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STRUCTURAL AND BIOCHEMICAL CHANGES IN LOBLOLLY PINE (*PINUS TAEDA* L.) SEEDS DURING GERMINATION AND EARLY SEEDLING GROWTH. II. STORAGE TRIACYLGLYCEROLS AND CARBOHYDRATES

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Triacylglycerols (TAGs) comprised 59% of the total storage reserve in mature loblolly pine (*Pinus taeda* L.) seeds; 80% of these TAGs were stored in the megagametophyte. The TAG breakdown in the seedling was initiated before radicle emergence (during germination), while in the megagametophyte breakdown occurred after radicle emergence (during early seedling growth). In both seed tissues, the majority of TAG breakdown took place during early seedling growth. Within the seedling, the most rapid rate of TAG breakdown occurred in the radicle and hypocotyl. Unlike TAGs, there was very little carbohydrate stored in loblolly pine seeds at maturity. Levels of 80% ethanol-soluble carbohydrate in the megagametophyte and seedling decreased during germination and then increased during early seedling growth. This increase coincided with the period of rapid TAG depletion. Accompanying the increase in 80% ethanol-soluble carbohydrate level was a corresponding increase in 80% ethanol-insoluble carbohydrates, such as starch, in the seedling during early seedling growth. Accumulation of starch occurred in both the cotyledons and hypocotyl. Starch accumulation in the megagametophyte was more transient, occurring around germination. The megagametophyte was important for the growth and nutrition of the seedling. In the presence of the megagametophyte, the seedling accumulated sucrose during early seedling growth. However, in the absence of the megagametophyte, loblolly pine seedlings failed to accumulate carbohydrates to any great extent.

Keywords: loblolly pine, seed, megagametophyte, germination, early seedling growth, triacylglycerol, sucrose, D-glucose, D-fructose, starch, development.

Introduction

The primary storage reserves in mature, desiccated loblolly pine (*Pinus taeda* L.) seeds are triacylglycerols (TAGs) that are packaged into lipid bodies during Pinaceae seed development (Krasowski and Owens 1993; Owens et al. 1993). The majority of the TAGs are located in the large megagametophyte (Janick et al. 1991) enclosing the embryo (Stone and Gifford 1997). Loblolly pine megagametophytes also store a large amount of buffer-insoluble proteins (Groome et al. 1991; Stone and Gifford 1997) that are broken down to amino acids, which are then transported to the seedling during germination and early seedling growth (King and Gifford 1997). Similarly, megagametophytic TAG reserves are also thought to be broken down and converted into appropriate forms for export to the seedling. It has been hypothesized that during germination and early seedling growth, free fatty acids are released from megagametophyte TAGs by lipases (Ching 1968; Kovac and Wrischer 1984; Hammer and Murphy 1994). Released fatty acids are catabolized by the β-oxidation pathway and the glyoxylate cycle to generate succinate (Firenzuoli et al. 1968; Ching 1970; Lopez-Perez et al. 1974; Noland and Murphy 1984; Pinzauti et al. 1986; Mullen and Gifford 1995*a*, 1995*b*, 1997). The succinate, as well as glycerol released by the lipases, is then converted via gluconeogenesis into carbohydrates that are exported to the seedling, where they are utilized as carbon and energy sources for growth (Ching 1966). In our study, we have further tested the hypothesis that loblolly pine megagametophyte TAGs are broken down and converted into carbohydrates that are exported to the seedling during germination and early seedling growth.

Previous studies (Ching 1966; Kao 1973; Groome et al. 1991) have documented the changes in total lipids during germination and early seedling growth. However, an accurate picture of TAG reserve mobilization is difficult to demonstrate using these data, especially in the seedling, where membrane synthesis is occurring. Therefore, we have specifically investigated the mobilization of TAG reserves in both the megagametophyte and the seedling and have further correlated these observations with total carbohydrate levels in these tissues. Because we are interested in the carbohydrates that are transported from the megagametophyte to the seedling, we documented changes in the levels of sucrose, D-glucose, and D-fructose. These carbohydrates have been examined to a limited extent in pinyon pine seedlings (*Pinus edulis* Engelm.) (Murphy et al. 1992) and whole seeds of Japanese black pine (*Pinus*

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thunbergii Parlatore) (Hattori and Shiroya 1951) and Scots pine (*Pinus silvestris* L.) (Nyman 1969).

Material and Methods

Seed Material and Germination

Half-sibling loblolly pine seeds were a gift from Westvaco (Summerville, S.C.; Clone 11-9, open-pollinated and collected in the fall of 1992). All seeds were surface sterilized prior to stratification according to Groome et al. (1991). Seeds stratified in complete darkness for 35 d at 2°C (DAI_2 [days after imbibition at 2°C]) were transferred to freshly autoclaved Kimpack-lined germination trays. The trays were then placed in a germinator with continuous fluorescent light (19 μmol m^{-2} s^{-1}) at 30°C (DAI_{30} [days after imbibition at 30°C]) to germinate. After germination was completed by radicle emergence from the seed coat at 4 DAI_{30}, the trays were maintained in the same germinator for an additional 8 d. Early seedling growth was defined as the period after radicle emergence and before the seedling shed the megagametophyte. Seeds were staged as described in Mullen et al. (1996). "Embryo" was the term used to describe the sporophyte at maturity and during the period of stratification at 2°C. After the seeds were placed at 30°C, the sporophyte was termed a seedling. Megagametophytes, whole embryos/seedlings, sporophytic hypocotyls and radicles (root poles), and sporophytic cotyledons and epicotyls (shoot poles) were quick-frozen with liquid N_2 and stored at −75°C before biochemical analysis.

Isolated Embryos

The 35 DAI_2 embryos with and without their megagametophytes were cultured, under the conditions described in King and Gifford (1997). Plant material was quick-frozen after a specific number of days in culture and was stored at −75°C until the 80% ethanol-soluble and -insoluble carbohydrate analysis was completed.

Microscopy

Small pieces of tissue were fixed for 2 h at room temperature with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2, 0.5 M sucrose, and then postfixed with 2% OsO_4 in 0.1 M sodium cacodylate buffer pH 7.2, 0.25 M sucrose, for 1.5–4 h at room temperature for younger tissues or 4°C for older tissues. After dehydration in an ethanol series and infiltration by propylene oxide, tissues were embedded in Spurr's resin (Spurr 1969) and were ultrathin-sectioned to gold thickness. Sections were stained with 4% aqueous uranyl acetate for 1 h followed by lead citrate (Reynolds 1963) for 2 min and were examined with a Philips 201 transmission electron microscope at 60 kV. For water-insoluble carbohydrate investigations, thin transverse slices through seeds were fixed by freeze substitution (Stone and Gifford 1997), embedded in Spurr's resin (Spurr 1969), sectioned at 1.5 mm, stained with periodic acid–Schiff's reaction (Jensen 1962), and examined with an Olympus BX40 photomicroscope.

Triacylglycerol Analysis

Triacylglycerols were extracted using a modification of the method of Feirer et al. (1989). The 20–200-mg FW (fresh weight) of tissue were homogenized with 1.25 mL isopropanol (anhydrous, Fisher HPLC grade). Samples were gently mixed for 15 min and microfuged at 20,000 *g* for 15 min, and the crude supernatant was collected. The pellet was reextracted once with 1.25 mL isopropanol, and the crude supernatants were pooled. The 800 mL of the pooled crude supernatant were added to test tubes containing 0.8 g alumina (Activity Grade I, type WN-3, Sigma) and 1.8 mL isopropanol. Tubes were capped, gently mixed for 15 min, and spun in a bench-top centrifuge at 1240 *g* for 20 min, and the purified supernatant was collected. The alumina pellet was washed twice with 2.6 mL isopropanol, and the purified supernatants were pooled. The TAG quantification was determined by the method of Feirer et al. (1989), using triolein (Sigma) as a standard.

Carbohydrate Analysis

The 80% ethanol-soluble and -insoluble carbohydrates were extracted using a modification of the method of Joy et al. (1991). The 40–200 mg FW of tissue were homogenized with 1 mL 80% (v/v) ethanol, gently mixed for 1 h, and microfuged at 20,000 *g* for 15 min at room temperature, and the supernatant was collected. The pellet was reextracted twice with 1 mL 80% ethanol and 15-min incubations. Pooled supernatants contained the 80% ethanol-soluble carbohydrates. The 80% ethanol-insoluble carbohydrates were extracted by suspending the above pellet in 1 mL cold Milli Q H_2O (Millipore), adding 1.5 mL 52% perchloric acid, and incubating on ice for 20 min. After the incubation, 2.5 mL cold Milli Q H_2O were added before centrifuging at 17,400 *g* for 30 min at 4°C. Supernatants were collected and filtered through Whatman #1. Pellets were reextracted once by the same procedure, and the supernatants were pooled. Soluble and insoluble carbohydrates were determined using the method of Joy et al. (1991) and anhydrous D-glucose (BDH) as a standard.

Sucrose, D-glucose, and D-fructose were extracted from megagametophytes and seedlings. The 20–350 mg FW of tissue were homogenized with 20 mg polyvinylpolypyrrolidone (PVPP) and 1 mL 100 mM HEPES (Sigma), pH 7.5, 3 mM magnesium acetate (Sigma), and centrifuged at 20,000 *g* for 20 min at 4°C, and the crude supernatant was collected. To 0.6 mL of pooled crude supernatant, 75 μL Carrez I solution (3.60% [w/v] $K_4[Fe(CN)_6] \times 3\ H_2O$), 75 μL Carrez II solution (7.20% [w/v] $ZnSO_4 \times 7\ H_2O$), and 150 μL 0.1 M NaOH were added, vortexing after each addition. The sample was microfuged at 20,000 *g* for 20 min at 4°C, and the deproteinized supernatant was collected. Sucrose, D-glucose, and D-fructose were quantified from the deproteinized supernatant enzymatically using a Boehringer Mannheim kit (716 260).

Starch was extracted from megagametophytes and embryos/seedlings by homogenizing 0.2–3 g FW of tissue with 0.1 g PVPP and 10 mL 80% ethanol. The homogenate was centrifuged at 31,000 *g* for 20 min at room temperature, the crude supernatant was discarded, and the pellet was reextracted twice with 10 mL 80% ethanol. The final pellet was allowed to air dry and then was transferred to a 25-mL Erlenmeyer flask with 5 mL dimethyl sulfoxide (Fisher) and 1.25 mL 8 M

HCl. The flask was covered with Parafilm M (American National Can, Chicago) and incubated in a 60°C shaking water bath for 30 min. After cooling to room temperature, 1.25 mL 8 M NaOH and 15 mL 112 mM citrate buffer pH 4.0 (citric acid/sodium citrate) were added, stirring vigorously after each addition. After rinsing the flask with 2 mL of the citrate buffer, the slurry was centrifuged at 31,000 *g* for 20 min at room temperature, and the supernatant was filtered through Whatman #1 and was made up to a final volume of 25 mL with the citrate buffer. Native starch was quantified from this supernatant enzymatically, using a Boehringer Mannheim kit (207 748), and expressed in terms of D-glucose units.

Results

Triacylglycerols (TAGs)

TAGs comprise 59% of the total storage reserve in mature loblolly pine seeds, based on storage protein (Stone and Gifford 1997), TAG, and 80% ethanol-soluble and -insoluble carbohydrates. Eighty percent of the TAGs were stored in the megagametophyte, constituting 27% of the mature megagametophyte on a dry weight basis. However, TAGs were also an important reserve in the embryo since they composed 40% of the mature embryo's dry weight. These TAGs were stored in lipid bodies in the megagametophyte (fig. 1*A*), cotyledon (fig. 1*B*), and hypocotyl (fig. 1*C*) cells. During germination and early seedling growth, TAG reserves in both the megagametophyte and seedling were depleted (fig. 2*A*). Although TAG depletion was measurable at 5 DAI_{30} in the megagametophyte, the majority of TAG breakdown occurred following 6 DAI_{30}. The average TAG depletion rate between 7 and 11 DAI_{30} was 0.34 mg triolein equivalents/seed part/DAI. The TAG depletion in the seedling was evident following 3 DAI_{30} (fig. 2*B*). Although the amount of TAGs stored in the embryo's root and shoot pole was approximately equal on a per-seed-part basis in the mature seed, during imbibition at 30°C, TAG levels decreased more rapidly and to a lower level in the root pole than in the shoot pole (fig. 2*B*). The depletion of TAGs in the root pole was most linear between 4 and 8 DAI_{30}, with an average rate of 0.04 mg triolein equivalents/seed part/DAI. The shoot pole lost an average of 0.03 mg triolein equivalents/seed part/DAI between 6 and 10 DAI_{30}. By 12 DAI_{30}, seedling TAG levels were 47% that of the 12 DAI_{30} megagametophyte. Although 80% of the mature megagametophyte TAGs had been depleted at 12 DAI_{30}, it still contained 0.5 mg TAGs, which was approximately that stored in the mature embryo. Lipid bodies in the megagametophyte were still numerous (fig. 3*A*). In the seedling, by 12 DAI_{30}, 56% of the shoot pole and 80% of the root pole TAGs had been depleted, and lipid bodies were also reduced in number (fig. 3*B, C*).

Carbohydrates

Seventy-one percent of the 80% ethanol-soluble carbohydrate in mature seeds was stored in the megagametophyte (fig. 4*A*). During germination and into early seedling growth, megagametophyte soluble carbohydrates declined, reached minimum levels 6 DAI_{30}, and then increased until 10 DAI_{30}, when the level remained steady. The 80% ethanol-insoluble carbohydrate levels were threefold lower than soluble carbohydrates

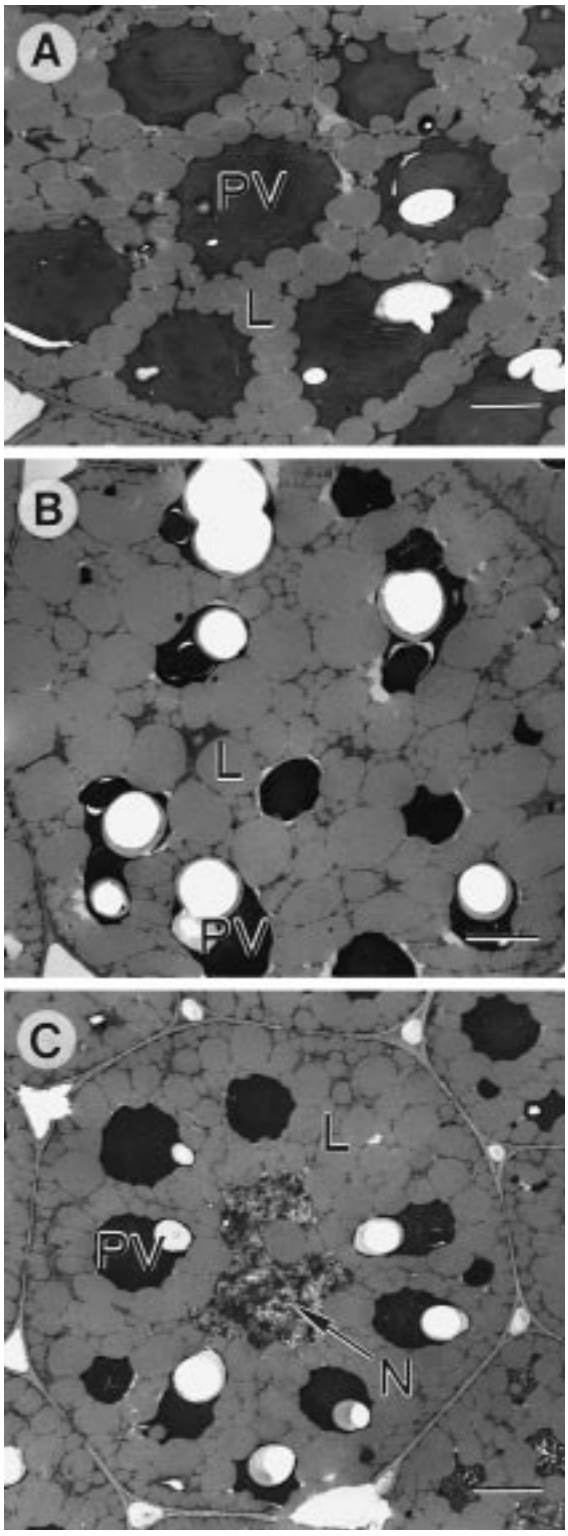

Fig. 1 35 DAI_2 seed material. Lipid body (*L*); protein vacuole (*PV*); nucleus (*N*). Bars = 3 μm. *A*, Megagametophyte cell. *B*, Cotyledon cell. *C*, Hypocotyl cell.

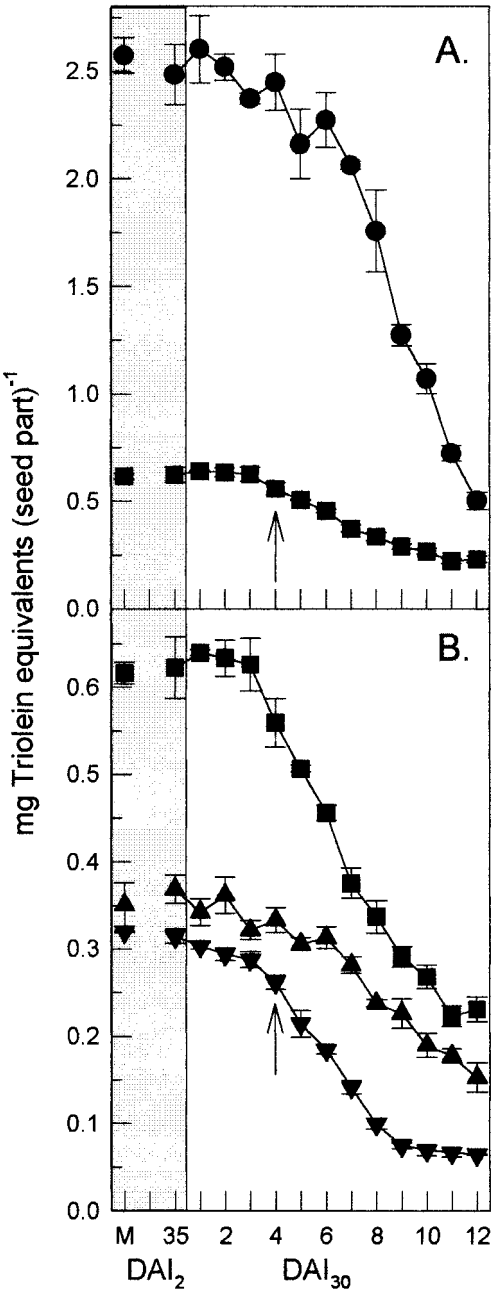

Fig. 2 Changes in TAGs during imbibition. *A*, Megagametophyte (circles) and seedling (squares). *B*, Whole seedling (squares), shoot pole (upward triangle), and root pole (downward triangles). Arrows indicate the completion of germination by radicle emergence from the seed coat. Each point was the mean of three separate determinations ±1 SE of the mean.

in the mature megagametophyte. The insoluble carbohydrate level increased slightly, reaching a maximum at 4 DAI$_{30}$, and then declined to levels similar to those of the mature megagametophyte.

Twenty-nine percent of the seed's 80% ethanol-soluble carbohydrates were stored in the embryo at maturity. The level of soluble carbohydrates remained constant until 5 DAI$_{30}$, after which the level increased in the seedling (fig. 4*B*). By 12 DAI$_{30}$, soluble carbohydrates in the seedling were sevenfold higher than the carbohydrate stored in the mature embryo. The 80% ethanol-insoluble carbohydrate levels behaved similarly to sol-

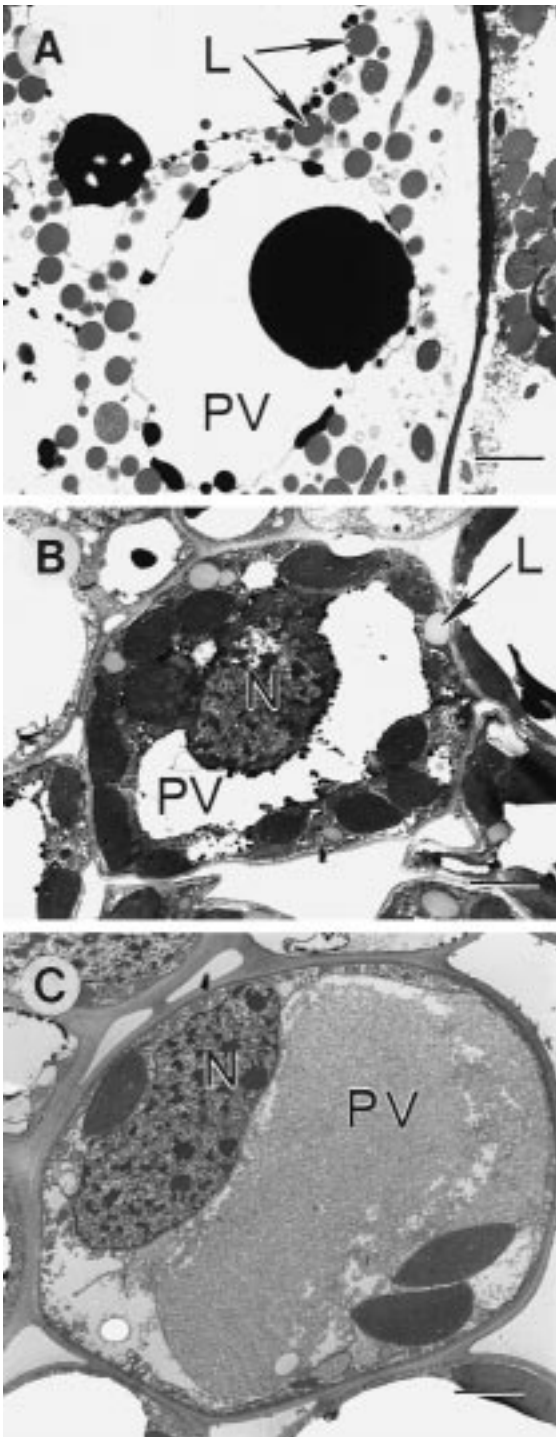

Fig. 3 12 DAI$_{30}$ seed material. Lipid body (*L*); protein vacuole (*PV*); nucleus (*N*). Bars = 3 μm. *A*, Megagametophyte cell. *B*, Cotyledon mesophyll cell. *C*, Hypocotyl cortical parenchymal cell.

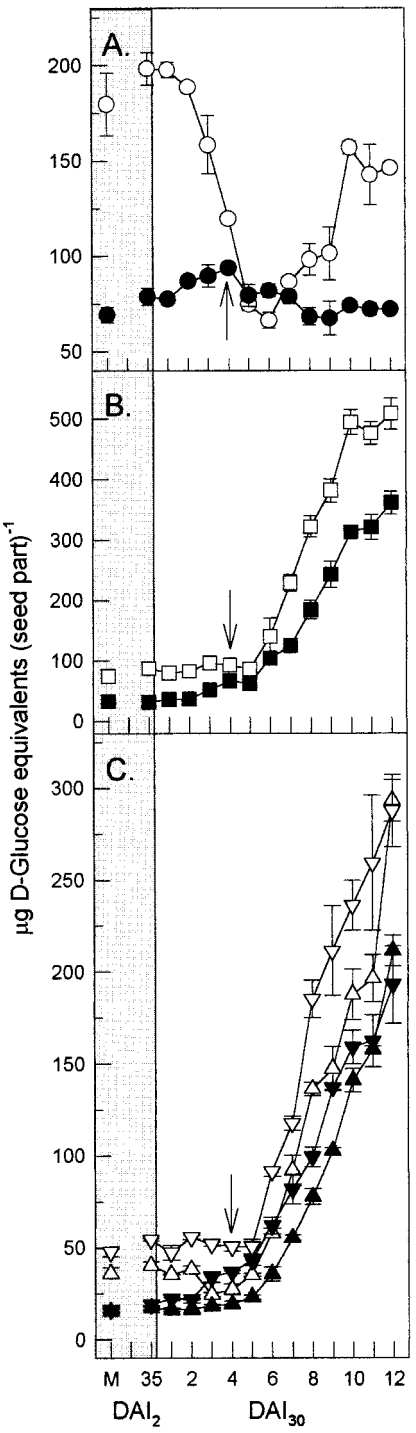

Fig. 4 Eighty percent ethanol-soluble and -insoluble carbohydrate levels during imbibition. *A*, Megagametophyte-soluble (open circles) and -insoluble (filled circles) carbohydrates. *B*, Embryo/seedling-soluble (open squares) and -insoluble (filled squares) carbohydrates. *C*, Shoot pole–soluble (open upward triangles), shoot pole–insoluble (filled upward triangles), and root pole–soluble (open downward triangles) and root pole–insoluble (filled downward triangles) carbohydrates. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each point was the mean of three separate determinations ±1 SE of the mean.

uble levels. There was very little increase in insoluble carbohydrates until 5 DAI_{30}, after which the level increased.

Within the mature embryo, the shoot and root poles contained 36 μg and 48 μg soluble carbohydrates per seed part, respectively (fig. 4*C*). Soluble carbohydrate levels in the root pole remained steady during germination and increased following 5 DAI_{30}. At 12 DAI_{30}, root pole–soluble carbohydrates were sixfold higher than at maturity. Unlike the root poles, shoot pole carbohydrate levels declined following 2 DAI_{30} and then increased. The majority of the increase occurred between 6 and 12 DAI_{30}. By 12 DAI_{30}, soluble carbohydrate levels were equal in the root and shoot poles. The 80% ethanol-insoluble carbohydrates were very low at maturity in both the root and shoot poles. Increase in these levels began following 2 DAI_{30} in the root pole and 5 DAI_{30} in the shoot pole. By 12 DAI_{30}, insoluble carbohydrate levels had increased 14-fold in the shoot pole and 12-fold in the root pole from mature levels.

In addition to changes in 80% ethanol-soluble and -insoluble carbohydrates, changes in sucrose, D-glucose, D-fructose, and starch were also measured. At maturity, the average megagametophyte and whole embryo contained 113 μg and 63 μg sucrose, respectively (fig. 5*A*). In the megagametophyte, sucrose levels increased from maturity until 35 DAI_2 and remained at that level until 3 DAI_{30}. Following 3 DAI_{30}, a decrease in the megagametophyte sucrose level occurred, remaining constant until 7 DAI_{30}. The sucrose level rose again to the level found in 35 DAI_2 megagametophytes and remained constant until 11 DAI_{30}. In the seedling, sucrose levels increased from 35 DAI_2 until 3 DAI_{30}. Following 5 DAI_{30}, sucrose levels rose rapidly to six times that of the mature embryo at 9 DAI_{30}. Unlike sucrose levels, D-glucose and D-fructose levels in both the mature megagametophyte and whole embryo were low. Although absolute amounts of these carbohydrates remained low until the end of early seedling growth, D-glucose and D-fructose levels increased relatively greatly after 5 DAI_{30} (fig. 5*B*, *C*).

Starch accumulated in the megagametophyte only transiently, peaking at 3 DAI_{30} (fig. 5*D*), while in the seedling rapid accumulation occurred following 9 DAI_{30}. By 11 DAI_{30}, the seedling contained 98% more starch than at maturity. The starch that accumulated in the seedling was stored in both the cotyledons and the hypocotyl. At 12 DAI_{30}, starch was visible in the mesophyll cells of the cotyledon (fig. 6*A*) and in the cortex cells that were just to the outside of the vascular ring of the hypocotyl (fig. 6*B*).

Both the megagametophyte and seedling, when cultured together, show 80% ethanol-soluble carbohydrate levels (fig. 7) similar to their *in vivo* grown counterparts (fig. 5). In the presence of its megagametophyte, the cultured seedling accumulated soluble carbohydrates between 3 d and 10 d in culture. However, without its megagametophyte, the level of soluble carbohydrate declined following 1 d in culture in the isolated seedling and then rose only slightly following 4 DAI_{30}. By 10 d in culture, the soluble carbohydrate level was 87% less in isolated seedlings, compared with seedlings cultured with their megagametophytes.

Discussion

There were both striking differences and similarities between the storage parenchymal cells in the embryo and the mega-

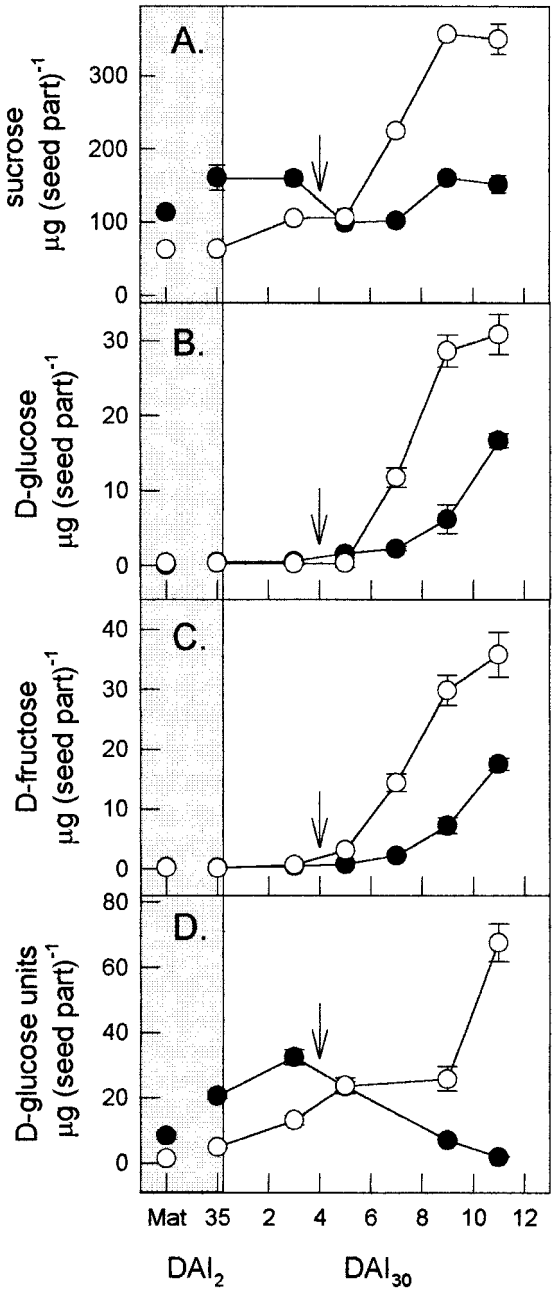

Fig. 5 Specific carbohydrate levels during imbibition. Megagametophyte (filled circles) and embryo/seedling (open circles). *A*, Sucrose. *B*, D-glucose. *C*, D-fructose. *D*, Starch. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each point was the mean of two separate determinations ±1 SD of the mean.

gametophyte of 35 DAI_2 loblolly pine seeds. Not only was the megagametophyte composed of cells that were much larger than those in the embryo, but it also contained 84% of the total seed reserves based on mature seed protein (Stone and Gifford 1997), TAGs, and general carbohydrate levels. It was evident from this study, as well as from our previous study (Stone and Gifford 1997), that the protein vacuoles were also larger in the megagametophyte than in the embryo. In spite of these differences, the lipid bodies were actually quite similar in their size range throughout all the tissues examined. Generally, mature loblolly pine seed cells were filled with a prominent nucleus surrounded by protein vacuoles and numerous small lipid bodies, which is typical of other Pinaceae seeds (Durzan et al. 1971; Simola 1974, 1976; Gori 1979; De Carli et al. 1987; Owens et al. 1993).

Breakdown of TAG Reserves

The majority of TAG reserve breakdown in loblolly pine occurred during early seedling growth. Fatty acids subsequently released from conifer megagametophytic TAGs via lipase activity (Ching 1968; Kovac and Wrischer 1984; Hammer and Murphy 1994) during this period are thought to be ox-

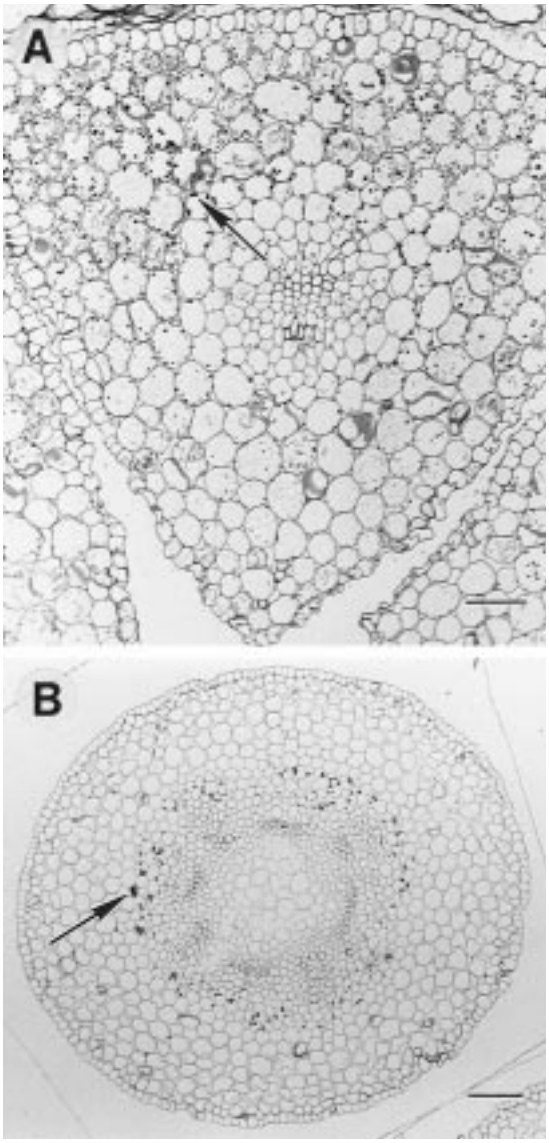

Fig. 6 12 DAI_{30} seedlings. Arrow indicates starch. *A*, Cotyledon; bar = 50 µm. *B*, Hypocotyl; bar = 100 µm.

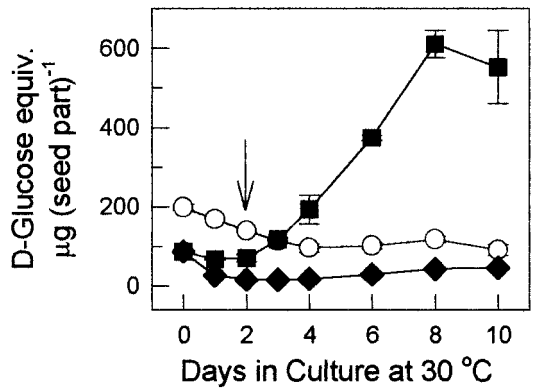

Fig. 7 Eighty percent ethanol-soluble carbohydrate levels in intact megagametophytes (circles), intact seedlings (squares), and isolated seedlings (diamonds) after being cultured at 30°C. Arrow indicates completion of germination by radicle emergence from the megagametophyte. Each point was the mean of three separate determinations ±1 SE of the mean.

idized by the β-oxidation pathway followed by the glyoxylate cycle (Firenzuoli et al. 1968; Ching 1970; Lopez-Perez et al. 1974; Noland and Murphy 1984; Pinzauti et al. 1986; Mullen and Gifford 1995*a*, 1995*b*, 1997). In relation to this, the activity of isocitrate lyase and malate synthase, two key enzymes of the glyoxylate cycle, increased in cell-free extracts during the later part of early seedling growth in loblolly pine megagametophytes (Mullen and Gifford 1995*b*, 1997). The timing of high activity of these glyoxylate cycle enzymes correlated well with decreasing TAG levels noted in this study.

Unlike their activity in the megagametophyte, isocitrate lyase and malate synthase activities were extremely low in loblolly pine seedlings (Mullen 1995; Mullen and Gifford 1995*b*), indicating that there may not be an active glyoxylate cycle. Low or undetectable glyoxylate cycle enzyme activities have also been reported in seedlings of Italian stone pine (*Pinus pinea*) (Firenzuoli et al. 1968), ponderosa pine (*Pinus ponderosa* Laws) (Ching 1970), silver fir (*Abies alba* Mill.) (Kovac and Wrischer 1984), and sugar pine (*Pinus lambertiana*) (Noland and Murphy 1984). Our study showed that embryonic TAG reserves were broken down. It is possible that loblolly pine seedlings do not possess an active glyoxylate cycle linked to gluconeogenesis. Instead, TAG breakdown may occur through the glyoxysomal β-oxidation pathway, releasing energy through the mitochondrial Krebs cycle. This chain of events is thought to occur in non-lipid-storing tissues. For example, glyoxysomes isolated from germinating barley embryos contained β-oxidation enzyme activity but no glyoxylate cycle enzyme activity (Holtman et al. 1994), while in the aleurone layer, which contained relatively more lipid, both β-oxidation pathway and glyoxylate cycle enzyme activities were detected. An alternative possibility is that loblolly pine seedlings do indeed contain a glyoxylate cycle, but one that operates at a low rate. The rate of TAG breakdown in the seedling was slower than in the megagametophyte. We are currently using loblolly pine megagametophytic isocitrate lyase and malate synthase antibodies (Mullen and Gifford 1995*a*, 1995*b*) to determine the presence of these key glyoxylate cycle enzymes in loblolly pine seedlings.

The activities and processes that occur in the seedling and megagametophyte of loblolly pine are highly regulated as the seed undergoes germination and early seedling growth. For example, we have previously demonstrated that starting during germination, storage protein reserves in the seedling were depleted from the seedling root pole at a faster rate than in the shoot pole (Stone and Gifford 1997). In our study, we have shown that TAG reserves were also broken down in the root pole faster than in the shoot pole and the onset of breakdown occurred during germination. Despite these similarities, the patterns of TAG and protein breakdown were different, since TAG reserves in the seedling were not broken down as completely as protein reserves. Thus, the breakdown of storage reserves in loblolly pine is regulated with respect to timing and tissue type, as well as type of storage reserve. The timing and tissue coordination of lipid body depletion was also noted in Scots pine (Simola 1974) and white spruce (*Picea abies*) (Simola 1976); in both species, lipid body depletion occurred in the radicles before the cotyledons. The rapid and early breakdown of protein and TAG reserves in the root poles of loblolly pine may have resulted from the large amount of energy and building materials the radicle required for the rapid growth and elongation that occurs during and after germination.

Although TAG reserves were broken down in the shoot pole of loblolly pine seedlings, the rate of breakdown was much slower, and the reserves were not depleted as extensively as in the root pole. Several factors may have contributed to this slow rate of breakdown. During early seedling growth, when the majority of TAG breakdown occurred in the megagametophyte, only the cotyledons of the seedling were in contact with the megagametophyte. The constant flow of carbohydrates, amino acids (King and Gifford 1997), and other metabolites from the megagametophyte into the seedling may inhibit key enzymes of TAG catabolism. For instance, the key β-oxidation pathway enzyme, acyl CoA oxidase, has been shown to be regulated by catabolite repression in yeast (Wang et al. 1992). It was also during the later stages of early seedling growth that a portion of the green cotyledons emerged from the megagametophyte and seed coat. It is possible that photosynthesis occurred during these later stages at low levels that provided additional carbohydrates and energy to the cotyledons and contributed further to slow TAG breakdown. The relatively large pool of TAGs remaining in the cotyledons at 12 DAI_{30} may be a basal level required for the synthesis of new membrane components by the cotyledons.

Transport of Carbohydrates to the Seedling

In this study, we hypothesized that loblolly pine megagametophyte TAGs were broken down and converted into carbohydrates that were exported to the seedling during germination and early seedling growth. Supporting evidence was the correlation of the timing of the TAG pool decrease in the megagametophyte and the increase in the seedling's 80% ethanol-soluble carbohydrate pool. A similar correlation between megagametophyte TAG losses and gains in seedling carbohydrates have also been observed in Douglas fir (*Pseudotsuga menziesii* Franco) (Ching 1966), Taiwan red pine (*Pinus tai-*

wanensis Hayata) (Kao 1973), and Chinese fir (*Cunninghamia lanceolata* [Lamb] Hook.) (Kao 1973). The decrease in loblolly pine megagametophytic TAGs was not accompanied by a large increase in megagametophyte 80% ethanol-soluble carbohydrates, which indicated that the carbohydrates produced were respired, incorporated into other products, or were exported to the seedling. Furthermore, loblolly pine embryos isolated and cultured *in vitro* did not accumulate carbohydrates to the same extent as embryos cultured with megagametophytes. Ponderosa pine megagametophytes were capable of exporting carbohydrates when isolated from their seedlings (Ching 1970). A similar observation has been made for amino acid export to the seedling of loblolly pine (King and Gifford 1997). These studies demonstrated that the relationship between the megagametophyte and seedling of loblolly pine is dynamic with respect to the transport of amino acids (King and Gifford 1997), as well as carbohydrates, from megagametophyte to seedling.

Sucrose is generally considered to be the carbohydrate that is transported to the seedling during the germination of oilseeds (Bewley and Black 1994), such as castor bean (Kriedemann and Beevers 1967). It has long been speculated that the end product of gluconeogenesis in the germinated conifer megagametophyte is also sucrose (Ching 1972; Kao 1973; Murphy and Hammer 1994). Because of the relatively large amount of sucrose present in the megagametophyte during early seedling growth, we too hypothesized that sucrose was the primary product of TAG breakdown in loblolly pine megagametophytes. Instead of increasing 36-fold above mature levels during early seedling growth, as it did in castor bean endosperms (Huang and Beevers 1974), the loblolly pine megagametophyte sucrose level increased by only 1.4-fold and remained constant throughout early seedling growth. This maintenance at a constant level may have resulted from a rapid removal of sucrose from the megagametophyte that occurred at a rate that paralleled TAG breakdown. Because the seedling accumulated sucrose during early seedling growth, it seems likely that sucrose produced in the megagametophyte is exported to the seedling of loblolly pine. In relation to this, sucrose accumulated during early seedling growth in sugar pine (Murphy and Hammer 1988), pinyon pine (Murphy et al. 1992), and castor bean (Kriedemann and Beevers 1967) seedlings. A similar sucrose increase was also noted in Japanese black pine (Hattori and Shiroya 1951), although it was unclear whether the seedling alone or the seedling with its megagametophyte was analyzed. In castor bean, the level of sucrose remained high in the seedling because the sucrose transported from the endosperm to the cotyledons of the seedling was maintained as sucrose when transported to the radicle (Kriedemann and Beevers 1967), but it is unknown whether this is also the case for seedlings of loblolly pine or other conifer seedlings.

Despite its probable importance in conifer seed germination, sucrose transport to the conifer seedling has received limited attention. Murphy and Hammer (1994) delivered ^{14}C-sucrose to the corrosion cavity of 10-d-incubated pinyon pine seeds and demonstrated that both the seedling and megagametophyte tissues were capable of sucrose uptake. However, they did not provide any evidence that sucrose was the carbohydrate normally transported from the megagametophyte to the seedling *in vivo*. Carrier and coworkers (1997) also showed that sucrose was a requirement for the germination of excised zygotic and somatic embryos of interior spruce (*Picea glauca engelmannii* complex). However, the lack of a megagametophyte made it difficult to assess the importance of sucrose delivery to the germinating seedling *in vivo*. To ascertain what carbohydrates are transported from the megagametophyte to the developing seedling, pulse-chase experiments with radiolabeled substrates applied to the outside of intact megagametophytes with seedlings must be conducted. These experiments are ongoing in our laboratory.

During early seedling growth, loblolly pine seedlings accumulated starch to a limited extent. In contrast, it was previously shown that pinyon pine seedlings accumulated large stores of starch during early seedling growth (Murphy and Hammer 1994). Pinyon pine seedlings were grown in a dark chamber with only periodic exposures to light for watering (Murphy and Hammer 1994; J. B. Murphy, personal communication), excluding the possibility that photosynthesis was a factor in starch accumulation. In contrast, the loblolly pine seedlings in this study were grown under continuous low light levels and may have accumulated some starch as a result of low-level photosynthesis. Even with the different growth conditions, it is unlikely that they account for the differences in starch accumulation noted here. It is more likely that these changes in starch accumulation are due to variation in TAG reserve levels and subsequent carbohydrate metabolism. Pinyon pine megagametophytes contained relatively more TAG reserves than loblolly pine. The TAGs comprised 58% of the dry weight of mature pinyon pine megagametophytes (Hammer and Murphy 1994), compared to 27% of the dry weight in loblolly pine megagametophytes.

In light of our findings, we are continuing our investigations into the transport of specific carbohydrates as well as other aspects related to storage TAG and carbohydrate metabolism in loblolly pine seeds.

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