recovery from mature tissues of olive cultivars *(Olea europaea* L.) "Canino" and "Moraiolo." Plant Cell Rep. 14: 257-260.

Santamour, F.S. 1972. Chromosome number in *Liquidambar.* Rhodora, 74: 287-290.

SAS Institute Inc.. 1994. RAP statistics and graphics guide, version 3 edition. SAS Institute Inc., Cary, N.C.

Sharp, W.R., S6ndahl, M.R., Caldas, L.S., and Maraffa, S.B. 1980. The physiology of in vitro asexual embryogenesis. Hortic. Rev. 2. pp. 268-310.

Sommer, H.E. 1981. Organogenesis in woody angiosperms: applications to vegetative propagation. Bull. Soc. Bot. Fr. Actual. Bot. 130(2): 79-85.

Sommer H.E., and Brown, C.L. 1980. Embryogenesis in tissue cultures of sweetgum. For. Sci. 26(2): 257-260.

Sutter, E.G., and Barker, P.B. 1985. In vitro propagation of mature *Liquidambar styracifua.* Plant Cell Tissue Organ Cult 5: 13-21.

Teixeira, 1.B., S6ndahl, M.R., and Kirby, E.G. 1994. Somatic em

bryogenesis from immature inflorescences of oil palm. Plant Cell Rep.13: 247-250.

Wann, S.R. 1994. Cloning famous trees. *In* Proceedings of the 2nd International Symposium on Applications of Biotechnology to Tree Culture, Protection, and Utilization, 2-6 Oct. 1994, Bloomington, Minn. *Edited by* C.H. Michler, M.R. Becwar, D. Cullen, W.L. Nance, R.R. Sederoff, and J.M. Slavicek. USDA For. Serv. Gen. Tech. Rep. NC-175. p. 21.

Westcott, R.J. 1994. Production of embryogenic callus from nonembryonic explants of Norway spruce *Picea abies* (L.) Karst. Plant Cell Rep. 14: 47-49.

Wilde, H.D., Meagher, R.B., and Merkle, S.A. 1992. Expression of foreign genes in transgenic yellow-poplar plants. Plant. Physiol. 98:114-120.

Witham, F.H., Blaydes, D.F., and Devlin, R.M. 1971. Experiments in plant physiology. Van Nostrand-Reinhold Co., New York.

Effects of megagametophyte removal on DNA yield and early seedling growth in coastal Douglas-fir

K.V. Krutovskii, S.S. Vollmer, F.C. Sorensen, M.T. Adams, and S.H. Strauss

Abstract: Stratified seeds of coastal Douglas-fir *(Pseudatsuga menziesii* (Mirb.) Franco var. *menziesii)* were germinated, sown in soil, and seed coats and megagametophytes were removed at various stages of early seedling development. Yield and quality of DNA extracted from the megagametophytes were related to several morphological traits of the seedlings after 2 months of growth in a controlled environment chamber. Regression and analysis of variance demonstrated nonlinear associations between stage of megagametophyte removal and seedling size traits, DNA yield and quality, and RNA presence. Megagametophyte removal when cotyledons had extended one-quarter of their length (about 4 nun) outside the seed coat (our stage 4) resulted in sufficient DNA for construction of saturated PCR- (polymerase chain reaction) based genome maps and had little effect on seedling development.

Resume : Les auteurs ont fait germs et ont ensemencd dans le sol des semences stratifides de sapin de Douglas de la C6te *(Pseudotsuga menziesii* (Mirb.) Franco var. *menziesu)* en enlevant le t6gument et le mdgagamdtophyte A diffdrents stades de ddveloppement prdcoce des semis. La quantitd et la qualitd de PADN extrait a partir des mdgagamdtophytes dtaient assocides a quelques caracteres morphologiques des semis 2 mois apres leur mise en croissance dans une chambre a environnement contr616. Les analyses de variance et de regression ont ddmontrd une relation non lindaire entre le stade oil le mdgagamdtophyte dtait recoltd et la taille des semis, la quantitd et la qualitd de PADN ainsi que la presence d'ARN. La rdcolte du mdgagamdtophyte alors que les cotylddons affichaient le quart de leur elongation maximale (environ 4 mm) a 1'extdrieur du tdgument (stade 4 des auteurs) a rdsultd en suffisamment d'ADN pour la construction de cartes gdnomiques saturdes a Faide de la technologie PCR (reaction en chene de polymerisation de 1'ADN). La rdcolte du mdgagamdtophyte a ce stade n'avait que peu d'effet sur le ddveloppement des semis. [Traduit par la Rddaction]

Introduction

The megagametophytes of conifers are haploid and originate mitotically from the same meiotic product as the megaspore. Because they allow maternal genes to be mapped directly in progeny of single trees without complications of dominance, megagametophytes are widely used for genome mapping (e.g., Binelli and Bucci 1994; Gocmen et al. 1996; Kaya and Neale 1995; Krutovskii et al. 1995; Nelson et al. 1993, 1994; Plomion et al. 1995; Tulsieram et al. 1992) and quantitative trait loci (QTL) mapping (e.g., Grattapaglia et al. 1992) using random amplified polymorphic DNA (RAPD) markers. The maternal contributions to the genotypes of individual seedlings can be determined by analysis of megagametophytes rescued during germination, allowing segregation of QTL heterozygous in maternal parents to be related to traits expressed in seedlings or later stages. However, because megagametophytes provide nourishment for seedlings during early stages of development, their premature removal may impair seedling growth and survival. Late removal, on the other hand, may sacrifice the quality and yield of DNA derived from exhausted megagametophytes, especially in species such as Douglas-fir *(Pseudotsuga menziesii* (Mirb.) Franco) with seeds of modest size (8.5 to 18.5 mg fresh weight; Owsten and Stein 1974). To identify the most desirable stage for megagametophyte removal, we studied the effects of megagametophyte removal on seedling growth and DNA yield during several stages of germination and early growth.

Material and methods

Plant material

Origin and germination of seeds
Seeds collected from three native trees located in a low-elevation stand in the western Cascades of Oregon (44°35N, 122°42'W, 300 m elevation, near Lacomb) were stratified for 60 days at 2-3°C and germinated at 23°C. The three open-pollinated families were evenly distributed among the various treatments described below. When radicles were 1 cm in length, the seeds were planted in soil in individual Leach tubes and the seedlings were grown for 2 months in a controlled environment under a 16 h : 8 h light:dark photoperiod at 23°C. Megagametophytes were removed from 14 seedlings at each of six stages of development (Fig. 1):

Stage l: Megagametophytes were carefully removed using a dissecting scope when the radicle was 1 cm in length. The germinants were planted immediately after megagametophyte removal.
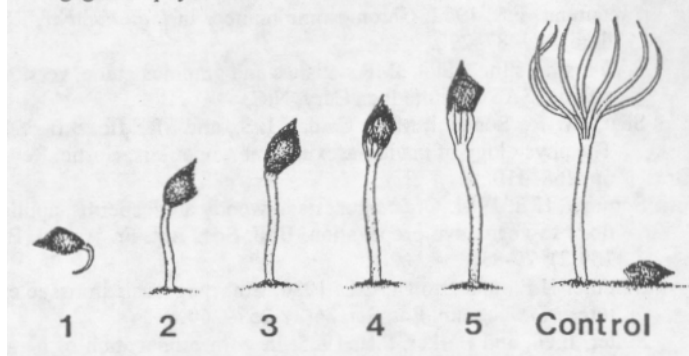
Stage 2: Megagametophytes were removed approximately 3 days after planting. Cotyledons were not visible at this time. Because the megagametophyte does not slip off easily at this stage extreme care was taken to prevent injury to the seedlings.

Stage 3: Megagametophytes were removed when cotyledons were first visible beneath the seed coat, approximately 4 days after planting.

Stage 4: Megagametophytes were removed when one-quarter of the total length of the cotyledons was visible below the seed coat (relative to their cotyledon length at the time of natural seed coat shed: stage 6 (control)), approximately 5 days after planting.

Stage 5: Megagametophytes were removed when one-half of the length of the cotyledon was visible beneath the seed coat, approximately 6 days after planting.

Fig. 1. Seedling morphology during the six stages of megagametophyte removal studied.

Stage 6 (control): Megagametophytes were collected after they fell to the ground, approximately 1 week after planting.

Harvesting and measurement of seedlings
A total of 84 two-month-old seedlings were harvested and the following traits were measured: seedling height and hypocotyl length (SH and HL), cotyledon number (CN), cotyledon and root lengths (CL and RL), and fresh and dry seedling weights (FW and DW). SH (to the tip of the primary needles) and RL were measured from soil level. Total seedling length (TL) was the sum of SH and RL.

Megagametophyte and DNA traits
A total of 72 megagametophytes (removed from 12 of the 14 seedlings analyzed for growth traits at each of the six stages) were measured for weight (MW), yield, and quality of DNA isolated (DNA and DNA-Q), and RNA presence in the DNA sample (RNA-P). DNA was isolated following a modified CTAB procedure for Douglas-fir (Tsumura et al. 1996) and quantified using a Hoefer TKO 100 fluorometer. Based on the appearance of the DNA when run (undigested with restriction enzymes) for 3-4 h on a 0.8% agarose gel (Tris-acetate/EDTA electrophoresis buffer, pH 8.3) and stained with ethidium bromide, DNA quality was scored as either 1 (no evidence of degradation) or 0 (partially degraded). During these analyses the presence of RNA remaining after RNase treatment of the samples (4 μg RNase A, 37°C for 30 min) was also noted from the same gels, scored as 1 for presence or 0 for absence. PCR amplification was tested on all six stages using four RAPD primers.

Statistical analyses

All statistical analyses were carried out using the SYSTAT (1992) computer package. Based on both scatterplot analysis of standardized residuals and Studentized residual values, we identified six outliers that were excluded from further analysis. They were 3-4 standard deviations from the means for their traits at a given stage of seedling development and differed statistically (P < 0.01) from other samples. Four seedlings were deformed and had extremely low values for several morphological traits. Another seedling appeared to have suffered from insect attack. The last outlier had normal seedling development, but its megagametophyte gave a very low yield of DNA, a possible result of an error during DNA isolation.

We used simple linear and multiple linear regression analysis and factorial multivariate analysis of variance (MANOVA) under the multivariate general linear hypothesis (MGLH) to test whether seedling traits depend significantly on stage of megagametophyte removal and family origin. The model for simple linear regression was $y = \alpha + \beta x + e$, and for multiple linear regression was $y = \alpha + \beta_1 x_1 + \beta_2 x_2 + e$, or $y = \alpha + X\beta + e$, where y is a dependent variable vector, a is a constant coefficient, X is a vector or matrix of independent variables x (stage and family origin), is a vector of regression coefficients, and a is a vector of random errors. Because most of the traits measured were curvilinearly related to stage of

Fig. 2. Effect of megagametophyte removal on seedling growth at 2 months, cotyledon number, and weight and DNA yield of megagametophytes. Each point, bracketed by one standard error of the mean, is the average of 11 to 14 seedlings.
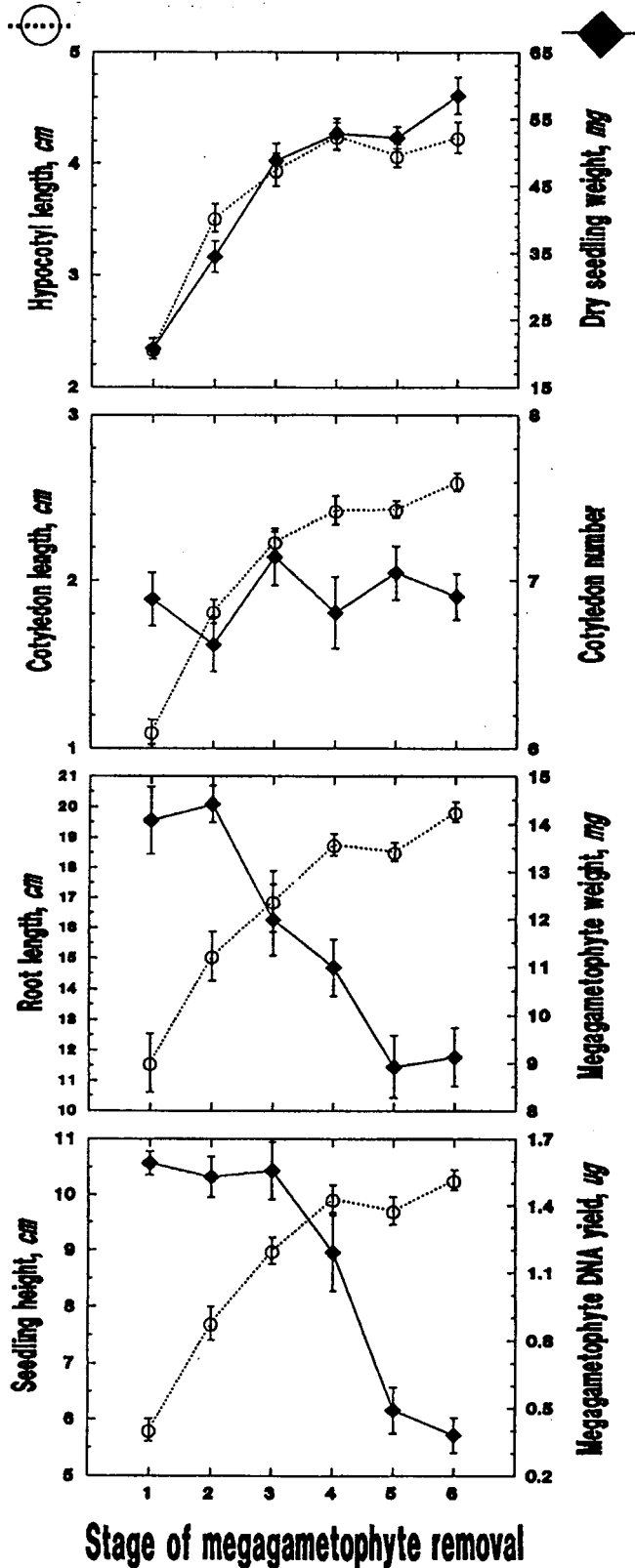
removal (Fig. 2), we used quadratic (including x^2 term) as well as linear functions to model their associations. The Kohnogorov-Smimov (K-S) one-sample test was used to compare the shape and location of the sample distributions with a normal distribution. Because some of the samples failed to conform to a normal distribution even after logarithmic and other transformations were applied; we also used the nonparametric Kruskal-Wallis (K-W) test and one-way analysis of variance (ANOVA) of ranked data. The K-S and Lilliefors (on standardized data) two-sample tests were applied to compare two-sample cumulative distributions (Sokal and Rohlf 1981). Pearson's product moment coefficient of linear correlation, and the nonparametric Spearman's coefficient of rank correlation, were calculated between stage of megagametophyte removal and all dependent variables. Means were tested for pairwise differences across the six stages and three open-pollinated families using the Bonferroni and Scheffe's multiple tests, and t-tests. Linear contrasts were used to test mean differences between selected stages or groups of stages. Because of the virtual identity in significance of results from the multivariate versus univariate analyses, and in the Scheffe versus Bonferroni tests, only the univariate analyses and Bonferroni tests are presented below.

Results

Early removal of megagametophytes decreased all size measures of 2-month-old seedlings except for number of cotyledons (Table 1). Cotyledon number, but not the other traits measured, varied significantly among families (data not shown), as reported previously (Sorensen 1966, 1967). Family by treatment interactions were nonsignificant for all traits (data not shown). Seedlings from the three families were therefore pooled for further analysis. Late removal of megagametophytes led to reductions in megagametophyte. size and DNA yield and quality, but increased RNA presence (Table 1). All six stages showed good PCR amplification using four RAPD primers.

The responses of both seedling size and megagametophyte traits to stage of megagametophyte removal were nonlinear (Table 1 and Fig. 2). The greatest impact on size traits was in the early stages of removal (stages 1-3); the greatest impact on DNA yield and quality was in the last stages (stage 5 and control). Statistical support for nonlinearity is provided by the significant quadratic regression coefficients for many of the traits (Table 1), and by pairwise contrasts among stages, which indicated few significant differences between stages 3 through 5 for the growth traits (data not shown).

Discussion

Removal of seed coats and megagametophytes at stage 4 appears to have minimal impact on seedling growth and development. Removal at stage 3 would be preferable for maximizing DNA yield, but had somewhat more impact on size, especially seedling height (Fig. 2). The DNA yield from stage 4 megagametophytes averaged 1.2 ± 0.2 lag, which is sufficient for approximately 500-700 RAPD reactions using 2 ng of template DNA per reaction (Krutovskii et al. 1995). This number of reactions should be sufficient to produce a saturated linkage map for genome and QTL mapping. Therefore, for QTL studies we recommend removal of megagametophytes of Douglas fir when cotyledons are one-quarter, or a little less, of the expected length at the time of natural seed coat shed, or when seeds of average size have 3-4 mm of cotyledon extending from the seed coat.

Table 1. Effect of stage of megagametophyte removal on several seedling and megagametophyte traits.

(A) Seedling traits

Stage	Hypocotyl length, cm	Cotyledon length, cm	Root length, cm	Seedling height, cm	Dry seedling weight, mg
1	2.34±0.09(11)a	1.10±0.07(11)a	11.58±0.97(11)a	5.81±0.21(11)a	20.73±1.61(11)a
2	3.52±0.15(13)b	1.82±0.07(13)b	15.06±0.80(13)b	7.71±0.29(13)b	34.46±2.36(13)b
3	3.94±0.12(14)bc	2.24±0.06(14)c	16.88±1.02(14)bc	8.99±0.24(14)c	48.79±2.59(14)c
4	4.25±0.13(13)c	2.43±0.09(14)cd	18.76±0.36(14)cd	9.92±0.25(14)cd	52.86±2.31(14)cd
5	4.07±0.10(14)c	2.44±0.05(14)cd	18.52±0.32(13)cd	9.71±0.25(14)cd	52.21±1.64(14)cd
Control	4.24±0.14(14)c	2.60±0.05(14)d	19.83±0.33(13)d	10.26±0.17(13)d	58.50±2.73(14)d
F	29.84***(79)	61.80***(80)	17.67***(78)	45.18***(79)	34.96***(80)
r_P	0.68***	0.81***	0.70***	0.80***	0.77***
r_x	2.72***	2.52***	1.81	2.37***	2.16***
r_{x^2}	−2.08***	−1.74***	−1.14	−1.60***	−1.42***

(B) Megagametophyte traits and cotyledon number

Stage	Cotyledon number	Megagametophyte weight, mg	DNA yield, μg	DNA quality	RNA presence
1	6.91±0.21(11)a	14.08±0.71(12)a	1.59±0.05(12)a	1.00±0.00(11)a	0.00±0.00(12)a
2	6.64±0.23(14)a	14.42±0.38(12)a	1.53±0.09(12)a	1.00±0.00(12)a	0.00±0.00(12)ab
3	7.21±0.24(14)a	11.98±0.76(12)ab	1.56±0.13(11)a	0.75±0.13(12)a	0.50±0.15(12)c
4	6.71±0.24(14)a	10.98±0.58(12)bc	1.19±0.17(12)ab	0.83±0.11(12)a	0.42±0.15(12)abc
5	7.00±0.21(14)a	8.93±0.65(12)c	0.49±0.10(12)b	0.67±0.14(12)a	0.92±0.08(12)c
Control	7.00±0.18(14)a	9.13±0.61(10)c	0.38±0.08(9)b	0.70±0.15(10)a	0.60±0.16(10)c
F	0.93(81)	14.01***(70)	22.96***(68)	1.78(69)	10.49***(70)
r_P	0.07	−0.69***	−0.74***	−0.31**	0.57***
r_x	0.36	−0.71	0.37	−0.58	1.20*
r_{x^2}	0.30	0.02	−1.13**	0.28	−0.64

Note: Data given are means ± SE (sample size). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; F, F-tests of stage differences from one-way analysis of variance; r_P, linear Pearson's correlation coefficient, and r_x and r_{x^2} standardized regression coefficients for linear (stage) and quadratic terms (stage^2). Lowercase letters indicate means that are not statistically different in pairwise Bonferroni tests.

The strongly curvilinear relationships between time of megagametophyte removal (as characterized by our stages) and seedling and megagametophyte traits is in agreement with earlier observations on biology of young seedlings. At the time of natural seed coat shed (stage 6 (control)), the megagametophyte contents are exhausted and the megagametophyte appears to consist of dry, papery tissue. This is consistent with their low yield and quality of DNA. Observations on albino Douglas-fir seedlings have shown that the megagametophyte tissue has just enough stored energy to enable the seedling to reach the stage of shedding the seed coat (F. Sorensen, unpublished observations). Additionally, Douglas-fir cotyledons have high photosynthetic rates compared with needles on seedlings just a few weeks older (Sorensen and Ferrell 1973). These observations suggest that if the megagametophyte is removed when cotyledons are significantly extended (our stage 4), there will be sufficient megagametophyte tissue remaining for DNA extraction and the high photosynthetic rate of the cotyledons will compensate for most of the energy potential lost due to removal of the megagametophyte. If, however, the seed coat is removed before the cotyledons have elongated to the point where they can supply significant photosynthate (e.g., our stages 1-2), removal of the megagametophyte will stunt seedling development.

The positive association observed between RNA presence and stage of megagametophyte removal may result from vari-ation in stage of development. Megagametophytes from later stages may have a higher proportion of their cells actively involved in germination, and thus have more mRNAs and rRNAs to support intensive transcription and translation.

The window of time when megagametophyte removal has tolerable impacts on both DNA content and seedling development is narrow. Megagametophyte removal must therefore be carefully standardized in studies of early seedling development and QTL analysis. Megagametophytes can be uniformly removed early, which would substantially retard seedling growth and might reduce maternal environmental (seed weight) effects, but also can lead to abnormal early development. Alternatively, uniform removal at stage 4 will have negligible retarding influence on growth, but still yield enough DNA for hundreds of PCR reactions.

Acknowledgments

References

Binelli, G., and Bucci, G. 1994. A genetic linkage map of Picea abies Karst., based on RAPD markers, as a tool in population genetics. Theor. Appl. Genet. 88: 283-288.

Gocmen, B., Jermstad, K.D., Neale, D.B., and Kaya, Z. 1996.

Development of random amplified polymorphic DNA markers for genetic mapping in Pacific yew (Taxus brevifolia). Can. J. For. Res. 26: 497-503.

Grattapaglia, D., Chaparro, Jx, Wilcox, P., McCord, S., Werner, D. et al. 1992. Mapping in woody plants with RAPD markers: applications to breeding in forestry and horticulture. *In* Proceedings of the Symposium on Applications of RAPD Technology to Plant Breeding, 1 Nov. 1992, Minneapolis, Minn. Joint Plant Breeding Symposia Series. Crop Science Society of America - American Society for Horticultural Science - American Genetic Association. pp. 37-40.

Kaya, Z., and Neale, D.B. 1995. Utility of random amplified polymorphic DNA (RAPD) markers for linkage mapping in Turkish red pine *(Pinus* brutia Ten.). Silvae Genet. 44: 110-116.

Krutovskii, K.V., Vollmer, S.S., Sorensen, F.C., Adams, W.T., and Strauss, S.H. 1995. RAPD linkage map of a Douglas-fir racial hybrid (poster P131, p. 55). Plant Genome IV, 15-19 Jan. 1995, San Diego, Calif. Scherago Associates, New York.

Nelson, C.D., Nance, W.L., and Doudrick, R.L. 1993. A partial genetic linkage map of slash pine *(Pinus elliottii* Engelm. var. elliottii) based on random amplified polymorphic DNAs. Theor. Appl. Genet. 87: 145-151.

Nelson, C.D., Kubisiak, T.L., Stine, M., and Nance, W.L. 1994. A genetic linkage map of longleaf pine *(Pinus palustris* Mill.) based on random amplified polymorphic DNAs. J. Hered. 85: 433-439.

Owsten, P.W., and Stein, W.I. 1974. Pseudotsuga. *In* Seeds of woody plants in the United States. Compiled by C.S. Schopmeyer. U.S. Dep. Agric. Agric. Handb. 450. pp. 674-683.

Plomion, C., O'Malley, D.M., and Durel, C.E. 1995. Genomic analysis in maritime pine (Pinus pinaster): comparison of two RAPD maps using selfed seeds and open-pollinated of the same individual. Theor. Appl. Genet. 90: 1028-1034.

Sokal, R.R., and Rohlf, F.J. 1981. Biometry. 2nd ed. W.H. Freeman and Company, San Francisco.

Sorensen, F.C. 1966. Inheritance of cotyledon number in Douglas-fir. For. Sci. 12: 175-176.

Sorensen, F.C. 1967. Two-year results of a west-east trmsect provenance test of Douglas-fir in Oregon. USDA For. Serv. Pac. Northwest For. Range Exp. Stn. Res. Note 72.

Sorensen, F.C., and Ferrell, W.K. 1973. Photosynthesis and growth of Douglas-fir seedlings when grown in different environments. Can. J. Bot. 51: 1689-1698.

SYSTAT. 1992. SYSTAT for Windows: statistics, version 5 edition. SYSTAT, Inc., Evanston, Ill.

Tsumura, Y., Ohba, K., and Strauss, S.H. 1996. Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). Theor. Appl. Genet. 92: 40-45.

Tulsieram, L.K., Glaubitz, J.C., Kiss, G., and Carlson, J.E. 1992. Single tree genetic linkage mapping in conifers using haploid DNA from megagametophytes. Biotechnology, 10: 686-690.