Developmental Changes in Seeds of Loblolly Pine

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Abstract. Mature seed weight of loblolly pine (*Pinus taeda* L.) averaged 25 mg (dry weight), of which 55.0% was seedcoat, 38.5% megagametophyte, and 6.4% embryo. Fatty acid (FA) content (dry-weight basis) was 17.5% for whole seed, 0.4% for seedcoat, 36.2% for megagametophyte, and 51.2% for embryo. Distribution of FAs (16:0; 18:0; 18:1; 18:2; 18:3 Δ 5,9,12; 18:3 Δ 9,12,15; 20:0; and 20:3) differed in seedcoat, megagametophyte, and embryo, but 18:2 was the predominant FA in all tissues. Seed development was analyzed for 110 days from 25 July, the year following pollination. Embryos could be macroscopically observed on or about day 30. Embryo dry weight, length, and FA accumulation increased until about day 50 and then remained constant. Embryo density decreased from day 30 to 50 and then stabilized at $\approx 1.0366 \text{ g}\cdot\text{ml}^{-1}$ or 10% sucrose equivalent. Excised zygotic embryos did not germinate in vitro until after day 51; germination increased linearly after this date, reaching 80% by day 72.

Recent interest in somatic embryogenesis as a potential means of propagating loblolly pine (*Pinus taeda*) and other forest trees (Durzan, 1988; Gupta and Durzan, 1986, 1987; Wann, 1988) has focused attention on embryo development. In this study, we examined seed development in loblolly pine. Our objective was to obtain morphological, metabolic, and physiological markers of zygotic embryo development.

Materials and Methods

Plant material. Mature seed and developing cones of three open-pollinated families of loblolly pine from the coastal plain of North Carolina were obtained the year following pollination from the Southern Forestry Research Dept., Weyerhaeuser Corp., Hot Springs, Ark. Mature seed of family 1 was obtained in 1987. Developing cones were obtained weekly from family 2 and family 3 from 17 Aug. to 5 Nov. in 1987 (80 days), 25 July to 7 Nov. in 1988 (105 days), and from 10 Aug. to 13 Oct. in 1989 (64 days).

Dry matter and fatty acid analysis. Mature seed of loblolly pine (family 1) collected in 1987 was dissected upon arrival into seedcoat (excluding nucellus), megagametophyte, and embryo; the embryo was further separated into cotyledon and hypocotyl, weighed, and frozen. Fatty acid (FA) analyses were carried out on five samples of 10 seed each.

In the developmental study in 1987 and 1988, three samples of 10 seeds each were analyzed for each harvest date and family for lipid determination. Only the megagametophyte and embryo were analyzed since seedcoat lipids of mature seeds are present in negligible amounts. Embryos were not visible before day 28 (day 0 = 25 July) and on day 28, 35, and 43, 10 embryo samples were too small for reliable analyses so sample size was increased to 30. All data are presented on a per embryo or megagametophyte basis.

Lipids were extracted from seed parts using a procedure modified from Folch et al. (1957). Samples were homogenized in 5 ml of 2 chloroform : 1 methanol (v/v) and solvent was added to bring the total volume to 20 ml. Pentadecanoic acid (15:0) was added (0.5 mg) as an internal standard for gas chromatographic analysis. Samples were sonicated for 30 min, 5 ml of aqueous 0.58% NaCl was added, and samples were resonicated

for 10 min. After centrifugation for 5 min at $180 \times g$, the chloroform phase was removed with a pipette and dried using an N-evap analytical evaporator (Organomation, Northborough, Mass.).

Triglycerides were saponified and soaps were methylated using the procedure of Metcalfe and Schmitz (1961). Samples were boiled for 5 min in 0.5 ml toluene and 2.0 ml 0.5 N NaOH in methanol. Boron trifluoride (2.5 ml) was added and samples were placed in a hot water bath for 3 min. When the samples were cool, 15 ml petroleum ether and 20 ml distilled water were added. Following sonication for 10 min, the petroleum ether phase was filtered through Whatman No. 1 filter paper with 1 g Na_2SO_4 to remove water. Samples were dried in the evaporator and resuspended in 1 ml petroleum ether.

Fatty acid composition was analyzed with a Varian 3700 gas chromatograph (Varian, Palo Alto, Calif.) using a 30 m $\times$ 0.32-mm fused silica capillary column with SP-2330 as the stationary phase. Initial column temperature was 160°C for 1 min and increased by 4°C/min to 204°C. Peak areas of FAs were determined with a Hewlett Packard 3390A integrator (Hewlett Packard, Palo Alto, Calif.). The order of peak elution was: 15:0 (internal standard); 16:0; 18:0; 18:1; 18:2; 18:3 Δ 5,9,12; 20:0; 18:3 Δ 9,12,15; and 20:3. Peaks were identified by comparing retention times with pure standards. The identity of each FA was confirmed by gas chromatography (Finnigan 9610; San Jose, Calif.) and mass spectroscopy (Finnigan 4000) using electron impact analysis.

Density determination. Relative densities of whole seed, megagametophyte plus embryo, megagametophyte, and embryo were determined for 1988 seeds using a continuous sucrose gradient (Price, 1982). The gradient was prepared by pipetting 6 ml of a high-sucrose (10% to 70%) solution into a 15-ml screw-top vial and then carefully layering an equal volume of a low-sucrose (0% to 40%) solution. The precise combination differed by tissue and age. The capped vials were set in a horizontal position for 30 min and then returned to vertical. Each sample to be measured was gently placed below the surface of the sucrose solution with a small spatula, and the vial was immediately centrifuged at $45 \times g$ for 5 min. At this time, the tissue had migrated to its equilibrium position and a small volume ($\approx 0.1 \text{ ml}$) of solution next to the tissue sample was withdrawn with a micropipette; the refractive index was determined with a refractometer (Bausch and Lomb, Rochester, N.Y.). The mean of five refractive index readings of each sample was converted into relative density (percent sucrose at 25°C) and true density ($\text{g}\cdot\text{ml}^{-1}$). Density measurements were first made on intact seeds from day 21 to 105 in 1989. In the first 3 weeks, some seeds floated and these were considered to have a relative density of

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Table 1. Dry weight and fatty acid (FA) distribution ($\pm$ SE) of mature seed of loblolly pine (family 1). Each mean is based on five samples of 10 units.

Seed part	Dry wt		Total FAs		
	mg	Percent of seed	mg	Percent dry wt	Percent of seed
Seedcoat	13.6 $\pm$ 0.3	55.0 $\pm$ 0.8	0.1 $\pm$ 0.0	0.4 $\pm$ 0.0	1.5 $\pm$ 0.1
Megagametophyte	9.5 $\pm$ 0.3	38.5 $\pm$ 0.9	3.4 $\pm$ 0.2	36.2 $\pm$ 0.7	79.8 $\pm$ 1.1
Embryo	1.6 $\pm$ 0.0	6.4 $\pm$ 0.1	0.8 $\pm$ 0.1	51.2 $\pm$ 3.2	18.8 $\pm$ 1.1
Cotyledon	0.8 $\pm$ 0.0	3.4 $\pm$ 0.1	0.4 $\pm$ 0.0	48.9 $\pm$ 2.2	9.6 $\pm$ 0.4
Hypocotyl	0.7 $\pm$ 0.0	3.0 $\pm$ 0.1	0.4 $\pm$ 0.0	53.7 $\pm$ 4.6	9.2 $\pm$ 0.8
Total seed	24.7 $\pm$ 0.4	100	4.3 $\pm$ 0.2	17.5 $\pm$ 0.6	100

0% sucrose or 0.9977 g·ml⁻¹. The density of the megagametophyte plus embryo was obtained by removing the seedcoat only. The megagametophyte was split with a scalpel and density measurements were then taken on the embryo and the two parts of the megagametophyte. The length of each seed part was measured throughout the 15 weeks.

Embryo growth in vitro. The capacity of developing zygotic embryos to germinate in vitro was determined in 1989 over 10 sampling periods, from day 16 to 79 (10 Aug. to 13 Oct.). The intact megagametophyte and embryo were isolated from the seedcoat, disinfested by soaking for 5 min in a solution of 1% NaOCl [20% (v/v) commercial bleach] with a drop of Tween 20, and rinsed five times with sterile distilled water. The intact embryo was then excised from the split megagametophyte. For each family, 50 embryos were cultured (five plates of 10 embryos each) with the cotyledonary end embedded in half-strength Murashige and Skoog salts (1962) with 2% sucrose and 0.6% agar. The dishes were sealed with Parafilm and inverted according to Carpita et al. (1983). Plates were incubated at 25°C under a 16-h photoperiod of 45 μ mol·s⁻¹·m⁻² photosynthetic photon flux provided by cool-white fluorescent lamps. In vitro development of zygotic embryos was measured after 4 weeks by: 1) increase in length of all embryos achieved 4 weeks after explantation (total length - original length), 2) percent germination 4 weeks after explantation. An embryo was considered germinated by the following: a) elongation of the hypocotyl and radicle, b) expansion, separation, and greening of cotyledons.

Results and Discussion

Dry matter and FA distribution in mature seed. Mature seed averaged 24.7 mg (dry weight) and consisted of seedcoat (13.6 mg), megagametophyte (9.5 mg), and embryo (1.6 mg) (Table 1). Thus, seedcoat constituted more than half the dry weight ($\approx$ 55%) of mature seed, with megagametophyte 38.5% and embryo only 6.4% of mature seed weight, equally divided between cotyledon and hypocotyl.

Mature seed contained an average of 4.3 mg FAs, 1.5% in the seedcoat (0.4% of seedcoat dry weight), nearly 80% in the megagametophyte (36.2% of megagametophyte dry weight), and 19% in the embryo (51.2% of embryo dry weight). Total FAs were similar in cotyledons and hypocotyl.

Eight FAs were identified in seeds (in order of elution) as 16:0 (palmitate); 18:0 (stearate); 18:1 (oleate); 18:2 (linolate); 18:3 Δ 5,9,12 (an isomer of α -linolenate); 20:0 (arachidate); 18:3 Δ 9,12,15 (α -linolenate); and 20:3 (Table 2). The FA 18:3 Δ 5,9,12, an unusual isomer of linolenate Δ 9,12,15, is common in the Pinaceae (Plattner et al., 1975). Time-course studies of FA accumulation and distribution conducted in 1987 and in 1988 had similar results, but only 1988 data are presented because collection dates were more extensive (105 days).

Although family 2 had higher dry weight and FA accumu-

Fig. 1. Accumulation of dry weight (A), total fatty acids (FA) (B), and total FA as percent dry weight (C) in the embryo and megagametophyte of two families of loblolly pine during development.

lation, the two families responded similarly over time (Fig. 1 A and B). Total FAs, as a percentage of dry weight in megagametophyte and embryo, were virtually identical in both families (Fig. 1C). Megagametophyte and embryo dry weight reached a peak at day 84 and then declined (Fig. 1A), but changes in megagametophyte were more pronounced.

FA accumulation followed dry weight accumulation (Fig. 1B). FA content of megagametophytes was initially 4.7% dry weight (0.03 mg). Total FAs of megagametophytes eventually surpassed 30% of dry weight (Fig. 1C). This is the same percentage as observed in mature seed (Table 1).

Table 2. Fatty acid (FA) composition ($\pm$ SE) of mature seed of loblolly pine (family 1). Each mean is based on five samples of 10 units.

Seed part	FA distribution (% of total)							
	16:0	18:0	18:1	18:2	18:3 $\Delta 5,9,12$	18:3 $\Delta 9,12,15$	20:0	20:3
Seedcoat	15.3 $\pm$ 1.2	17.7 $\pm$ 1.8	14.2 $\pm$ 0.7	34.7 $\pm$ 2.2	13.8 $\pm$ 1.2	3.5 $\pm$ 1.4	0.4 $\pm$ 0.4	0.0 $\pm$ 0.0
Megagametophyte	4.1 $\pm$ 0.0	2.6 $\pm$ 0.1	17.0 $\pm$ 0.3	50.3 $\pm$ 0.3	20.9 $\pm$ 0.2	0.4 $\pm$ 0.0	0.5 $\pm$ 0.0	3.2 $\pm$ 0.0
Embryo	8.9 $\pm$ 1.2	5.7 $\pm$ 0.9	22.3 $\pm$ 2.0	41.8 $\pm$ 3.3	14.9 $\pm$ 2.3	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	3.2 $\pm$ 0.6
Cotyledon	7.5 $\pm$ 0.1	4.3 $\pm$ 0.2	26.3 $\pm$ 0.3	45.3 $\pm$ 0.2	12.3 $\pm$ 0.0	0.5 $\pm$ 0.1	0.5 $\pm$ 0.0	2.0 $\pm$ 0.0
Hypocotyl	10.3 $\pm$ 2.4	7.5 $\pm$ 1.5	18.2 $\pm$ 3.2	38.4 $\pm$ 6.6	17.4 $\pm$ 4.5	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2	4.3 $\pm$ 1.1
Total seed	5.0 $\pm$ 0.2	3.3 $\pm$ 0.1	18.2 $\pm$ 0.8	49.0 $\pm$ 2.3	19.4 $\pm$ 0.7	0.4 $\pm$ 0.0	0.6 $\pm$ 0.0	3.1 $\pm$ 0.1

Table 3. Fatty acid (FA) composition ($\pm$ SE) of developing embryos and megagametophytes of loblolly pine (family 2). Each mean is based on three samples of 10 units, except embryos at day 28, 35, and 42, which consist of three samples of 30.

Days from 25 July	FA distribution (% of total)							
	16:0	18:0	18:1	18:2	18:3 $\Delta 5,9,12$	18:3 $\Delta 9,12,15$	20:0	20:3
<i>Embryo</i>								
28	14.9 $\pm$ 0.4	11.0 $\pm$ 2.2	17.7 $\pm$ 1.5	43.0 $\pm$ 0.6	9.4 $\pm$ 0.8	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	3.9 $\pm$ 0.6
35	14.4 $\pm$ 0.8	7.0 $\pm$ 1.3	21.0 $\pm$ 0.7	44.6 $\pm$ 0.9	7.9 $\pm$ 0.5	1.2 $\pm$ 0.2	0.3 $\pm$ 0.1	3.8 $\pm$ 0.2
42	10.0 $\pm$ 0.3	3.5 $\pm$ 0.0	25.3 $\pm$ 0.1	47.1 $\pm$ 0.1	10.5 $\pm$ 0.3	0.3 $\pm$ 0.1	0.0 $\pm$ 0.0	3.2 $\pm$ 0.2
49	8.0 $\pm$ 0.0	3.4 $\pm$ 0.1	26.5 $\pm$ 0.1	46.5 $\pm$ 0.1	11.8 $\pm$ 0.1	0.6 $\pm$ 0.1	0.2 $\pm$ 0.0	3.1 $\pm$ 0.0
56	7.6 $\pm$ 0.2	3.5 $\pm$ 0.0	26.6 $\pm$ 0.2	46.5 $\pm$ 0.3	11.9 $\pm$ 0.2	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1	3.0 $\pm$ 0.0
63	7.5 $\pm$ 0.2	3.7 $\pm$ 0.1	26.2 $\pm$ 0.1	46.3 $\pm$ 0.0	12.4 $\pm$ 0.1	0.7 $\pm$ 0.0	0.4 $\pm$ 0.0	2.9 $\pm$ 0.1
70	7.3 $\pm$ 0.1	3.1 $\pm$ 0.0	26.8 $\pm$ 0.3	47.2 $\pm$ 0.4	12.0 $\pm$ 0.0	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	2.8 $\pm$ 0.0
77	7.2 $\pm$ 0.1	3.1 $\pm$ 0.1	26.4 $\pm$ 0.1	47.4 $\pm$ 0.2	12.1 $\pm$ 0.1	0.4 $\pm$ 0.0	0.2 $\pm$ 0.0	3.0 $\pm$ 0.0
84	7.3 $\pm$ 0.1	3.0 $\pm$ 0.0	26.6 $\pm$ 0.3	47.4 $\pm$ 0.1	12.0 $\pm$ 0.1	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	2.9 $\pm$ 0.0
91	7.8 $\pm$ 0.1	3.1 $\pm$ 0.1	25.4 $\pm$ 0.3	47.7 $\pm$ 0.4	12.1 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.0	3.1 $\pm$ 0.0
98	7.2 $\pm$ 0.1	3.1 $\pm$ 0.0	26.6 $\pm$ 0.3	47.1 $\pm$ 0.2	12.1 $\pm$ 0.1	0.6 $\pm$ 0.1	0.3 $\pm$ 0.0	3.0 $\pm$ 0.1
105	7.0 $\pm$ 0.1	2.9 $\pm$ 0.1	26.3 $\pm$ 0.6	47.3 $\pm$ 0.4	12.6 $\pm$ 0.3	0.5 $\pm$ 0.0	0.4 $\pm$ 0.0	2.9 $\pm$ 0.1
<i>Megagametophyte</i>								
0	21.9 $\pm$ 2.7	19.0 $\pm$ 3.6	17.4 $\pm$ 0.3	35.4 $\pm$ 3.5	6.2 $\pm$ 3.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
7	17.0 $\pm$ 1.0	12.1 $\pm$ 1.2	19.5 $\pm$ 0.4	36.9 $\pm$ 0.9	14.5 $\pm$ 0.9	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
14	11.2 $\pm$ 0.5	5.9 $\pm$ 0.7	15.1 $\pm$ 0.2	46.6 $\pm$ 0.9	18.0 $\pm$ 0.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	3.3 $\pm$ 0.5
21	8.4 $\pm$ 0.1	2.7 $\pm$ 0.1	14.8 $\pm$ 0.2	49.4 $\pm$ 0.1	20.1 $\pm$ 0.1	0.6 $\pm$ 0.0	0.0 $\pm$ 0.0	4.1 $\pm$ 0.2
28	7.1 $\pm$ 0.1	2.0 $\pm$ 0.0	16.6 $\pm$ 0.3	50.2 $\pm$ 0.1	19.7 $\pm$ 0.2	0.5 $\pm$ 0.0	0.0 $\pm$ 0.0	4.0 $\pm$ 0.1
35	6.1 $\pm$ 0.2	1.9 $\pm$ 0.1	15.6 $\pm$ 0.7	50.9 $\pm$ 0.4	20.9 $\pm$ 0.4	0.4 $\pm$ 0.1	0.3 $\pm$ 0.0	3.9 $\pm$ 0.0
42	5.0 $\pm$ 0.0	1.9 $\pm$ 0.0	15.9 $\pm$ 0.1	52.2 $\pm$ 0.1	20.4 $\pm$ 0.2	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	4.0 $\pm$ 0.0
49	3.4 $\pm$ 1.2	1.9 $\pm$ 0.1	16.0 $\pm$ 0.2	53.4 $\pm$ 0.7	20.6 $\pm$ 0.2	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	4.1 $\pm$ 0.0
56	4.9 $\pm$ 0.1	1.7 $\pm$ 0.0	16.8 $\pm$ 0.1	52.7 $\pm$ 0.2	19.5 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	3.9 $\pm$ 0.1
63	5.0 $\pm$ 0.1	1.8 $\pm$ 0.1	17.1 $\pm$ 0.2	52.1 $\pm$ 0.2	19.5 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	3.9 $\pm$ 0.1
70	4.9 $\pm$ 0.1	1.6 $\pm$ 0.0	17.8 $\pm$ 0.2	52.6 $\pm$ 0.1	19.0 $\pm$ 0.2	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	3.5 $\pm$ 0.0
77	4.7 $\pm$ 0.1	1.6 $\pm$ 0.1	17.7 $\pm$ 0.3	53.0 $\pm$ 0.2	18.8 $\pm$ 0.2	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	3.7 $\pm$ 0.1
84	4.8 $\pm$ 0.1	1.6 $\pm$ 0.0	18.5 $\pm$ 0.3	52.4 $\pm$ 0.3	18.4 $\pm$ 0.4	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1	3.8 $\pm$ 0.1
91	4.9 $\pm$ 0.1	1.6 $\pm$ 0.0	17.8 $\pm$ 0.3	53.4 $\pm$ 0.3	18.0 $\pm$ 0.1	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	3.7 $\pm$ 0.1
98	4.2 $\pm$ 0.0	1.7 $\pm$ 0.0	16.5 $\pm$ 0.2	52.5 $\pm$ 0.2	20.4 $\pm$ 0.1	0.3 $\pm$ 0.0	0.6 $\pm$ 0.0	3.8 $\pm$ 0.0
105	4.4 $\pm$ 0.1	1.7 $\pm$ 0.0	17.2 $\pm$ 0.2	52.9 $\pm$ 0.1	19.6 $\pm$ 0.1	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	3.7 $\pm$ 0.1

The greatest changes in FA composition of the megagametophyte occurred during the first 3 weeks following 25 July in both families (Tables 3 and 4). Percent 16:0 and 18:0 of total FAs decreased, while all other FAs increased as a percentage of total FAs. The predominant FA was 18:2, followed by 18:3 $\Delta 5,9,12$ and 18:1.

Embryos were large enough at day 28 to analyze, when they averaged 0.05 mg FAs, 30.8% dry weight. Substantial changes in embryo FA distribution occurred between 22 Aug. (day 28) and 12 Sept. (day 49), at which time embryo FAs had surpassed 50% of dry weight with little change thereafter (Fig. 1C). Percent total FAs of 16:0, 18:0, and 20:3 decreased; 18:1; 18:2; 18:3 $\Delta 5,9,12$; and 20:0 increased as a percentage of total FAs. Although accumulation of 18:3 $\Delta 9,12,15$ was inconsistent, final concentration was the same in both families. The largest

portion of total FAs was 18:2, followed by 18:1 and 18:3 $\Delta 5,9,12$. This difference suggests that FA composition may be a significant marker for early development. Although mature embryos of loblolly pine are more than 50% FA, as compared to megagametophytes (36% FA), it is the megagametophyte that contains the greatest absolute amount of FAs in mature seed (79.8%) and is the major nutritive source for the developing embryo in vivo (Carpita et al., 1983).

Density and length changes in developing seed. Seeds from both families behaved similarly in regard to density and length (Fig. 2), but only density data for family 2 are discussed.

Relative density of intact seeds increased from <0% sucrose (0.9977 g·ml⁻¹) to 56% sucrose (1.2611 g·ml⁻¹) and then declined to as low as 27% sucrose (1.1108 g·ml⁻¹). Maximum density was achieved by day 70 and then stabilized (Fig. 2A).

Table 4. Fatty acid (FA) composition (+ SE) of developing embryos and megagametophytes of loblolly pine (family 3). Each mean is based on three samples of 10 units, except embryos at day 28, 35, and 42, which consist of three samples of 30.

Days from 25 July	FA distribution (% of total)							
	16:0	18:0	18:1	18:2	18:3 $\Delta 5,9,12$	18:3 $\Delta 9,12,15$	20:0	20:3
<i>Embryo</i>								
28	11.6 $\pm$ 0.2	3.7 $\pm$ 0.2	23.1 $\pm$ 0.1	46.9 $\pm$ 0.2	10.1 $\pm$ 0.1	1.0 $\pm$ 0.0	0.2 $\pm$ 0.0	3.3 $\pm$ 0.1
35	10.3 $\pm$ 0.5	3.5 $\pm$ 0.3	23.3 $\pm$ 0.2	47.4 $\pm$ 0.4	11.1 $\pm$ 0.4	0.6 $\pm$ 0.1	0.4 $\pm$ 0.0	3.3 $\pm$ 0.1
42	8.2 $\pm$ 0.1	3.0 $\pm$ 0.1	26.1 $\pm$ 0.2	47.2 $\pm$ 0.1	11.9 $\pm$ 0.1	0.5 $\pm$ 0.0	0.3 $\pm$ 0.0	2.7 $\pm$ 0.0
49	7.4 $\pm$ 0.1	3.2 $\pm$ 0.1	25.9 $\pm$ 0.3	46.8 $\pm$ 0.2	12.5 $\pm$ 0.1	0.8 $\pm$ 0.1	0.3 $\pm$ 0.0	3.2 $\pm$ 0.0
56	7.1 $\pm$ 0.2	3.3 $\pm$ 0.1	27.3 $\pm$ 0.6	46.2 $\pm$ 0.7	12.0 $\pm$ 0.1	0.7 $\pm$ 0.2	0.4 $\pm$ 0.0	3.0 $\pm$ 0.0
63	6.8 $\pm$ 0.0	3.1 $\pm$ 0.1	27.9 $\pm$ 0.5	46.3 $\pm$ 0.1	12.3 $\pm$ 0.2	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	3.0 $\pm$ 0.1
70	6.8 $\pm$ 0.2	3.0 $\pm$ 0.1	28.2 $\pm$ 0.2	45.8 $\pm$ 0.1	12.4 $\pm$ 0.1	0.5 $\pm$ 0.0	0.2 $\pm$ 0.0	3.0 $\pm$ 0.0
77	6.8 $\pm$ 0.2	3.0 $\pm$ 0.1	26.8 $\pm$ 1.3	47.4 $\pm$ 0.9	12.3 $\pm$ 0.1	0.4 $\pm$ 0.0	0.2 $\pm$ 0.0	3.0 $\pm$ 0.0
84	6.5 $\pm$ 0.1	2.1 $\pm$ 0.6	26.9 $\pm$ 0.4	47.5 $\pm$ 0.3	13.1 $\pm$ 0.2	0.4 $\pm$ 0.0	0.2 $\pm$ 0.0	3.3 $\pm$ 0.0
91	6.7 $\pm$ 0.2	3.3 $\pm$ 0.1	27.6 $\pm$ 0.1	46.0 $\pm$ 0.1	12.3 $\pm$ 0.2	0.5 $\pm$ 0.1	0.4 $\pm$ 0.0	3.3 $\pm$ 0.1
98	6.9 $\pm$ 0.1	3.1 $\pm$ 0.1	27.2 $\pm$ 0.3	46.2 $\pm$ 0.3	12.5 $\pm$ 0.2	0.5 $\pm$ 0.0	0.4 $\pm$ 0.0	3.1 $\pm$ 0.1
105	6.7 $\pm$ 0.2	3.0 $\pm$ 0.1	27.8 $\pm$ 0.1	46.5 $\pm$ 0.3	12.6 $\pm$ 0.2	0.5 $\pm$ 0.0	0.4 $\pm$ 0.0	2.7 $\pm$ 0.3
<i>Megagametophyte</i>								
0	21.1 $\pm$ 0.9	18.8 $\pm$ 1.9	16.4 $\pm$ 1.6	33.4 $\pm$ 1.5	10.2 $\pm$ 0.8	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
7	14.5 $\pm$ 0.8	12.5 $\pm$ 2.4	19.3 $\pm$ 0.5	36.0 $\pm$ 0.6	14.0 $\pm$ 1.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	3.7 $\pm$ 1.1
14	8.6 $\pm$ 0.2	3.4 $\pm$ 0.4	17.3 $\pm$ 0.2	46.5 $\pm$ 0.3	19.2 $\pm$ 0.1	0.5 $\pm$ 0.2	0.0 $\pm$ 0.0	4.5 $\pm$ 0.1
21	6.5 $\pm$ 0.1	2.2 $\pm$ 0.2	16.4 $\pm$ 0.3	48.9 $\pm$ 0.1	21.2 $\pm$ 0.1	0.5 $\pm$ 0.1	0.1 $\pm$ 0.1	4.1 $\pm$ 0.1
28	5.1 $\pm$ 0.1	2.5 $\pm$ 0.2	19.7 $\pm$ 0.0	47.3 $\pm$ 0.2	20.5 $\pm$ 0.3	0.5 $\pm$ 0.1	0.3 $\pm$ 0.0	4.1 $\pm$ 0.1
35	4.6 $\pm$ 0.1	2.1 $\pm$ 0.1	18.1 $\pm$ 0.3	49.6 $\pm$ 0.4	21.0 $\pm$ 0.2	0.5 $\pm$ 0.1	0.3 $\pm$ 0.0	3.8 $\pm$ 0.2
42	4.4 $\pm$ 0.1	2.0 $\pm$ 0.0	18.9 $\pm$ 0.2	50.0 $\pm$ 0.5	20.9 $\pm$ 0.2	0.4 $\pm$ 0.0	0.4 $\pm$ 0.1	3.0 $\pm$ 1.0
49	4.1 $\pm$ 0.0	1.9 $\pm$ 0.0	18.8 $\pm$ 0.2	50.0 $\pm$ 0.3	20.5 $\pm$ 0.2	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	4.0 $\pm$ 0.1
56	4.0 $\pm$ 0.0	2.2 $\pm$ 0.0	19.2 $\pm$ 0.3	49.8 $\pm$ 0.2	20.2 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	4.0 $\pm$ 0.1
63	3.9 $\pm$ 0.0	2.0 $\pm$ 0.1	20.0 $\pm$ 0.3	49.6 $\pm$ 0.2	19.7 $\pm$ 0.3	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	4.2 $\pm$ 0.1
70	3.9 $\pm$ 0.0	2.1 $\pm$ 0.0	20.9 $\pm$ 0.1	49.1 $\pm$ 0.2	19.5 $\pm$ 0.1	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	3.9 $\pm$ 0.1
77	3.9 $\pm$ 0.1	2.1 $\pm$ 0.1	20.8 $\pm$ 0.2	49.5 $\pm$ 0.0	19.0 $\pm$ 0.0	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	4.2 $\pm$ 0.1
84	3.8 $\pm$ 0.0	1.9 $\pm$ 0.0	20.8 $\pm$ 0.1	49.9 $\pm$ 0.2	18.9 $\pm$ 0.3	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	4.1 $\pm$ 0.1
91	3.7 $\pm$ 0.1	1.8 $\pm$ 0.1	19.3 $\pm$ 1.2	50.3 $\pm$ 0.8	19.9 $\pm$ 0.4	0.3 $\pm$ 0.0	0.5 $\pm$ 0.1	4.2 $\pm$ 0.1
98	3.7 $\pm$ 0.0	1.6 $\pm$ 0.0	20.9 $\pm$ 0.2	49.4 $\pm$ 0.1	19.7 $\pm$ 0.2	0.4 $\pm$ 0.0	0.2 $\pm$ 0.0	4.1 $\pm$ 0.1
105	3.8 $\pm$ 0.0	1.6 $\pm$ 0.0	20.5 $\pm$ 0.4	49.8 $\pm$ 0.3	19.9 $\pm$ 0.1	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	3.8 $\pm$ 0.2

The megagametophyte + embryo density increased from 15% sucrose (1.0574 g·ml⁻¹) at the first sampling to 44% sucrose (1.1953 g·ml⁻¹) at the seventh sampling and then remained constant (Fig. 2 B and C). The same trend was observed with the megagametophyte alone, but density had increased from $\approx$ 30% to 60% sucrose (1.1250 to 1.2842 g·ml⁻¹) by day 35. The density difference between megagametophyte + embryo and megagametophyte alone may be an artifact. To obtain density of the megagametophyte, the megagametophyte + embryo was dissected with a corresponding loss of liquid endosperm surrounding the embryo, which may have affected the results. This situation is clearly seen at day 0, where there is a marked difference in density between megagametophyte + embryo vs. megagametophyte alone (Fig. 2 B and C). Until day 21, embryos were too small to measure. Thus, the only difference between megagametophyte + embryo and megagametophyte was that megagametophyte density was determined from split halves. Embryo density declined during development, with a maximum at the first sampling (day 28) of 51% sucrose (1.2331 g·ml⁻¹) then remained relatively constant (10% sucrose or 1.0366 g·ml⁻¹) after day 49 (Fig. 2D). The decrease in embryo density was inversely related to embryo length and percent total FA (Figs. 1C and 2D). This relationship suggests that the decrease in embryo density is related to FA accumulation.

Intact seed had a constant length of 6 mm (Fig. 2A). Length of megagametophyte + embryo increased from 3 to 4 mm (Fig. 2B). Embryo length increased markedly for 3 weeks (day 28 to 49) and then stabilized at 4 mm (Fig. 2D).

In vitro germination of developing zygotic embryos. The two families behaved similarly in respect to in vitro embryo growth and germination (Fig. 3 A and B). Growth of zygotic embryos was negligible in the first six sampling periods (day 16 to 51) (Fig. 3A), but increased markedly from the seventh to the 10th sampling period (day 58 to 79). Similar results were observed for zygotic embryo germination (Fig. 3B). In vitro germination was nil until day 58 but increased from day 58 to 79. At the last sampling date, the average increase in length during 4 weeks in culture was 12.9 mm with 70% germination.

In summary, by day 49, embryo size, FA content, and density were close to that achieved in mature seed. Germination of excised embryos, however, did not occur until day 59 but increased rapidly thereafter. These results suggest that embryo maturity is not achieved until it is 4 mm long, FA content reaches 50%, and embryo density declines to the equivalent of 30% sucrose. Decreasing embryo density is a characteristic feature of embryo maturation, suggesting that density separation could be an effective technique for isolation of mature somatic embryos.

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Fig. 2. Relative density, density and length of seed (A), megagametophyte + embryo (B), megagametophyte (C), and embryo (D) of two families of loblolly pine during development.

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Fig. 3. Embryo length increase (A) and in vitro germination based on elongation of hypocotyl and radical plus cotyledon development (B) of excised zygotic embryos of two families of loblolly pine during development.

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