The after-effects of reproductive environment in shortleaf pine

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Summary

Thirteen identical controlled crosses were made among 22 shortleaf pine (*Pinus echinata* Mill.) clones growing in two contrasting reproductive environments: a seed orchard in central Arkansas, USA (34.6° N) and a clone bank in south Mississippi (30.5° N). The resulting seedlings from both reproductive environments were planted at both locations where the seed was produced. After 4 years in the field (5 years from seed), trees from seed produced in the two environments differed significantly in height. The reproductive environment × family interaction was also significant, however, indicating that the effect depended upon the genetic background of the parent trees. By age 9 years, differences were no longer statistically significant. Allozymes were assayed in seed produced in the two reproductive environments. Chi-squared tests of heterogeneity of segregation ratios for polymorphic loci showed significant differences between the two environments for many cross/loci combinations. Although the pattern of differences was not obviously linked to differences in growth, gametophytic selection is suggested as a possible explanation for the after-effects.

Introduction

Seed orchards for forest trees, especially boreal species, have often been located south of the origin of the ortets, because flowering and seed production are often greater in the warmer locations (Werner, 1975). Moving southern pine seed orchards to warmer climates also appears to increase seed production (Schmidtling, 1987).

When trees from seed of the northern ecotypes produced in seed orchards in southern environments are planted in the northern environments, however, their vegetative phenology and growth resembles that of southern ecotypes, rendering them susceptible to cold damage (Johnsen, 1989 a, b; Johnsen *et al*., 1989). This change in behaviour of genetically determined traits, usually called 'after-effects' appears to be permanent in Norway spruce (*Picea abies* (L.) Karst.) (Skrøppa, 1994). Greenwood and Hutchison (1996) found that increased temperature during gametogenesis and embryogenesis induced 'after-effects' in larch (*Larix* spp.). They also observed segregation distortion for a chlorophyll *a*/*b*–protein associated with differences in environment.

This study was initiated to determine if reproductive environment affects growth in shortleaf pine (*Pinus echinata* Mill.) and if the effect can be attributed to segregation distortion.

Materials and methods

Field

Ramets of 22 shortleaf pine clones were established in 1967 in two contrasting reproductive environments: (1) a seed orchard in central Arkansas, USA (34.6° N), near the source of the ortets; and (2) a clone bank in south Mississippi 30.5° N) (Figure 1). The ortets were first-generation selections for a breeding programme for central Arkansas. Thirteen identical controlled crosses were made at both locations in 1987. The resulting seed from both reproductive environments were planted in a nursery at the southern location in March 1991. Seedlings were lifted from the nursery in January 1992 and planted in the two locations where the seed was produced.

The field plots were randomized complete blocks of five replications with three-tree plots. Spacing in the plantings was 1 m × 3.1 m.

Heights were measured at ages 1, 4 and 9 years at the southern planting and at age 4 and 9 years at the northern planting. Branch nodes were counted on all trees living at age 9 years. This was used as an estimate of growth phases. Bud elongation of the first flush was measured on a sample of trees at the southern location. The sample consisted of two buds on two trees on each of 10 crosses from two reproductive environments for a total of 80 buds. Four trees from a similar-aged planting of a local shortleaf pine source were included as a control. Buds were measured three times a week for the complete elongation of the first flush.

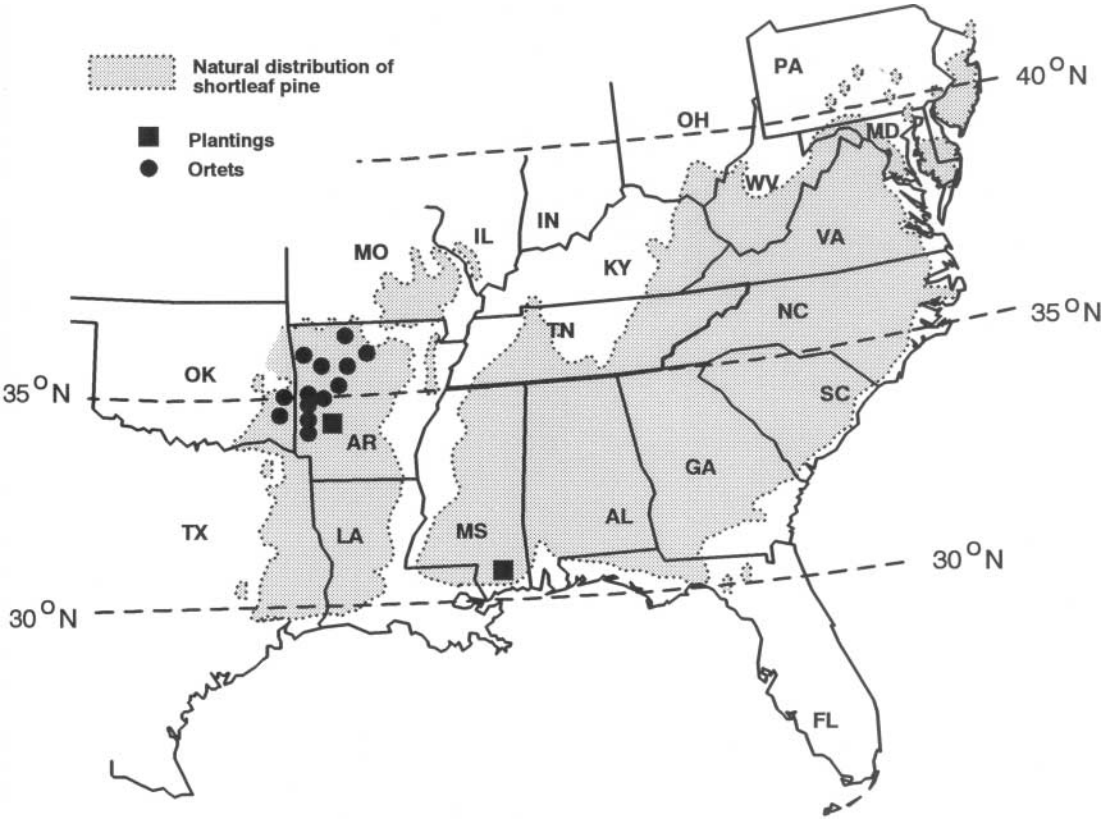

Figure 1. Map of the south-eastern United States showing the natural shortleaf pine distribution and the location of seed sources and planting sites.

In December 2000, a major ice storm damaged the northern planting. In March 2001, ~4 months after the ice storm, the planting was examined for damage. A score of 0–5 was used to quantify damage:

(0) No damage apparent
(1) Upper stem bent 45° or less
(2) Upper stem bent >45° or tip broken (1 inch in diameter or less
(3) Upper stem bent between 45° and 90° or top broken (up to 2 inches in diameter) but within the live crown
(4) Stem bent >90° or stem broken up to 3 inches in diameter
(5) Tree uprooted, on the ground, or stem broken below live crown.

The scale is meant to reflect the severity of damage and the probability of survival. A score of 5 indicates no possibility of survival; a score of 4 might survive but growth would be severely curtailed; a score of 0 indicates a tree unaffected in growth or survival.

Height and d.b.h. (diameter at breast height, or 4.5 feet) were measured in autumn 2000, after 9 years in the field. Least-squares analysis of variance was used to test differences among treatments (SAS, 1985).

Laboratory

Enough seed was available from 10 of the crosses from both reproductive environments for allozyme analysis. Megagametophyte and embryo tissue from a total of 1310 shortleaf pine seed (between 24 and 70 seed per cross) were genotyped at 21 allozyme loci using 15 enzyme systems. Seeds were germinated at room temperature until visible radicles were between 0.2 and 0.5 cm in length. Megagametophytes and embryos were dissected into 0.2 M phosphate buffer, pH 7.5, and frozen at –70°C. At the time of electrophoresis, tissue was thawed, ground, and saturated onto 2- and 2.5-mm-wide wicks prepared from Whatman 3MM chromatography paper, for megagametophytes and embryos, respectively. Methods of electrophoresis follow the general methodology of Conkle *et al.* (1982), with some modifications (USDA Forest Service, 1995). Enzyme extracts were resolved on 11 per cent starch gels (Sigma Chemical Co.) that accommodated 21 seed along the longitudinal axis. The following enzymes were examined: phosphoglucomutase (PGM), aconitase (ACO), phosphoglucose isomerase (PGI), malic enzyme (ME), alcohol dehydrogenase (ADH), leucine aminopeptidase (LAP), fluorescent esterase (FEST), phosphogluconate dehydrogenase (6PGD), triosephosphate isomerase (TPI), uridine diphosphoglucose pyrophosphorylase (UGPP), glucose-6-phosphate dehydrogenase (G6PDH), glutamate-oxaloacetate transaminase (GOT), glycerate-2-dehydrogenase (GLYDH), malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH). A lithium borate electrode buffer (pH 8.3) was used with a Tris citrate gel buffer (pH 8.3) (USDA Forest Service, 1995) to resolve PGM-1, PGM-2, ACO-1, PGI-2, ME7, ADH, LAP-1,2 and FEST-2. A sodium borate electrode buffer (pH 8.0) was used with a Tris citrate gel buffer (pH 8.8) (USDA Forest Service, 1995) to resolve 6PGD-1, TPI-1, TPI-2, UGPP-1, G6PD-2, GOT-2,3 and GLYDH. A morpholine citrate electrode and gel buffer (pH 8.0) (USDA Forest Service, 1995) was used to resolve MDH-2,4, 6PGD-2 and IDH-1. Enzyme stain recipes follow USDA Forest Service (1995) except that GOT was stained using the recipe from Wendel and Weeden (1989). Two people independently scored each gel. When they disagreed, a third person resolved the conflict. For quality control, 10 per cent of the individuals were run and scored twice.

Genetic interpretations were inferred directly from isozyme phenotypes, based on knowledge of the generally conserved enzyme substructure, compartmentalization and isozyme number in higher plants (Gottlieb, 1981, 1982; Weeden and Wendel, 1989).

Chi-squared analysis was used to test for 1 : 1 segregation of polymorphic loci in megagametophytes in each cross. Differences in allozyme allele ratios between reproductive environments in the crosses were examined using chi-squared tests of heterogeneity in $2 \times n$ contingency tables (2 environments $\times$ n genotypes) for the embryos.

Results

Growth and survival

Germination of the seeds produced varied widely, ranging from 69 per cent for seeds produced in the north to 85 per cent for seeds produced in the south. Germination of the crosses was not very well correlated across the two reproductive environments (r = 0.34). Storage conditions may have affected germination. The seeds were stored at their point of origin rather than at the same location before planting.

Survival after 4 years averaged 79 per cent in the northern planting and 86 per cent in the southern planting. Only the differences between plantings were significant (P = 0.024). There were no significant differences among crosses or reproductive environments.

After the first year in the field in the southern planting, the crosses differed significantly from one another in height ($P \leq 0.0001$). Seedlings from crosses made in the southern reproductive environment averaged slightly taller than those from the northern environment, 43 *vs* 40 cm, but the cross × reproductive environment interaction was significant (P = 0.0004), indicating that families responded differently to reproductive environment. Results after 4 years in the field also showed that the influence of reproductive environment depended on the particular cross (Table 1 and Figure 2). Overall, crosses made in the warmer environment grew slightly taller in both plantings, but in a few of the crosses, seedlings produced from crosses made in the northern environment were taller. The reproductive environment effect was significant (P = 0.036), but the interaction of cross with reproductive environment was significant at a higher level (P = 0.002).

The interactions of planting site with cross and reproductive environment were not statistically significant (Table 1). It is apparent in Figure 2 that the performance of the crosses made in the two reproductive environments was consistent over the two plantings for all but one cross. After 9 years in the field, mean heights by planting, cross and reproductive environment were similar to those at 4 years (Spearman rank correlation = 0.70), but statistical significance was lost (Table 1). This may be a result of the close spacing, which resulted in suppression and mortality by age 9 years, contributing to higher error.

Reproductive environment appeared to have no effect on the timing of growth in the spring (Figure 3). The bud elongation of trees from seeds formed in the southern reproductive are indistinguishable from those from seeds formed in the northern environment. The local source appears to begin growth a little earlier than the others, but this may be due to the fact that the trees for the local source are located some 300 m distant, and at a slightly higher elevation (~5 m). In any case, the differences are not statistically significant at any measuring date. It is not surprising that there were no effects on vegetative phenology, since even geographic races of shortleaf pine

Table 1: Analysis of variance of fourth-year and ninth-year heights

		Fourth-year height			Ninth-year height		
Source of variation	d.f.	S. Sq.	*F*	Pr > *F*	S. Sq.	*F*	Pr > *F*
Planting	1	18.3	8.33	0.0040	35.7	4.02	0.0455
Block (plant)	8	94.7	5.39	0.0001	360.2	5.08	0.0001
Cross	12	71.8	2.72	0.0014	363.7	3.41	0.0001
Repro. environment	1	9.7	4.43	0.0357	21.9	2.47	0.1168
Cross × environment	12	71.1	2.70	0.0015	179.0	1.68	0.0675
Plant × cross	12	34.3	1.30	0.2142	252.8	2.37	0.0055
Plant × environment	1	0.4	0.20	0.6581	0.1	0.00	0.9756
Plant × cross × environment	12	11.1	0.42	0.9557	94.6	0.89	0.5588
Error	562	122.4			460.3		

SAS (1985) type III sums of squares are used.

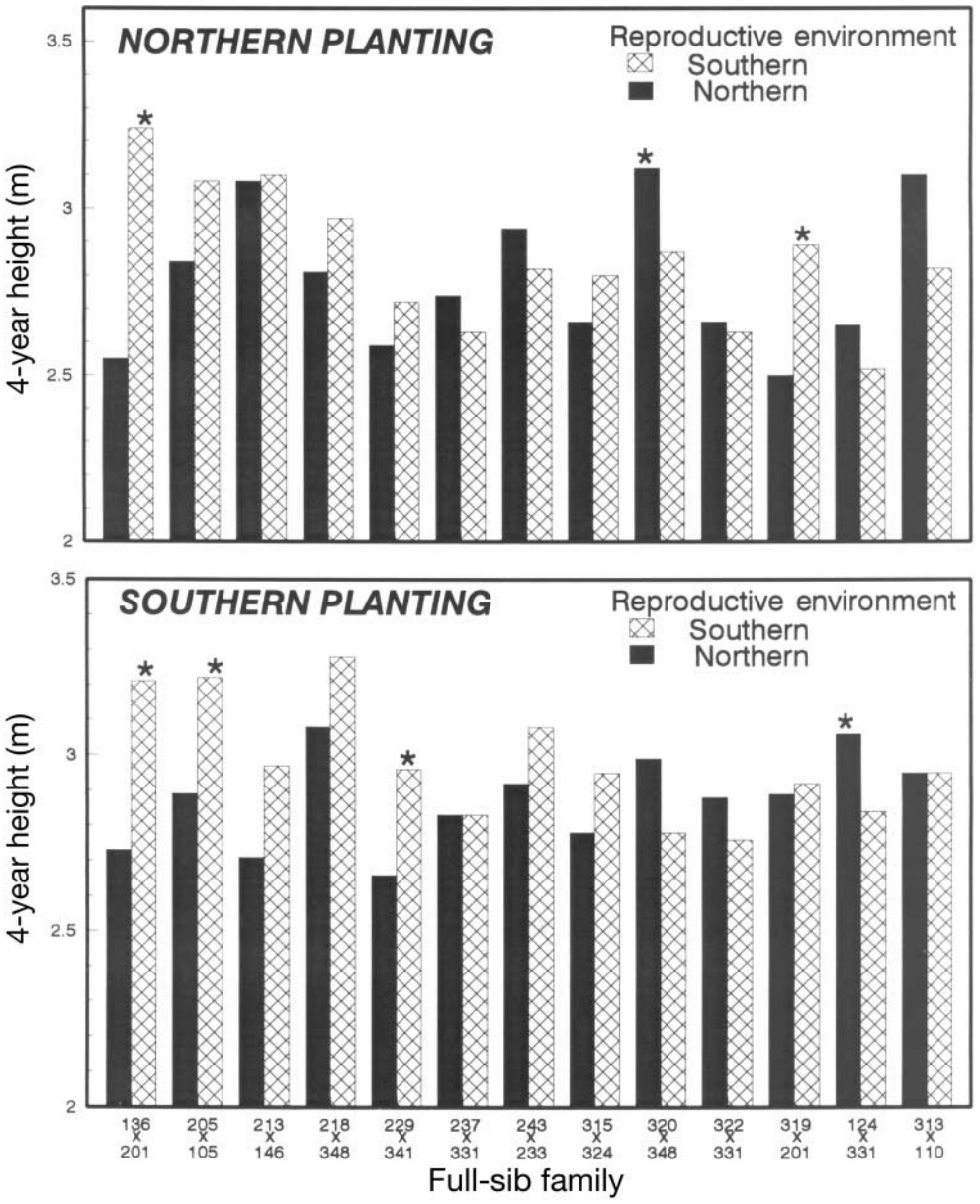

Figure 2. Fourth-year height of trees produced in northern *vs* southern reproductive environments, planted in two locations. *The difference between environments was statistically significant at $P \leq 0.05$.

differ little in phenology in common garden experiments (Schmidtling, 1971).

The branch node counts averaged 27.7 in the northern planting and 28.7 in the southern planting. This indicates that the trees in both plantings averaged around three growth phases, or flushes, per year over the 9 years of the study. In the statistical analysis of branch node counts, the planting location and cross (genetic) effects were statistically significant ($P \leq 0.001$). The

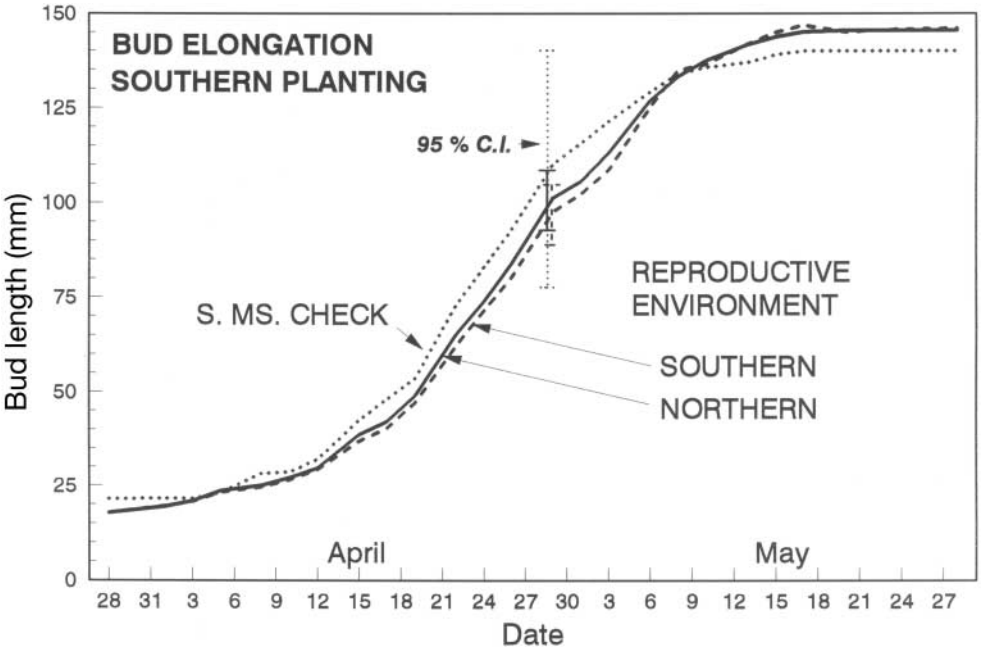

Figure 3. Spring bud elongation of seedlings produced in a northern reproductive environment *vs* those produced in a southern environment.

reproductive environment (P = 0.13), and the cross × reproductive environment interaction (P = 0.82) were not significant.

Ice damage

In the northern planting, the average ice-damage class for trees produced in the northern reproductive environment was 2.38 *vs* 2.62 for those produced in the southern reproductive environment. The difference, though small, was statistically significant (P = 0.018). Differences among crosses were also significant (P = 0.047) for ice damage. The cross × reproductive environment interaction was not significant (P = 0.965). Trees in the highest damage classes tended to have greater diameters: d.b.h. ranged from 10.8 cm in the '0' damage class to 12.4 cm in the '5' damage class. Crown size is generally correlated positively with d.b.h., and it is logical to assume that larger crowns accumulate more ice and subject trees to more stress. The trees produced in the southern reproductive environment averaged only slightly greater in diameter than those from the northern environment, 11.5 *vs* 11.2 cm, but the difference was statistically significant (P = 0.025). Differences in diameter in the combined analysis were not significant (P = 0.097), nor were they significant in the southern planting (P = 0.53).

Allozymes

For five of the 21 allozyme loci (PGM-2, TPI-1, TPI-2, MDH-2 and MDH-4) no polymorphisms were detected. The 16 polymorphic loci averaged 2.7 alleles per locus.

Segregation distortion, or deviation from a 1 : 1 ratio for polymorphic loci in megagametophyte tissue was fairly common in our material as indicated by chi-squared tests. In data pooled over both reproductive environments, significant segregation distortion was found in eight out of the 62 combinations of female parents and loci in megagametophyte tissue. Three out of 62 significant chi-squared values would be expected by chance alone.

Linkage analysis of our data indicated that

eight of the 16 loci are linked. They are listed first, in order of linkage, in Tables 2 and 3. The remaining eight loci appeared to segregate independently.

In chi-squared tests of heterogeneity, allele frequencies in gametophytic tissue differed according to reproductive environment in eight out of 62 combinations of female parents and loci (Table 2). This analysis did not test for a 1 : 1 ratio, but rather tested for differences among environments. The significant female parent/locus combinations in Table 2 were not the same combinations that differed significantly from a 1 : 1 segregation in the overall megagametophyte analysis.

The effect of linkage is apparent in Table 2. In female 315, three of the linked loci, Got-3, Pgi-2 and 6pgd-1, showed significant allele heterogeneity. Similarly, in female 322, two of the linked loci, Lap-1 and Me7, together show significant allele heterogeneity.

In the embryo analysis many, but not all, of the same locus/female combinations showed significant allele heterogeneity as in the gametophyte analysis (Table 3). One would expect some similarity between Tables 2 and 3 since the females are the same. The pattern is somewhat more complex, since two, three or four different combinations of genotypes are possible in crosses involving polymorphic parents, depending on their genotypes. In spite of this added complexity, and the higher chi-squared values often required, seven out of 76 locus/cross combinations showed significant allele heterogeneity (Table 3).

The environmental irregularities in allozyme alleles do not relate very well to differences in growth. In the one cross where the southern reproductive environment produced significantly greater growth in both plantings, 136 × 201 (Figure 2), no significant deviations from the expected allozyme ratios were found in embryo tissue (Table 3). In the one cross where there were many significant departures from expected allozyme ratios, 315 × 324, differences in height, although favouring the southern reproductive environment in both plantings, were not statistically significant.

Table 2: Chi-squared tests of heterogeneity for differences in segregation ratios of allozyme alleles due to reproductive environment in megagametophyte tissue of shortleaf pines

	Female									
Enzyme	136	205	213	218	229	237	243	315	320	322
Lap-1	1.3	1.9	.	0.2	0.7	5.3*	.	2.2	0.9	4.1*
Me7	.	.	.	.	.	0.3	.	.	.	14.0*
Got-3	2.1	0.1	1.2	0.1	1.8		0.1	4.0*		0.8
Pgi-2	.	.	.	0.1	.	.	1.9	5.3*	0.1	1.6
Got-2	.	1.8	.	.	1.1	0.2	.	.	0.1	.
Tpi-1	.	.	2.2	.	.	.	.	.	.	.
6pgd-1	0.1	.	3.4	0.5	.	.	0.3	4.4*	0.3	2.0
Aco-1	.	0.5	0.1	0.7	2.5	.	0.2	.	.	.
Lap-2	.	.	.	2.1	.	.	.	.	2.1	.
Pgm-1	.	.	.	0.3	.	.	0.3	.	.	.
Adh	.	.	.	.	.	.	.	0.3	.	.
G6pd-2	.	.	3.7*	.	.	.	.	0.5	0.1	.
Glydh	.	4.3*	0.3	.	.	.	.	0.5	0.1	.
Ugpp-1	1.0	.	.	0.1	.	0.4	0.1	0.5	0.1	.
Idh-1	.	0.8	.	.	.	.	1.2	.	.	2.3
6pgd-2	.	.	3.0	0.3	.	.	.	.	.	0.1

*Chi-squared test significant at $P \leq 0.05$.
H_o: Alleles from polymorphic loci segregate the same in either reproductive environment.

Table 3: Chi-squared tests of heterogeneity for differences in segregation ratios of allozyme alleles due to reproductive environment in embryos of controlled crosses of shortleaf pines

	Cross									
Enzyme	136× 201	205× 105	213× 146	218× 348	229× 341	237× 331	243× 233	315× 324	320× 348	322× 331
Lap-1	1.0	1.7	.	7.8*	2.1	3.4	.		7.7*	5.7*
Me7	0.5	.	.	.	.	1.1	.	.		6.8*
Got-3		5.4	0.9	0.1	0.1	0.1	0.1	6.5		1.5
Pgi-2	0.1	.	.	0.1	.	.	1.9	5.4*	0.1	1.6
Got-2	.	1.5	.	.	0.7	0.1	0.2	.	0.1	.
Tpi-1	.	.	2.2	.	.	.	.	.	.	.
6pgd-1	0.8	.	3.1	2.1	.	.	0.2	21.0*	1.5	2.0
Aco-1	.	0.7	0.1	2.7	1.1	.	0.2	.	0.2	.
Lap-2	.	0.01	4.6*	4.7	.	.	.	.	7.5*	.
Pgm-1	.	.	.	0.5	.	.	0.2	.	.	.
Adh	.	.	.	.	.	.	.	0.3	.	.
G6pd-2	.	.	3.4	.	.	.	.			.
Glydh	0.03	2.1	0.4	0.1	.	.	.	3.3	0.6	.
Ugpp-1	1.0	3.5	2.1	0.1	.	3.6	0.1	0.1	0.2	0.6
Idh-1	.	0.2	.	.	.	.	1.2	.	.	2.3
6pgd-2	0.1	.	3.4		0.2	.	.	.	.	0.1
Fest-2	.	.	.	.	.	0.1	.	.	.	0.1

*Chi-squared test significant at $P \leq 0.05$.
H_o: Alleles from polymorphic loci segregate the same in either reproductive environment.

Discussion and conclusions

It does not appear that reproductive environment has consistent long-term effects on adaptability of shortleaf pine seedlings, with the possible exception of resistance to ice damage. We did not observe the plethora of long-term adaptive changes associated with reproductive environment seen by Johnsen (1989a, b) in *Picea abies* (L.) Karst. Our growth results are remarkably similar to those of Greenwood and Hutchison (1996) in that significant effects of reproductive environment on growth varied according to the genetic background of the seedling. The genotype × reproductive environment interaction was significant in both studies. In a large study in Douglas-fir (*Pseudotsuga menziessii* (Mirb.) Franco), Wheeler *et al.* (1999) found that there were important differences induced by a warmer reproductive environment, but the effects were transitory, and not of great importance to their tree-improvement programme.

The allozyme data in this study suggest that there is some form of gametophytic selection induced by differing reproductive environments, similar to that found by Greenwood and Hutchison (1996). Enzymes are known to have temperature optima that differ among enzymes. It is reasonable to assume that some selection may occur for allozymes somewhere in the reproductive process, also affecting closely linked loci. It is also reasonable to assume that this differential selection would not be the same for all genotypes, as we have observed here.

Certainly other explanations are possible for the observed effects, such as temperature or photoperiodic effects on fertilization, multiple archegonia, and meiotic processes. It seems likely that factors not explained by traditional genetics are operating.

Our study is limited by the inclusion of only two planting sites, but the two sites represent a major part of climatic conditions that would be experienced by shortleaf pine. We conclude that after-effects of reproductive environment do exist in shortleaf pine, but they are not as clear-cut nor as important as in Norway spruce. The north–south distance in our study amounts to

only 4.1 degrees latitude rather than the 5–8 degrees latitude difference in the Norway spruce studies (Johnsen, 1989a, b), so we might not expect such large after-effects. These after-effects in shortleaf pine do not appear to have any consequence for tree-breeding programmes, and may not be important for other species of the Australes group, considering their similarities in climatic requirements.

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