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# Osmotic Measurements of Whole Ovules During Loblolly Pine Embryo Development

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# OSMOTIC MEASUREMENTS OF WHOLE OVULES DURING LOBLOLLY PINE EMBRYO DEVELOPMENT

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## ABSTRACT

The osmotic environment has been shown, both in angiosperms and gymnosperms, to play an important role in embryo development. In 1993 and 1994 the osmotic environment in ovules of loblolly pine (*Pinus taeda*) was measured. Cones were collected weekly from late June to October and shipped overnight on ice to IPST. Ovules were prepared for measurement by removing the seed coat, integument, and nucellus. Three to five whole ovules from a cone were placed in the osmometer sampling pan of a Wescor 5500 Vapor Pressure Osmometer modified with a cycle hold switch and allowed to equilibrate for 30 minutes. After osmotic readings were obtained, each ovule was opened and the embryo rated for developmental stage. Cones from three trees were processed during 1993 and again in 1994 providing six sets of osmotic profiles during embryo growth and development. Most cone collections showed a consistent water potential pattern: high readings of 400-500 mmol/Kg during early embryo development in late June to early July when embryo stages 1-2 occur; a reduction for one to several weeks to levels of 200-300 mmol/Kg, correlating with stages 4-5; followed by a steady increase in osmotic level from 350 to 700-800 mmol/Kg for stages 6 onwards from late August onwards. We are actively testing whether mimicking natural changes in water potential within the tissue culture environment will promote somatic embryo development.

## INTRODUCTION

The U.S. forest products industry accounts for nearly 7% of the total U.S. manufacturing output. However, much of the industry faces reduced availability of future timber resources. If the industry is to continue to grow, it must secure reliable sources of raw materials. Clonal propagation of high-value, fast-growing trees, in a crop-like setting offers the potential to meet future needs for high quality raw materials.

Somatic embryogenesis is a type of plant tissue culture which starts with a piece of donor plant and forms new embryos. In conifers, somatic embryogenesis currently involves the culture of zygotic seed embryos, usually from breeding programs, to start or initiate a culture. The major advantage of this technology lies in its ability to rapidly multiply highly valuable genetic material forming an unlimited number of identical somatic seedlings. In, addition, somatic embryogenesis provides some of the necessary tools for the genetic engineering of forest trees. Currently a major limiting factor for the scientific or commercial use of somatic embryogenesis is found in the quality of embryos produced. Few, if any, somatic embryogenesis systems in coniferous plant species or angiosperms produce embryos similar in biochemistry or vigor to natural (zygotic) seed embryos. Many embryogenic systems produce somatic embryos which are capable of germination, but which do not fully mature, resulting in slow germination and initial seedling growth.

In 1990 researchers at the Institute of Paper Science and Technology began an approach towards embryo quality improvement which focused on the comparison of zygotic and somatic embryo development. It was expected that somatic embryo quality would improve through imitation of the hormonal, nutritional, and physical environments during zygotic embryo development. Over the past years a staging system was developed for comparison of zygotic and somatic pinaceous embryos (1) and abscisic acid levels in developing embryos were measured in loblolly pine(2).

Water potential is a property of considerable importance to our understanding of water movement in the plant and soil, including developing embryos. If water potential differs in various parts of a system, water will move to the point where water potential is the lowest as free energy per mole. Water potential ($\Psi$) is the sum of three components: osmotic or solute potential ($\Psi s$), matrix potential ($\Psi m$), and pressure potential ($\Psi p$). Osmotic potential is due to solutes dissolved in water, matrix potential is due to the ability of colloidal hydrophylic surfaces to hold water, and pressure potential is due to the addition of positive or negative pressure. This paper reports results which describe the water potential of whole ovules of loblolly pine during development.

## MATERIALS AND METHODS

Work was begun in the summer of 1993 to understand the osmotic environment during loblolly pine zygotic embryo development. Loblolly pine (*Pinus taeda*) cones were collected weekly from Boise Cascade (Tree BC-1) and Union Camp (Trees UC-10-1003 and UC-10-1015) breeding orchards near Lake Charles, Louisiana and Rincon, Georgia, respectively. Cones were shipped on ice and received within 24-48 hours of collection. Precautions were taken to

accurately measure water potential (3). Initial experiments showed that ovules and embryos dried rapidly during excision, therefore, all opening of seeds and isolation of embryos and female gametophytes was done in a moist chamber. A moist chamber which housed a dissecting scope was created by partially lining a transparent plastic box with wet paper towels. Two entry ports were covered with plastic flaps which allowed hands to enter the box for dissections under magnification. Initial isolations of embryos from the female gametophyte also showed fluids leaking from the cut surfaces. Since water potential is composed of solute or osmotic potential, matric potential, and turgor pressure components, it was feared that the cut ovule surfaces would cause the solute potential to be measured rather than the water potential. It was decided, therefore, to measure the water potential of the whole ovule containing the female gametophyte and embryo. This decision was also based on the assumption that we are most interested in the osmotic environment surrounding the developing embryo which should be reflected by the water potential of the whole structure.

A Wescor 5500 Vapor Pressure Osmometer was fitted with a 7 mm diameter x 2.5 mm deep sample holding pan, and modified with a cycle hold switch which allowed long-term sample equilibration. Three to five whole ovules were isolated from the seed coat and integuments and rapidly placed in the osmometer holding pan. Samples were allowed to equilibrate for 30 minutes and two osmotic measurements as mmol/Kg were taken. The higher the mmol/Kg, the lower the free energy/mole of water. Water moves to the area of highest mmol/Kg. If osmotic values were not within 10 mmol/Kg of each other, additional equilibration was allowed until two similar consecutive readings were obtained. In most cases a 30 minute equilibration period was sufficient to obtain consistent readings. This process was repeated for four cones from each collection time and mother tree. After osmotic readings were obtained, each ovule was opened and the embryo was rated for developmental stage (1). When embryos reached stage 9 they continued to increase in size weekly. Therefore embryos were additionally staged by the week they were collected; 9.1 (stage 9 week 1), 9.2 (stage 9 week 2), etc.

In order to obtain ovule fresh and dry weights and moisture contents, five whole ovules containing female gametophyte and embryo, were isolated in the moist chamber, enclosed in small pre-weighed aluminum weighing containers, and weighted on a five place Mettler balance. Containers and embryos were dried overnight at 70°C and re-weighed to obtain dry weights. Data was obtained from embryos for each of four cones for each collection time and mother tree. From this data moisture content, ovule fresh weight and ovule dry weight could be calculated.

## RESULTS

### 1993 Ovule Measurements

Early season measurements for all cone collections showed high water potential levels of 400-550 mmol/Kg (Figure 1a-c). To be sure that these high levels were not an artifact of drying during shipping, a local loblolly pine tree was chosen with cones that could be reached with a tree pole pruner. This local tree, was used to collect three cones weekly until no more cones could be reached. The local cones were collected early in the morning (~7am) and rushed to the lab on ice for osmotic measurements. Osmotic measurements from the local tree cones showed similar high osmotic levels during early embryo development, indicating that the shipping period was not causing erroneous measurements.

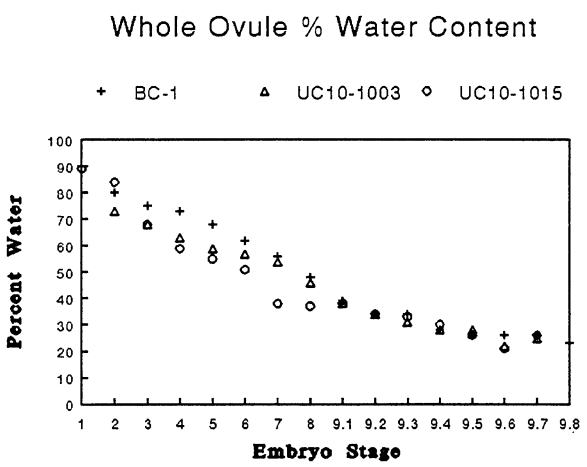
All three cone collections, including the local tree cones showed a consistent pattern of high water potential of 400-550 mmol/Kg during early embryo development in late June to early July when embryo stages 1-2 occurred. All cone collections then showed a reduction in mmol/Kg for one to several weeks (Figure 1 a-c)). BC-1, UC10-1003, UC10-1015, and local ovules reached low water potential levels of 256, 334, 318, and 200 mmol/Kg respectively. In all collections the decrease in mmol/Kg correlated with embryo stages 4 and 5 (Figure 3 a-c). All cone collections except local, which ran out of reachable cones in mid-season, then showed a steady increase in ovule osmotic measurements from late August onwards. From late August onwards water potential increased from approximately 350 to 700-800 mmol/Kg where they leveled off until cone harvest.

Embryo stage showed a lag for several weeks at stage one during mid to late June and early July (Figure 3 a-c). Observations of embryos showed that stage one embryos cleaved due to cleavage polyembryogeny followed by suspensor elongation. This repeated several times with each new set of stage one embryos located farther from the micropylar end until embryos reached a mid-point within the ovule. From then on one dominant embryo progressed rapidly through stages 2-8 within 4-5 weeks. Stages 4-6 correlated well with the observed dip in water potential. Embryos then remained morphologically at stage nine but continued increasing in size and weight for an additional 6-8 weeks while water potential increased and leveled off at 700-900 mmol/Kg.

Percent water content of the whole ovule is shown in Figure 5 a-c. Percent water content showed a sigmoidal curve with a

short lag period at approximately 90% water content as stage 1 persisted. A linear decrease in water content then occurred over most of embryo development. Approximate water content by embryo stage is shown in Figure 7. Water contents by stage were similar for all four tree collections. All embryos continued to accumulate fresh and dry weight while decreasing water contents. Over a period of 6-8 weeks, stage 9 embryos continued to increase in size and weight while water potential varied from 350 to 900 mmol/Kg and average water content varied from 38-26% at maturity.

Figure 7. 1993 whole ovule moisture content during seed development. Cones from trees BC-1, UC10-1003, and UC10-1015.




Data for fresh and dry weight accumulation were also monitored throughout embryo development. Fresh and dry weight also showed sigmoidal curves with a short lag, a linear increase, and then a plateau at maturity. Fresh weight per ovule was consistently 2-4 mg greater than dry weight. The difference between fresh and dry weight ranged towards the higher values for the larger seeded collections such as BC-1 and UC10-1051. UC10-1051 was small seeded and the local collection cones were large seeded. The difference between fresh and dry weight remained fairly constant over the entire season. This indicates that a constant amount of water was present in the ovule regardless of the % water contents. Dry weight per ovule started around 0.5-1.0 mg and ended at 6, 8, and 13 mg for UC10-1003, UC10-1051, and BC-1, respectively. It is interesting to note that during the weeks of September, when water potential rose above 500-600 mmol/Kg, embryo dry weight still continued to increase.

1994 Ovule Measurements

During 1994 the above study was repeated. The same Boise Cascade tree, BC-1, was again used for cone collections. However, the Union Camp trees used in 1993 were not available for collection. Trees, UC5-1036 and UC7-1051, collected from a seed orchard near Bellville, GA were substituted. Weekly collections were made and shipped as described earlier. The same procedures for water potential, embryo stage, and fresh and dry weight determinations were followed. In addition, three cones of BC-1 and UC5-1036 each were used to isolate 10-20 embryos and female gametophytes per cone for fresh and dry weight determinations.

Water potential measurements for whole ovules of BC-1 showed the same pattern of osmotic change through embryo development as was measured during 1993 (Figure 6a). The water potential curve for UC5-1036 showed a early declining osmotic level followed by a rise, a level region, and then the late development osmotic rise (Figure 6b). The water potential curve for UC7-1051 showed a slightly different pattern than was measured in other tree collections during 1993 or 1994, a slowly rising pattern was observed starting from about 325 mmol/Kg (Figure 6c). For both tree collections UC5-1036 and UC7-1051 collections began on July 5, 1994 and missed the 2-3 weeks of stage l.

1994 embryo stage, percent water content, and dry weights all showed developmental patterns through the season similar to those observed in developing embryos during 1993 (Figures 4a-c, 6a-c).

Both BC-1 and UC10-1036 showed the decline in water potential around embryo stage 4 similar to 1993 observations (Figure 2a-b). However, UC7-1051 showed an osmotic increase during stages 4-5 (Figure 2c). Out of seven tree collections followed during 1993-1994, UC7-1051 was the only one to show a osmotic rise during stages 4-5.

Embryo and female gametophyte dry weights are shown in Figures 7 and 8. Embryo fresh and dry weights showed a typical sigmoidal pattern of weight increase with the greatest mass accumulation during stages 8-9.2. Note that in all cone collections the high rate of dry matter accumulation at stages 8-9.2 correlated with water potential readings of 300-500 mmol/Kg. During the last four weeks of measurements, UC5-1036 embryo weights leveled off and BC-1 increased slightly. Female gametophytes showed a similar pattern of weight accumulation with the greatest mass accumulation when embryos were at stages 7 through the fourth week of stage nine.

## Conclusions

Two years of water potential evaluations during loblolly pine ovule development have provided valuable information to guide us in somatic embryogenesis. Water potential, measured as mmol/Kg, was measured throughout embryo development. Measurements show a consistent developmental pattern: start high, drop, and then increase. The water potential drop usually occurred when embryos developed to stages 4-5, at the same time as apical dome formation. A drop in water potential may be critical for apical dome formation. The greatest fresh and dry embryo weight accumulation occurred during embryo stages 8-9.2 when water potential measured 300-500 mmol/Kg. Mass accumulation of embryo and female gametophyte continued to increase during late embryo development, when water potential levels remained above 500 mmol/Kg.

Water relations parameters have been investigated for zygotic and somatic embryos of red pine (4) and western larch, white spruce, and loblolly pine (5). In red pine, the osmotic potential (Mpa) of gametophytic supernatant increased (became less negative) only slightly between the zygote and proembryo stages of embryo development and remained constant thereafter, water potential was not measured (4). The osmotic potential was most negative at the earliest of four stages of gametophyte development tested. For western larch, loblolly pine, and white spruce bound water, elastic modulus, osmotic potential at full turgor, and relative water content at turgor loss point, were determined for zygotic and somatic embryos at two developmental stages (5).

Little information on water potential throughout embryo development is available. Most studies have concentrated on measuring osmotic potential as a component of water potential. However, in *Phaseolus vulgaris*, bush bean, water potential patterns similar to those reported here for loblolly pine ovules, were found in developing bean embryos and pod tissue (6). Other systems such as soybean have shown continuous rise (data converted to mmol/Kg) in water potential in embryo and pod tissues (8). Water relations are important because they likely regulate solute (nutrient) availability to developing embryos and female gametophyte tissues.

The obtained water potential measurements over ovule development begin to provide us with targets to mimic natural embryo development in a tissue culture system. For example, the drop in osmotic levels during stages 4-6 gives us some insight as to why maltose provides an optimal maturation environment for loblolly pine (7). Sucrose breaks down in the medium into glucose and fructose during embryo growth thus increasing the osmotic environment. Maltose does not increase osmolality of the medium (unpublished data). Thus, the use of sucrose causes osmolality of the embryonic environment to rise while maltose allows osmotic levels to remain static or slowly decline. The use of maltose as an energy source more closely resembles the natural osmotic profile in developing ovules of loblolly pine.

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Figure 1. *Pinus taeda* whole ovule osmolality during 1993 embryo development. Cones from tree: A) BC-1, B) UC10-1003, and C) UC10-1015. Standard error bars are shown for each set of replicates.

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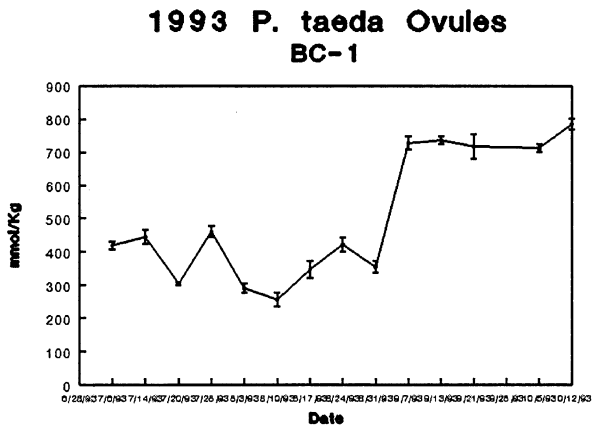


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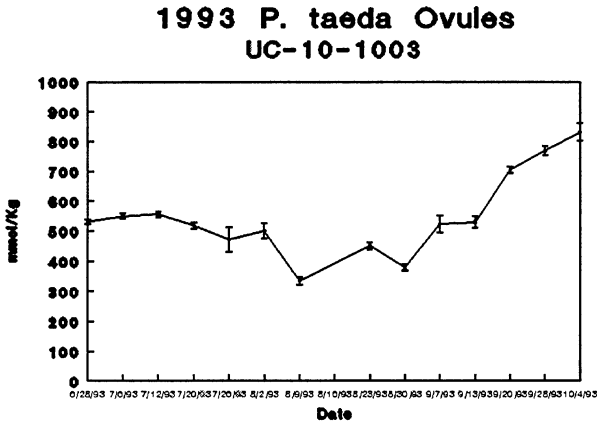


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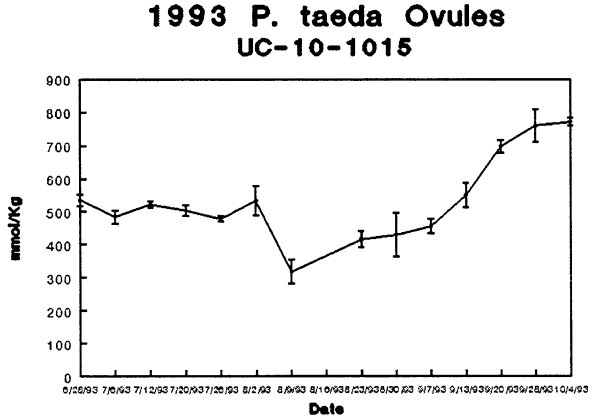


Figure 2. *Pinus taeda* whole ovule osmolality during 1994 embryo development. Cones from tree: A) BC-1, B) UC5-1036, and C) UC7-1051. Standard error bars are shown for each set of replicates.

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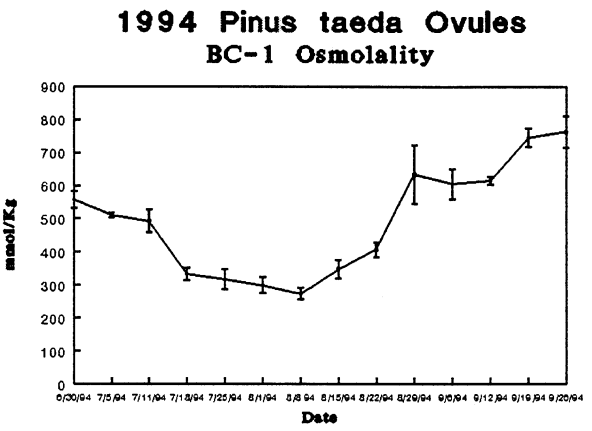


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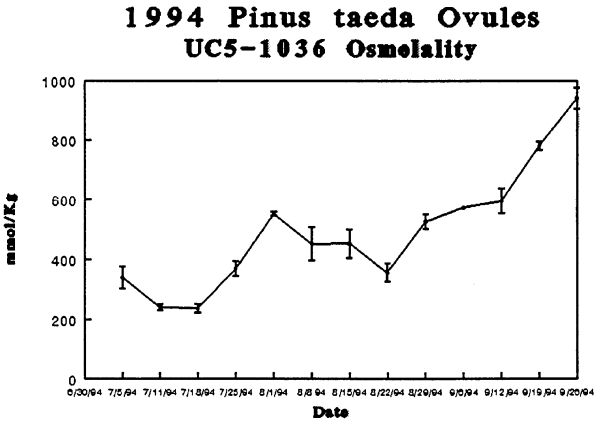


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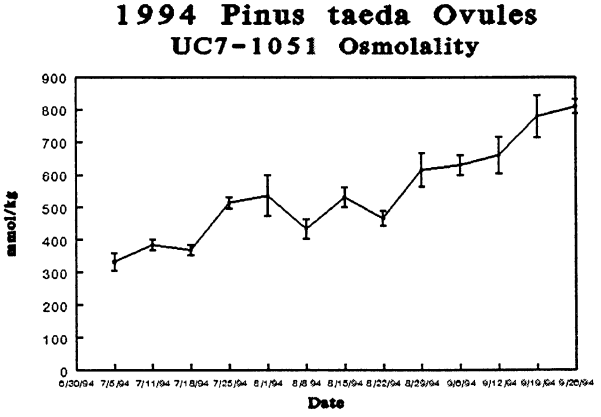

Figure 3. *Pinus taeda* embryo stage during 1993 embryo development. Cones from tree: A) BC-1, B) UC10-1003, and C) UC10-1015.

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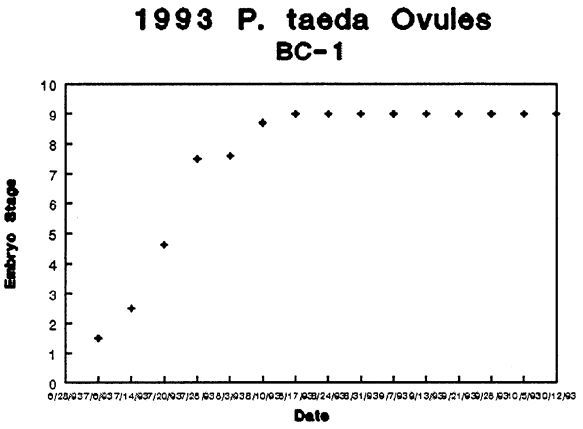


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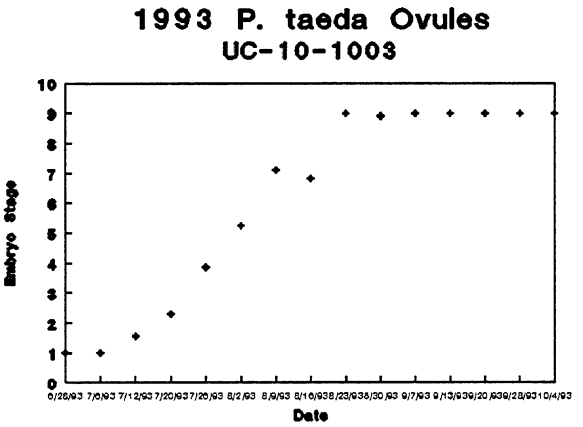


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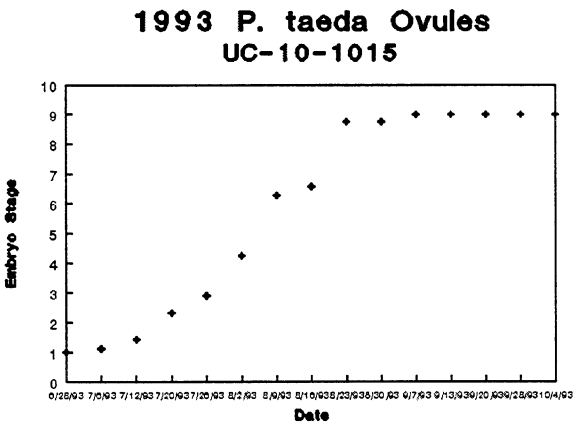


Figure 4. *Pinus taeda* embryo stage during 1994 embryo development. Cones from tree: A) BC-1, B) UC5-1036, and C) UC7-1051.

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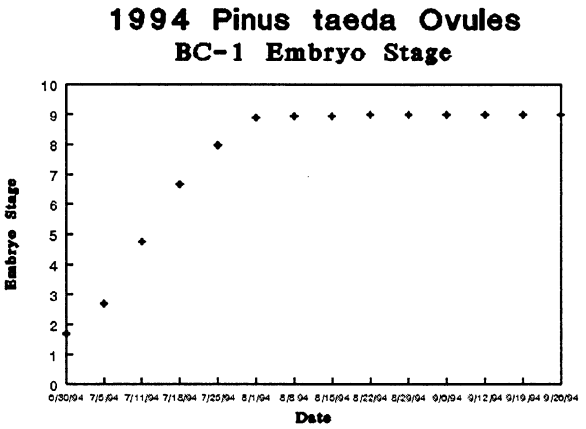


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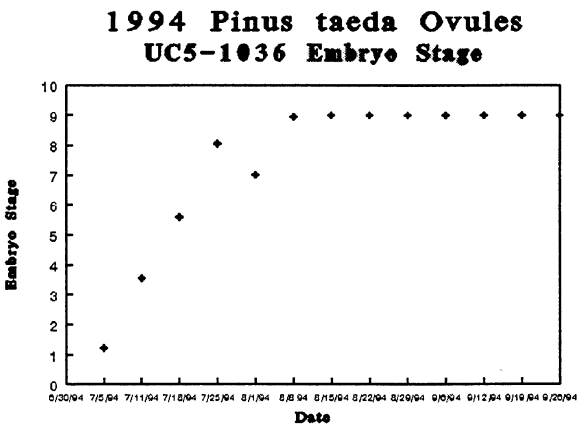


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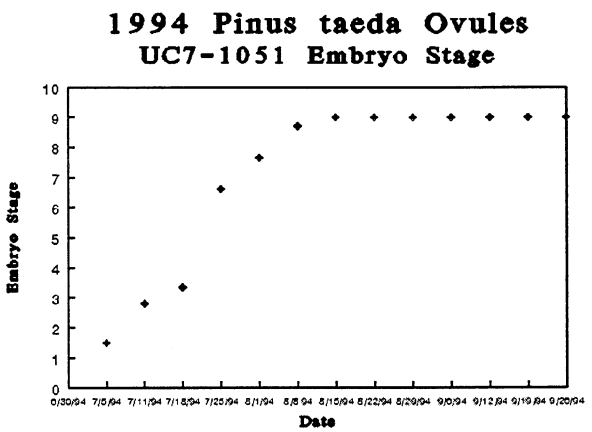

Figure 5. *Pinus taeda* percent water content of whole ovules during 1993 embryo development. Cones from tree: A) BC-1, B) UC10-1003, and C) UC10-1015. Standard error bars are shown for each set of replicates.

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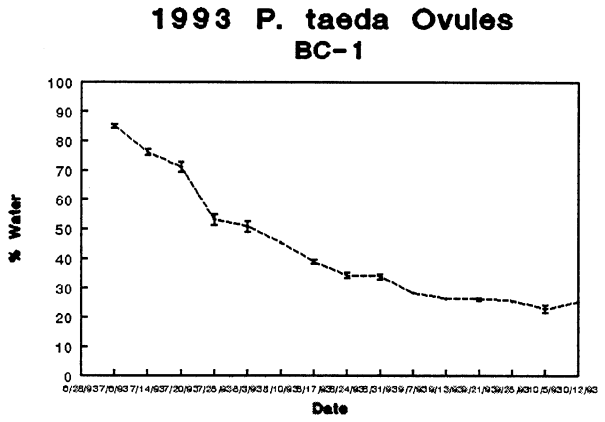


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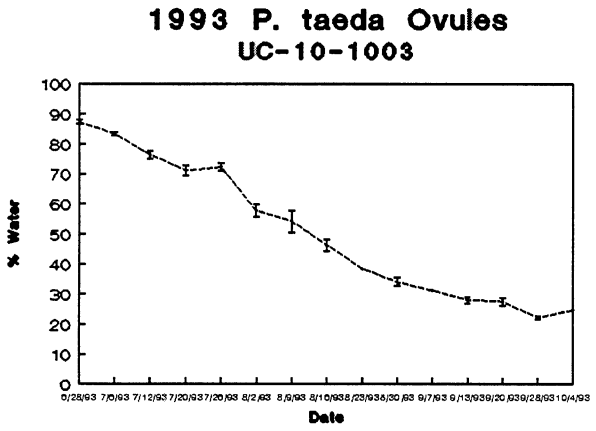


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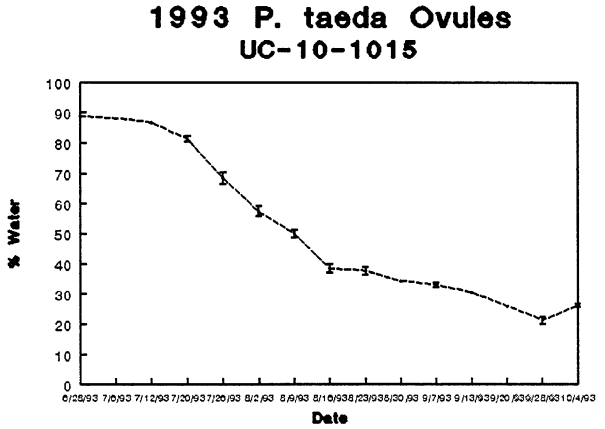


Figure 6. *Pinus taeda* percent water content of whole ovules during 1994 embryo development. Cones from tree: A) BC-1, B) UC5-1036, and C) UC7-1051. Standard error bars are shown for each set of replicates.

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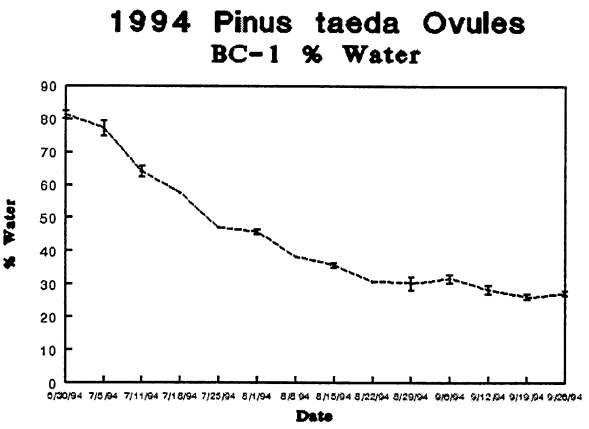


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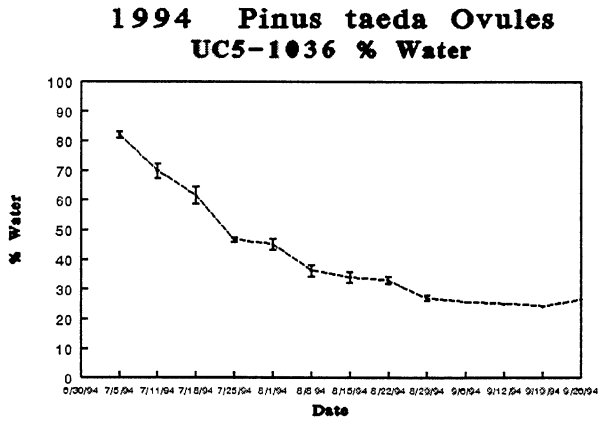


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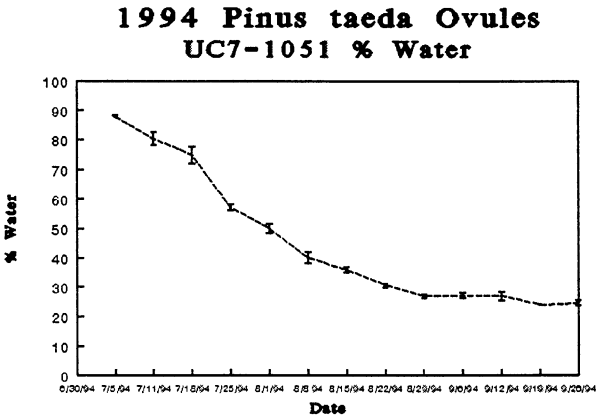

Figure 7. *Pinus taeda* embryo & female gametophyte dry weights during 1994 for Boise Cascade BC-1 cones. A) Embryo dry weight. B) Female gametophyte dry weight.

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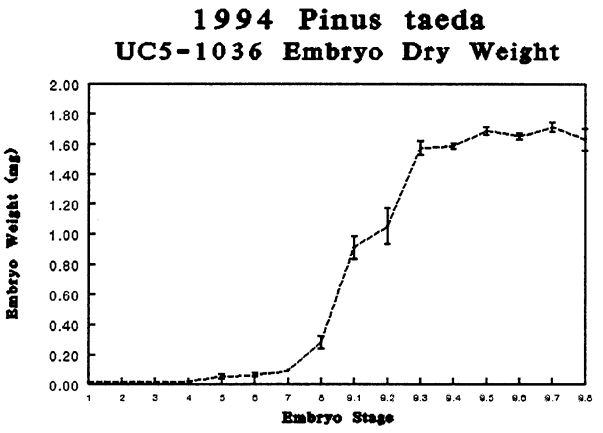


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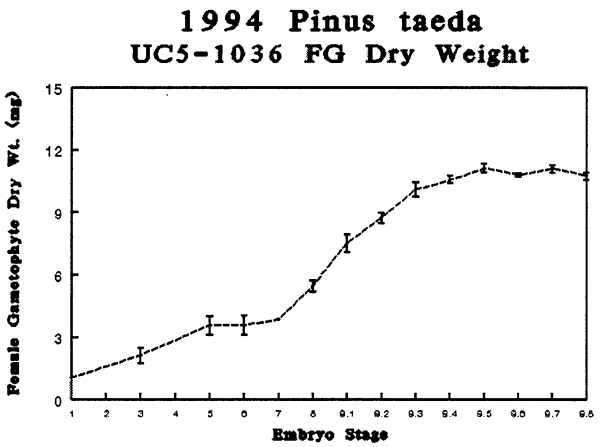
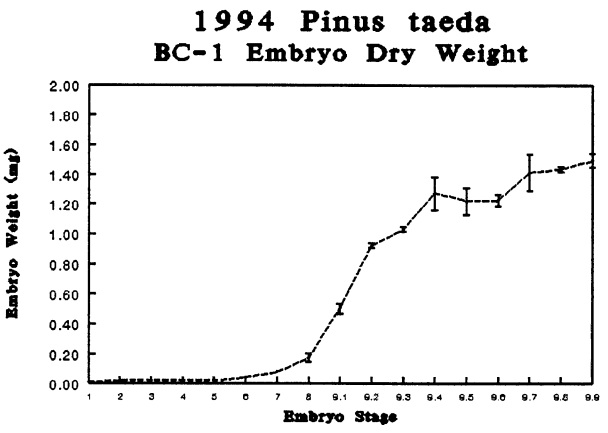



Figure 8. *Pinus taeda* embryo and female gametophyte dry weights during 1994 for UC5-1036 cones. A) Embryo dry weight. B) Female gametophyte dry weight.

A




B