

OVULE AND EMBRYO DEVELOPMENT, SEED PRODUCTION AND  
GERMINATION IN ORCHARD GROWN CONTROL POLLINATED  
LOBLOLLY PINE (*PINUS TAEDA* L.) FROM COASTAL SOUTH CAROLINA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE  
in the Department  
of Biology


We accept this thesis as conforming  
to the required standard

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### ABSTRACT

This study utilized four clones to trace the anatomical development of the coastal loblolly pine (*Pinus taeda* L.) ovule and embryo, using light microscopy. This study covered the period from the spring following initiation of the reproductive structures until cone maturity, a total of 19 months spread over two growing seasons. Particular attention was directed toward determining the time of fertilization, the number of archegonia per ovule, the number of pre-cleavage and post-cleavage embryos produced by each ovule and the time of subordinate post-cleavage embryo degeneration. In addition, these four clones plus one other clone were used to determine the number of ovuliferous scales in total and by type, the seed production capacity and seed germination of the species.

Ovule and embryo development in this species deviates in only one respect from the accepted pattern in other temperate pines, this being the delay in the onset of free nuclear division, within the female gametophyte, until the start of the second growing season. Loblolly pine had one to four archegonia in each ovule. If each was fertilized (between June 6 and 13) a maximum of four pre-cleavage embryos and sixteen post-cleavage embryos could be produced. By July 28 subordinate post-cleavage embryos were no longer visible within the corrosion cavity.

Seed production capacity can vary widely. Cones may have between 138.55 and 171.55 ovuliferous scales, seed potentials of 161.4 to 193.0 and seed efficiencies of 31.6 to 84.4 percent. However, in seeds that contain viable embryos germination potential was consistently high. All five clones had germination percentages of 96.0% or better and very similar germination profiles, with the maximum (non-cumulative) number of germinated seeds occurring 11 days after stratification.

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## CHAPTER 1

### INTRODUCTION

The southern pine region is a twelve state block extending from Virginia to east Texas within which the forest industry has been a significant, if not locally dominant, part of the economy for more than 250 years (Hughes, 1990). Currently there are approximately 72.9 million hectares of forested land in the southern pine region, down from 80.5 million hectares in 1963 (Hughes, 1990). The natural range of loblolly pine (*Pinus taeda* L.) extends in a broad arch from Delaware to west Texas, where it is continuous except for the Mississippi River valley and south Florida (Dorman, 1976). Throughout most of this range it grows in mixed pine and mixed pine-hardwood stands. However, single species stands are located in Louisiana, Arkansas, east Texas, Mississippi, Alabama as well as coastal North and South Carolina (R. Schultz, Southern Forest Experiment Station, *in press*).

Since the first pine plantations were established in the 1920s, loblolly pine has held an increasingly prominent position in southern plantation forestry so that currently it is the most often planted pine species (Hughes, 1990). Between 1985 and 1989, 1.2 to 1.5 billion loblolly pine bare-root seedlings were produced and planted each year. This was approximately 80 percent of all the pines planted in the southern pine region (R. Schultz, Southern Forest Experiment Station, *in press*). The attributes which allowed loblolly pine to be chosen as the major plantation species, over slash and longleaf pines, were its acceptable wood properties for lumber and pulp as well as its large genetic diversity. The latter point is very important considering that loblolly pine is planted on sites varying from dry, rocky hills to wet, peat swamps (Hughes, 1990).

In 1988 more than 90 percent of the loblolly pine seedlings produced were from genetically improved stock (R. Schultz, Southern Forest Experiment Station, *in*

*press*). It is through genetic improvement (in concert with intensive forest management practices) that the forest industry hopes to increase production, thereby compensating for both the shrunken forest land base and increased demand for building products. To this end much effort has been expended to develop tree breeding programs to improve the planting stock. However, with the exception of Mathews (1932), Greenwood 1980 and Brown and Bridgewater (1987) little work has been directed toward describing the reproductive process of the southern pines. To this end the purpose of this study was to document ovule and embryo development from anthesis until maturation in loblolly pine. Particular attention was given to determining the time of fertilization, the time of embryo cleavage, the number of cleavage embryos formed and the time of subordinate post-cleavage embryo degeneration. In addition, at cone maturity, the mean number of ovuliferous scales, by type and per clone; the mean seed production per clone; and, the mean seed potential and seed efficiency per clone were ascertained. Finally, viable stratified seeds were subjected to a germination test to determine germination rate and total germination percentage.

## CHAPTER 2

### LITERATURE REVIEW

The literature review will be presented in two sections. Konar and Oberoi's review paper in 1969 summarized the information available, until that time, on the development of conifer reproductive structures. Because of the general nature of most publications prior to 1969 they will be presented in chronological order. Other publications, from this and subsequent years, are more focused and, as such, do not lend themselves to a historical discussion. For this reason they will be presented by area of research.

Due to the abundance of information on conifer reproductive structures and processes the literature review is restricted to the Pinaceae with emphasis on *Pinus*.

#### **2.1 Early Research**

The earliest investigations into pine reproduction were completed by three German botanists; Hofmeister, Goroschankin and Strasburger. The first of these to publish was Hofmeister in 1848 with subsequent papers from 1849 to 1867. Both Goroschankin and Strasburger published between 1869 and 1904.

Hofmeister's 1851 paper made the first reference to the development of the female gametophyte of gymnosperms (*see* Ferguson, 1904). Although many of his observations were inaccurate, he pointed the way for those that followed. In 1861 and 1862 Hofmeister noted the presence of pits between the archegonial jacket cells and the egg cell and more carefully described the archegonial chamber (*see* Ferguson, 1904).

Strasburger's papers comprise the largest body of early work on conifer reproduction. He began publishing details of fertilization in the *Abietineae* in 1869

(see Ferguson 1904). This investigation continued and in 1872 a paper was published in which he described details of the egg cell nucleus and the separation of the egg cell and ventral canal cell (see Ferguson, 1904). His 1877 paper, also devoted to fertilization in the *Abietineae*, included a description of male and female gamete fusion (see Ferguson, 1904). The next paper, published in 1878, was devoted to the early development of the female gametophyte while in 1879 he reviewed reproduction in angiosperms and gymnosperms (see Ferguson, 1904). His 1880 publication carried an erroneous description of multinucleate female gametophyte cells (see Ferguson, 1904). By 1884 he concentrated on detailing the appearance of the egg nucleus at maturity and determined that while both male gametes enter the egg cell, only one fuses with the egg nucleus (see Ferguson, 1904). In 1888 Strasburger opined that mixing of the nuclear material of the male and female gametes was an important part of the fertilization process. Through the 1890s he published three papers on the process of fertilization. In 1892 the first of these described the reduction in size of the zygote nucleus between fertilization and the first division, while papers from 1895 and 1897 concentrated on the participation of the nucleolus in mitotic spindle formation (see Ferguson, 1904). By 1900 he once again concentrated on female gametophyte development, with a detailed description of megaspore mother cell division, central cell, spindle formation and proembryo development (see Ferguson, 1904).

In three papers published between 1880 and 1883 Goroshankin made two significant observations; that the archegonial jacket cells had both thick cell walls and interconnecting pores and that both male gametes enter the egg from the ruptured pollen tube (an observation made by Strasburger in 1894) (see Ferguson, 1904).

Many other researchers of note published during the late 1800s. Dixon

(1894) examined the process of fertilization in *Pinus sylvestris*. His work was extensive, encompassing male gamete formation, pollen tube growth, the fates of the stalk cell and tube nucleus and the events that take place during fertilization. In addition, considerable effort was expended to explain why there was variation in the number of chromosomes between the 1N and 2N tissues within the integument. In 1897 Coulter summarized work done by his graduate students on the approach of the pollen tube to the egg, entry of male gametes into the egg, fusion of male and female gametes and early embryo development. The majority of this work was done on *Pinus* with comparisons to other conifer species. In 1898 Blackman, working on *P. sylvestris*, traced the formation and maturation of the egg cell and ventral canal cell and the processes of fertilization, nuclear division and cell wall formation in the proembryo. Once again, there was speculation and confusion about the number and structure of chromosomes in both maternal and paternal reproductive structures. In 1899 Chamberlain described the series of events that produce the mature egg cell and ventral canal cell in *P. laricino*. Although fertilization was discussed briefly, more emphasis was directed toward characterizing proembryo formation. As is the case with many papers of this vintage, particular attention was paid to the shape, size and action of chromosomes during division. Another area of interest to Chamberlain was the formation of and changes to the mitotic spindle during nuclear and cell divisions.

In 1900 Murrill described ovule development from archegonial initial formation through fertilization, free nuclear division and proembryo formation in *Tsuga canadensis*. Particular attention was directed toward the structure, number and movement of the chromosomes during division. Miyake (1903) described female reproductive development in *Picea excelsa* from archegonial initial formation to the mature proembryo and the progress of the pollen tube through the nucellus to

the egg cell.

Early work is much more diverse than what is presented here. During this period other researchers were delving into the reproductive systems of a variety of conifer genera. In addition, many early researchers either wrote in German, or their work was not readily available, or both. It should also be said of this pioneering work that although some erroneous assumptions were made, the basic pattern of events was correctly described. The elegant manner with which their findings were presented cannot be denied, especially with regard to the detailed drawings that accompanied most of these early publications.

Ferguson's extensive survey of sexual reproduction in pine (1904), marked a great leap forward in the understanding of this process. Contained within its 202 pages were an excellent summary of the work completed until that date, including descriptions of: pollen formation, pollination, pollen tube elongation, female gametophyte development (from megaspore mother cell appearance to the mature egg cell), fertilization, and proembryo formation. A series of 262 hand-drawn figures illustrated the text. The thoroughness with which she made her observations make this one of the premier extant publications on the subject.

In 1907 Kildahl studied the order of cell wall formation in the proembryo of *Pinus laricino*. Prior to this time there was some confusion amongst researchers regarding these events. While this was not completely solved, she confirmed that transverse walls appear before longitudinal walls and that cell wall formation occurs after the first division of the four free nuclei. However, the author incorrectly observed that the second nuclear division can occur in either tier and this division may not be simultaneous in the four nuclei that make up the tier. It has since been determined that in most conifers the primary upper tier divides before the primary embryonal tier (Singh, 1978).

It was in 1918 that Buchholz began publishing his long series of papers on conifer embryology. His first work discussed the development of the corrosion cavity, proembryo, embryo and suspensor. The most significant part of this paper was its exploration of the changes occurring to the proembryo and embryo as they mature. While some of his conclusions are incorrect, the number of conclusions indicates the thoroughness of the author's observations. The most obvious error was his belief that the rosette tier of the proembryo divides to form four rosette embryos. Because of this Buchholz believed that each archegonium produced eight embryos. His description of cleavage polyembryony, embryo maturation and suspensor elongation was more correct. He determined that cleavage polyembryony produced one embryo from each of the four cells in the proembryo's embryonal tier. Distinct regions appeared within the enlarging embryo in the following order: root apex, root cap, shoot apex and cotyledons. The author correctly assumed that the suspensor came from the tier of cells located immediately adjacent those that produced the embryo. However, he thought that elongation continued until the embryo was mature rather than until it reached the chalazal end of the corrosion cavity. In 1920 Buchholz compared embryo development and polyembryony in eleven conifer groups. In the section on *Pinus* he reiterated the steps necessary for proembryo formation and the potential for the rosette tier to form embryos. He also mentioned the formation of the suspensor, initially from the suspensor tier followed by the elongation of cells at the base of the enlarging embryo. Cleavage polyembryony was described and distinguished from simple polyembryony. Because only one embryo survives to maturity, he recognized that this may represent "survival of the fittest" due to competition between the embryos for dominance in the corrosion cavity.

Sethi (1928) was the first of the Indian researchers to publish on *Pinus*. This

large work, on *P. longifolia*, described normal and abnormal male and female reproductive development. The description of the ovulate strobilus included ovule development, fertilization and embryo maturation. The most detailed description was of the events occurring between the appearance of the megaspore mother cell and the end of proembryo formation.

The first publication devoted to reproduction in a southern pine was authored by Mathews in 1932. Both male and female portions of the reproductive cycle of *P. palustris* were described, with equal emphasis. Early female gametophyte development (megaspore mother cell to cellular female gametophyte) received less extensive coverage than did the development of the; archegonial initial, central cell, neck cell, ventral canal cell and egg cell. Fertilization was also extensively covered. While E development was only mentioned briefly, Mathews did state that the post-cleavage embryo which attains dominance in the corrosion cavity is the one which survives to be dispersed with the seed. The belief that rosette embryos were formed in addition to those from the embryo tier was perpetuated by Mathews.

Emig 1935 proved that there was still some confusion regarding the development of the pine female gametophyte. He erroneously believed that the megaspore mother cell divided to form three daughter cells. However, he did correctly state that the sporogenous tissue surrounding the megaspore mother cell, once digested, had a nutritive function and that most of the nucellus was used for this purpose. This resulted in a cavity in which the enlarging female gametophyte developed. Emig reported that the free nuclei within the female gametophyte resemble "...a finely granulated matrix very much like the suspension of fruit in a gelatine desert" and that wall formation between these nuclei proceeds from the micropylar end to the chalazal end of the female gametophyte. These cells had a more recognizable organization at the micropylar end of the female gametophyte

than at the chalazal end. Emig was one of the first authors to speculate on why ovules abort. He thought first year ovule abortion was caused by reduced nucellar digestion supplying fewer food-stuffs to the developing female gametophyte. Emig felt that second year ovules could abort before or after fertilization resulting in empty but full-size seed.

In 1939 Stockwell indicated that there were at least six stages at which selection, affecting the fate of the embryos in the mature pine seed, can occur. These were the following events: withdrawal of pollen into pollen chamber; formation of pollen tubes by grains in the pollen chamber; resumption of pollen tube elongation after overwintering; the number and position of the archegonial initials; the number of fertilized eggs; and, competition between embryos for the dominant position in the corrosion cavity.

Haupt (1941) described maturation of the female reproductive structures in *P. lambertiana*, beginning with central cell formation formation and ending with the mature egg cell. An effort was made to describe the appearance of the nuclear material during division. Also covered was the entrance of the male gametes into the egg cell, syngamy and the two subsequent mitotic divisions. The author described the egg cell's large cytoplasmic inclusions as "deeply staining food granules".

Cone and seed production in ponderosa pine was explored by Roeser (1941). He followed conelet and cone abortion throughout the two year reproductive cycle. Results indicated that up to 90 percent of the abortion occurred during the first growing season.

One of the first attempts to classify emerging conelets of slash pine, allowing more accurate controlled pollination, was presented by Snow *et al.* (1943). Four stages were identified which would enable breeders to isolate first year conelets

prior to contamination with unwanted pollen, perform a controlled pollination and remove the isolation bags after the receptive period had passed. Spurr (1949) described late embryo development in *Pinus* with particular attention directed toward describing the formation of tissues within the embryo. The first recognizable tissues were the root apex and root cap followed by the shoot apex, which in combination with the root apex delimited the hypocotyl. Last to develop were the cotyledons. These observations were the same as those of Buchholz's (1918).

The first significant advancement in our knowledge of pine reproduction in the 1950s came in Thomas's 1951 Ph.D thesis, in which she traced male and female reproductive development in *P. virginiana*. Her description of ovulate strobilus development revealed that the functional megaspore was the innermost of a triad of cells. Maturation of the female gametophyte and fertilization followed the pattern described for other pines. The proembryo of *P. virginiana* was unique because the open tier was either partially or completely isolated from egg cytoplasm by cell walls. Feulgen staining of female gametophyte and proembryo nuclei indicated that their enlargement during maturation was not due to increased DNA content.

Lyons (1956) used *P. resinosa* to introduce the concepts of seed production capacity and seed efficiency. Lyons was the first to divide the seed cone into "nil, undeveloped and productive" regions and gave an indication of the size of these regions and their characteristics. First and second year ovule abortion was examined, as was the relationship between seed production efficiency and seed-cone location on the tree (ie. location in the crown and on the branch). Hakansson (1956) described seed development in *Pices abies* and *Pinus sylvestris*. Some doubts were expressed about the validity of Buchholz's argument, from 1950, that cleavage polyembryony was beneficial. He felt that since three of the embryos degenerated in a matter of days (compared to six weeks in Buchholz's study) there could hardly

be time for competition between them.

The first study of reproductive structure initiation in a southern pine was published by Mergen and Koerting in 1957. In slash pine, staminate<sup>1</sup> strobili were initiated during late June and developed until microspore mother cells were present. Ovulate strobili were initiated in late August. Development continued until individual ovules could be identified. Wareing (1958) explored the initiation of ovulate and staminate strobili, in *P. sylvestris*, in relation to changes in daylength, age of the tree, position within the crown and vigor of the branch bearing the strobili. Konar and Ramchandani (1958) followed internal and external changes in staminate and ovulate strobili of *P. wallichiana*, from the start of the first growing season until strobilus maturity. The authors concluded that the developmental stages of the female reproductive structures paralleled that of other temperate pines. One unique feature of this paper was its description of the seed coat which was found to have a middle stoney layer surrounded by inner and outer fleshy layers. McWilliam and Mergen (1959) were the first to photograph the act of fertilization in *P. nigra*. Previously the only pictorial representations were camera lucida drawings or diagrams. Their paper described the interval between male gamete release into the egg and the first zygotic division. No apparent deviations from the steps described by earlier investigators were uncovered by these authors.

Based on material collected from only one ponderosa pine at Placerville, California, Gifford and Mirov (1960) concluded that, unlike vegetative apices, newly initiated ovulate strobili do not become completely dormant over the winter. This result was in agreement with that of Mergen and Koerting (1957) for slash pine grown in Florida. In Konar's (1960) comparison of the morphology and embryology

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<sup>1</sup> Staminate strobilus is the companion term to ovulate strobilus which accurately describes the coniferous seed cone. Although not botanically correct, it will be used to describe the coniferous pollen cone.

of *P. roxburgii* with *P. wallichiana*, no significant new stages in the development of the female reproductive structures, fertilization or embryo were uncovered. However, he did confirm the presence of a three layered seed coat. A second publication from 1960 was Goddard's Ph.D thesis describing factors influencing vegetative and reproductive responses in *P. taeda*. While most of this work was devoted to the effect of photoperiod on vegetative growth, the author did examine the development of longshoot buds throughout the year. He determined that staminate strobili were recognizable in mid-September and ovulate strobili in mid-October. While development of these structures slowed, the author felt that there was some activity during the winter. This may have been a factor of location (east Texas) or a general occurrence for the species.

The early 1960s provided a wealth of information on strobilus initiation, seed production, proembryology and frost damage in southern pines. Egger (1961) determined the time of cone initiation in three species (longleaf, slash and loblolly) of southern pines, growing in southern Louisiana and Mississippi. Earliest recognition dates for staminate and ovulate strobili were presented for each species. For loblolly pine these were October 15 and 29 for ovulate and staminate strobili, respectively. While some variation in the date of strobilus initiation is expected, it was interesting that the order in which the strobili were initiated was not consistent between species. Both longleaf and loblolly pines initiated ovulate strobili before staminate strobili, while the reverse was true for slash pine. Variation in the order of strobilus initiation was not found by Goo (1961) in his examination of *P. densiflora* and *P. thunbergii*. In both species staminate strobili were initiated by early to mid-September and ovulate strobili by late September to early October. Sarvas (1962) produced a much cited work on the flowering and seed crop of *P. sylvestris* that determined "factors" on which the Scots pine seed crop depended, with

particular attention directed toward the importance of pollination. He determined that unpollinated ovules deteriorated during the first year and that if a sufficient number of ovules on a conelet remained unpollinated (about 20) it senesced. The effect of self-fertilization on seed production was also investigated and he concluded that formation of homozygous recessive alleles was the leading cause of embryo abortion. Together, lack of pollination and self-pollination reduced the potential seed crop to less than 20% of seed potential.

Doyle (1963) surveyed proembryology of *Pinus* and related it to other conifer families. He concluded that, at some point the suspensor function had been transferred from the rosette tier to the (new) suspensor tier. Doyle claimed that this was possible because of the distinct separation between proembryo tiers which is not present in many of the other conifer families. The last of the early 1960s publications was co-authored by Hutchinson and Bramlett (1964). Pictorial evidence was presented of complete conelet necrosis on shortleaf pine 11 days after a late spring frost. The authors concluded that because post-receptive Virginia pine conelets, exposed to the same frost, exhibited a lesser degree of necrosis, the extensive damage was due to the fleshy nature of the ovulate strobili at the time of receptivity.

Konar and Oberoi (1969) summarized literature covering development of the microsporangium, female gametophyte, pollination, fertilization and embryos of the seven conifer families. The authors pointed out that the existence of a tetrad of megaspores was not universally accepted, and in fact had only been shown in *P. roxburghii*. Confusion still existed regarding the embryology in the Pinaceae. Specifically, movement of the four free nuclei and the order in which they divided to form a proembryo was questioned and the existence of rosette embryos had not yet been refuted.

## **2.2 1969 To The Present**

### **2.2.1 Pollination**

Brown (1971) used Scots pine to determine, the effectiveness of controlled pollinations, the effect of pollen quality, and the effect of isolating ovulate strobili, in order to prevent contamination by unwanted pollen during pollination. Results indicated that, regardless of the amount of pollen applied to an ovulate strobilus, 20% of the ovules remained unpollinated and, that the application of fresh pollen was most effective in increasing the number of filled seed per cone. Aspect played a role in conelet abortion with the largest percentages of these occurring on the north side of the tree. Lill and Sweet (1977) examined pollination in *P. radiata* in a paper based on the former author's 1974 Ph.D thesis. Results indicated that the micropyle could hold up to seven pollen grains and that the length of conelet receptivity depended on the rainfall pattern at the time. The latter result, the authors concluded, was due to rain induced premature swelling of the ovuliferous scales restricting pollen grain's access to the micropylar arms. Their final conclusion was that late arriving pollen, if it landed on the micropylar arms, was as likely to be drawn into the micropyle as pollen that arrived earlier. Brown and Bridgewater (1987) examined pollination in *P. taeda* in a paper based on the former author's 1984 Masters thesis. The authors followed the pattern of pollination drop appearance on individual ovulate strobili and how this affected the movement of pollen from the micropylar arms to the nucellus. They concluded that pollination drops did not reappear once pollen was present on the nucellus. In addition, it was opined that rain could act as a pollinating agent before the appearance of the drops; making their formation redundant.

### 2.2.2 Pollination and Seed Development

Lill (1976) examined ovule and seed development in *P. radiata* and concluded that postmeiotic development, fertilization and embryology did not differ significantly from the accepted pattern in pine. In addition, a similarity existed between this and other pine species regarding the length of time necessary to produce a morphologically mature embryo. Owens and Molder (1977) traced the development of staminate and ovulate strobili in *P. monticola* from initiation through pollen and seed shed. No significant deviation from the accepted pattern of ovule development in pine was noted, although the length of time necessary for the completion of certain events may vary between species. Two papers by Owens *et al.* (1981, 1982) described sexual reproduction in *P. contorta*. The first described pollen development, the pollination mechanism, and early ovule development (ovulate strobilus initiation to post-pollination dormancy). One of the significant observations from this period was the two month delay between pollination and pollen tube elongation. The second publication described post-dormancy ovule, embryo and seed development spanning the interval between the resumption of free nuclear division within the female gametophyte and seed shed. No radical departure from the accepted pattern of ovule development in pine was reported.

Seed development in cedar pine (*P. siberica*) was described by Nekrasova (1970). She traced ovular development from division of the megaspore mother cell to seed shed, with no deviation from the accepted pattern being reported. Also described were the quality of the seeds produced and the effect that weather had on the seed crop.

### 2.2.3 Seed Development and Abortion

A significant publication from 1969 was Mikkola's examination of

interspecific sterility in *Picea*. This extensive work discussed development of fertilized ovules and the degeneration of those that remained unfertilized. The developmental stages were often compared to *Pinus*, which closely resembles the pattern of reproductive development in spruce. One of the more pertinent parts of this discussion was the description of degenerating unfertilized egg cells. In these, loss of cytoplasmic integrity followed nuclear degeneration, resulting in an empty egg cell. The final step in this process was the melding of the egg cell cavity and the degenerating micropylar end of the female gametophyte. Mikkola also utilized X-ray analysis to examine the seeds produced from interspecific crosses, concluding that small empty seeds were the result of abortion soon after pollination while full-size empty seeds were caused by the lack of successful fertilization or post-fertilization embryo degeneration. Four possible causes of abortion at this later stage were presented: no pollen within the pollen chamber; pollen within the pollen chamber did not germinate; the pollen germinated but the pollen tube did not penetrate the nucellus or neck cell; and, lastly, fertilization occurred but the embryo aborted.

Cecich (1978) described ultrastructurally, ovule development and abortion and outlined a possible mechanism for the enlargement of the cavity containing the female gametophyte, in *P. banksiana*. The mechanism involved the transport of a substance into the cavity from plastids in the surrounding sporogenous tissue via endoplasmic reticulum. The author felt that his description of the female gametophyte's eventual separation from the sporogenous tissue was unique. When the relative rate of abortion was examined, it appeared that the distal and median portions of the ovulate strobilus contained fewer aborting ovules than did the proximal region. In addition, the outer curvature of the first year conelet had greater pollination success and fewer abortions than did the inner.

#### 2.2.4 Ovulate Strobilus and Ovule Abortion

One of the first papers devoted to ovulate strobilus abortion was co-authored by Sweet and Thulin (1969). Conelet abortion between pollination and fertilization was investigated in *P. radiata*. It was believed that abortion began during receptivity, increased through pollination and then decreased over the next six weeks. Compared to healthy conelets, aborting ovulate strobili displayed reduced size and lack of plumpness. A conelet's vertical position within the crown was also given some importance as a cause of abortion. Conelet drop in *P. radiata* was explored by Sweet and Bollman in 1970. Examination of nutrient partitioning in developing ovulate strobili and the adjacent long shoot lead the authors to hypothesize that there were two stages to conelet drop; receptive and post-receptive. Abortion during receptivity was thought to be caused by the inability of a strobilus to compete with the adjoining vegetative shoot for carbohydrates and possibly mineral nutrients. Abortion of post-receptive ovulate strobili was thought to be caused by incomplete pollination. Bramlett (1972) demonstrated that the greatest percentage of ovulate strobilus abortion occurred during the first year after anthesis. Other than insects and frost, the cause of this abortion was not identified. However, the author did refer to an "imbalance of specific growth regulators" as a possible cause. Sweet (1973) outlined further investigations into abortion of pine ovulate strobili. He reiterated that most abortion occurs between anthesis and fertilization and stressed the importance of pollination and pollen tube elongation in the prevention of abortion. The series of events leading to the abortion of a first year ovule were described. Tissue degradation began in the region of the megaspore mother cell and spread to the surrounding sporogenous tissue which, along with the adjacent nucellar cells, became a formless mass leading to the collapse of the ovule. Sweet speculated that the pollen grain might supply a protein or a substance with a

protein component to trigger ovular development. In turn the developing female gametophyte might produce a hormone (or hormones), perhaps an auxin, preventing abscission of the entire ovulate strobilus. Kormanik (1974) supported Sweet's (1973) use of the terms conelet and cone to describe the pine ovulate strobilus during the period from anthesis to fertilization and fertilization to seed shed, respectively. He also affirmed the belief that the bulk of abortion occurs during the 10 to 12 weeks immediately following pollination and the importance of successful pollination in preventing this. In addition to pollination, insect infestation was discussed as an important cause of conelet abortion. White *et al.* (1977) reported conelet abortion rates as high as 80%, in longleaf pine. However, they did not consider lack of pollen to be a major cause of this phenomenon. The authors found that isolating pollinated and unpollinated ovulate strobili in sausage casing pollination bags hurried the onset of conelet abortion by about three weeks. This was attributed to increased temperature and humidity within the bags. Kuznetsova (1988) used Scots pine for an ultrastructural examination of developing seeds after self and open-pollination, concentrating on the relationship between pre-cleavage embryos and the surrounding female gametophyte tissue. Transmission electron microscopy revealed a thick callose layer between self-fertilized embryos and the gametophyte. This structure was believed to prevent the transfer of nutrients from the female gametophyte to the embryo, causing the latter to abort.

### **2.2.5 Abortion, Seed Efficiency and Seed Potential**

Bramlett (1974) determined seed potential, seed efficiency and total number of ovuliferous scales per cone, in shortleaf, Virginia, loblolly and slash pines. The amount of ovule abortion during both years of the reproductive cycle was determined. During the first growing season, lack of pollen and insect infestation

were confirmed as the major causes of ovule abortion. Two stages of ovule abortion were described during the second growing season. If abortion occurred prior to the formation of the mature seed coat, collapsed resinous seeds were the result. If abortion occurred after a mature seed coat was present, full-size empty seeds were produced. In both cases, second year ovule abortion was attributed to insect infestation, although self-pollination was also thought to contribute to the formation of empty seeds.

### 2.2.6 Phenology

A system of classifying the ovulate strobilus of *P. keyisia* during its receptive period was developed by Pattinson *et al.* (1969). Increased seed efficiency due to controlled pollen application when the ovules were at maximum receptivity was the aim of this work. The receptive period was partitioned into five stages, based on the external morphology of the conelet. This approach was not unusual except that these stages were also correlated with the conelet's internal morphology. Greenwood's 1980 paper on the initiation of staminate and ovulate strobili in loblolly pine was an extension of the work begun by Goddard (1960). Study trees were located in North Carolina, yet they exhibited roughly the same pattern of staminate and ovulate strobilus initiation, microsporophyll and bract-scale initiation and detection without the aid of a microscope, as in Texas. Greenwood felt that staminate strobili were capable of continued development over the winter as long as environmental conditions were favorable, whereas ovulate strobili appeared to require a cold period before development could resume in the spring. To increase the accuracy with which controlled and supplemental mass pollinations could be made, Bramlett and O'Gwynn (1980) divided the early development of southern pine ovulate strobili into six stages based on degree of bud burst, conelet elongation

and increasing or decreasing distance between the ovuliferous scales. When applied at the appropriate stage they found that pollen had a much greater likelihood of adhering to the micropylar arms and being drawn down to the nucellus, effecting a successful pollination.

## CHAPTER 3

### MATERIALS AND METHODS

#### **3.1 Use of the Terms Conelet and Cone**

Throughout the thesis the terms conelet and cone will be used to describe the different periods in the development of the ovulate strobilus. The term conelet refers to the interval from anthesis to fertilization while cone refers to the ovulate strobilus between fertilization and seed shed. This follows the scheme used by Sweet (1973) and Kormanik (1974).

#### **3.2 Selection of Sample Trees**

Preparations for this study began in the spring of 1988 with the controlled pollination of more than 100 conelets on each of five clones at the Westvaco Corporation's 1.5 generation Coastal Loblolly Pine seed orchard in Ravenel, South Carolina. Controlled pollinations using the same pollen sources were again performed in the spring of 1989 on at least another 100 conelets on each of the same five clones. Two or three ramets of each clone were pollinated to ensure that a minimum 100 conelets were available for collection. This strategy insured sufficient control pollinated ovules to cover each of the developmental stages from anthesis to maturity.

The five clones were chosen on the basis of their seed efficiency. These included one with excellent, one with poor and three with intermediate seed efficiencies. Throughout the thesis these will be referred to as clones 1, 2, 3, 4 and 5 respectively. The detailed study of ovule and seed development used only the first four clones. Determination of seed potential, seed efficiency and the seed germination test used all five.

### **3.3 Pollinations**

Most conelets chosen for the study were located in the upper crown because it is here that longshoots bear multiple conelets, thus minimizing the number of isolation bags required. To ensure controlled pollination the conelets were isolated from background pollen well before peak receptivity (stage 5) (Bramlett and O'Gwynn, 1980) by placing them in cellulose sausage casings held open by a wire support and closed at the base by twist ties and polyester batting. This is the standard method used in the southern United States.

Pollinations were made with a polymix composed of pollen from all 15 clones in the seed orchard. The mix was made up by volume, although each clone's contribution was adjusted slightly if its performance in a germination test was less than 95%. Pollen used in 1988 was collected in 1987 and pollen used in 1989 was collected in 1988, except for two clones where 1987 pollen was used to bulk up a small 1988 collection. Pollen cones were collected before pollen shed, placed in paper bags and dried to less than 10% moisture content in a forced air chamber. After drying the pollen was extracted and placed in glass bottles which were stored in a freezer, at -20°C, until needed. When the conelets were receptive a "cyclone" pollinator was used to introduce the pollen into the bags. Within ramet variation in peak receptivity meant that two pollinations per bag were required. Between ramet and between clone variation extended the pollination period to five days in 1988 and eight days in 1989. When the conelets were no longer receptive (stage 6) (Bramlett and O'Gwynn, 1980) the isolation bags were removed.

### **3.4 Harvesting the Conelets and Cones**

Conelets and cones were harvested on a twice weekly, weekly or biweekly schedule depending on the suspected state of ovule or embryo development.

Collections of both first and second year structures began in February of 1989 and ended in September of that year. The pollination and sampling strategy allowed field collections of specimens covering the two year reproductive cycle to be completed in one year. The harvest of first year conelets began before pollination (late February) and continued on a twice weekly basis until after the functional megaspore began to enlarge (late March). After this, first year conelets were harvested biweekly until the end of collections in early September. Second year conelets were harvested biweekly, from late February, until just before the free nuclear female gametophyte began cell wall formation in early May. From this time until the embryos were visible at the end of their elongating suspensor (mid June), collections of second year conelets and cones were made twice weekly. Then two weekly collections of cones were made followed by biweekly collections from July until early September when the embryos were anatomically mature.

### **3.5 Microtechnique, Observations and Photomicrographs**

Entire first year conelets were fixed in chromic acid, acetic acid, formalin (CRAF) (Johansen, 1940; Berlyn and Miksche, 1976) until the end of the period of twice weekly collections in late March. Once individual ovules could be dissected 10 ovules per clone per collection were removed from their scale with a double edged razor blade and fixed in CRAF. This type of dissection was used for the rest of the collection period; except where it was suspected that the seed coat had hardened to the point where it would cause the sections to tear. In this case the seed coat was removed prior to fixation. All fixed specimens were rinsed in water, dehydrated in the Johansen Series (Johansen, 1940) and embedded in Tissue Prep (Fisher Scientific Catalog Number T555). Embedded specimens were softened for a week at 35°C in Gifford's solution (Gifford, 1950). Sections were cut on a rotary

microtome at 6 or 8 $\mu$  and stained with Safranin and Hematoxylin. Observations were then made using a Leitz Laborlux S microscope. Photomicrographs were taken on a Leitz Orthoplan microscope with a Leitz Vario-Orthomat photographic system. Most of the microtechnique was done at the research facilities of the Westvaco Corporation in Summerville, South Carolina. Some microtechnique and all of the microscopy and photomicrography were completed at the University of Victoria.

### **3.6 Seed Potential, Efficiency and Germination**

Twenty mature, but unopened, cones were collected from each clone and dried in a greenhouse until their scales reflexed. Seeds were then removed from each cone and counted. Also calculated were the number of fertile, sterile and rudimentary scales. The latter two scale types were located distally and proximally on the cone. The seed potential for each cone was calculated as the number of fertile scales times two, while seed efficiency was calculated as the number of filled seeds divided by the seed potential, times 100. The number of filled and empty seed per cone were determined by X-ray analysis using the Hewlett Packard Faxitron X-Ray System, (Model 4355A) located at the Pacific Forestry Centre, Victoria BC. Filled seed were those which appeared to have a well developed embryo and gametophyte tissue while in empty seeds these were either reduced or lacking. Filled seeds were all full-size while empty seed were full or half-size. The seed potential and seed efficiency for each of the 20 cones per clone were used to calculate the mean for each clone.

Germination tests were performed on 400 seeds from each of the five clones. Seeds chosen for use in this test were those which sank ("sinkers") after a 24 hour hydration in distilled water rather than those remaining on the surface ("floaters").

The "sinkers" were then sterilized in 10% sodium hypochlorite for 15 minutes, rinsed three times (for five minutes each) in distilled water then stratified at 4°C for 28 days in a refrigerator. Fifty seeds per clone were placed in each of eight germination boxes and these were put into a growth chamber set at a constant 22°C, with an eight hour photoperiod. The germination boxes were 11.3 x 11.3 x 3.5 cm, made of clear plastic and had a tight fitting lid to prevent evaporation. While not actually allowed to sit in the distilled water the seeds were kept moist throughout the test. This was accomplished by placing the seeds on a sheet of Whatman's no.1 filter paper which was atop a cellulose sponge. Seeds were considered to have germinated if the radicle was equal to or greater than twice the length of the seedcoat. Germinants were counted five times a week (Monday through Friday) and on each date new germinants were removed from the boxes. For each clone the test was terminated if no new germinants had appeared for a week. Ungerminated seeds left at the end of the test were dissected to determine the cause. The five categories used to describe these seeds were; inviable (shriveled brown embryo and gametophyte tissue), sound (firm white embryo and gametophyte), empty (no embryo or gametophyte tissue), abnormal (cotyledons emerge first during germination) and infected (embryo and gametophyte destroyed by fungal contamination).

### **3.7 Statistical Analysis**

Results were analysed by one-way Anova to determine if there were clonal differences in seed potential, seed efficiency, germinated and ungerminated seed, the number of sterile, rudimentary, fertile and total scales. Clonal production of empty, filled and empty plus filled seed was also analysed statistically. If clonal differences existed the Fisher Protected Least Significant Difference test (Fisher,

1949) was used to determine, more precisely, where these occurred. For both tests an  $\alpha$  level of 0.05 was used. As the raw data was not always uniformly distributed a transformation was necessary, which in all but three cases was  $\text{Log}(X+1)$ . Where the values were percents (seed efficiency, germinated and ungerminated seeds) an arcsine transformation was chosen (Zar, 1984). If the one-way Anova indicated that there was no difference in the clonal means the second test was not invoked and it was assumed that they were part of the same population.

## CHAPTER 4

### RESULTS

#### **4.1 Development of the First Year Conelets**

##### **4.1.1 Appearance of the Megaspore Mother Cells**

By the first collection (February 23) each of the four clones possessed a few ovules with megaspore mother cells, indicating that mitotic divisions had resumed within the ovules prior to this date. If present, the megaspore mother cells of clones 1 and 2 were at the earliest stage of development, each having the light granular appearance characteristic of an interphase nucleus and dimensions similar to the surrounding sporogenous tissue (Fig. 1). Some megaspore mother cells from clones 3 and 4 were larger than the sporogenous tissue cells but still had a light granular appearance (Fig. 2). On March 3 a few recently formed megaspore mother cells were still present in clones 1 and 2, however most were beginning to enlarge and clones 3 and 4 contained only enlarging, light granular, megaspore mother cells (Fig. 3). After this collection all ovules contained a single enlarging megaspore mother cell.

##### **4.1.2 Development of the Sporogenous Tissue**

There were clonal differences in sporogenous tissue development. On February 23 clone 1 had ovules which did not show any sporogenous tissue, as well as those with early sporogenous tissue without megaspore mother cells. The latter condition was also present in clone 2. These sporogenous tissue cells appeared little different from the surrounding nucellar tissue except for their location and a slight increase in cytoplasmic stainability (Fig. 1). Clones 3 and 4 had more advanced sporogenous tissue to compliment their megaspore mother cells. In this case the

sporogenous tissue was recognizable not only by location and cytoplasmic stainability but by shape as well. Several concentric layers of sporogenous cells formed a sphere surrounding the megaspore mother cell. The sphere increased in number of cells as the megaspore mother cells matured. By the time megaspore formation was complete (March 24) sporogenous tissue cytoplasm was much darker than that of the surrounding nucellar cells (Fig. 8).

#### 4.1.3 Nucellus Development

Divisions within the nucellus were present from the first collection (February 23) until May 5. During this 10 week period the size of the nucellus rapidly increased in both width and length so that it attained maximum size.

Another facet of nucellar development was the formation of a pollen chamber, resulting from the degeneration of cells at the tip of the nucellus (Fig. 4). This structure was first noticed in clones 2, 3 and 4 on March 3 and in clone 1 on March 10. The pollen chambers continued to increase in width and depth through March 21. Beginning on March 7 germinated and ungerminated pollen grains were found in a few pollen chambers of clones 3 and 4. These were not present the pollen chambers of clone 1 until March 10 and March 14 in clone 2. By March 17 the majority of ovules examined had one or more germinated pollen grains in their pollen chamber (Fig. 8).

Pollen-tube elongation began soon after the grain settled into the pollen chamber and continued until it had penetrated one-half the length of the nucellar tip. The collection made on May 5 was the last in which there was evidence of pollen-tube elongation. The limit of pollen-tube advancement was indicated by a region of disrupted nucellar cells (Fig. 10).

#### 4.1.4 The End of Conelet Receptivity

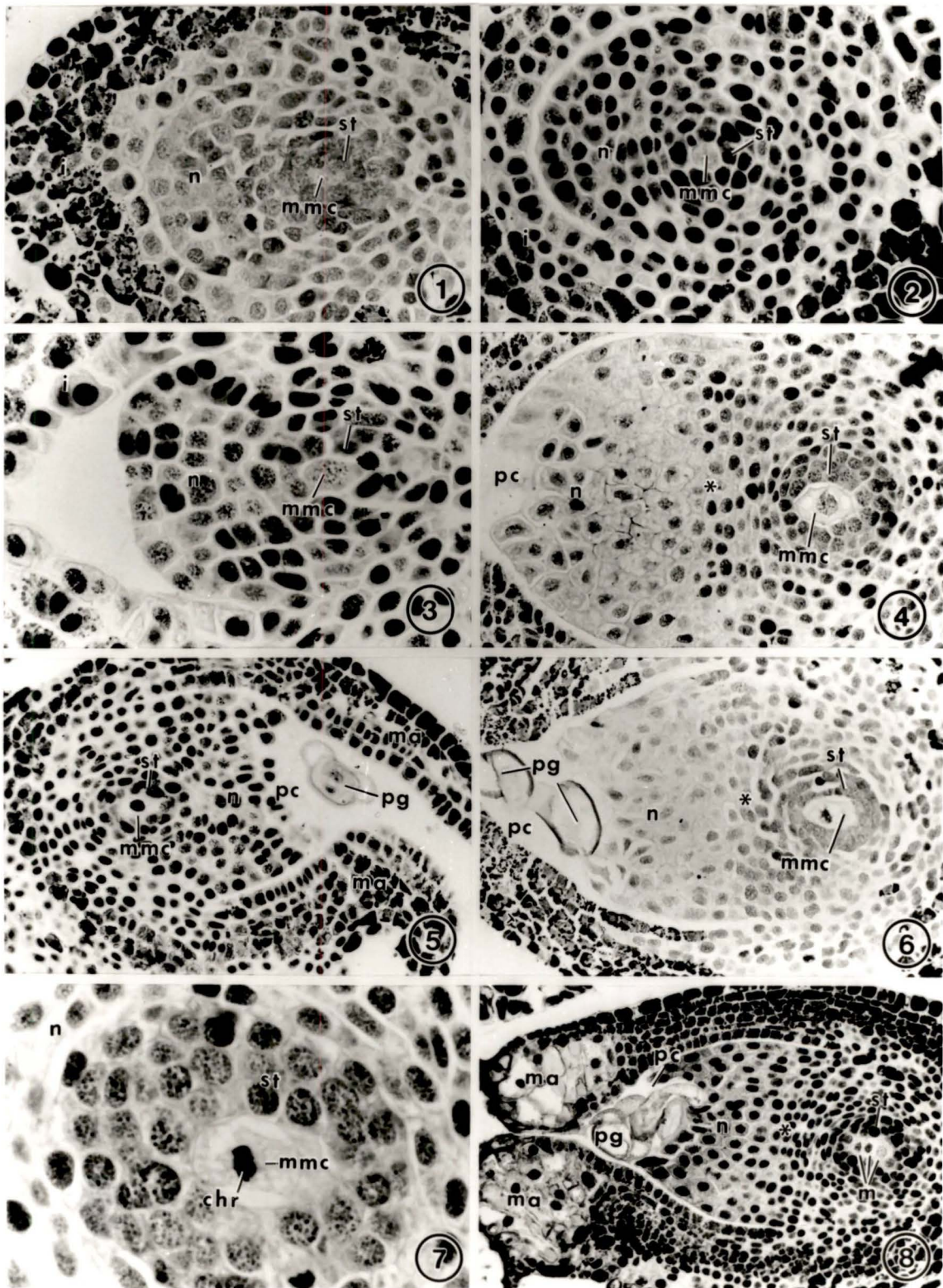
The formation of a collar of thickened cells near the base of the micropylar arms signaled the end of conelet receptivity. This thickening took place at 90° to the long axis of the arms due to the elongation of cells lining the micropyle (Fig. 8). By March 10 the developing collar had partially sealed the micropyle of clone 2 and by March 14 the micropyle of this and clone 4 were completely closed. On the latter date clones 1 and 3 had either partially or completely closed micropyles. Conelet receptivity ended prior to March 17 because on this date the micropyle of every ovule examined was closed.

#### 4.1.5 Maturation of the Megaspore Mother Cells

Enlarging megaspore mother cells were present between March 7 and 24. Generally megaspore mother cell enlargement was completed first in clone 4 followed by clones 2, 3 and 1. In addition to enlargement, condensation of the nuclear material occurred in preparation for meiosis. While still uniformly distributed, the nuclear material changed from light to dark granular, indicating the onset of meiotic prophase (Fig. 4). The nuclear material then pulled back from the megaspore mother cell wall, while retaining a dark granular appearance and regular outline (Fig. 5). This was followed by further condensation so that the granular appearance was lost and an irregular outline developed (Fig. 6). This alteration of the nuclear material's appearance pointed to a shift from early to late meiotic prophase. The appearance of distinct chromosomes at the megaspore mother cell's metaphase plate (Fig. 7) indicated that the first meiotic division was about to begin.

**Figs. 1-8. First year ovules: late February to late March, 1989**

- Fig. 1.** Ovule from late February showing megaspore mother cell (mmc) surrounded by sporogenous tissue (st), nucellus (n) and integument (i). x136.
- Fig. 2.** Ovule from late February/early March showing light granular mmc surrounded by st, n and i. x160.
- Fig. 3.** Ovule from late February showing enlarging light granular mmc surrounded by st, n and i. x200.
- Fig. 4.** Ovule from early March showing enlarged mmc, with dark granular nucleus, surrounded by st and n. Cavity at tip of n is a shallow pollen chamber (pc). (\*) indicates limit of degenerating n cells. x128.
- Fig. 5.** Ovule from early March showing mmc, with dark granular nucleus, surrounded by st and n. Also visible are micropylar arms (ma) surrounding a pc with a pollen grain (pg). x120.
- Fig. 6.** Ovule from early March showing mmc, with a condensed, irregular, nucleus, surrounded by st and n. Two pg's are in the pc. (\*) indicates limit of degenerating n cells. x100.
- Fig. 7.** Ovule from mid-March showing mmc, with chromosomes (chr) at metaphase plate, surrounded by st and n. x315.
- Fig. 8.** Ovule from late March showing tetrad of megaspores (m) (only three are visible). The two outer m's with dark nuclei are non-functional. The inner functional m has a light granular nucleus. Surrounding the m's is st and the n. The (\*) indicates the limit of degenerating n cells. Two germinating pg's lie in the pc. The distal portions of the ma's have shriveled and the micropyle is swollen shut. x85.



#### 4.1.6 Megaspore Formation and Degeneration

The first evidence of megaspore formation was in clone 4 (March 17) followed by clones 2 and 3 (March 21) and clone 1 (March 24). This meant that both between clones and within a clone meiotic divisions were not synchronized. Thus a collection might contain megaspore mother cells and megaspores with a variety of internal conditions. For example, on March 21, every ovule from clone 4 contained megaspores while the March 24 harvest was a mixture of megaspore mother cells and megaspores. After two meiotic divisions a linear tetrad of haploid megaspores (Fig. 8) was formed. Only the megaspore nearest the chalazal end of the ovule remained functional. The three non-functional megaspores degenerated and had no further role to play in the reproductive process. The first sign of nonfunctional megaspore degeneration was evident on March 24. At this time these cells had denser nuclear material than the functional megaspore (Fig. 8). Nuclear condensation continued until the nonfunctional megaspores were reduced to small black dots (Fig. 9). By April 21 no trace of the nonfunctional megaspores could be found (Fig. 10). Concurrent with nonfunctional megaspore degeneration was enlargement of the functional megaspore and the resulting separation of the surrounding sporogenous tissue. The innermost sporogenous tissue cells were disrupted in this way, whereas the outer sporogenous tissue cells lost their dense cytoplasm and increased in volume until they were larger than the adjacent nucellar cells (Fig. 10). Most of these cells eventually degenerated as the functional megaspore enlarged. Degeneration of the sporogenous tissue was complete by May 5. Enlargement of the functional megaspore continued until June 2 at which time it was many times larger than any of the other cells in the ovule (Fig. 11). The functional megaspore remained uninuclear until the end of the sampling period (September 8) (Fig. 12).

## **4.2 Development of the Second Year Conelets and Cones**

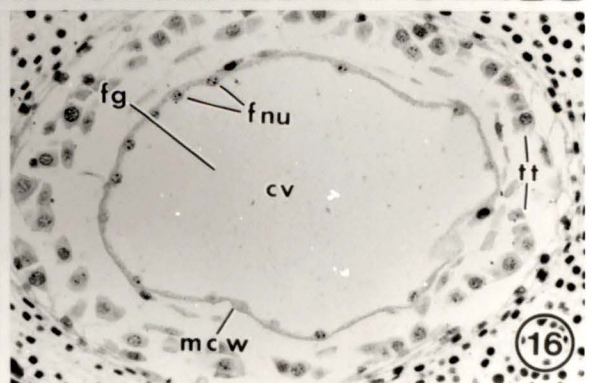
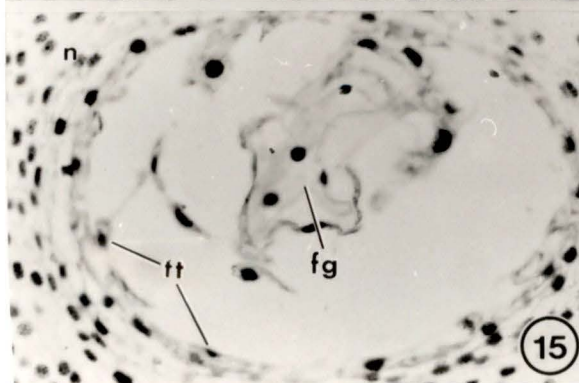
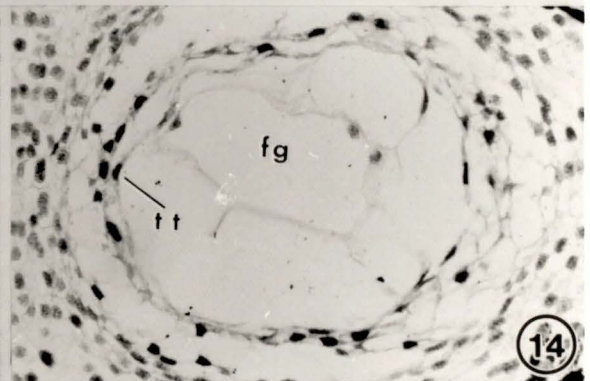
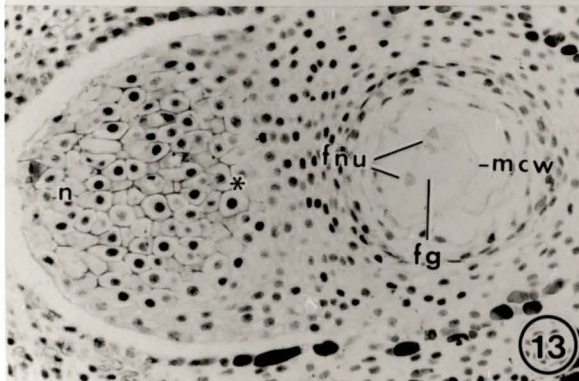
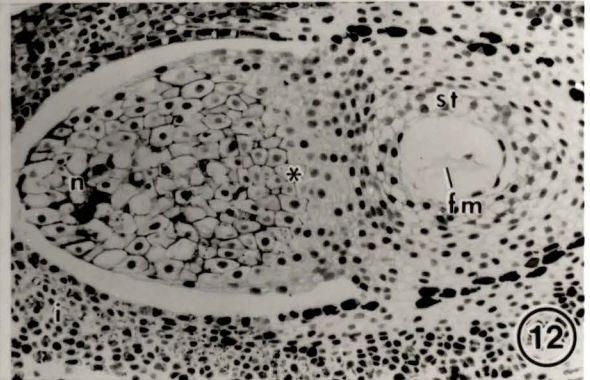
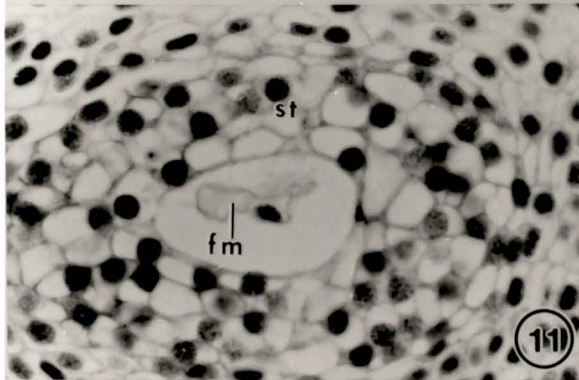
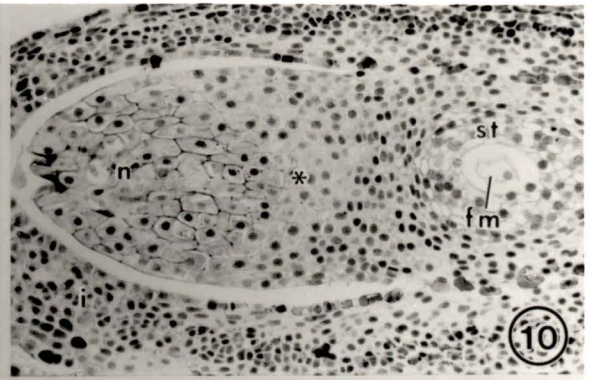
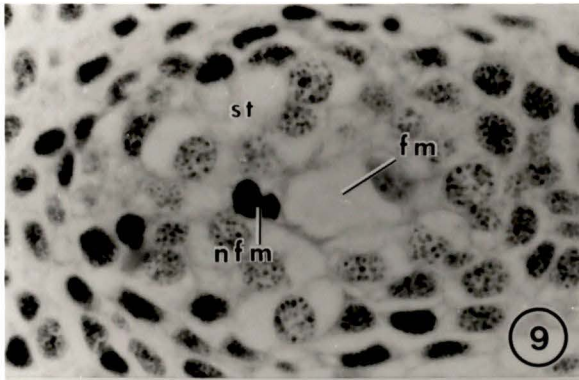
### **4.2.1 Changes in the Enlarging Free Nuclear Female Gametophyte**

Nuclear division within the megaspore cell wall began before the first collection of second year conelets was made on February 23 (Fig. 13). All clones exhibited a similar pattern of development for the next two biweekly harvests. There was an increase in the size of the female gametophyte and the number of free nuclei it contained (Figs. 14,15). By April 7 the free nuclei, which previously were evenly distributed throughout the free nuclear female gametophyte, formed a monolayer against the megaspore cell wall. Inside this was a large central vacuole (Figs. 16,17). Except for continued free nuclear division and an increase in volume there was no change in the free nuclear female gametophyte's appearance until cell wall formation.

### **4.2.2 Cell-Wall Formation**

Cell-wall formation (Fig. 18) was first noticed in clone 2 on May 5. In the next collection (May 12) cell wall formation was occurring in some of the ovules from clones 2, 3 and 4. There were clonal differences in the rate of cell-wall formation so the size of the central vacuoles varied from quite large (surrounded by one or a few layers of cells) to absent. On May 16 most clone 1 female gametophytes showed early cell-wall formation while clone 3 possessed a few free nuclear female gametophytes and cellular female gametophytes with no central vacuole (Fig. 19). Also each female gametophyte in clones 2 and 4 was completely cellular and had begun to differentiate specialized cells. Throughout this period the size of the free nuclear female gametophytes and cellular female gametophytes continued to increase.

- Figs. 9-12. First year ovules: early April to early September, 1989.**
- Fig. 9.** Ovule from early April showing enlarging functional megaspore (fm) and degenerating non-functional megaspores (nfm), all surrounded by degenerating sporogenous tissue (st). x315.
- Fig. 10.** Ovule from mid-April showing enlarging fm, degenerating st, nucellus (n) and integument (i). The (\*) indicates the limit of n disrupted by pollen tube growth. x80.
- Fig. 11.** Ovule from early June showing enlarging fm surrounded by degenerating st. x200.
- Fig. 12.** Ovule from early September showing enlarging fm surrounded by degenerating st and n. The (\*) indicates the limit of n disrupted by pollen tube growth. x75.
- Figs. 13-16 Second year ovules: late February to late April, 1989.**
- Fig. 13.** Ovule from late February showing enlarging free nuclear female gametophyte (fg). Free nuclei (fnu) are surrounded by the megaspore cell wall (mcw). The fnfg is surrounded by the n. The (\*) indicates the limit of n disrupted by pollen tube growth. x80.
- Fig. 14.** Ovule from early March showing enlarging fg surrounded by developing tapetal tissue (tt) and n. x110.
- Fig. 15.** Ovule from late March showing enlarging fg surrounded by developing tt and n. x125.
- Fig. 16.** Ovule from late April showing fnu of fg, arranged in a mono-layer against the mcw, surrounding an central vacuole (cv). Also visible is prominent tt. x80.



### 4.2.3 Changes in the Amount of Tapetal Tissue

Tapetal tissue was first evident on March 10 as a layer of cells located between the enlarging free nuclear female gametophyte and the outer compact cells of the nucellus (Fig. 14). The tapetal tissue increased in cell number as the free nuclear female gametophyte enlarged and reached a maximum on April 21. At this time tapetal tissue cells could be identified by their dark cytoplasm, angular outline and larger size than the surrounding nucellar cells (Fig. 17). Degeneration of the tapetal tissue began about May 5 and continued for roughly one month. When development of the cellular female gametophyte was complete no trace of the tapetal tissue could be found (Fig. 25).

### 4.2.4 Differentiation Within the Female Gametophyte

One to four cells at the micropylar end of the female gametophyte did not undergo nuclear division or form cross-walls as often as the rest of the cells. These became the archegonial initials (Fig. 19). There were clonal differences in the first appearance of the archegonial initials. Clones 2, 3 and 4 had archegonial initials in some of their female gametophytes by May 16, whereas it was not until May 19 that clone 1 reached this stage. As the archegonial initials enlarged, the nucleus remained at the micropylar end of the cell, adjacent to the megaspore cell wall (Fig. 20).

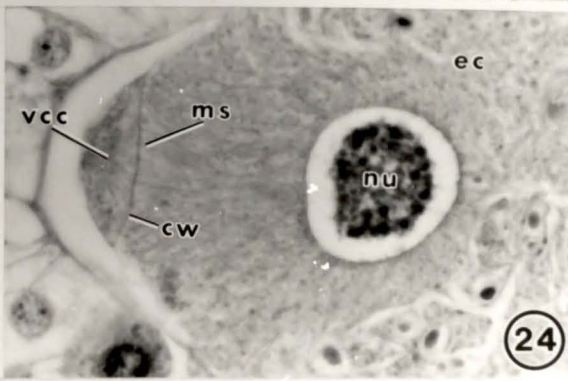
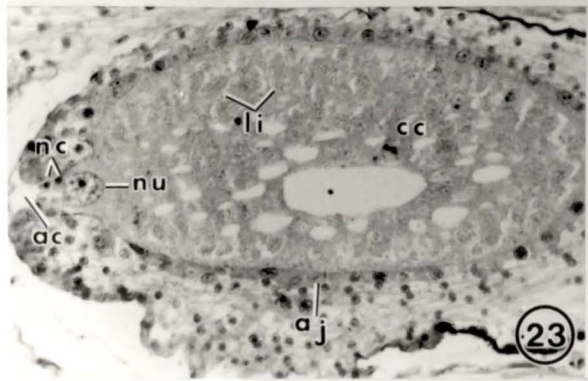
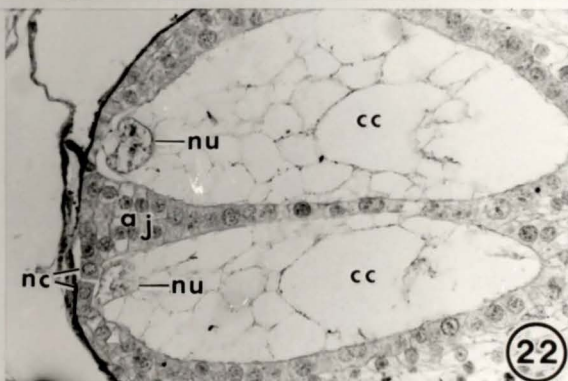
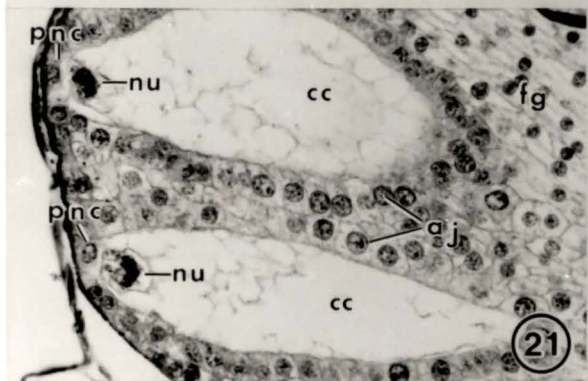
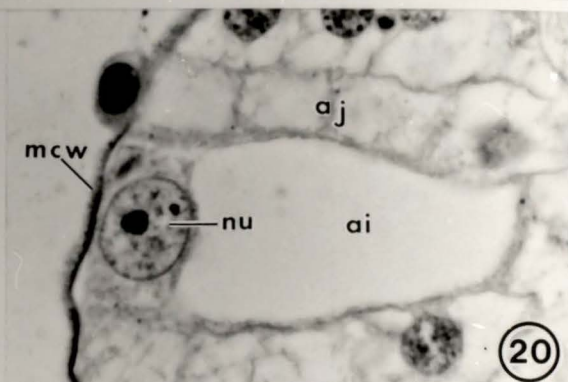
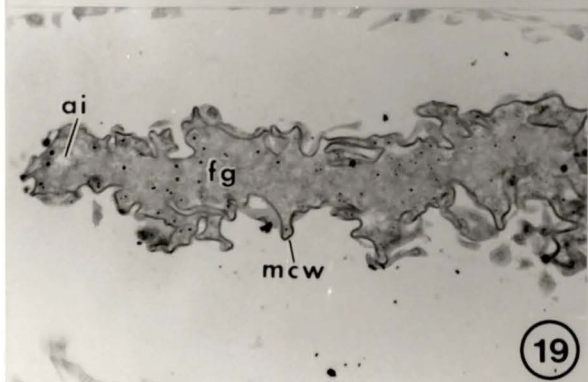
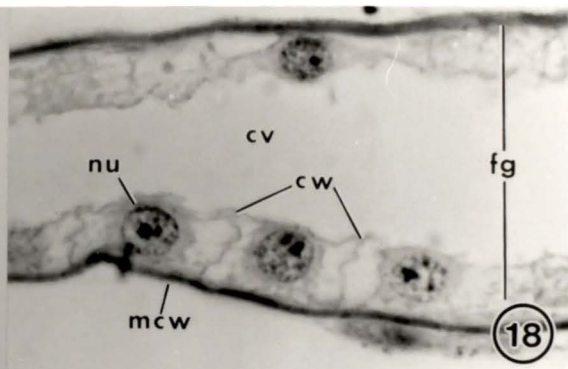
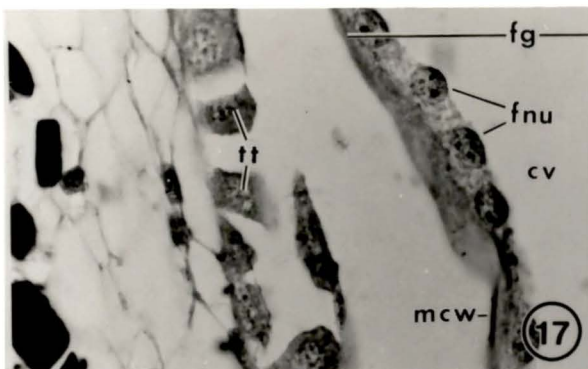
Division of the archegonial initial began in clones 2, 3 and 4 by May 19. Cell division was unequal and the two daughter cells had distinctly different sizes, shapes and functions. The inner was the central cell and the outer the primary neck cell (Fig. 21). By May 23 the archegonial initial in all but one female gametophyte (in clone 3), had undergone division and many of the primary neck cells had undergone anticlinal division forming one tier of neck cells (Fig. 22). By June 2 further

periclinal division had formed a second tier of neck cells (Fig. 23), which in all clones appeared to be the maximum number of tiers produced. The central cells began to increase in volume and cytoplasmic density until May 26 to June 6, depending on the clone. By June 2 the central cell cytoplasm of all clones was nearly avacuolate and numerous large inclusions began to appear (Fig. 23). Throughout central cell development the nucleus remained at the micropylar end adjacent to the neck cells (Figs.21,22,23).

Between June 2 and 9 unequal division of the mature central cell formed the egg cell and ventral canal cells (Fig. 24). The central cells of clone 2 were the first to undergo division and clone 1 the last. Maturation of the egg cells did not involve further enlargement, rather the appearance and position of the nucleus changed. At the time of division the egg cell nucleus was composed of condensed (dark granular) material and lay adjacent to the ventral canal cell (Fig. 24). The nucleus then migrated to the centre of the egg cell and underwent simultaneous enlargement. At maturity the egg cell nucleus had a light granular appearance (Fig. 25) and was surrounded by a finely granular perinuclear zone. The ventral canal cell was small and lens-shaped (Fig. 24) and while it underwent no obvious change during egg cell maturation, a slight reduction in cell size and increase in cytoplasmic density may have occurred.

The cells immediately outside the archegonial initials, central cells and egg cells made up the archegonial jacket. These were first evident as a single layer of isodiametric cells, in clones 2, 3 and 4, on May 19. On this date the only difference between archegonial jacket cells and the rest of female gametophyte was the former's apparently isodiametric shape (Fig. 20). Subsequently, they began to stain more darkly than the other female gametophyte cells and increased their cell and nuclear volumes until they were larger than adjacent female gametophyte cells. The

- Figs. 17-24**      **Second year ovules: early May to early June, 1989.**
- Fig. 17.**      Ovule from early May showing a free nuclear female gametophyte (fg) with a mono-layer of free nuclei (fnu), against the megaspore cell wall (mcw), between the central vacuole (cv) and tapetal tissue (tt). x265.
- Fig. 18.**      Ovule from mid-May showing a female gametophyte (fg) during early cell wall (cw) formation. Nuclei (nu) are still arranged in a mono-layer against the mcw and around a cv. x378.
- Fig. 19.**      Ovule from mid-May showing a completely cellular fg with an archegonial initial (ai), all enclosed by the mcw. x70.
- Fig. 20.**      Ovule from mid-May showing an ai with its nu against the mcw. The ai is encased by archegonial jacket (aj) cells. x378.
- Fig. 21.**      Ovule from late May showing two central cells (cc) with each nu against a primary neck cell (pnc). The cc's are encased by aj cells within the fg. x144.
- Fig. 22.**      Ovule from late May showing two vacuolate cc's with each nu against a single tier of neck cells (nc). x120.
- Fig. 23.**      Ovule from early June showing a single, nearly avacuolate, cc (encased by aj cells) with large inclusions (li) in the cytoplasm. The nu is now located against two tiers of nc's and the nc's are recessed in an archegonial chamber (ac). x80.
- Fig. 24.**      Ovule from early June showing cell division forming the ventral canal cell (vcc) and egg cell (ec), the nu of which is clearly visible. The cell wall (cw) separating the two cells is visible, as are the remnants of the mitotic spindle (ms). x315.



archegonial jacket was fully developed in all clones by June 6 when it was composed of isodiametric cells with large nuclei and darkly staining cytoplasm (Fig. 25).

The female gametophyte continued to enlarge, with clone 4 being the first to fully enlarge by June 6. By the next collection (June 13) the female gametophytes of all clones were fully enlarged. The cessation of female gametophyte enlargement coincided, roughly, with egg cell maturation. The last change occurring in the female gametophyte was a thickening of the tissue at the micropylar end around the neck cells. This recessed the neck cells of each archegonium in separate archegonial chambers (Fig. 23). In all clones archegonial chambers were evident by June 2.

#### **4.2.5 Pollen-Tube Growth and Fertilization**

Pollen-tubes resumed growth by May 19, in clones 2, 3 and 4, and by May 23 in clone 1. Pollen-tube growth proceeded slowly until the end of May. By June 2 clones 2 and 4 displayed more active pollen-tube growth so that the male gametes were near the megaspore cell wall as the egg cell completed its maturation (Fig. 26). By June 6 all clones had actively advancing pollen-tubes and evidence of fertilization was seen in clone 4. By June 9 all clones contained a few fertilized eggs (Fig. 27) and it appeared that clones 1 and 4 contained more fertilized than unfertilized eggs (9 of 14 and 10 of 12 viable ovules examined, respectively). In clones 2 and 3 the number of fertilized eggs either equalled or was less than the number of unfertilized eggs (5 of 10 and 2 of 5 viable ovules examined, respectively). No newly fertilized eggs were seen after June 13. Owens and Morris (1991) state that at the point of gamete fusion the cytoplasm of the perinuclear zone becomes neocytoplasm. This is due to the addition of the nucleoplasm from the male gamete and, eventually, a "cluster" of male organelles to the mitochondria rich

cytoplasm of the perinuclear zone.

#### 4.2.6 Proembryo Development

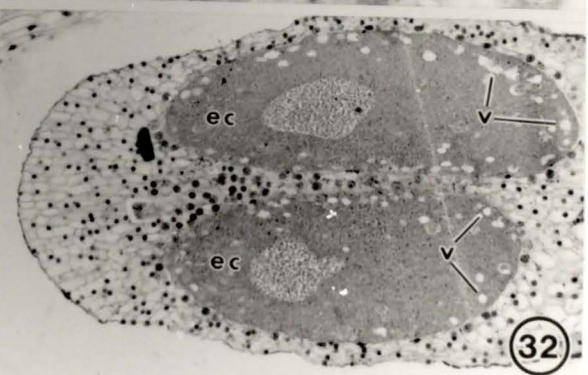
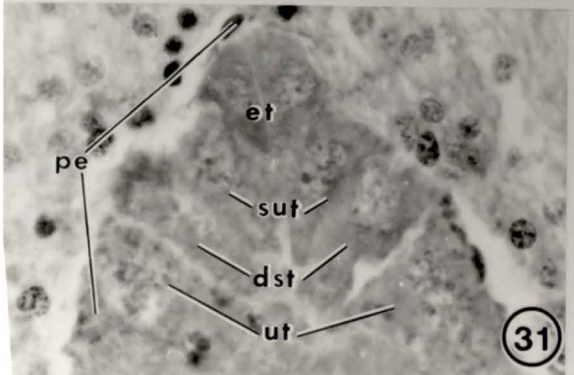
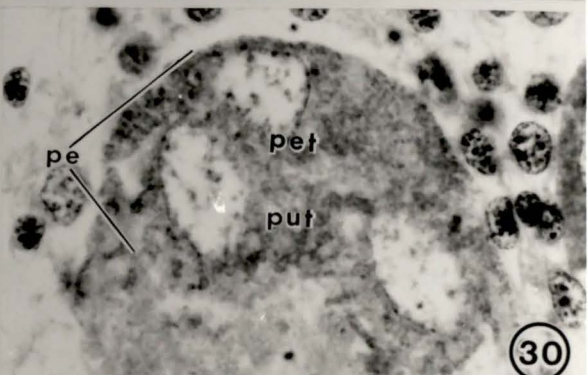
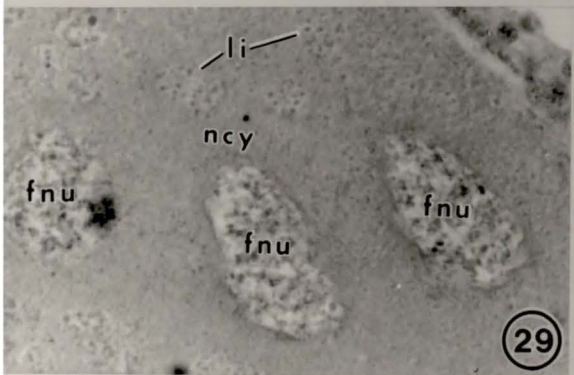
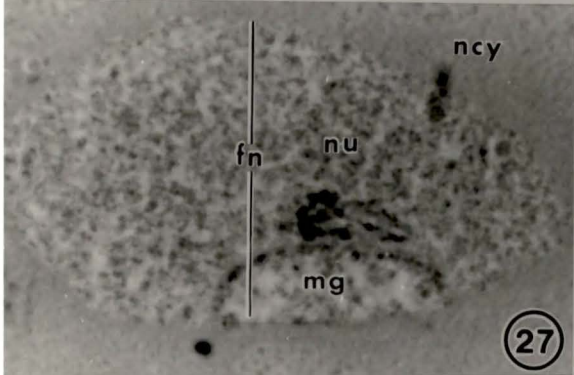
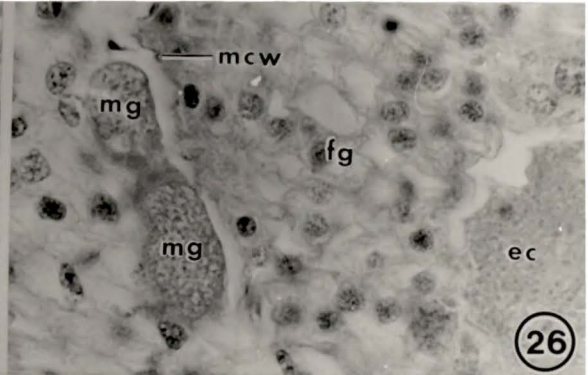
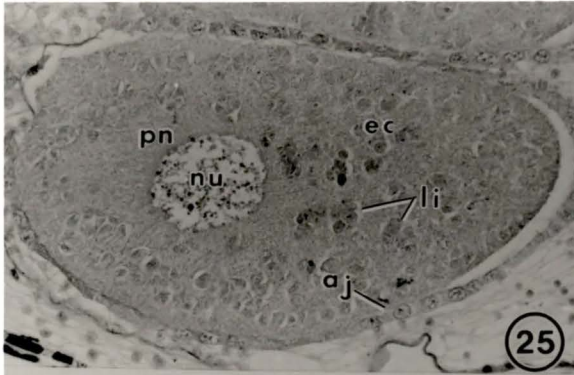
The membranes separating the male and female gametes within the fusion nucleus broke down and the nuclear material combined. The zygote nucleus then underwent two mitotic divisions (Fig. 28), forming four free nuclei (Fig. 29) which migrated, along with the surrounding neocytoplasm, to the chalazal end of the archegonium. Free nuclei appeared first in clone 4 on June 6 and by June 9 they were present in all clones at either the centre or the chalazal end of the archegonium. At the chalazal end the free nuclei formed a single tier. In *Pinus* it is at this time that the cluster of male organelles (primarily undifferentiated plastids and a few mitochondria) joined the neocytoplasm (Bruns and Owens, 1989). Nuclear division followed by cell-wall formation created two tiers of four cells each (Fig. 30). All cells in both tiers then divided and underwent cell-wall formation to form the mature proembryo with four tiers of four cells each (Fig. 31). From the chalazal end these were the embryonal, embryonal suspensor, dysfunctional suspensor and upper tiers. The first three tiers were comprised of individual cells while the cytoplasm of the last was continuous with the egg cell cytoplasm. Mature proembryos appeared by June 9 in clone 4 and by June 13 in clones 1, 2 and 3. However, not all the proembryos were mature by this date as one to four-tiered proembryos were present in all clones except number 4 where fertilized ovules contained embryos at the end of elongating suspensors.

#### 4.2.7 Degeneration of Unfertilized Eggs and Surrounding Female Gametophyte Tissue

Unfertilized eggs soon developed many small cytoplasmic vacuoles (Fig. 32) and no fertilized eggs with these vacuoles were seen. This stage was reached in

**Figs. 25-32**      **Second year ovules: early June to mid-June, 1989.**

- Fig. 25.**      Ovule from early June showing a mature, avacuolate, egg cell (ec) with the ec nucleus (nu) near the centre of the cell. The nu is surrounded by the perinuclear zone (pn) and an outer region of cytoplasm containing large inclusions (li). The entire ec is surrounded by the archegonial jacket (aj). x80.
- Fig. 26.**      Ovule from early June showing two male gametes (mg) lying just outside the megaspore cell wall (mcw). The ec is just visible in the lower right. Between the mcw and the ec are female gametophyte (fg) cells. x208.
- Fig. 27.**      Ovule from early June showing the fusion nucleus (fn) with two discrete gametes (mg and egg nu). The fn is surrounded by neocytoplasm (ncy). x315.
- Fig. 28.**      Ovule from early June showing zygote nucleus (znu) during first mitotic division. Chromosomes (chr) are visible near the metaphase plate. The znu is immediately surrounded by the ncy. x353.
- Fig. 29.**      Ovule from early June showing three of four free nuclei (fnu) produced by division of the znu and surrounding ncy, descending to the chalazal end of the ec. Outside the ncy are many li's. x200.
- Fig. 30.**      Ovule from mid-June showing a two-tiered proembryo (pe) composed of the primary embryonal tier (pet) and the primary upper tier (put). The nuclei of three of four cells are visible. x302.
- Fig. 31.**      Ovule from early June showing a mature, four-tiered, pe composed of the embryonal tier (et), suspensor tier (sut), dysfunctional suspensor tier (dst) and the upper tier (ut). The nuclei of most cells are just visible. x227.
- Fig. 32.**      Ovule from early June showing reappearance of small vacuoles (v), indicating that these unfertilized ec's are degenerating. x48.



clones 2 and 4 by June 13 and in clones 1 and 3 by June 16. These vacuoles enlarged and coalesced until the egg was reduced to an empty shell (Fig. 33). This occurred in unfertilized eggs of all clones by June 22. Cellular degeneration followed in the surrounding female gametophyte tissue causing these cells to lose their cytoplasm and shrivel. This resulted in the collapse of the micropylar end of the female gametophyte by about August 25 (Fig. 40).

#### 4.2.8 Embryo Development

The change from proembryo to embryo occurred when the embryonal tier was forced through the archegonial jacket into the enlarging corrosion cavity by the elongating embryonal suspensor tier (Fig. 34). This began by June 9 in clone 4 and in all others by June 13. Initially the suspensor consisted of only the elongating embryonal suspensor cells. Prior to cleavage these were supplemented by cells that originated by division of the embryonal unit forming the secondary embryonal suspensors (Singh, 1978). Cleavage was not directly observed, but observations of pre- and post-cleavage stages suggest that it happened between June 16 and 22. Multiple embryos present within the ovules prior to June 16 were each comprised of four files of cells, indicating that they were from separate fertilizations (Fig. 35). Within a female gametophyte multiple post-cleavage embryos could be identified because they consisted of only a single file of cells (Fig. 36). Prior to cleavage the only embryo enlargement was due to the formation of the embryonal units. Post-cleavage embryos underwent cell division and enlarged slowly at first but when one attained a dominant position at the distal end of the corrosion cavity (Fig. 33) it began more rapid growth. Dominant embryos were evident on June 30 in clones 3

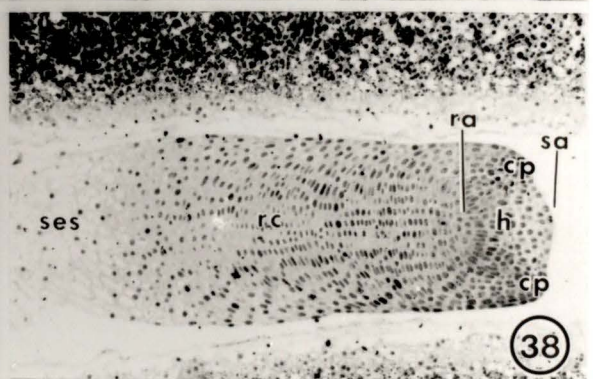
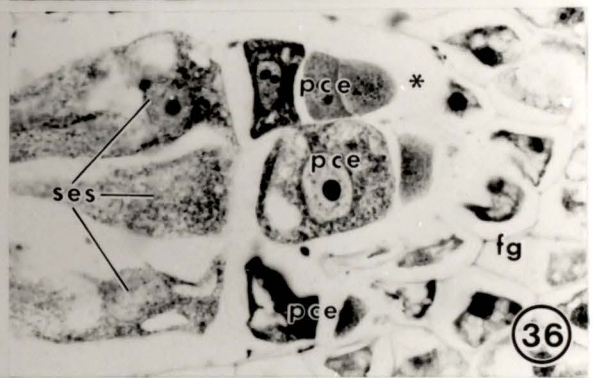
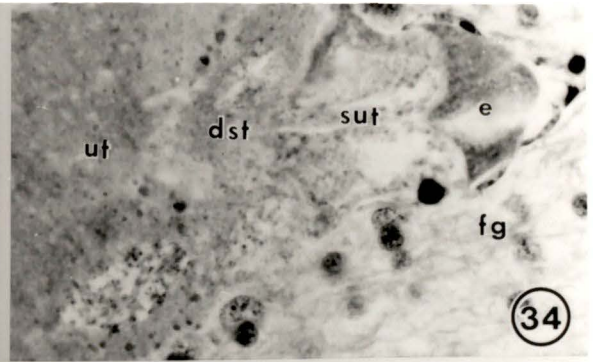
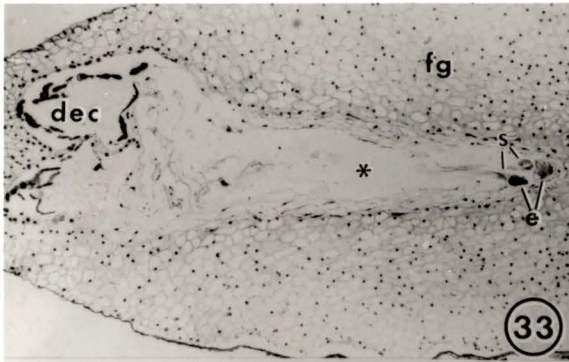
and 4, and July 14 in clones 1 and 2. These were located in corrosion cavities that were nearly 60% the length of the female gametophyte. Subordinate embryos ceased to enlarge and by July 28 they were no longer present in the corrosion cavity. By July 28 the corrosion cavities had enlarged to between 75% and 80% the length of the female gametophyte. As the dominant embryo was forced deeper into the female gametophyte by its elongating secondary embryonal suspensor, it took on a globular appearance due to active cell division within the embryo (Fig. 37). Every clone had a few globular embryos on July 14. Tissue organization was not apparent until July 28 when many of the embryos had reached the large globular stage at which time a large root cap, a root apex, a very short hypocotyl, a shoot apex and often several small cotyledon primordia were recognizable (Fig. 38). By August 11 the embryos had enlarged and grown further into the female gametophyte. They consisted of a root cap (half the embryo's length), root apex, short hypocotyl, a large dome-shaped shoot apex and several cotyledons (Fig. 39).

By August 25 embryo development was essentially complete. The embryos occupied the entire length of the corrosion cavity (which was up to 90% the length of the female gametophyte on August 11) and were divided into thirds: the cotyledons and shoot apex; the hypocotyl; and, the root apex and rootcap (Fig. 40). If any change occurred in structure between this collection and the final one made on September 8, it involved only a slight enlargement of the cotyledons in some of the slower developing embryos.

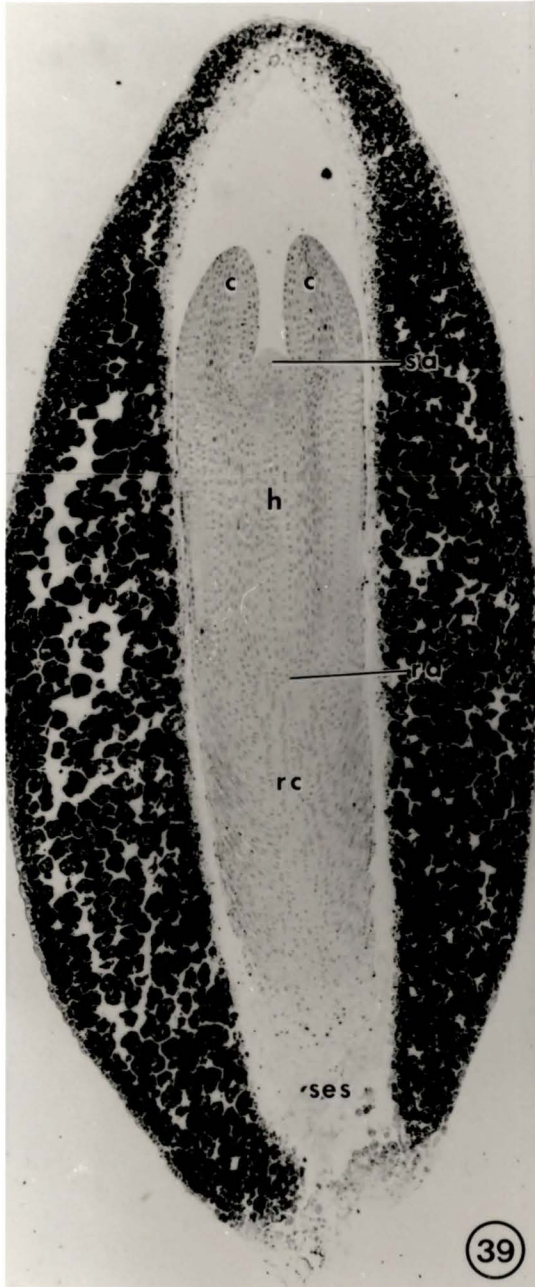
#### **4.3 Mean Number of Ovuliferous Scales per Cone**

The mean number of ovuliferous scales per cone was 139.55, 149.70, 151.25, 155.45 and 171.55 in clones 5, 4, 3, 1 and 2, respectively (Table 1, Fig. 41). A one-way Anova of the mean number of ovuliferous scales showed a significant

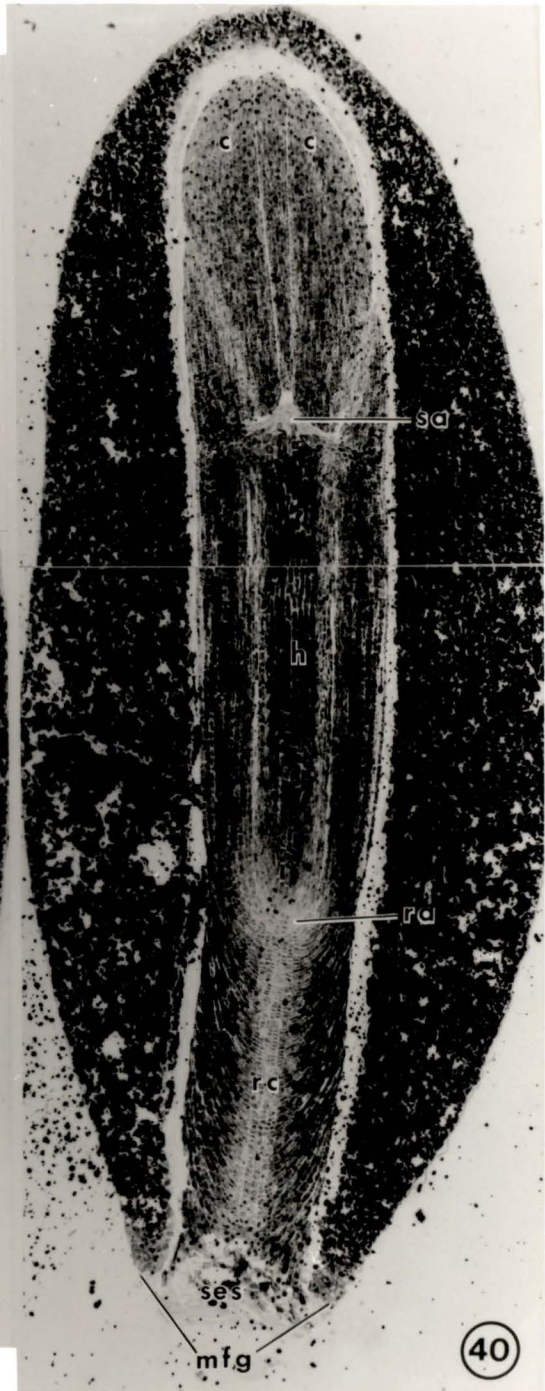
- Figs. 33-38**      **Second year ovules: mid-June to late July, 1989.**
- Fig. 33.**      Ovule from mid-June showing female gametophyte (fg) with a completely degenerated egg cell (dec) and dominant and subdominant embryos (e) at the end of their suspensors (s) near the apex of the corrosion cavity (\*). x24.
- Fig. 34.**      Ovule from mid-June showing an e being pushed, by the elongating suspensor tier (sut) of the proembryo, into the fg. Just visible are the proembryo's dysfunctional suspensor tier (dst) and upper tier (ut). x239.
- Fig. 35.**      Ovule from late June showing dominant (de) and subdominant (se) pre-cleavage embryos, at the end of their secondary embryonal suspensors (ses), near the apex of the corrosion cavity (\*). x160.
- Fig. 36.**      Ovule from mid-June showing three post-cleavage embryos (pce), at the end of their ses, at the apex of the corrosion cavity (\*). x176.
- Fig. 37.**      Ovule from mid-July showing a globular e, with no obvious tissue organization, near the apex of the corrosion cavity (\*). Also visible is the ses. x90.
- Fig. 38.**      Ovule from late July showing a e with distinct tissues; cotyledon primordia (cp), shoot apex (sa), hypocotyl (h), root apex (ra) and root cap (rc). The ses is also visible. x40.



- Figs. 39-40**      **Second year ovules: late August to early September, 1989.**
- Fig. 39.**        Ovule from late August showing an e with small cotyledons (c), sa, h, ra and rc and crushed ses. x12.6.
- Fig. 40.**        Ovule from early September showing an anatomically mature e with large c's, sa, h, ra, rc and crushed ses as well as a collapsed micropylar end of the fg (mfg). x12.6.



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**TABLE 1: RESULTS OF A ONE-WAY ANOVA AND FISHER PROTECTED LEAST SIGNIFICANT DIFFERENCE TEST: clones are ordered by increasing mean value and standard error (SE) terms; as indicated by the FPLSD test. Populations to which the clones belong are indicated by a horizontal dashed line.**

MEAN NUMBER OF DISTAL STERILE SCALES PER CONE PER CLONE.

	CLONE 5	CLONE 2	CLONE 3	CLONE 1	CLONE 4
mean	2.95	3.90	4.30	4.55	4.70
SE	0.20	0.19	0.23	0.32	0.18

MEAN NUMBER OF DISTAL RUDIMENTARY SCALES PER CONE PER CLONE

	CLONE 1	CLONE 2	CLONE 5	CLONE 3	CLONE 4
mean	1.20	1.45	1.90	1.95	3.10
SE	0.27	0.14	0.12	0.21	0.45

MEAN NUMBER OF FERTILE SCALES PER CONE PER CLONE

	CLONE 4	CLONE 5	CLONE 3	CLONE 1	CLONE 2
mean	80.70	85.10	89.95	93.15	96.50
SE	1.44	1.67	1.66	1.88	0.96

MEAN NUMBER OF PROXIMAL RUDIMENTARY SCALES PER CONE PER CLONE

	CLONE 5	CLONE 1	CLONE 4	CLONE 3	CLONE 2
mean	27.35	41.20	44.20	45.70	52.35
SE	0.96	1.50	0.95	1.88	1.46

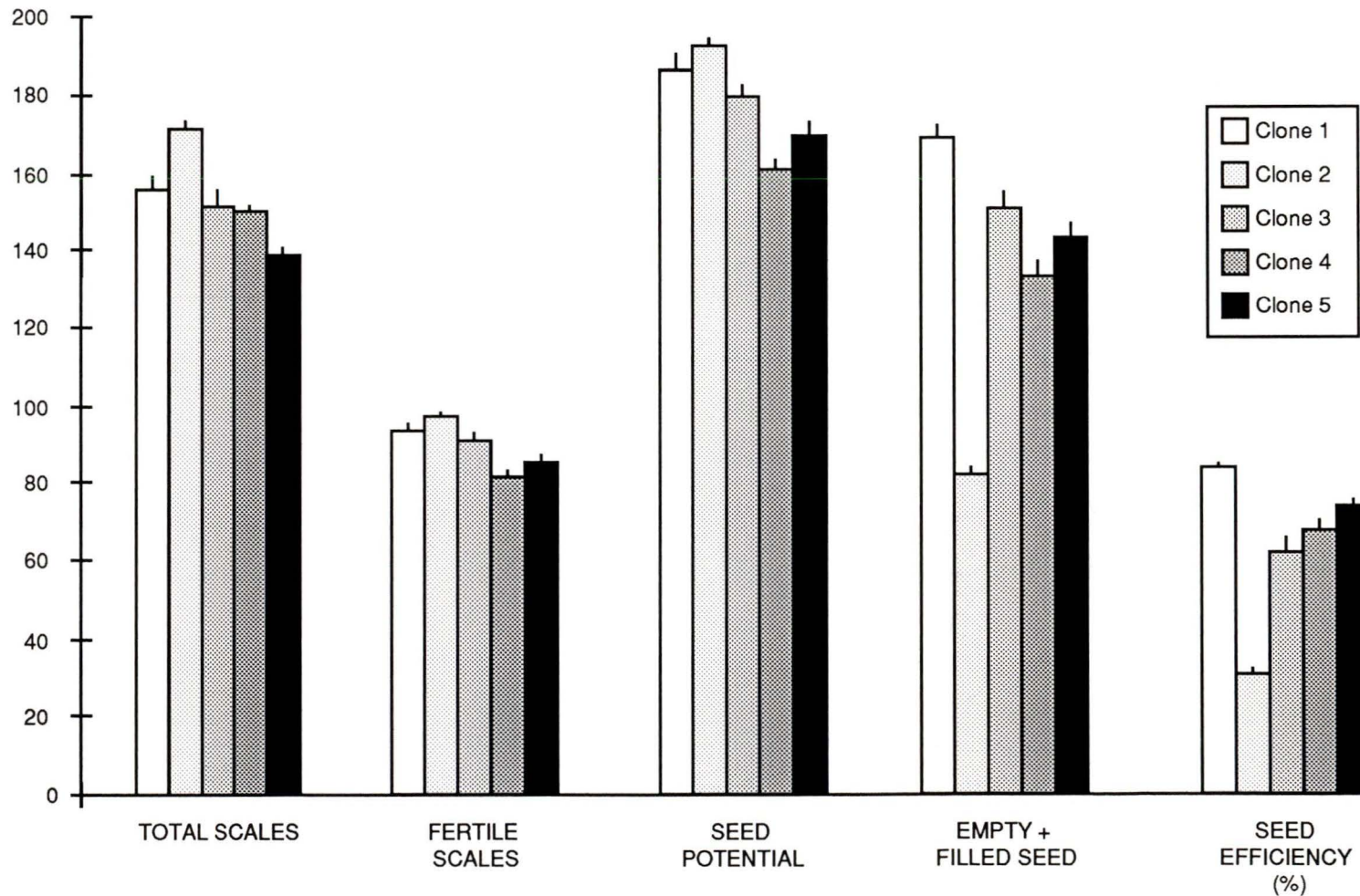
MEAN NUMBER OF PROXIMAL STERILE SCALES PER CONE PER CLONE

	CLONE 1	CLONE 3	CLONE 4	CLONE 2	CLONE 5
mean	15.35	15.60	17.00	17.35	21.95
SE	0.61	0.55	0.29	0.57	0.43

MEAN TOTAL NUMBER OF OVULIFEROUS SCALES PER CONE PER CLONE

	CLONE 5	CLONE 4	CLONE 3	CLONE 1	CLONE 2
mean	138.55	149.70	151.25	155.45	171.55
SE	2.08	1.54	4.47	3.05	1.58

Figure 41. Interclonal variation in the second-year-cone and seed performance of control pollinated coastal loblolly pine growing in South Carolina during 1989. Note: Total Scales = ovuliferous scales per cone; Fertile Scales = scales bearing fertile ovules; Seed Potential = the number of fertile scales per cone x 2; Empty + Filled Seed = total seeds per cone; Seed Efficiency = realized seed potential (%). Shown are the means  $\pm$  SE (n=20).



difference between the clones ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test separated the clones into three populations. Clones 5 and 2 made up two uni-clonal populations while clones 4, 3 and 1 made up a single tri-clonal population (Table 1).

#### **4.4 Ovuliferous Scale Types**

Each seed cone was divided into five regions based on the degree of ovule development. At the distal end there is an area of sterile scales which did not possess recognizable ovules. Below this is a region of distal rudimentary scales where ovules will not develop sufficiently to produce viable seed. Fertile scales bear ovules which develop fully and may produce mature seed. These scales are more numerous than any other group and occupy the mid-portion of the cone. Scales at the proximal end of the cone are partitioned in the same manner as those located distally. Immediately proximal to the fertile scales are a group bearing only rudimentary ovules. As with the distal rudimentary scales these ovules will not produce viable seed. Because they lack recognizable ovules the most proximal scales on a cone are sterile. The number of ovuliferous scales within the five regions can vary significantly between clones.

The mean number of distal sterile scales per cone was 2.95, 3.90, 4.30, 4.55 and 4.70 in clones 5, 2, 3, 1 and 4, respectively (Table 1). A one-way Anova indicated the presence of significant differences in the number of distal sterile scales between clones ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test indicated that the five clones came from three separate populations, two of which overlapped. Clone 5 stood as a uni-clonal population whereas clones 2, 3 and 1, and clones 3, 1 and 4 formed two tri-clonal Fig. 41 populations (Table 1).

The mean number of distal rudimentary scales per cone was 1.20, 1.45, 1.90,

1.95 and 3.10 in clones 1, 2, 5, 3 and 4, respectively (Table 1). Analysis by one-way Anova confirmed the presence of a significant difference between the mean number of distal rudimentary scales in each of the five clones ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test indicated that the five clones made up two distinct populations. The largest of these contained clones 1, 2, 3 and 5 whereas clone 4 made up a uni-clonal population (Table 1).

The mean number of fertile scales per cone was 80.70, 85.10, 89.95, 93.15 and 96.50 in clones 4, 5, 3, 1 and 2, respectively (Table 1, Fig. 41). The one-way Anova confirmed that there was a significant difference between clones with respect to the mean number of fertile scales ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test indicated that the clones were divided into four populations. Clones 4 and 5 made up two uni-clonal populations whereas clones 1 and 3, and 1 and 2 formed two bi-clonal populations (Table 1).

The mean number of proximal rudimentary scales per cone was 27.35, 41.20, 44.25, 45.70 and 52.35 in clones 5, 1, 4, 3 and 2, respectively (Table 1). The one-way Anova showed a significant clonal difference in the mean number of proximal rudimentary scales ( $P=0.0001$ ). When the Fisher Protected Least Significant Difference test was applied it indicated that the clones were partitioned into three populations. Two uni-clonal populations contained clones 2 and 5, and a tri-clonal population consisted of clones 1, 3 and 4 (Table 1).

The mean number of proximal sterile scales per cone was 15.35, 15.60, 16.50, 17.35 and 21.95 in clones 1, 3, 4, 2 and 5, respectively (Table 1). The one-way Anova showed significant differences in the mean number of proximal sterile scales per clone ( $P=0.0001$ ). When the Fisher Protected Least Significant Difference test was employed it indicated that three populations were present amongst the clones. The two bi-clonal populations were made from clones 1 and 3, and 2 and 4, whereas

clone 5 made up the uni-clonal population (Table 1).

#### **4.5 Clonal Comparison of Seed Production, Potential and Efficiency**

The mean number of empty seed per cone was 11.80, 18.25, 20.80, 25.95 and 52.40 in clones 1, 5, 2, 4 and 3, respectively (Table 2). A one-way Anova showed a significant difference in the mean number of empty seed produced by each of the five clones ( $P=0.0001$ ). Three separate populations were identified by the Fisher Protected Least Significant Difference test. Clone 1 and 3 made up two uni-clonal populations whereas the tri-clonal population contained clones 2, 4 and 5 (Table 2).

The mean number of filled seed per cone was 60.60, 107.65, 110.25, 125.65 and 157.05 in clones 2, 4, 3, 5 and 1, respectively (Table 2). Analysis by one-way Anova showed significant differences between clones in the mean number of filled seed produced ( $p=0.0001$ ). The Fisher Protected Least Significant Difference test ascertained that the five clones were distributed into four populations. Three uni-clonal populations were made of clones 1, 2 and 5, whereas a bi-clonal population consisted of clones 3 and 4 (Table 2).

The mean number of empty plus filled seed per cone represents the total number of seed produced by each clone on a cone. This was 81.60, 133.60 143.90, 159.15 and 168.85 in clones 2, 4, 5, 3 and 1, respectively (Table 2, Fig. 41). A one-way Anova showed significant differences in the mean number of empty plus filled seed produced by each of the five clones ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test showed that the five clones were distributed in three populations. A uni-clonal population contained clone 2 whereas clones 4 and 5, and 1 and 3 made up two bi-clonal populations (Table 2).

The mean seed potential was 161.40, 170.20, 179.90, 186.20 and 193.00 seeds

**TABLE 2: RESULTS OF A ONE-WAY ANOVA AND FISHER PROTECTED LEAST SIGNIFICANT DIFFERENCE TEST:** clones are ordered by increasing mean value and standard error (SE) terms; as indicated by the FPLSD test. Populations to which the clones belong are indicated by a horizontal dashed line. When the Anova indicated no significant difference between the means of all five clones (ie. a single population), the FPLSD test was not invoked.

MEAN NUMBER OF EMPTY SEED PER CONE PER CLONE

	CLONE 1	CLONE 5	CLONE 2	CLONE 4	CLONE 3
mean	11.80	18.25	20.80	25.95	52.40
SE	1.15	1.75	1.85	3.40	8.08

MEAN NUMBER OF FILLED SEED PER CONE PER CLONE

	CLONE 2	CLONE 4	CLONE 3	CLONE 5	CLONE 1
mean	60.60	107.65	110.25	125.65	157.05
SE	2.44	4.08	8.30	4.09	3.52

MEAN NUMBER OF EMPTY PLUS FILLED SEED PER CONE PER CLONE

	CLONE 2	CLONE 4	CLONE 5	CLONE 3	CLONE 1
mean	81.40	133.60	143.90	159.15	168.85
SE	2.22	3.68	3.74	4.83	3.62

MEAN SEED POTENTIAL PER CONE PER CLONE

	CLONE 4	CLONE 5	CLONE 3	CLONE 1	CLONE 2
mean	161.40	170.20	179.90	186.20	193.00
SE	2.80	3.33	3.31	3.77	1.92

MEAN SEED EFFICIENCY (%) PER CONE PER CLONE

	CLONE 2	CLONE 3	CLONE 4	CLONE 5	CLONE 1
mean	31.60	61.75	66.85	73.90	84.40
SE	1.39	4.50	2.60	1.87	1.15

MEAN PERCENTAGE OF SEED GERMINATION PER CLONE

	CLONE 5	CLONE 4	CLONE 2	CLONE 1	CLONE 3
mean	96.00	96.50	97.25	98.50	98.75
SE	0.76	1.24	0.75	0.82	0.65

MEAN PERCENTAGE OF UNGERMINATED SEEDS PER CLONE

	CLONE 3	CLONE 1	CLONE 2	CLONE 4	CLONE 5
mean	1.25	1.50	2.75	3.50	4.00
SE	0.65	0.82	0.75	1.24	0.76

NOTE: MEAN NUMBER OF EMPTY SEED includes half and flat half-size seed.

per cone in clones 4, 5, 3, 1 and 2, respectively (Table 2, Fig. 41). The one-way Anova showed a significant difference in the mean seed potential of the five clones ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test showed that the five clones were separated into four populations. Two uni-clonal populations were made of clones 4 and 5 whereas two bi-clonal populations were made of clones 1 and 3, and 1 and 2 (Table 2).

The mean seed efficiency per cone was 31.60%, 61.75%, 66.85%, 73.90% and 84.40% in clones 2, 3, 4, 5 and 1, respectively (Table 2, Fig. 41). A one-way Anova showed significant differences in the mean seed efficiency of the five clones ( $P=0.0001$ ). The Fisher Protected Least Significant Difference test showed that the clones were separated into four populations. Two uni-clonal populations were made up of clones 1 and 2, and two bi-clonal populations were made up of clones 3 and 4, and 4 and 5 (Table 2).

#### **4.6 Seed Germination**

Of the original 400 seeds the number that germinated and the mean seed germination percentage were 384 (96.00%), 387 (96.50%), 389 (97.25%), 393 (98.50%) and 395 (98.75%) in clones 5, 4, 2, 1 and 3, respectively (Table 2). A one-way Anova showed that there was no significant difference in the number of germinated seeds in the five clones ( $P=0.1249$ ). Because it was assumed that all five clones belonged to the same population, it was not necessary to use the Fisher Protected Least Significant Difference test. All five clones recorded their first germinants on day 7 of the test. The maximum mean germination rate for each clone occurred on day 11, after which there was a rapid decline so that by day 12 in no clone was it greater than 3.50 seeds per box. By day 13 the mean seed germination rate had decreased to less than 1.29 seeds per box. A further decrease

was noticed on day 14, at which time the seed germination rate was only 0.40 seeds per box. For the remainder of the test, the mean seed germination rate fluctuated between 0.00 and 0.75 seeds per box (Fig. 42). The seed germination test was run for 22, 25, 28 and 29 days in clones 3, 1 and 4, 2 and 5, respectively (Fig. 42). The test was terminated when no new germinants had been recorded for seven days.

After termination of the seed germination test 52, or 2.60%, of the 2000 seeds in the test remained ungerminated. Dissection revealed that between the five clones there were 42 inviable seeds, four sound seeds, four empty seeds, one abnormal seed and one infected seed. By clone, the total number of ungerminated seeds and the percentage they represent were as follows: clone 3, 5 seeds (1.25%); clone 1, 7 seeds (1.50%); clone 2, 11 seeds (2.75%); clone 4, 13 seeds (3.50%) and clone 5, 16 seeds (4.00%) (Tables 2 and 3). A one-way Anova showed that there was no significant difference between the clones in the percentage of ungerminated seeds ( $P=0.1249$ ). Because of this the Fisher Protected Least Significant Difference test was not required and it was assumed that all five clones belonged to the same population (Table 2).

Figure 42. Mean germinant count ( $\pm$  SE) of control pollinated coastal loblolly pine following 28 days of stratification at 4 C. Note: n = 8.

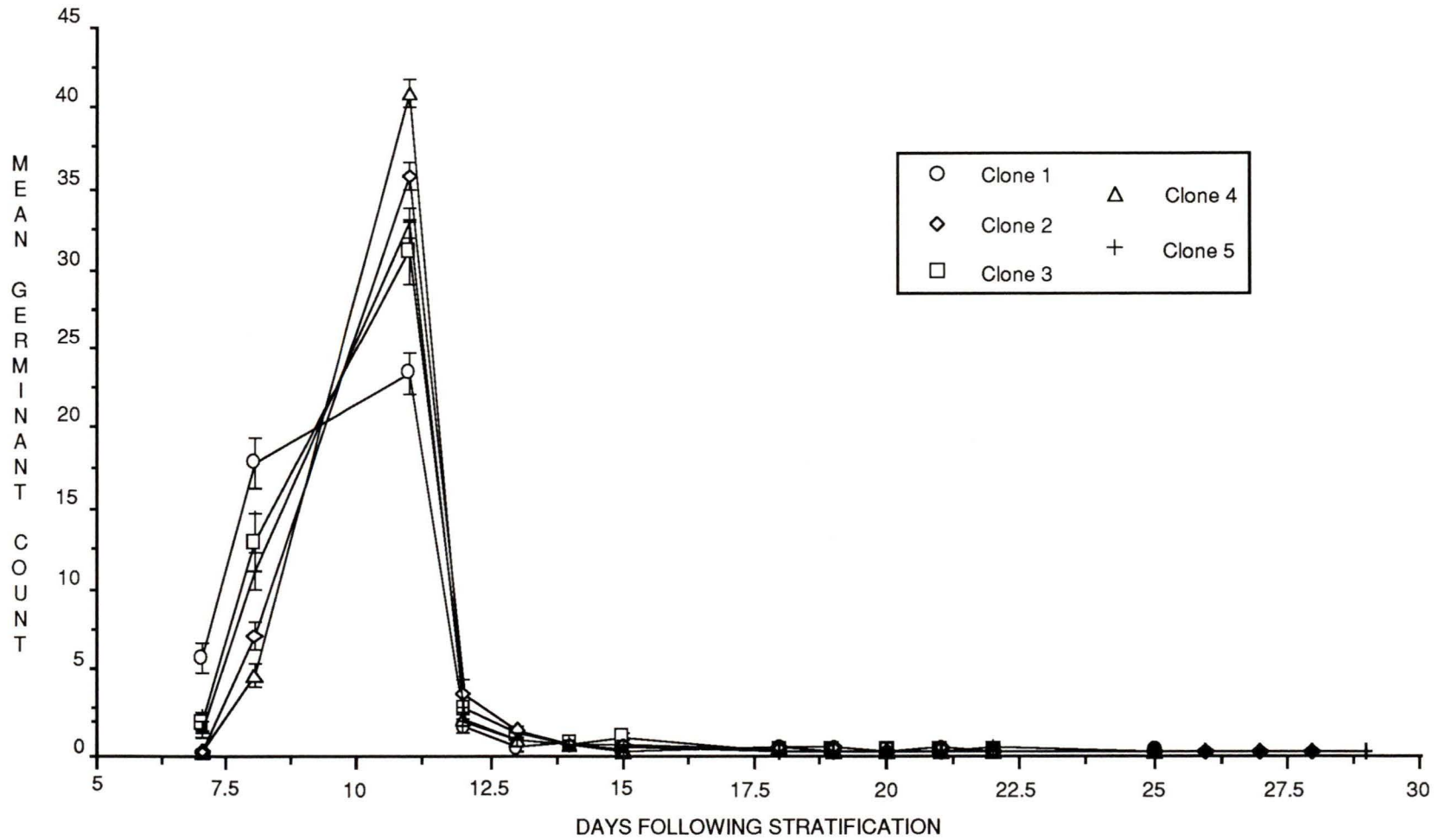


TABLE 3: NUMBER AND INTERNAL CONDITION OF UNGERMINATED SEEDS, BY CLONE.

	CLONE 1	CLONE 2	CLONE 3	CLONE 4	CLONE 5	$\Sigma$
INTERNAL CONDITION						
DEAD	5.00	6.00	4.00	11.00	16.00	42.00
FRESH	2.00	0.00	0.00	2.00	0.00	4.00
EMPTY	0.00	3.00	1.00	0.00	0.00	4.00
ABNORMAL	0.00	1.00	0.00	0.00	0.00	1.00
INFECTED	0.00	1.00	0.00	0.00	0.00	1.00
$\Sigma$ UNGERMINATED SEEDS	7.00	11.00	5.00	13.00	16.00	$\Sigma\Sigma$ 52.00

## **CHAPTER 5**

### **DISCUSSION**

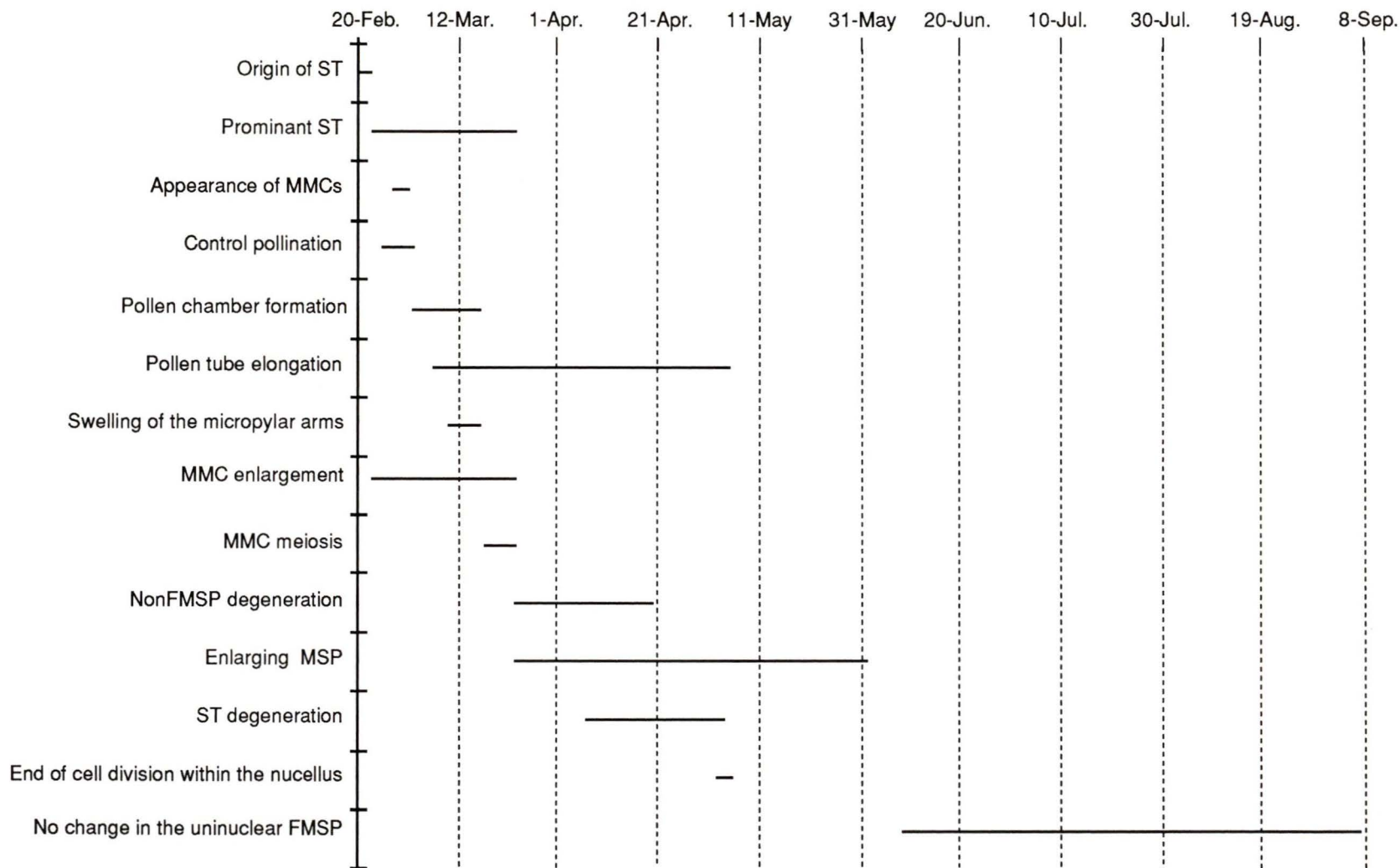
Mitotic activity within the ovules of the first year conelet's resumed prior to the first collection on February 23, 1989. Without knowing when cell division resumed, degree-days could not be used to "time" significant events of the reproductive cycle (Sarvas, 1968). For this reason stages in the reproductive cycle are discussed by the dates, or interval on or during which they occurred.

#### **5.1 Development of the First Year Conelets**

The duration of the major events in the development of first year loblolly pine conelets, from the four clones used in this study, are summarized in Figure 43.

The first recognizable tissue inside the post-dormancy ovule was the sporogenous tissue. Appearing shortly after, and at the centre of the sporogenous tissue, was a megaspore mother cell, which was distinguished from the sporogenous tissue by its slightly larger size and lighter staining, more granular, cytoplasm. The sporogenous tissue increased in prominence as the megaspore mother cell enlarged and underwent meiotic division. Two meiotic divisions (identified as MMC meiosis in Fig. 43) produced a linear tetrad of four megaspores. At this time the sporogenous tissue was most prominent, consisting of several concentric layers of slightly enlarged cells with dense cytoplasm. This pattern of development was consistent throughout the five clones examined. Comparing the pattern of sporogenous tissue development in loblolly pine to other pines, or other conifers, is difficult because few researchers offer a detailed description. Lill (1976) did not consider sporogenous tissue development separately from tapetal tissue development, therefore, no direct comparison can be made. Singh and Owens

Figure 43. Megasporangial development of orchard grown, control pollinated, first year conelets from coastal loblolly pine growing in South Carolina, in 1989.



Abbreviations: ST, sporogenous tissue; MMC, megaspore mother cell; MSP, megaspore; NonFMSP, nonfunctional megaspore; FMSP, functional megaspore.

(1981b) described sporogenous tissue and tapetal tissue development in *Abies lasiocarpa*, where the former tissue increased in prominence through megaspore formation, as it did in *P. taeda*.

The nucellus of the first year conelets showed active mitotic division from February 23 to May 5 (Fig. 43), allowing it to attain maximum size by the end of this period. The tip of the nucellus began to degenerate (March 3) forming the pollen chamber and pollination drop. The process of pollination drop formation was not followed carefully on *P. taeda* but appeared similar to that in *Picea engelmannii* (Owens *et al.* 1987). In that species, before pollination drop secretion, the cells at the base of the nucellus enlarged and their cytoplasm became restricted to a parietal layer surrounding a central vacuole. Degeneration began in the outermost nucellar cells and progressed inward deepening the pollen chamber. The secreted material apparently formed the pollination drop. In this study, the appearance of the loblolly pine nucellus after pollination drop secretion was very similar to that described by Owens *et al.* (1981) for *P. contorta* and by Brown and Bridgewater (1987) for *P. taeda*. In *P. taeda* pollen tube elongation began soon after the pollen grain settled into the pollen chamber and continued until May 5, at which time pollen tubes had penetrated approximately one-half the length of the nucellar tip.

Pollen was applied to the conelets between February 25 and March 4 when they appeared to be at, or near, maximum receptivity (late stage 4 or stage 5 as defined by Bramlett and O'Gwynn, 1980). The end of receptivity was signaled (microscopically) by a swelling at the base of the micropylar arms. This swelling sealed off the micropyle preventing pollen from entering or exiting the pollen chamber. It was not determined whether this swelling occurred before or after the cone's external morphology signaled the end of receptivity. If it did occur before the shift from stage 5 late (declining receptivity) to stage 6 (no longer receptive)

(Bramlett and O'Gwynn, 1980) then the actual period of receptivity is shorter than it appears using Bramlett and O'Gwynn's system. If it occurred after the change in external morphology then it is of little consequence since the swollen ovuliferous scales would prevent further entry of pollen. Determining the length of the receptive period in pines is more complicated than just assessing when pollen chamber formation occurs. This is because (in early March) the micropylar arms and lip of the micropyle secrete small droplets to which the pollen adheres prior to pollination drop formation. The presence of these droplets was first described by Owens *et al.* in 1981 and their presence in *P. taeda* was confirmed when stage 4 conelets were observed under a dissecting microscope.

The megaspores in *P. taeda* were arranged in a linear tetrad, which is the most common megaspore arrangement in conifers (Singh, 1978). The three megaspores closest to the micropyle were non-functional and degenerated (Fig. 43). One month after degeneration began they were no longer visible due to disruption by the enlarging functional megaspore. The period of (uninuclear) functional megaspore enlargement lasted about 10 weeks, after which it was many times larger than any of the other cells in the ovule. No further change in the internal condition of the functional megaspore was noticed between June 2 and the final collection in September. This delayed the onset of free nuclear division within the megaspore cell wall until the start of the second growing season. This differentiates ovule development in *P. taeda* from other temperate and subtropical species of pine. This result could be due to the long period of favorable growing conditions (compared to what more northerly temperate pines have to endure) present in coastal South Carolina, so that, while the production of a mature seed fits comfortably into two growing seasons, there is no benefit to the initiation of free nuclear division prior to the dormant period separating the two growing seasons. The combination of a delay

in the onset of free nuclear division and the long growing season may divert nutrients to vegetative growth allowing multiple flushes (three were observed in 1989) to occur. It would be of interest to determine whether this pattern of free nuclear division and multiple vegetative flushes is consistent for the species or specific to coastal *P. taeda*.

The 19 months necessary to produce a mature seed, in *P. taeda*, is similar to most other temperate, subtropical and tropical pines. For example: 14 months in *P. sylvestris*, (Sarvas, 1962) and *P. contorta*, (Owens *et al.*, 1982); 16 months in *P. wallichiana*, (Konar and Ramchandani, 1958) and *P. banksiana*, (Cesich and Bauer, 1987); 17 months in *P. roxburgii*, (Konar, 1960); 18 months in *P. palustris*, (Mathews, 1932) and *P. monticola*, (Owens and Molder, 1977); 20 months in *P. echinata*, (Schopmeyer, 1974), *P. radiata*, (Lill, 1976) and *P. caribaeae*, (D.L.S. Harrison, University of Victoria, *personal communication*) and 21 months in *P. elliotii*, (Schopmeyer, 1974).

Enlargement of the functional megaspore coincided with degeneration of the inner sporogenous tissue cells and an increase in volume and decrease in cytoplasmic density of the outer sporogenous tissue cells. Previous authors (Ferguson, 1904; Thomas, 1951; Lill 1976) reported that degeneration began earlier, soon after megaspore formation. While these descriptions were not consistent in every detail, similarities in the process of degeneration outweighed the discrepancies. In all cases degeneration involved a flattening of the inner sporogenous tissue cells and enlargement of the outer sporogenous tissue cells that were closely opposed to a thin layer of flattened nucellar cells.

## **5.2 Development of the Second Year Conelets and Cones**

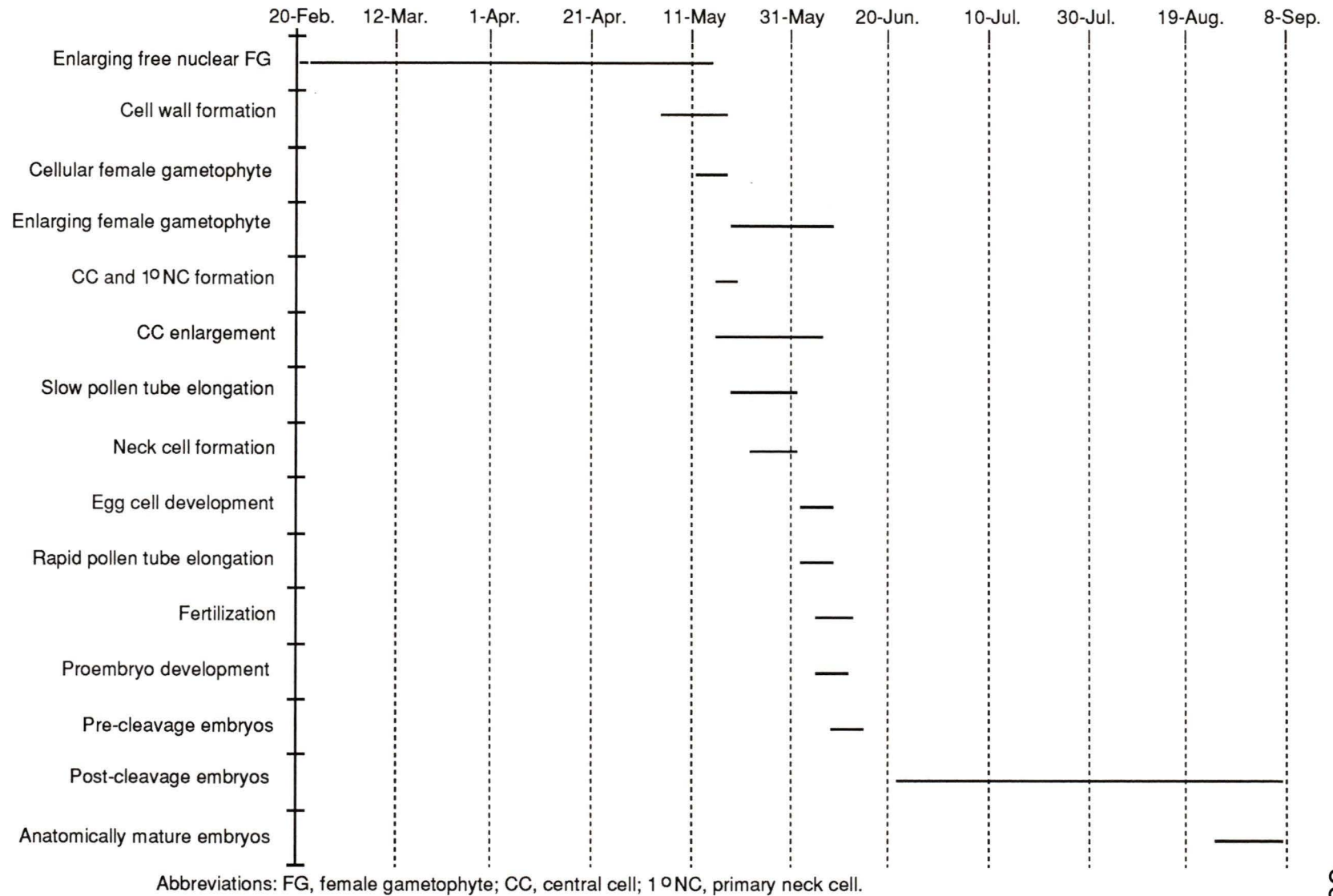
The duration of major events occurring within the conelets and cones of the

four loblolly pine clones used in this study, during the second full growing season, are summarized in Figure 44.

Several free nuclei were present inside the megaspore cell wall on February 23, (Fig. 44) indicating that mitotic activity had begun in the functional megaspore prior to this harvest. Free nuclear division continued in all four clones until May 16. Before April 7 the free nuclei were located throughout the female gametophyte's cytoplasm. After April 7 they formed a mono-layer, in the peripheral cytoplasm of the megaspore cell wall, surrounding a large central vacuole. It was from this peripheral cytoplasm that cell wall formation began, completely eliminating the central vacuole. Singh (1978) reviewed the process of cell wall formation in a number of conifers and the results of this study do not provide any reason to dispute his description. Unlike other pines studied thus far, free nuclear division in *P. taeda* takes place over, approximately, 10 weeks during the second growing season, making this stage of ovule development shorter than in other species of pine. Examples of the period of free nuclear division (including dormancy) in other temperate pines are as follows: 7 1/2 months in *P. virginiana*, (Thomas, 1951); 9 months in *P. strobus*, (Ferguson, 1904), *P. radiata*, (Lill, 1976), *P. monticola* (Owens and Molder, 1977) and *P. contorta*, (Owens *et al.*, 1981, 1982); and 12 months in *P. longifolia*, (Sethi, 1928).

Tapetal cells increased in number and prominence from March 10 until April 21. This increase coincided with the middle six weeks of observed free nuclear division within the female gametophyte. As cell wall formation began (May 5) so did tapetal tissue degeneration and this continued for about one month until the female gametophyte was mature. At this point no evidence of the tapetal tissue remained. It is speculated that tapetal tissue cells are derived from sporogenous tissue adjacent to the enlarging female gametophyte (Singh, 1978). Both Lill (1976)

Figure 44. Megasporangial development of orchard grown, control pollinated, second year conelets and cones from coastal loblolly pine growing in South Carolina, in 1989.



and Owens *et al.* (1982) reported that, at the height of development, the tapetal tissue in *P. radiata* and *P. contorta* was made up of bi-nucleate cells. While bi-nucleate cells were not observed in *P. taeda* it is assumed that they were present. In addition to being multi-nucleate tapetal tissue cells can be polyploid. In *Pinus* 8n (or even 16n) nuclei have been observed (Singh, 1978). The multi-nucleate, polyploid nature of the tapetal tissue cells may be indicative of a nutritive function within the developing ovule, because when the cells break down they would release more nuclear material into the fluid surrounding the megaspore cell wall. Lill (1976) suggested that these substances could then be adsorbed by the megaspore cell wall due to the pilloid nature of its outer layer.

One to four (but most commonly two or three) archeogonial initials, were present in the cellular female gametophyte from May 16 to 19 (Fig. 44). This compares favorably with other species that have been examined in this regard, i.e. 1 to 3 in *P. sylvestris* (Sarvas, 1962) and *P. siberica* (Nekrasova, 1970); 1 to 4 in *P. radiata* (Lill, 1976); 1 to 5 in *P. strobus*, *P. resinosa* and *P. rigida*, (Ferguson, 1904); 2 to 4 in *P. contorta* (Owens *et al.*, 1982); 2 to 7 in *P. virginiana* (Thomas, 1951); 3 in *P. monophylla* (Haupt, 1941); 3 to 5 in *P. monticola* (Owens and Molder, 1977) and 5 in *P. lambertiana* (Haupt, 1941).

With the nucleus always located at the micropylar end of the cell, slightly enlarged archeogonial initials underwent unequal division producing a large central cell and a small primary neck cell. Enlargement of the central cells began immediately and continued for about one month. The short interval over which central cell and primary neck cell formation took place indicates a high degree of synchronicity between all clones at this stage of ovule development. As the central cell enlarged its nucleus remained at the micropylar end of the cell and the cytoplasmic density changed from primarily vacuolate to avacuolate with many large

and small inclusions. Owens and Morris (1990) found in *Pseudotsuga menziesii* that large inclusions were formed by cytoplasmic inpocketing that caused considerable enlargement and modification of the plastid rendering these nonfunctional. Large inclusions then, are part of the process of cytoplasmic inheritance, and allow the plastid genome to be paternal in origin (Neale and Sederoff, 1989). While not seen in this study, small inclusions also occur during central cell development as a result of cytoplasmic nodules (with or without organelles) being surrounded by a portion of endoplasmic reticulum (Singh, 1978). They are thought to have an autophagic function, destroying the female cytoplasm soon after fertilization (Chesnoy, 1987a).

Anticlinal and periclinal division of the primary neck cell formed one and then two tiers (of four) neck cells (Fig. 44). This neck cell arrangement is not consistent amongst the pines, or other conifers. Variation exists in the number of tiers and the number of cells in each tier. Examples of pines that were found to have one tier of four neck cells are: *P. strobus* (Ferguson, 1904), *P. palustris*, (Mathews, 1932), *P. wallichiana* (Konar and Ramchandani, 1958), and *P. roxburgii* (Konar, 1960). In contrast, *P. monticola* was found to have one tier of four to six neck cells (Owens and Molder, 1977) and *P. contorta* one tier of several neck cells (Owens *et al.*, 1982). Thomas (1951) and Lill (1976) found that *P. virginiana* and *P. radiata* could have either one or two tiers of four neck cells, while *P. longifolia* was found by Sethi (1928) to have two tiers of four neck cells. Singh (1978) reviewed the neck cell arrangement in some of the less common conifers and found that neck cells need not be arranged in tiers. Rather they can form a pinwheel, a column and may only be distinguishable from the surrounding female gametophyte cells by location.

Early in June the central cell divided unequally forming a small lense-shaped ventral canal cell and a large avacuolate egg cell. There was a post-division

migration of the egg cell nucleus from the micropylar end to the centre of the cell and the nucleus took on the light granular appearance characteristic of mitotic interphase. From its inception the EC nucleus was surrounded by a cytoplasmic region, called the perinuclear zone which was devoid of large or small inclusions but rich in mitochondria, smooth endoplasmic reticulum, small vesicles and ribosomes (Bruns and Owens, 1989; Owens and Morris, 1990). Because of its contents the perinuclear zone is an important part cytoplasmic inheritance. The ventral canal cell, on the other hand, underwent no drastic changes during this period. Because of its ephemeral nature little attention has been directed toward the ventral canal cell. Most often researchers note that it is formed after division of the central cell nucleus and undergoes little or no internal development before being ruptured by the pollen tube (Ferguson, 1904; Mathews, 1932; Thomas, 1951; Lill, 1976; Owens and Molder, 1977; Singh and Owens, 1981a,b; Owens *et al.*, 1982). Recently, though, its transmission electron microscopy structure has been described by Owens and Morris (1990).

Throughout the development of its specialized cells the female gametophyte continued to enlarge. Cessation of this phase coincided with the completion of egg cell maturation. The last major development in female gametophyte maturation was a thickening of the micropylar end of the female gametophyte forming archeogonial chambers (with neck cells at their base). The purpose of the archeogonial chamber is unclear. However, it may serve to guide pollen tubes, that have penetrated the megaspore cell wall, to the neck cell. It may or may not be true for *Pinus* but in *Pseudotsuga menziesii* neck cells at the base of the archeogonial chamber are secretory and may cause the directional growth of the pollen tube into the archeogonial chamber. In addition the archeogonial chamber of *P. menziesii* is where the pollen tube containing the body cell settles and divides to form the two

male gametes (Owens and Morris, 1990).

Bounding the archegonial initials the archegonial jacket cells were recognizable, from other female gametophyte cells, because of their location, uniform isodiametric shape, greater cytoplasmic density and larger nuclei. Singh (1978) reported that archegonial jacket cells may supply nutritive materials to the developing egg cell and cites examples of mitochondria, plastids, dictyosomes, endoplasmic reticulum and nuclei passing through plasmodesmata connecting the two types of cells. Owens and Morris (1990) attributed these observations to artifacts from tissue preparation which caused thin primary pit fields to rupture.

There appeared to be two stages to pollen tube elongation during the second growing season (Fig. 44). Slow elongation occurred in the nucellus prior to central cell division (June 2 to 6). After central cell division pollen tubes increased their rate of elongation and reached the megaspore cell wall as the egg's interphase nucleus reached the centre of the cell (June 9). This period of pollen tube growth is longer than that reported by Sarvas (1962). His study indicated that, in *P. sylvestris*, pollen tube growth resumed just 10 days before fertilization compared to 20 days in *P. taeda*. Sarvas states that the pollen tube "...penetrates the second year growth of the nucellus..." so it is possible that he did not recognize a period of slow pollen tube growth. If this is the case then *P. taeda's* period of rapid pollen tube growth is shorter than that in *P. sylvestris* (7 verses 10 days, respectively). The period of slow growth may only have served to move the pollen tube into a position where it can make final rapid progress toward the egg cell, as the latter completes its maturation.

Newly fertilized eggs were seen between June 6 and 13 (Fig. 44), although in only one case was the fusion nucleus observed. The appearance of a dividing zygote nucleus or the free nuclei of the proembryo were taken to be signs of successful fertilization. With the addition of the nucleoplasm from the male gamete the

perinuclear zone became the neocytoplasm. However there appears to be some discrepancy over when this event occurs. Older literature (Chesnoy and Thomas, 1971) indicates that neocytoplasm is formed when the zygote undergoes its first division. While Owens and Morris (1991) feel that neocytoplasm forms when the gametes fuse, although they say that "true neocytoplasm" is created only after the cluster of paternal organelles joins the free nuclei and surrounding cytoplasm, at the chalazal end of the egg cell. The Owens and Morris version appears to be the pattern of events in *Pinus* (Bruns and Owens, 1989). It appears, however, that in the *Pinaceae* the pattern of cytological inheritance of plastids and mitochondria is constant. This means that in all species examined so far plastids are paternal in origin while mitochondria are primarily maternal in origin, although some are paternal (Owens and Morris, 1991). Once the free nuclei had reached the chalazal end of the egg cell they underwent two mitotic divisions and cell wall formation, ultimately producing the mature four tiered, 16-celled proembryo. The process of proembryo formation took place between June 6 and 13. Fertilization and proembryo development in *P. taeda* does not appear to differ from other pines investigated thus far.

The change from proembryo to early embryo occurred when the embryonal tier was forced through the archegonial jacket and into the female gametophyte by the proembryo's elongating embryonal suspensor tier. This began on June 13 (Fig. 44). The origin of the cells making up the suspensor changed as the embryo was carried deeper into the corrosion cavity. The primary suspensor was formed from cells of the embryonal suspensor tier which elongated until cleavage occurred. Two types of secondary suspensors were then formed from the embryo; the embryonal suspensor and embryonal tube (Singh, 1978). The embryonal suspensors were produced by transverse division of embryo cells after cleavage when the embryos

were still either a single file of cells or, at the most, a few cells wide. Once the embryos reached the embryonal mass stage they were supported on embryonal tubes, groups of unequally elongated cells formed from the base of the embryonal mass. The continuous production of embryonal suspensors and then embryonal tubes caused the suspensor to coil and the cells to become crushed into a mass at the micropylar end of the female gametophyte. This deformation of suspensor cells began at the start of embryonal suspensor production and became more extensive as the length of the secondary suspensor increased. The final deformation of the suspensor was caused by elongation of the embryo itself, because as it reached maximum size it occupied almost the entire corrosion cavity.

Since one to four archegonia were present in each female gametophyte, there was the possibility that four pre-cleavage embryos would be produced. Until cleavage, cells of the embryonal unit were organized in four columns that were pushed deeper, as a whole, into the corrosion cavity. The presence of multiple embryos, consisting of a single file of cells, suggested that cleavage occurred between June 16 and 22 (1 to 2 weeks after fertilization). The time of cleavage compares favorably with *P. sylvestris*, *P. monticola* and *P. contorta*, in which cleavage occurred 2, 1 to 2 and 2 to 3 weeks after fertilization, respectively (Hakansson, 1956; Owens and Molder, 1977; Owens *et al.*, 1982). If the maximum four pre-cleavage embryos were present and sufficiently developed to cleave, the possibility existed that, for a short time, 16 post-cleavage embryos would be present in the corrosion cavity. Implications for those attempting to tissue culture *P. taeda* are twofold. Firstly, because the embryos are from multiple fertilizations a variety of genotypes could be present within a female gametophyte, throwing the genetic homogeneity of the explants into doubt (Becwar *et al.*, 1991). Secondly, the rapid degeneration of subordinate post-cleavage embryos, within the corrosion cavity, limits the time

during which female gametophytes contain more than one embryo capable of initiating polyembryonic callus.

Once a post-cleavage embryo attained a position of dominance at the apex of the corrosion cavity (between June 30 and July 14) it began to increase in mass. Subordinate embryos enlarged only slightly, if at all, and were no longer visible within the corrosion cavity by July 28. As the dominant embryo enlarged and moved deeper into the corrosion cavity, it took on a globular appearance (approximately July 14). Individual tissues and meristems could not easily be identified until the embryo was at the large globular stage or had cotyledon primordia ("shoulders"). However, Singh (1978) and Allen and Owens (1972) indicate that organization of specific tissues occurs prior to what was observed here. They reported that the first indication of tissue organization in the young embryo was the formation of distal and proximal regions. The proximal region produces the secondary suspensor, root apex and root cap while the distal region produces the hypocotyl, shoot apex and cotyledons. The first distinct tissue to appear is the root apex followed in turn by the root cap, hypocotyl, shoot apex and cotyledon primordia. Subsequent enlargement resulted in anatomically mature embryos being present in all female gametophytes between August 25 and September 8. These were roughly divided into thirds by the cotyledons, hypocotyl and root cap and occupied approximately 90% of the length of the female gametophyte.

### **5.3 Seed Production**

It was known that, in the past, the five clones used in this portion of the study had either excellent (clone 1), intermediate (clones 3, 4, 5) or poor (clone 2) seed efficiency. The objective here was to quantify these parameters and determine what conditions produced them. To this end the mean number of ovuliferous scales were

determined in total and by type. Each mature cone was divided into five regions: distal sterile, distal rudimentary, fertile, proximal rudimentary and proximal sterile. This is similar to the system first introduced by Lyons (1956), in which he divided the cone into distal and proximal unproductive regions as well as a central productive region. Within the unproductive regions Lyons recognized that there were scales bearing "nil" and "undeveloped" ovules corresponding to the sterile and rudimentary scale classification used here. Bramlett (1974) determined that loblolly pine's mean number of ovuliferous scales was 135, second highest of the four southern pine species he investigated. His value was somewhat less than the 153.5 found in this study. When the mean number of ovuliferous scales of the five clones were compared, clone 2 had the greatest number; clone 5 the fewest and there was no significant difference between clones 1,3, and 4 (Table 1, Fig. 41). Unfortunately there is no direct relationship between mean number of ovuliferous scales and seed efficiency (Fig. 41). Therefore, if a clone has a high mean number of ovuliferous scales it does not mean that it will have a correspondingly high seed efficiency. An example of this is clone 2 which had the highest mean number of ovuliferous scales and the lowest seed efficiency. There is no simple explanation for this inconsistency. The most direct cause of reduced seed efficiency may be the rate of ovule abortion during the first and second growing seasons. Especially since control pollination and pollen testing, as used in this study, should have reduced pollen deprivation and inviability as a cause of reduced seed efficiency with a high mean number of ovuliferous scales.

There was a considerable difference between the number of sterile and rudimentary ovules borne by ovuliferous scales at the distal and proximal ends of the cones (Table 1). This can be attributed, in part, to a conelet's pattern of emergence from the bud scales. Using the drawings in Bramlett and O'Gwynn

(1980) as a guide, it is evident that the elongating conelet axis forces the distal 1/2 to 2/3 of the conelet out of the bud scales (stage 4) but it is not until stage 5 that the bud scales reflex, exposing the proximal ovuliferous scales. Therefore, it is likely that ovules on ovuliferous scales experiencing the longest period free of confinement within the bud scales will have the greatest chance of developing fully and becoming fertile. Unless, of course, the ovuliferous scales are too poorly developed to carry a fertile ovule as is the case in the distal and proximal sterile and rudimentary regions.

On a conelet the region of primary concern is the central portion which bears fertile ovules. The five clones examined had an overall mean of 89.08 fertile scales which is greater than the fertile region (77.5 ovuliferous scales) on the loblolly pine that Bramlett (1974) observed. The pattern of mean number of fertile scales was not exactly the same as that for mean number of ovuliferous scales (Fig. 41), the difference being that, while clone 4 had fewer fertile scales than clone 5, it had a greater mean number of ovuliferous scales. Clone 4 was no longer part of a population that included clones 1 and 3, and, in fact, stood on its own (as did clone 5). Also clone 2, which had a mean number of ovuliferous scales significantly different from any of the other clones, was now part of a biclinal population with clone 1. The latter clone was also part of a biclinal population with clone 3 (Table 1). By definition, variation in the number of fertile scales on a cone had a direct influence on that cone's seed potential. However, the number of filled seed produced by a cone is primarily related to the availability and germinability of the pollen reaching the nucellus. So, in a seed orchard where open pollination, rather than control pollination, is used to produce seed, pollen availability and quality, not the number of fertile ovules, would limit the production of filled seed.

Since filled seed and seed potential were, by definition, directly related, a

doubling of the number of filled seed gave the mean seed potential of each clone. Seed production was divided into two categories, empty and filled seed. Examined individually the following relationships, between the number of empty seed and filled seed, were evident. Clone 1 (excellent seed efficiency) had both the fewest empty seed and the most filled seed. However, clone 2 (poor seed efficiency) had the third lowest number of empty seed and the lowest number of filled seed (Table 1). The three clones (3, 4 and 5) with intermediate seed efficiency had the fifth, fourth and second highest number of empty seed, respectively, and the third, second and fourth highest number (clones 3, 4 and 5) of filled seed, respectively, (Table 1). Because the empty seed category is made up of both full-size and (if present) half-size empty seed, the results above indicate clonal differences in the rate of ovule abortion. The consensus of opinion seems to be that, aside from insect and frost damage, half-size empty seed are caused by post-pollination ovule abortion late in the first growing season, whereas full-size empty seed are the result of either pre- or post-fertilization ovule abortion during the second growing season (Lyons, 1956; Bramlett, 1972, 1974). If abortion occurs at pre-pollination or in the early post-pollination period no remnant of the ovule is present at the end of the reproductive cycle (Bramlett, 1972).

As early as 1939, Stockwell realized that there were three possible causes of pre-fertilization ovule abortion in pine. All of these had to do with pollen supply and pollen viability. This has since been confirmed as the major cause of first-year-ovule abortion in pine (McWilliam, 1959; Sarvas, 1962; Brown, 1971). In this study however, concerns about pollen supply and viability had been minimized because all the conelets had been hand pollinated using pollen with an *in vitro* germination percentage of at least 95%. Despite these measures an undetermined number of fertile ovules were not pollinated or had ungerminated pollen grains in their pollen

chamber. It appears that non-pollinated ovules are always present in the fertile region of a ovulate strobilus. Brown (1971) reported that in *P. sylvestris*, 24% of fertile ovules were not pollinated even though dissection revealed that control pollination delivered ample pollen to each conelet.

Despite being applied when the conelets are at peak receptivity, after reaching the nucellus, pollen may not germinate. There are two possible reasons for this. Self-pollination with viable pollen could result in an enzymatic incompatibility between the nucleus and pollen grain that prevents germination or pollen tube growth after germination (Pettitt, 1985). Because a pollen polymix was used in this study some self-pollination would have occurred, leading to a small reduction in seed efficiency. If this is ever proven to significantly reduce seed efficiency then it may be worth removing the possibility of self-pollination when making up a polymix. If cross-pollinated the grain may simply not have been capable of producing a pollen tube despite germinating during an in vitro germination test (Brown, 1971). It may never be possible to eliminate this as a cause of ovule abortion unless a more efficient germination test is devised. Pre-pollination and early post-pollination events that could lead to ovule abortion were not closely followed, however they appeared to be similar to those described by McWilliam (1959), Sarvas (1962) and Sweet (1973).

The reason empty plus filled seed were combined was because this represented the total seed production by each clone. When the number of empty plus filled seed were subtracted from a clone's seed potential it gave the amount of pre-pollination and early post-pollination ovule abortion per cone in that clone. Clone 1 had the lowest mean pre- and early post-pollination ovule abortion (17.35 ovules/cone), clone 2 the highest (111.4 ovules/cone), between these were clones; 3 (20.75 ovules/cone); 5 (26.3 ovules/cone) and 4 (27.3 ovules/cone). The mean

number of empty seed (Table 2) indicates the number of late post-pollination ovule abortions. The clones ranked from lowest to highest are: 1, 5, 2, 4, and 3. In all but clone 3 the rate of pre-pollination and early post-pollination ovule abortion was lower than late post-pollination ovule abortion, confirming that pollen supply and pollen viability were both optimized. In the case of clone 3 the large amount of early ovule abortion would have to be due to factors other than pollen supply and pollen viability. It is possible that the control pollinations were not made at peak receptivity or that the conelet's receptive period is unusually short. There may also have been an undetermined interaction between the integument or female gametophyte and the pollen tube which prevented or hindered pollen tube growth or the pollen's ability to fertilize during the second growing season.

It is notable that clone 2, which had the largest mean number of ovuliferous scales per cone and the second smallest fertile region (Table 1) had the greatest number of pre-pollination and early post-pollination abortions and the second smallest number late post-pollination abortions. This may indicate that about the time of pollination the conelet cannot compete for available photosynthate with the elongating vegetative shoot which may cause ovule abortion. This was reported for certain clones of *P. radiata* (Sweet and Bollmann, 1970) in which there was a high incidence of conelet drop. Another possibility is that there is enough difference in conelet structure between clones that some are less efficient pollen traps than others.

The seed efficiencies resulting from this study confirmed the earlier classification of the five clones according to excellent, intermediate or poor seed efficiency. Based on X-ray analysis, seed efficiencies from highest to lowest ranked the clones as: 2, 3, 4, 5 and 1 (Table 2). All clones had seed efficiencies greater than Bramlett's (1974) value of 24% for *P. taeda*, although it is assumed that he used

open pollinated trees. Significant differences in the seed efficiencies separated the five clones into four populations, with clones 1 and 2 separate from clones 3, 4 and 5 which were arranged in two overlapping biclinal populations (Table 1). Although controlled pollination, on a mass scale, is not practical these results indicate that significant gains in seed efficiency can be realized if pollen supply and pollen viability are optimized.

#### **5.4 Seed Germination**

There was no significant difference among clones in the total germination percentage (96.00 to 98.75%, Table 2) or the length of time each clone took to complete the germination test (22 to 29 days, Fig. 42). The uniform germination percentages may be a characteristic of the species but also resulted from selecting only "sinkers" for the test and discarding the "floaters". It would have been interesting to dissect (or X-ray) the "floaters" to determine their internal condition as some of these may have been filled and viable. If hydration is used operationally the major benefit would be to increase efficiency of sowing and germination while, perhaps, wasting some viable seed. Even though 24 hours is the recommended hydration period (Edwards, 1987), a reduction in the number of "floaters" may be achieved by lengthening the hydration to 48 hours. There was little fungal contamination of the seeds during stratification or the germination test probably as a result of sterilization in 10% sodium hypochlorite after hydration. Sterilization after, rather than before, hydration prevented sodium hypochlorite absorption by dehydrated seed (A.M. Colangeli, University of Victoria, *personal communication*), which would have been detrimental to germination. McLemore and Czabator (1961) recommended that loblolly pine seeds be stratified for up to 60 days for maximum germination percentages. However, 28 day stratification gave a uniform

and high germination percentage. All five clones had very similar germination profiles (Fig. 42), with significant differences in germination rate occurring only in the first 11 days after stratification. By day 12 no clone had more than 3.5 germinating seed per replicate and from this point until the end of the test there was no significant difference in the performance of the five clones. Although each clone had seeds which did not germinate, in total only 52 of 2000 seeds used in the germination test remained ungerminated (Table 3).

## CHAPTER 6

### CONCLUSIONS

#### **6.1 Reproductive Cycle**

1. The only major difference between the reproductive cycle of *P. taeda* and other temperate pines examined to date is the delay, until the start of the second full growing season, in the onset of free nuclear division within the female gametophyte.
2. Although the fusion nucleus was observed in only one case, preceeding and succeeding stages indicated that fertilization occurred between June 6 and 13.
3. From one to four, but most often two or three, archegonia are present within each ovule.
4. Each female gametophyte contains from one to four pre-cleavage embryos which, in turn, can produce from four to sixteen post-cleavage embryos.
5. Subordinate post-cleavage embryos (those which did not attain the dominant position at the apex of the corrosion cavity) were no longer recognizable by July 28.

#### **6.2 Seed Production**

1. Among the five clones the mean number of total ovuliferous scales was 153.5.
2. Among the five clones the mean number of fertile ovuliferous scales was 89.08 and ranged from a high of 96.50 (clone 2) to a low of 80.70 (clone 4). This variation was attributed to the pattern of strobilus emergence from the bud scales.

3. The difference between seed potential and empty plus filled seeds indicated the rate of pre-pollination and early post-pollination abortion. Clone 1 had the lowest number of abortions at this stage (17.35 ovules per cone) and clone 2 the highest (111.4 ovules per cone). This early abortion is most often due to a lack of pollen or the presence of inviable pollen. These factors were minimized by control pollination and pollen testing.

4. Classification of the five clones according to excellent, intermediate or poor seed efficiency was confirmed. The seed efficiencies were: clone 1 (excellent, 84.4%); clone 2 (poor, 31.6%); and, clones 3,4 and 5 (intermediate, 61.75%, 66.85%, 73.9%, respectively). Because control pollination and pollen testing were used these were assumed to be "best case" seed efficiencies.

### **6.3 Seed Germination**

1. Seed obtained from the five clones had the following germination percentages: clone 1, 98.50%; clone 2, 97.25%; clone 3, 98.75%; clone 4, 96.50%; clone 5, 96.00%.

2. The five clones had very similar germination profiles with maximum germination occurring 11 days after stratification. The only significant differences in germination rates were prior to this date. By day 12, germination rate had decreased dramatically with no significant difference between the clones.

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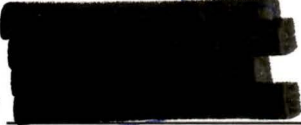
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**OVULE AND EMBRYO DEVELOPMENT, SEED PRODUCTION AND GERMINATION IN ORCHARD GROWN CONTROL POLLINATED LOBLOLLY PINE (*PINUS TAEDA* L.) FROM COASTAL SOUTH CAROLINA**

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April 10, 1992