses due to processing errors, and these were treated as missing data. Similarly, total seed set, percent full seed and full seed per cone data were treated as missing for four crosses that were affected by pine cone weevil (*Pissodes validirostris* Sahlberg).

2.3 Nursery germination

Seeds from both crossing series were sown in the greenhouse in 14th April, 2016, with field planting scheduled for 2017. All available seeds were sown, to a maximum of 100 per family-year combination (average of 40); seeds remaining were placed in long-term cold storage. The seeds were sown in 5-cell row plots in 45-cell plastic growing trays having 90 cc volume per cell. The plots were fully randomized throughout the growing area. No fungicide treatments were applied and no fungal infections were observed. Germination assessment was made in weeks 3–4 (9–11 May). Germination statistics were calculated for all crosses where 10 or more seeds were sown, and expressed as:

- Germination % (normal, viable)
- Abnormal or albino germination %

2.4 Statistical analysis

The seed and germination data were subjected to separate mixed-model analysis of variance, using the following linear model:

$$Y_{ijk} = \mu + C_{ij} + Y_k + F + e_{ijk} \tag{1}$$

where Y_{ijk} is the trait response of the cross between seed parent i and pollen parent j in year k ; μ is the overall mean; C_{ij} is the random effect of the ij th unique cross (parent combination); Y_k is the fixed effect of the k th year in which the cross was performed ($k = 1, 2$); F is a fixed covariate describing the expected inbreeding coefficient (coancestry between parents); and e_{ijk} is the residual error $N[0, \sigma_e^2]$. The mixed-model analysis was performed with the ASReml software package (Gilmour et al. 2015), where the pedigree of the seed and pollen parents was associated with cross C_{ij} . The significance of differences between years and among levels of inbreeding

were tested by a Wald approximate F -statistic constructed from the mean squares for the model. Variance component estimates were evaluated by the ratio of each estimate to its standard error.

3. Results

The mixed-model analysis results are presented in Table 3. The individual-cross data points for each response variable are plotted against the inbreeding coefficient and the covariance functions presented in Figure 1.

3.1 Response means

The mean number of seeds set per cone was 17, with 54% having fully developed embryos. The variation in seed set was large; individual crosses produced from 1.5 to 52 seeds per cone, with up to 32 of these being full. The 1000-seed weight varied from 3.3 to 8.7 g, with a mean of 5.4 g. Germination of the cleaned full seeds averaged more than 92%, with a range of 50 to 100%. Abnormal germination was generally very low, averaging 2.9%. Of these, about 23% were classified as “albino”, with most occurring in a single family (4671652x4671650) with expected inbreeding of 0.25. For the 2013 crossing, 18 of the 45 germinants from this family were classified as “abnormal”, as were 5 of 19 for the 2014 crossing (seen as outlier points in Figure 1F). All but one of the abnormal germinants in this family were recorded as “albino”.

3.2 Effect of cross pedigree

The variance component estimates for the random effect of family ($\hat{\sigma}_C^2$) were greater than twice their standard errors for all assessed seed traits. Total seed set and weight per 1000 seeds were

the only traits where the variance component estimates for the family effect exceeded three times the effect standard error.

3.3 Effect of crossing year

The year of crossing was highly significant for all traits, with the exception of abnormal germination. Compared with crosses made in 2013, those made in 2014 had higher seed set (21.4 versus 13.1), greater yield of full seed (72.3% versus 68.0%), more full seeds per cone (15.2 versus 10.1), and heavier seed (1000-seed weight of 5.5 g versus 5.2 g), but lower germination (92.3 versus 95.5%).

3.4 Effect of inbreeding

Inbreeding was modelled as a fixed covariate effect. It revealed a highly significant inbreeding effect ($p < 0.001$) on the percent full seed and number of full seed per cone, where the coefficient of determination (R^2) for the regression indicated that level of inbreeding explained 48% and 16%, respectively, of the variation in these traits. The effect of inbreeding on germination percent and rate of abnormal germination was much less dramatic, with the inbreeding depression weakly significant ($p < 0.05$ level), and explaining only 1.7% and 1.6%, respectively, of the variation in these traits. Level of expected inbreeding had no significant effect on total seed set or filled-seed weight.

4. Discussion

While the numbers of extractable seeds were unaffected, the proportion of seeds with fully developed embryos was strongly affected by inbreeding. This was expressed as inbreeding depression in the yield of full seeds per cone, but not in their average weight. While levels of germination of these full seeds were affected by inbreeding, the depression was rather small and only weakly significant. These results suggest that much of the inbreeding depression is expressed early in the seed development and leads to embryo abortion. Full seeds go on to produce seedlings whose viability is little different from that of outcrossed seed.

While our observations confirm that inbreeding depression can certainly lead to lower seed yields, we have not followed the resulting germinants long enough to know if their survival or growth will be significantly affected by inbreeding depression expressed later in life. To address this question, the germinants from this study were raised in our nursery for one full season in 90 cm³ containers and outplanted the following spring on three field test sites in northern Sweden, where they will be assessed in later years and inbreeding depression in vitality and growth traits evaluated using similar models.

While the coancestry between parents is equivalent to the average inbreeding among their progeny, the actual inbreeding under Mendelian sampling and expression of genetic load will vary from one sibling to the next. Obviously, inbreeding depression can lead to the intra-ovule death of the zygote and produce empty seed, but we also expect that inbreeding depression will affect the outcome of intra-ovule embryonic competition in a poly-embryonic context (Lindgren 1975) and affect the selection of the fertilization event that leads to a germinating embryo. So, it

is to be expected that there are mechanisms even in the case of controlled crossing of related parents to select against actual genetic load.

While the expectation of inbreeding at the family level can be calculated from standard pedigree records, as was done here, it is well recognized that there will be stochastic variation among individuals in their genetic load. Although beyond the present scope of this study, it is possible to use whole-genome characterization approaches with DNA markers to reveal the basis for inbreeding depression (Remington and Malley 2000). The use of higher-density SNP assays will facilitate this exploration of inbreeding depression at the individual genome level (Plomion et al. 2016).

In our crosses, the pollen available for fertilization of a given ovule all originated from a single male parent, so any competition among pollen grains or among multiple embryos was limited to that single male. In a seed orchard context, many other pollen sources provide additional opportunities for less-inbred embryos to be favoured (Williams 2009), thus mitigating the impact of inbreeding and inbreeding depression. To test this hypothesis, we have used a subset of the parents reported here to generate poly-mix crosses, where 50% of the mix consists of the related parent, and the remainder provided as a mixture of unrelated pollens. With the possibility of multiple fertilization of archegonia with unrelated pollen grains (Sarvas 1962), we will test the hypothesis that this can further reduce the expression of genetic load through intra-ovule selection. As in the study reported here, the new polycross study utilizes a mating design where there are strong connections among the families through pedigree. We expect to utilize paternity analysis with SNP markers to recover the male parent information for each seedling (Vidal et al. 2015, 2017).

Acknowledgments

Funding for this study was provided by: Föreningen Skogsträdsförädling (The Swedish Tree Breeding Foundation); and Skogforsk (The Swedish Forestry Research Institute). We are grateful for the valuable contributions by Skogforsk technical and field staff – Berit Gregorsson, Marie Lundström and Monica Lundström. We thank Harry Wu for helpful comments on a draft, and Rowland Burdon for his thorough review of the submitted manuscript. All trademark terms are the property of their respective owners.

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Table 1. Crossing design matrix, showing the inbreeding coefficient¹ of the family (coancestry between parents).

Seed	Pollen	4671036	4671040	4671118	4671119	4671123	4671124	4671183	4671186	4671345	4671352	4671373	4671376	4671614	4671615	4671630	4671632	4671650	4671652	4671655	4671656	4671668	4671670	4671671	4671673	4671681	4671685	4671686	4671693	4671697	4671744	4671751	4671755	4671757	4671765	4671772	4671844	4671847	4671948	4671950	4671961	4671970	4672014	4672015	4672027	4672028	4672103	4672105	4672108	4672115	assessed						
4671040		0.25	0.50													0.03				0.06					0.13																									6							
4671118		0.13		0.50	0.25															0.06					0.19																									6							
4671123			0.13			0.50	0.25													0.06	0.19																												6								
4671186							0.25	0.50																						0.00						0.03				0.16					0.09				0.00			6					
4671345									0.50	0.25								0.06		0.00												0.13																	0.16			6					
4671373								0.03			0.50	0.25													0.00									0.09		0.13														6							
4671614				0.00			0.09					0.03	0.50	0.25																																			0.13			6					
4671630								0.03								0.13	0.50	0.25																																0.06			5				
4671632								(0.03)								(0.13)	0.25	0.50																	0.00															0.06			4				
4671652									0.00									0.25	0.50		0.03																															7					
4671656							0.06												0.25	0.50		0.25	0.50		0.13				0.09		0.13																			0.03			6				
4671670																0.00		0.03						(0.25)	0.50	0.25						(0.06)																		5							
4671681								0.13													0.19	0.25	0.50												0.06															6							
4671685																													0.50	0.25	0.03																				0.16			7			
4671693																			0.13											0.03	0.50	0.25		0.00																	0.06			6			
4671744			0.00											0.16																		0.50	0.25		0.03				0.09													6					
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4671772												0.13																							0.16	0.25	0.50												6								
4671847													0.03																								0.25	0.50											0.00			6					
4671950															0.13		0.06													(0.00)								0.25	0.50											0.16			5				
4671961											0.09																											0.19	0.13													6					
4672014												0.09						0.03															0.16						0.50	0.25											0.50	0.25			6		
4672028											0.13																																										0.25	0.50			6
4672105																																		0.19	0.03																				6		
4672108																																																									1
4672115																																																			(0.50)	(0.25)					6
as pollen		3	2	2	3	2	3	3	2	3	3	3	3	3	3	3	4	5	4	2	3	4	2	1	3	3	2	3	2	4	3	3	3	4	4	4	4	3	3	2	3	2	2	2	3	2	3	3	5	2	3	142					

¹ Inbreeding coefficients in the table are rounded to two decimal places. Actual coefficients, from lowest to highest, are 0.00, 0.03125, 0.0625, 0.09375, 0.125, 0.15625, 0.1875, 0.25 and 0.5. Crosses indicated in parentheses were attempted at least once, but produced no harvestable cones.

Table 2. Individual genotypes used for pair crosses and their frequency as seed and pollen parents to produce successful families with different expected inbreeding.

Individual	Used as		Resulting expected inbreeding coefficient ¹									Total
	Seed	Pollen	0.0	0.03	0.06	0.09	0.13	0.16	0.19	0.25	0.50	
4671036		3	1				1			1		3
4671040	6	2	1	1	1		2			1	2	8
4671118	6	2	1		1		2		1	1	2	8
4671119		3	1		1					1		3
4671123	6	2	1		1		2		1	1	2	8
4671124		3	1	1						1		3
4671183		3	1			1				1		3
4671186	6	2	1	2		1		1		1	2	8
4671345	6	3	1		1	1	2	1		1	2	9
4671352		3	1			1				1		3
4671373	6	3	1	2		1	2			1	2	9
4671376		3		1		1				1		3
4671614	6	3	1	1		1	2	1		1	2	9
4671615		3	1				1			1		3
4671630	5	4		2	2		1			2	2	9
4671632	4	5	1	2	2		1			2	2	10
4671650		4	1			1	1			1		4
4671652	7	2	3	1		1	1			1	2	9
4671655		3	1		1					1		3
4671656	6	4	1	2	3		1			1	2	10
4671668		2	1						1			2
4671670	5	1	1	1			1			1	2	6
4671671		3					1		1	1		3
4671673		3	1						1	1		3
4671681	6	2	1		1		2		1	1	2	8
4671685	7	3	3	1		2		1		1	2	10
4671686		2		1						1		2
4671693	6	4	1	2	2		2			1	2	10
4671697		3	1		1					1		3
4671744	6	3	1	1		1	1	2		1	2	9
4671751		3	1						1	1		3
4671755		4	1	1		1				1		4
4671757	6	4	2	2		1		2		1	2	10
4671765		4	1	1			1			1		4
4671772	6	3	1	2			1	2		1	2	9
4671844		3	1						1	1		3
4671847	6	2	1	1		1	1		1	1	2	8
4671948		3	1					1		1		3
4671950	5	2			1		1	2		1	2	7
4671961	6	2	1			2		1	1	1	2	8
4671970		2							1	1		2
4672014	6	3	1	2		1	1	1		1	2	9
4672015		2	1					1		1		3
4672027		3	1					1		1		3
4672028	6	3	1	2		1	1		1	1	2	9
4672103		3	1					1		1		3
4672105	6	5	1	1	2	1	1	1		1	2	10
4672108	1	2					1				1	2
4672115		3	1	1				1				3
142		142	47	34	20	20	34	20	12	48	49	284

¹ Inbreeding coefficients in the table are rounded to two decimal places. Actual coefficients, from lowest to highest, are 0.00, 0.03125, 0.0625, 0.09375, 0.125, 0.15625, 0.1875, 0.25 and 0.5.

Table 3. Summary of results from the mixed-model analyses of variance. Component estimates that exceed twice their standard error and levels of significance above the 95% level are indicated in bold font.

	Trait					
	Total seed set	Full seed (%)	Number full seed/cone	Weight per 1000 seeds (g)	Germination (%)	Abnormal germination (%)
<i>Sample size</i>	222	216	230	225	173	173
<i>Mean</i>	17.06	53.71	9.616	5.396	92.4	2.9
<i>Minimum</i>	1.5	3.1	0.250	3.333	50.0	1.00
<i>Maximum</i>	52.0	100.0	32.0	8.714	100.0	39.1
<i>Variance components (estimate/standard error)</i>						
$\hat{\sigma}_c^2$	28.27(3.15)	104.2(2.70)	12.18(2.65)	0.4764(4.75)	20.08(2.42)	8.756(2.89)
$\hat{\sigma}_e^2$	52.43(7.40)	195.8(6.69)	29.64(7.54)	0.4057(7.26)	42.77(6.05)	12.52(5.66)
<i>Fixed effect p-level</i>						
Year	<0.001	<0.001	<0.001	<0.001	<0.001	0.008
Inbreeding (<i>F</i>)	0.837	<0.001	<0.001	0.409	0.026	0.033

Figure 1. Regressions describing the covariation of response traits against expected inbreeding coefficient (F): **(A)** Total seed set, **(B)** Percent full seed, **(C)** Number of full seed per cone, **(D)** Weight per 1000 seeds, **(E)** Percent germination, and **(F)** Percent abnormal germination.