TECHNIQUES TO STUDY THE CELL CYCLE IN THE SHOOT APEX OF CONIFERS

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Abstract

Techniques were developed to measure mitotic index (MI) and identify nuclei in G1, S, and G2 stages of the cell cycle in squash preparations of conifer shoot apices. Feulgen staining and sampling by horizontal scanning at fixed vertical intervals were used. These procedures allowed indices for the entire apex to be accurately determined with a minimum of 10 apices. Using this technique, the resumption of mitosis from a G2 population of cells was observed after 10-14 hours in loblolly pine (*Pinus taeda* L.) shoot apices which were removed from cold storage and placed in growth promoting conditions. The potential for using this procedure to determine the growth potential of dormant shoot apices is discussed.

A method was developed to identify nuclei in the interphase stages of G1, S, and G2. This involved labelling of shoot apices with tritiated thymidine (203.3 kBq/ml) in 1% DMSO for two hours to identify nuclei in S stage. Nuclei in G1 and G2 were distinguished visually. In loblolly pine, abnormal mitotic figures and a 26% reduction in MI resulted from this treatment, possibly due to the presence of DMSO. Labelling of western redcedar (*Thuja plicata* D.) shoot apices was significantly higher than loblolly pine apices and may have been due to exposure of vascular tissue to the treatment solution. Procedural modifications which may improve incorporation, decrease disruption to cells, and improve criteria for determining labelled nuclei are discussed.

The morphology of nuclei in G1, S, and G2 was examined using microdensiometry. This allowed G1 and G2 nuclei to be identified by their staining intensity and area. Mitotic figures which have a known DNA content (2C or 4C) assisted in these determinations. Nuclear area varied greatly in different apical

stages of the cell cycle, but this still allowed an accurate identification of cell cycle stage. Loblolly pine seedlings in rapid neoformed growth had 75% of their cells in G1, 14.8 % in S, 4.7% in G2, and 4.6 in mitosis, and is correlated with the relative duration of each cell cycle stage. The G1/S transition appeared to be limiting cell cycle progression in these apices. These findings are discussed in relation to apical zonation and previous studies which used microdensiometry to determine cell cycle stage.

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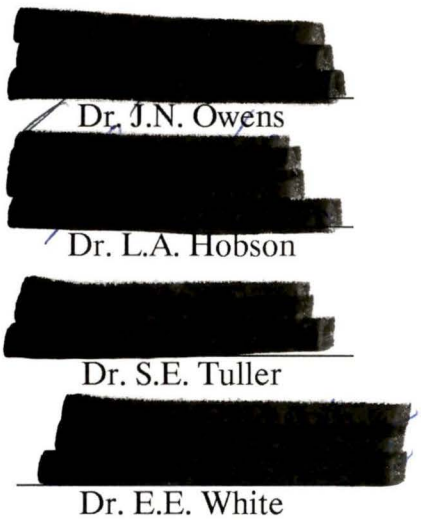

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Chapter I

INTRODUCTION

The cell cycle is a fundamental concept in biology, and is a basic unit of time at the cellular level (Mitchison 1971). The cell cycle is responsive to both internal physiological states and external environmental conditions (Rost 1977, Van't Hof 1985) and is involved in the coordination of developmental processes (Owens and Molder 1973, Francis and Lyndon 1985, O'Farrell *et al.* 1989). Based on its fundamental role in biological activities, the cell cycle is an important area of basic research which has recently been the focus for an issue of Science (Vol. 246, Nov. 1989).

Few cell cycle studies have been conducted on shoot apices of conifers, although the measurement of mitotic index (MI), the percentage of cells in mitosis, is increasingly used as a measure of cellular activity and as an anatomical criterion for dormancy (Carlson 1985a, Colombo *et al.* 1988, Fielder and Owens 1989, O'Reilly *et al.* 1989). However, the preparation, sampling and interpretation of MI determined from squash preparations is varied and needs standardization.

In at least one study which has used MI (Carlson 1985a) further questions related to the cell cycle have resulted. Studies which have been conducted on the cell cycle in *Picea* (Cecich *et al.* 1972) and *Pseudotsuga* (Owens and Molder 1973) showed dramatic changes in interphase (G1, S and G2) populations during the annual cycle of growth. Unfortunately, further study has been hampered by the labor intensive nature of microdensiometric measurement, and the highly protected nature of the shoot apex which makes experimental manipulation difficult (pers. comm. J.N. Owens).

A more comprehensive understanding of conifer biology is required to assist research in reforestation and biotechnology. This must include an understanding of

activities at the cellular level, and therefore of the cell cycle. The objectives of this study were to improve procedures for determining MI in squash preparations, and develop an appropriate technique to study the cell cycle in conifer shoot apices using loblolly pine (*Pinus taeda* L.) as a model species.

CHAPTER 2

LITERATURE REVIEW

2.1 Organization of the shoot apex of conifers

The terms shoot apex, apical meristem and apical dome are commonly used to describe that portion of the shoot tip above the last definable foliar primordia (Parke 1959). They have been defined as a "collection of meristematic cells from which, by repeated divisions, all primary tissues of the shoot are derived" (Gregory and Romberger 1972). The term "shoot apex" will be used in this discussion.

The shoot apex of conifers is a dome-like structure which exhibits cytological variation among cells from different zones. The description of this zonation by Foster (1939) and later by Owens and Molder (1973, 1977) will be used. The apex is organized into 4 zones: 1. Apical Initials (AI)- A group of large cells with large nuclei at the summit of the apex; 2. Central Mother Cells (CMC)- Located below the apical initials and consisting of larger ovoid, vacuolate cells, also with large nuclei. At certain stages of development the AI and CMC zones cannot be easily separated and form a single cup-shaped region, referred to as the apical zone; 3. Peripheral Zone (PZ)- Covering the remainder of the apex is a single layer of protoderm and immediately below this several layers of small cells with small nuclei and dense cytoplasm. These and protoderm cells are highly meristematic and are the site of primordia initiation; 4. Rib meristem-Pith (RM)- The central region of cells surrounded by the PZ and derived from the CMC and PZ. These cells are also meristematic and produce cell files of the future pith.

The cause and functional significance of zonation in conifer apices has not been explained, although several studies have examined the ultrastructural and biochemical differences among the zones. Cecich (1977, 1979) and Cecich and

Horner (1977) examined the shoot apex of *Pinus banksiana* L. (jack pine) seedlings ultrastructurally. The CMC zone had cytoplasm with large vacuoles containing lipid and large nuclei which generally lacked heterochromatin and were diluted by a large nuclear volume, whereas cells in the peripheral zone had cytoplasm with a high concentration of ribosomes and small nuclei which were heterochromatic.

Fosket and Miksche (1966) and Riding and Gifford (1973) have indicated that the apical zone is metabolically active. These studies have suggested that the CMC zone might be influencing cells in the peripheral zone and acting in some ways as differentiated cells (Fosket and Miksche 1966) or even controlling apical development (Riding and Gifford 1973).

Owens and Molder (1973) have shown that these cells are not quiescent, are not held at a specific cell cycle stage throughout the year, and do proceed through the cell cycle and mitosis, but at a lower rate than the peripheral zone and rib meristem. This study concluded that the size and prominence of zonation changes during the annual growth cycle and is related to particular developmental stages of the apex and shoot.

2.2 The cell cycle

2.2.1 Definition and stages of the cell cycle

Mitchison (1971) defined the cell cycle as "the period between the formation of the cell by the division of its mother cell and the time when the daughter cell divides to form two daughter cells, and is the fundamental unit of time at the cellular level since it defines the life cycle of the cell". The cell cycle was first partitioned into distinct stages by Howard and Pelc (1953) who observed four stages by autoradiographic techniques. These consisted of two temporal "gap" (Yeoman

1981) stages, (G1 and G2), a stage of DNA synthesis (S), and mitosis (M). G1, S and G2 are often grouped together and called interphase. G2 principally differs from G1 by a 4C (versus 2C) level of DNA which is required for nuclear division. G1 and G2 have been found to be points in the cell cycle where cells are held under adverse conditions due to an inability to enter S and M, respectively (Van't Hof 1968, Webster and Van't Hof 1969). The proportion of cells which are held in G1 and G2 under such conditions has been shown to be species specific (Van't Hof 1985).

The cell cycle can be diagramatically represented as a circular path which cells traverse. The length of the perimeter of the circle is equal to the duration of the cell cycle, and the length of the line in each stage is equal to the duration of each stage and proportional to the percentage of the cell cycle spent in that stage. In addition to the 4 stages observed by Howard and Pelc (1953) there is an additional stage, G0, which represent cells which are not traversing the cell cycle (Mitchison 1971) or are traversing the cycle very slowly (Gonthier *et al.* 1985). G0 cells greatly influence measurements by altering the growth fraction (GF), or percentage of cells in a population actively traversing the cell cycle (Gonthier *et al.* 1985).

In addition to this view of the cell cycle, which is centered around the timely progression of the nucleus through the cell cycle by DNA synthesis and karyokinesis, Mitchison (1974) and Cavalier-Smith (1985) have proposed an alternative view of the cell cycle. Both authors describe the cell cycle as consisting of two independent components, cell division and cell growth. This view stresses cell growth and volume characteristics in an equal manner to DNA synthesis and karyokinesis. Cavalier-Smith (1985) proposed that cell volume characteristics are an epigenetic factor which, along with genetic and environmental factors, control cell cycle characteristics.

2.2.2 Measurement of cell cycle parameters

Studies of the cell cycle began in plant and animal cells with the work of Howard and Pelc (1953). Since that time four principal methodologies have been used to examine the cell cycle: mitotic index, use of tritiated thymidine, microdensiometry of DNA content, and analysis of changes in the size and structure of nuclei and chromatin.

2.2.2.1 Mitotic index

Mitotic index (MI), the percentage of cells in mitosis, is the simplest and most frequently used cell cycle parameter. It is a measure of the percentage of cells undergoing nuclear division at time t (time of fixation) and can be measured on squash preparations or sectioned material without any experimental manipulation. Mitotic figures are identified by the visual presence of chromosomes (Carlson *et al.* 1980) and can be rapidly assessed. Mitotic frequency (MF), the number of observed mitotic figures in the median 6% of the apex in sectioned material, has also been used as a measure of mitotic activity and is similar to measurements of MI (Owens and Molder 1973, Owens *et al.* 1985). MI will be discussed in the following discussion since MF is no longer used.

MI has been found to be proportional to the rate of cell division and inversely proportional to the length of the cell cycle in many studies (Lyndon 1973). Walker (1954) demonstrated theoretically that MI is altered by either a disproportional change in the duration of interphase or mitosis or an alteration of the GF. However, Walker (1954) and later Burholt and Van't Hof (1971) also showed that MI is a reflection of several cell cycle variables, and may remain constant despite changes in cell cycle duration, the rate of cell division or an alteration of the GF.

Cell cycle studies in conifer shoot apices have primarily used MI to determine a relative measure of cellular activity. These studies have correlated the magnitude of mitotic activity with developmental stage (Colombo *et al.* 1988, Fielder and Owens 1989, O'Reilly *et al.* 1989), frost hardiness (Colombo *et al.* 1988), nutrient availability (Carlson *et al.* 1980), external temperature and photoperiod (Carlson 1985a, Colombo *et al.* 1989) and respiratory activity (Fielder 1986) in seedlings, and developmental stage (Owens and Molder 1973) and water relations (Owens *et al.* 1985, Owens and Simpson 1988) in more mature trees.

Many of the studies which have used MI, have specifically examined changes in seedlings to ascertain dormancy status. The absence of mitotic activity has been used as an anatomical and cytological basis for dormancy in northern temperate conifers (Owens and Molder 1973, Carlson *et al.* 1980, Fielder and Owens 1989). Carlson (1985a) observed an annual cycle of mitotic activity similar to northern temperate conifers in *Pinus taeda* L. (loblolly pine) seedlings during winter in Arkansas, U.S.A., but did not observe an absolute absence of mitosis. In this study, the period of lowest MI (2.8%) in mid winter corresponded with the period of most successful transplanting, while periods of elevated MI in fall and early spring coincided with times of reduced transplanting success. Changes in MI were thought to be affected by changes in temperature and photoperiod (Carlson 1985a).

Currently, many of the studies which measure MI in conifer shoot apices use squash preparations (Carlson *et al.* 1980, Carlson 1985a, Colombo *et al.* 1989, O'Reilly *et al.* 1989). The anatomical portion removed for squashing as well as the procedure for determining MI varies among these studies, and is quite different from the MI determined from sectioned material which is a small sub-sample (Fielder and Owens 1989). This has made comparison between studies

difficult, especially in terms of the magnitude of MI.

2.2.2.2 Labelling with tritiated thymidine

Thymidine, a nucleotide, containing a tritium atom most often in the methyl group (Taylor and Clowes 1978) can be incorporated into cell nuclei when the cell is undergoing DNA replication (synthesis) and identified by autoradiographic analysis. Due to the low energy emitted by tritium disintegration, grains produced by tritium decay occur within 1 μm of the source making this a very precise method for identifying labelled structures (Evans 1974).

Penetration and incorporation of tritiated thymidine into plant cell nuclei in either tissue culture systems (Van't Hof and McMillan 1969) or in roots growing in aerated hydroponic systems (Wimber 1966, Taylor and Clowes 1978) have been most frequently used since dissection is not necessary and thymidine is easily absorbed. In both systems, low concentrations of radioactive nucleotide (18.5-37.0 kBq/ml) are incorporated in 30 minutes.

Incorporation into shoot apices has been more difficult to achieve (Gifford *et al.* 1963, Lyndon 1973) because of the relatively non-absorptive nature of shoot apices versus roots, and the less exposed, more protected nature of many plant shoot apices by leaves and scales (Lyndon 1973). Various applications of labelled nucleotides have been made to leaves (Gifford *et al.* 1963), injected into stem and internodes (Clowes 1958, Gifford *et al.* 1963), applied to undissected buds (Gifford *et al.* 1963, Bernier and Bronchart 1963) or applied directly to exposed apices with nearby primordia removed (Taillander 1965, Jacqmard 1970). The results of these initial experiments and more recent studies (Ormrod and Francis 1985, Gonthier *et al.* 1985) indicate that the most efficient method to label cells of the shoot apex is by direct application to the dome after removal of some primordia near the apex, and

use of surfactants when necessary. The concentration and specific activity of the thymidine used, as well as the duration of exposure has varied greatly among different studies. The current range of treatment conditions used for angiosperm shoot apices ranges from 4-10 μCi (148-370kBq)/ml for 2-4 hours (Ormrod and Francis 1985, Gonthier *et al.* 1985). Radioactive labelling of conifer shoot apices has not been reported, except for a brief note by Taillander (1965) on *Pinus pinea* L. (stone pine) where a detailed description of the labelling method was not provided.

Tritiated thymidine has been used in long duration treatments to identify non-cycling cells (Gonthier *et al.* 1985) and is commonly used to identify S cells in structural studies of interphase nuclei (Nagl and Scherthan 1985). In addition, the duration of the cell cycle and it's component phases can be calculated by following a population of S cells through the cell cycle which have been pulse labelled with a short period of exposure to tritiated thymidine. This is accomplished by sampling apices at regular intervals after exposure and observing labelled mitotic figures in autoradiographs (Quastler and Sherman 1959). A graph is then constructed and the duration of the cell cycle and it's component phases is calculated by the "rhythmic appearance and disappearance of the labelled cells (in mitosis) with time" (Van't Hof and McMillan 1969). Pulse labelling with tritiated thymidine can also be used in conjunction with C-14 labelled thymidine to identify cells at different times and determine the duration of the cell cycle and its components by changes in the percentage of doubly labelled mitotic figures (Nougarede and Rembur 1977).

Certain requirements and conditions must be met to measure cell cycle duration using pulse labelling with tritiated thymidine. These include: cells in the population have roughly the same cell cycle duration, the population is asynchronous with no diurnal peaks of mitotic activity, and the dose of thymidine does not disturb the duration of the cell cycle, its components, or the DNA in the

nucleus (Wimber 1959, Natarajan 1961, Mitchison 1971). Additionally, when protective foliar structures which protect succulent tissue are removed, and this tissue is placed in liquid solution, the effects of damage, desiccation and anoxia should be minimized and monitored throughout the period of treatment and subsequent collections (Bernier and Bronchart 1963, Evans *et al.* 1957, Nougarede and Rembur 1977).

The only study of cell cycle duration that could be found in conifers is a brief report by Miksche (1967) on roots of jack pine grown in water soaked perlite at 22°C. The cell cycle duration was 25.7 h, and the component stages were: G1-15.3, S-7.6, G2-1.4, and M-1.4 hours.

2.2.2.3 Microdensiometry

The cell cycle stage of nuclei in shoot apices can be estimated by staining nuclei by the Feulgen-reaction and measuring the amount of light that the nucleus absorbs. The wavelength used in this determination has varied from 560-580 nm (Fox 1969, Cecich *et al.* 1972, Greilhuber 1986, Peet and Sahota 1984). Since the amount of stain present is proportional to the amount of DNA present (Jenson 1962), and DNA doubles during the cell cycle, a histogram of total absorbancies for nuclei will separate into three groupings (2C, 3C, 4C) if all three cell cycle stages (G1, S and G2, respectively) are present.

The methodology and equipment used in determining the Feulgen absorbence varies and has gradually improved since the early 1970's. The process has been called microphotometry (Owens and Molder 1973), cytophotometry (Cecich *et al.* 1972) and more recently microdensiometry (Goldstein 1981, Ormrod and Francis 1985). Microdensiometry will be utilized for the purposes of this discussion. Early studies used the plug method (Swift and Rasch 1956) and one wavelength of light,

and was later improved by the use of two wavelengths (Patua 1952) which removed some distributional error. Currently the most accurate method is scanning microdensiometry which is more instrumentally complicated (Goldstein 1981). This method measures the absorbance of the nucleus by summing the absorbances of very small areas or pixels (.125-.25 μm^2) over the entire area of the nucleus using computer technology (Peet and Sahota 1984). Such systems can also measure other nuclear parameters such as nuclear area, average staining intensity of the nucleus, and other structural characteristics which affect chromatin distribution patterns (Nagl and Scherthan 1985, Peet and Sahota 1984).

The histogram created by microdensiometric analysis indicates the number of cells in the different interphase stages at the time of fixation, and is often accompanied by MI determination. However, sampling for DNA content and MI are frequently done separately and at different sampling frequencies (Cottignies 1979, Ormrod and Francis 1985) due to the labor intensive nature of microdensiometry versus the rapid visual assessment of MI.

While results obtained by microdensiometry do not measure the duration of the cell cycle or it's component phases, it has been suggested that the proportion of nuclei in each cell cycle stage may be proportional to the time spent in that stage (Howard and Pelc 1953, Sparvolli *et al.* 1966). While Walker (1954) generally agreed that the number of cells in a histogram interval (cell cycle stage) is proportional to the time spent in that interval, he pointed out that this is not a simple relationship in dividing cells. Since two cells are created at each division, a higher proportion of younger versus older cells will be found in any numerically increasing population.

Few studies have used microdensiometry to examine changes in the cell cycle in conifer shoot apices. Cecich *et al.* (1972) intensively examined changes in

nucleic acids and proteins in *Picea glauca* V. (white spruce) during the transition from bud scales to initiation of pre-formed leaf primordia. The results indicated that the apex may go through a series of periods of intense DNA synthesis and an ordered progression of developmental and cytological sequences which result in the transition to preformed leaf initiation.

Owens and Molder (1973) examined vegetative shoot apices of Douglas fir over the annual growth cycle and observed changes in DNA content and mitotic activity. The results obtained were generally similar to Cecich *et al.* (1972), i.e. a rapid increase in DNA content and mitotic activity at late bud-scale initiation just prior to the transition to preformed leaf initiation. However, this was not thought to be due a "prolonged" duration of DNA synthesis (Cecich *et al.* 1972) but an increase of cells moving through S and into G2 which were released from G1. During the period of rapid apical enlargement, maximal mitotic activity and DNA content coincided with the most active primordia initiation. In addition, an inhibition of mitosis and an increase in the proportion of cells in G1 stage (to 50%) was observed in late November as cells became mitotically inactive. This differs from observations on microsporangia of jack pine (Cecich 1984) and the dormant shoot apex of *Fraxinus excelsior* L. (Cottignies 1979) where all cells of the apex remained in G1 during winter.

2.2.2.4 Structural changes in nuclei during the cell cycle

Changes in the size and morphology of nuclei during interphase have been observed in several plants species and tissues using light and electron microscopy in conjunction with microdensiometry and autoradiography. Changes in nuclei have been related to interphase stage (Nagl 1970 a,b, Nagl 1977), elapsed time in interphase (Woodard *et al.* 1961), the transition from G1 to S (Mitchell *et al.* 1983),

stages of DNA replication (Lafontaine and Lord 1974, Barlow 1976, 1977), proliferative capacity (Mitchell *et al.* 1983, Armstrong and Francis 1987), cellular differentiation (Mitchell *et al.* 1983) and patterns of cytological zonation (Owens and Molder 1973).

Nagl (1970 a,b,) was the first to suggest that a correlation between morphological features of nuclei and cell cycle stage was possible by visual criteria using light microscopy and squash preparations. This was accomplished by treating nuclei from the root apex of two species of *Allium* with tritiated thymidine to identify S nuclei, and measuring total DNA content and nuclear volume to initially distinguish between G1 and G2 nuclei (Nagl 1970 a,b). Nagl (1970b, 1977) diagramatically represented G2 nuclei as both larger and more darkly staining than G1 nuclei. Lord and Lafontaine (1976) and Kuroiwa and Tanaka (1971) have used similar procedures on sectioned material.

Nagl and Scherthan (1985) have most recently stated that particularly clear alterations between G1 and G2 are visible in DNA-(chromatin) rich species and/or species which exhibit the chromonematic type of nuclear organization. Conifers have a relatively large amount of DNA (Cavalier-Smith 1978); loblolly pine has 26 pg of DNA per nucleus at the 2C level (Renfroe and Berlyn 1984). Additionally, Kupila-Ahvennemi (1978) have stated that nuclei from bud tissues of *Pinus sylvestris* (Scots pine) exhibit reticulate-like (chromonematic) organization.

Nuclear volume or area (Woodard *et al.* 1961, Mitchell *et al.* 1983) has also been used to determine nuclear progress through the cell cycle. This is based on the assumption that nuclear volume will increase with time and that the frequency of nuclei with a particular nuclear volume is proportional to the duration of time taken by that nucleus to reach that size (Woodard *et al.* 1961). Mitchell *et al.* (1983) have used this model to examine changes within cell cycle stages, specifically G1. The

basis for increased nuclear volume during interphase has two potential causes. It has been suggested that increased levels of decondensed chromatin, due to increased nuclear volume, is satisfying transcriptional needs as cells increase in size (Mitchell *et al.* 1983). This supports Cavalier-Smith's nucleoskeletal hypothesis (1978), which states that as cells enlarge so must the nucleus to increase the number of nuclear pores available to transport transcriptional products. Secondly, nuclei will enlarge as synthesis increases the amount of DNA in the nucleus, although volume does not necessarily need to double with a doubling in DNA (Nagl and Scherthan 1985).

Webster (1979) disputed the conclusions of Woodard *et al.* (1961) and showed that nuclei exhibit heterogeneity in nuclear volume both within and between cell cycle stages which precluded its use to determine percentage elapsed interphase time. Mitchell *et al.* (1983) have also pointed out that nuclear volume as a indicator of cell cycle progression is not universally applicable to all cells and tissues, and should be documented experimentally.

Studies examining the relationship between nuclear size and cell size have also produced variable results. Thomas and Davidson (1983) found that although cell and nuclear size were tightly coupled in roots growing under constant growth conditions, the two factors were very plastic and independent of each other when environmental conditions changed. Armstrong and Francis (1987) found that nuclear and cellular parameters can act independently, and that large increases in cell size when placed in culture will alter the nuclear:cytoplasmic size ratio, while Lyndon (1967) showed that cell and nuclear volume relationships varied among different cell files in roots.

Owens and Molder (1973) examined vegetative shoot apices of Douglas fir over the annual cycle of growth and observed changes in nuclear volume independent of cell cycle stage which were related to zonal origin and cell size. This

represents the only study which has directly examined nuclear volume and cell cycle stage in vegetative conifer shoot apices. During the period of rapid apical enlargement, maximal mitotic activity, DNA content, and nuclear volume were correlated with the most prominent apical zonation and most active primordia initiation. This was due to decreases in cell and nuclear volumes in the peripheral zone and increases in cell and nuclear volumes in the apical zone. Nuclear volume was correlated with both DNA content (cell cycle stage) and the zonal origin of the nucleus. Due to the independent effect of zonal origin on nuclear volume, it was suggested that nuclear volume alone was not a good indicator of DNA content. This agrees with the findings of Webster (1979), except that the heterogeneity in nuclear volume observed by Owens and Molder (1973) could be related to a specific cause, zonation in the shoot apex. This could be expected given the fundamental differences in cytology and mitotic activity among cells from these regions. Cecich (1984) suggested that nuclear volume could be used to estimate cell cycle stage, however this suggestion was based on a study of morphologically uniform, G1 nuclei of jack pine microsporangia which were beginning to synthesize DNA in the spring.

Because of the relationship between MI and developmental, physiological and environmental conditions in conifer shoot apices, a more intensive study of the cell cycle is needed. This requires development of techniques specific to conifer shoot apices, due to the protection of these structures by layers of primordia and suberized bud-scales or cataphylls. Pulse labelling studies with tritiated thymidine and detailed structural analysis of nuclei have never been attempted on conifer shoot apices, while microdensiometry has been successful but imposes severe restraints on experimental size and sampling intensity.

CHAPTER 3

MITOTIC INDEX OF CONIFER SHOOT TIPS

3.1 Introduction

Mitotic index (MI), the percentage of cells in mitosis, has been successfully used to observe changes in seedling and mature shoot apices of conifers under natural and experimental growth conditions. These studies have correlated MI with developmental stage (Colombo *et al.* 1988, Fielder and Owens 1989, O'Reilly *et al.* 1989), frost hardiness (Colombo *et al.* 1988), nutrient availability (Carlson *et al.* 1980), external temperature and photoperiod (Carlson 1985a, Colombo *et al.* 1988), and respiratory activity (Fielder 1986) in seedlings, and reproductive development (Owens and Simpson 1988) in more mature trees. Mitotic frequency (MF), the number of cells in division in the median 6% of the apex (Owens *et al.* 1985) from longitudinally sectioned apices has also been used, and is similar to measurements of MI (Owens and Molder 1973).

Some developmental and anatomical studies have used paraffin embedded, sectioned and stained apices (Owens and Molder 1973, Fielder 1986, Fielder and Owens 1989). This technique allows assessment of MI or MF for each of the zones of the apex (apical, peripheral, rib meristem and pith) and/or the entire apex. However, sampling using longitudinal median sections is not a complete sample of the apex. Also, processing and sampling included many steps and results are not available for several weeks. Carlson *et al.* (1980), Carlson (1985a) and Colombo *et al.* (1989) decreased the time from fixation to MI determination by using acetocarmine staining of either squashed embryonic shoots or excised apices. This allowed all cells to be potentially sampled but destroyed structural and anatomical features. In both cases MI was determined for one grid area over the most

mitotically active region. Recently, the Feulgen technique has been used to stain nuclei in squash preparations and MI has been assessed on either all the cells in the apex (O'Reilly *et al.* 1989), or in three randomly selected grid fields (pers. comm. M. Krasowski).

The purpose of this study was to develop a standardized method for squash preparation and MI determination of shoot apices. Specifically, a practical squash technique should allow one to process many samples easily and quickly, allow short term storage at certain stages in the process, and consistently produce high quality permanent preparations. The sampling procedure should allow rapid and objective sample selection of a minimum number of cells per squash, adequately represent the mitotic activity of the whole apex, allow sampling of large and small apices without major procedural modification, and produce a small standard error.

This was accomplished with improved Feulgen staining techniques and fixed interval horizontal scanning. Using these techniques the resumption of mitosis was observed in shoot apices of loblolly pine seedlings removed from cold storage and placed in a lighted 25°C environment. The relationship between the results of this preliminary experiment and cell cycle populations, and the growth potential of seedlings are discussed.

3.2 Methods and Materials

3.2.1 Fixation and staining

Shoot apices of first year loblolly pine seedlings in neoformed leaf initiation were dissected leaving only the last layer of primordia covering the apical dome. This was done using either a triple 0 insect pin mounted on a needle probe, a microscalpel (Rudolf Beaver Inc., Waltham, MA) or a scalpel, depending on the size of primary needles or other primordia being removed. Apices and 2-3 cm of the subtending shoot were fixed in 10% formalin (pH adjusted to 7.0 with 1N NaOH) in Tissue-tek polypropylene processing capsules (Miles Laboratories, Naperville, Ill.) at 0-4°C for a minimum of 2 hrs (Fox 1969, Teoh and Rees 1976, Greilhuber 1986). Longer fixation did not affect shoot apices as long as this low temperature was maintained (Greenwood and Beryln 1968). Several apices were also fixed in FAA (formalin-acetic acid-alcohol) at room temperature. Fixation for 24 hours was commonly used. Specimens were washed 3 times in distilled water at 4°C over a 24-hr period to remove formalin then washed in 20°C water for 10 minutes prior to hydrolysis. Apices were hydrolyzed with 5N HCl at 20°C for 40-60 minutes and resulted in good staining (Fox 1969), but 50 minutes produced the best monolayer preparations of cells. Following hydrolysis samples were washed once in distilled water and stained in Schiff's reagent (Fox 1969) for 2 hrs in the dark at room temperature. Specimens were then rinsed 3x10 minutes in SO_2 water, then in several distilled water rinses and stored in distilled water at 4°C for several days until needed.

3.2.2 Squash preparation

Shoot apices stained more intensely than surrounding tissues and were easily

located under 40x magnification of a dissecting microscope. The last primordia which had protected the apex during processing were removed, followed by any smaller primordia at the base of the apical dome (Fig 1). The apex was then dissected from the shoot (Fig. 2) with an insect pin and placed in a drop of 45% acetic acid on a microscope slide using a microscalpel. Under the dissecting microscope the apex was oriented upright and any remaining primordia were carefully removed. A 22 X 22 mm #1 coverslip was lowered from one side until the apex was initially squashed. The eraser end of a pencil was used to apply direct vertical pressure to the coverslip and gently squash the cells into a monolayer (Fig. 3,4). The slide was then placed on a flat piece of dry ice for 30 seconds or until completely frozen (Conger and Fairchild 1953). A thin edge razor blade was used to pry one corner of the coverslip from the slide while still on the dry ice. The slide, with cells still attached, was immediately immersed in 95%, then 100% ethanol for 2-3 minutes each and mounted in euparal (Carolina Biological Supply, Gladstone, Ore). Slides were dried face up in the dark for several days. Slides were stored in the dark to prevent fading of the stain.

3.2.3 Sampling of squashes

Observations were made through a Leitz Orthoplan light microscope with a 10X ocular and 40X objective lens. Squash sampling by horizontal scanning was conducted with a 10 X 10 mm, square grid ocular micrometer with divisions at 1-mm intervals. The intersection of the median horizontal and vertical lines of the grid produced a center point. Sampling began by placing the top horizontal line at the top of the rounded squash preparation. The centerpoint was then moved horizontally to the left until it was beyond the periphery of the squash. A scan was then made from left to right. Nuclei or chromosomes which made contact with the

center point were recorded as mitotic or interphase. Vertical deviation of horizontal movement was adjusted, when necessary, since fixed horizontal movement was a basic assumption of the technique. Only one anaphase or telophase figure per pair was counted irrespective of whether both made contact with the scan line. Determination of prophase and telophase figures was often difficult due to the continuum of morphological change as interphase nuclei enter prophase, and telophase figures enter interphase. Prophase figures were identified by the presence of visible chromosomes which in early stages appeared granular with a lobed perimeter, while telophase figures were identified by paired asymetrically shaped, reforming nuclei which often had a convoluted perimeter (Fig. 5). Additionally, despite careful dissection, squashes were routinely contaminated with differentiating procambial cells (Fig. 6) and tannin filled pith cells (Fig. 7), neither of which were counted.

Following completion of a horizontal scan, the center point was moved down to the position of the bottom horizontal line, a 200 μm distance. The center point was then moved to the left side of the squash and scanning recommenced at the center point. Scans were made until the squash was fully sampled. MI was calculated by the following equation:

Mitotic Index= (# Mitotic figures/ Total counted (mitotic and interphase)) x 100

The accuracy of horizontal scanning was examined by measuring the MI, as described above, of 10 shoot apices of loblolly pine seedlings during free growth and then re-measuring the same apices at five times the vertical sampling frequency (every 40 μm). This did not result in the sampling of every nucleus but was sufficiently intensive to indicate sampling deficiencies if they existed. The mean and

standard error of MI, and the ratio of cells counted by the intensive versus less intensive method were calculated on untransformed data. In addition, a paired t-test was conducted on MI data after arcsin transformation (Zar 1984).

3.2.4 Resumption of mitosis after cold storage

Second year loblolly pine seedlings were grown in 1987 under greenhouse conditions in plastic tubes (Cone-tainer Nursery, Canby, Oregon) with frequent fertilization with 20-20-20 (N, P, K) at 100 ppm nitrogen. On October 2 seedlings exhibiting well formed terminal buds were placed outdoors in a lathhouse and allowed to accumulate chilling hours (the number of hours between 0°C and 8°C, Carlson 1985b), under ambient conditions at Victoria, B.C. On February 12, 1988 these seedlings were moved to a growth chamber with a 10-hr. photoperiod, and 5°C day/night temperature. On February 20th they were placed in the dark at 1°C until April 6th to simulate storage conditions. Thus seedlings were exposed to an unknown amount of natural chilling plus 1296 continuous chilling hours in growth chambers (1056 hrs. of which were in continuous darkness).

On April 6th seedlings were placed into a 25°C growth chamber with continuous light (300 μmol) for 20 hrs. Seven apices were sampled while still in cold storage (Time 0) and 5-7 apices every 2 hrs thereafter. Six seedlings were not sampled during the experiment. These were placed in a greenhouse after the experiment to insure that buds could flush and were not damaged by either cold storage or rapid exposure to 25°C temperatures.

Apices were prepared as described above. Between 3 and 6 squashes of apices were measured at each collection time. Squashes were scanned at 200 μm intervals except at Time 0, when they were sampled at 80 μm intervals to insure the initial absence of mitotic activity. In addition to mitotic and interphase counts, the

number of G2 nuclei were also recorded and G2 index (G2I), the percentage of cells in G2, calculated. Cells in G2 occupy the last portion of interphase prior to mitosis (Van't Hof 1968). These cells were qualitatively identified in squash preparations by a large, darkly staining nucleus (Fig. 3) due to a fully replicated 4C level of DNA. The ability to distinguish G2 from G1 nuclei had been reported previously (Nagl 1970a,b) and a similar system of identification had been developed for pine nuclei from the shoot apex (Chapter 4). Nuclei in S cannot be identified by this method without treatment with tritiated thymidine. If present, nuclei in S were identified as either G1 or G2 depending on the amount of DNA synthesis which had been completed when fixed.

3.3 Results

3.3.1 Staining and squash preparation

Squash preparations of loblolly pine shoot apices using 10% neutral formalin fixation, 20°C hydrolysis with 5N HCl and staining in Schiff's reagent produces visually excellent preparations of nuclei without background cellular staining (Figs. 3,4). Nuclei were vivid magenta except for small, lightly stained areas which were the nucleoli (Fig. 3). Apices prepared in the same manner but fixed with FAA also produced well stained nuclei but cytological detail was significantly reduced. Two stages in the processing of apices were amenable to storage: during initial fixation in formalin and during storage in distilled water prior to squashing. Apices were routinely stored for 1-3 days at both of these stages.

3.3.2 Sampling of squashes

Due to losses during dissection, and preparations with excessive contamination

Fig. 1. Scanning electron micrograph of loblolly pine shoot apex in neoformed leaf initiation showing shoot apex (a) and primordia (pm). X 77.

Fig. 2. Median longitudinal section of Feulgen-stained loblolly pine shoot tip. The area inside the triangle indicates the region of the shoot tip removed for squash preparations, apex (a), primordia (pm). X 61.

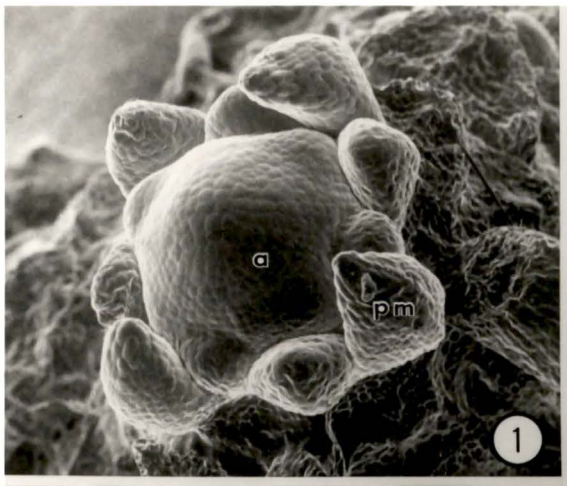
Fig. 3. Squash preparation of Feulgen-stained shoot apex in cold storage showing 3 G2 nuclei (2), 3 G1 nuclei (1) and a nucleolus (n). X 757.

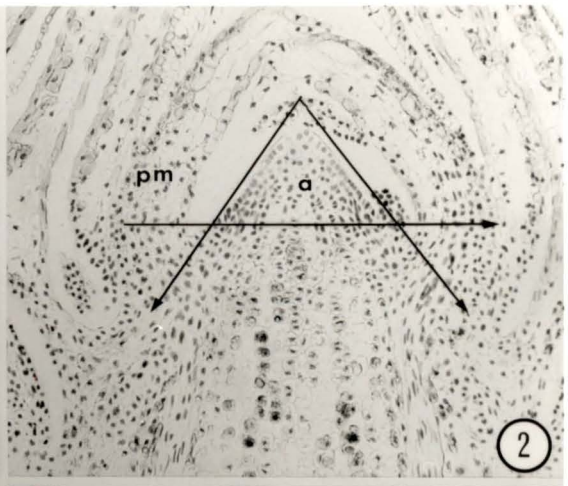
Fig. 4. Squash preparation of Feulgen-stained shoot apex resuming mitosis in growth promoting conditions after 10 hours, showing prophase (p), metaphase (m), and anaphase (an) figures. X 757.

Fig. 5. Squash preparation of Feulgen-stained shoot apex showing prophase (p) and telophase (t) figures. X 757.

Fig. 6. Squash preparation of Feulgen-stained shoot apex showing three nuclei of the procambium (pc) X 757.

Fig. 7. Squash preparation of Feulgen-stained shoot apex showing pith cells (pi) containing tannins. X 757.


a
pm
1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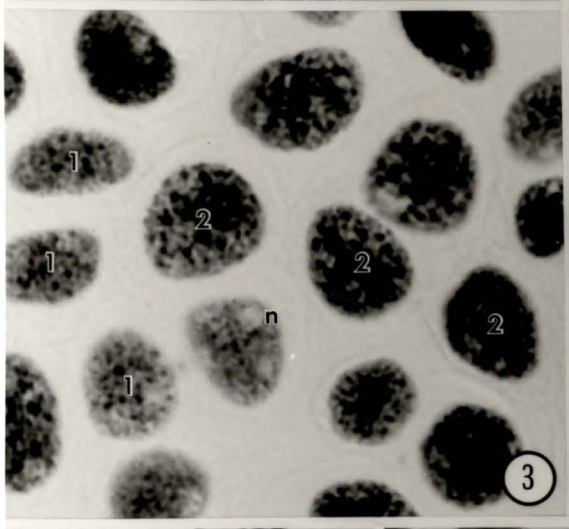
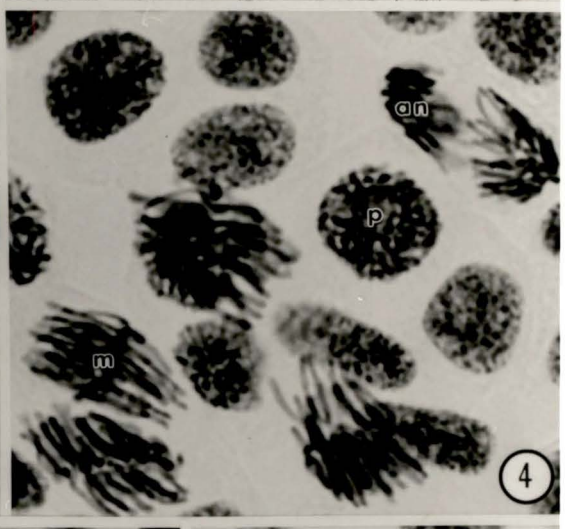
2


1
2
1
2
n
2
1
3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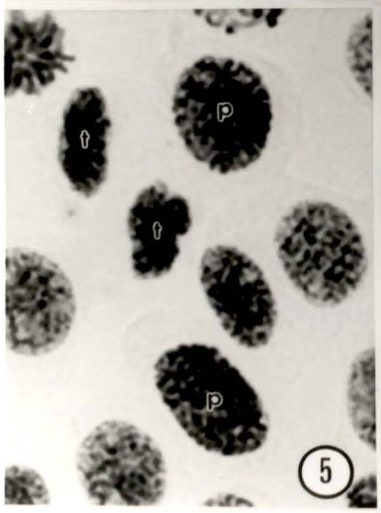
P
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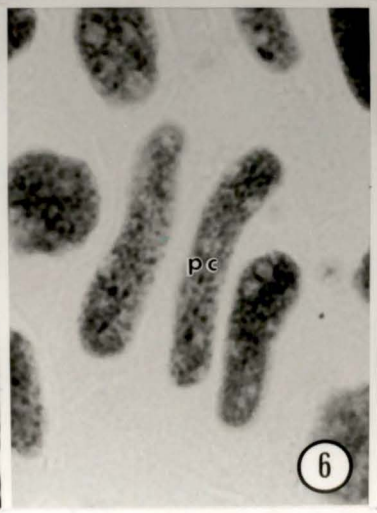
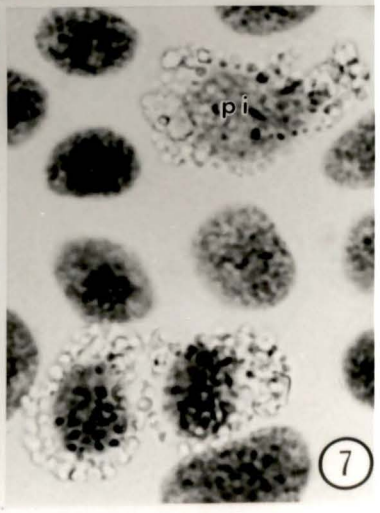

pc
6


pi
7

of pith and procambial cells, a collection size of 15-20 apices was required to produce a minimum of 10 examinable squash preparations. Sampling using the horizontal scan technique at 200 μm intervals involved 8-14 horizontal scans and counts of 350-650 nuclei. This method proved to be both rapid and objective for determining MI.

The intensive scan technique (40 μm intervals) was very time consuming and required 45-75 scans/squash and counts of 1800-3100 cells, 5.1 times the number of nuclei counted by the less intensive method. Untransformed data and paired t-test comparison of the two methods after arcsin transformation is presented in Table 1. Differences were not found ($p = .192$). By increasing the sampling intensity over five fold the already low standard error of the mean was reduced by only half. Therefore, sampling by horizontal scanning at 200 μm gave an accurate measure of MI of the shoot apex with an acceptable level of variance when the sample size of apices was 10 or greater.

3.3.3 Resumption of mitosis in shoot apices after cold storage

Second year loblolly pine seedlings were mitotically inactive in cold storage but rapidly resumed mitotic activity when placed in growth promoting conditions. MI was zero in cold storage except for four metaphase figures. However, these did not appear to be actively dividing cells since the chromosomes were extremely condensed. The MI remained at or near zero during the first 6 hours in growth promoting conditions (Table 2, Fig. 8). A few early prophase figures were observed, but no anaphase or telophase structures were present. G2I indicated that apices varied in the percentage of cells in G2 between 3-27% during this period (Table 1, Fig. 9). The first prominent prophase figures appeared at 8 hr and all mitotic stages were present by 12 hr. The highest mean MI (9%) occurred at 14 hr and coincided

Table 1. Mitotic index assessed by horizontal scanning at two vertical intervals

Vertical Interval (μm)	Number of Samples	Mean Number Cells Counted/Apex	Mean Mitotic Index (%)	Standard Error (%)
200	10	430	6.42	.40
40	10	2199	5.99	.23

Ratio Cells counted (200/40)= 5.1

Paired T-test t =1.41 D.F.= 9 p= .192

Fig. 8-10. The resumption of mitosis in loblolly pine shoot apices placed in growth promoting conditions after removal from cold storage.

Fig. 8. Individual mitotic index measurements (closed circles).

Fig. 9. Individual G2 index measurements (closed triangles).

Fig. 10. Mean mitotic index (closed circles) and G2 index (open triangles) for each collection period.

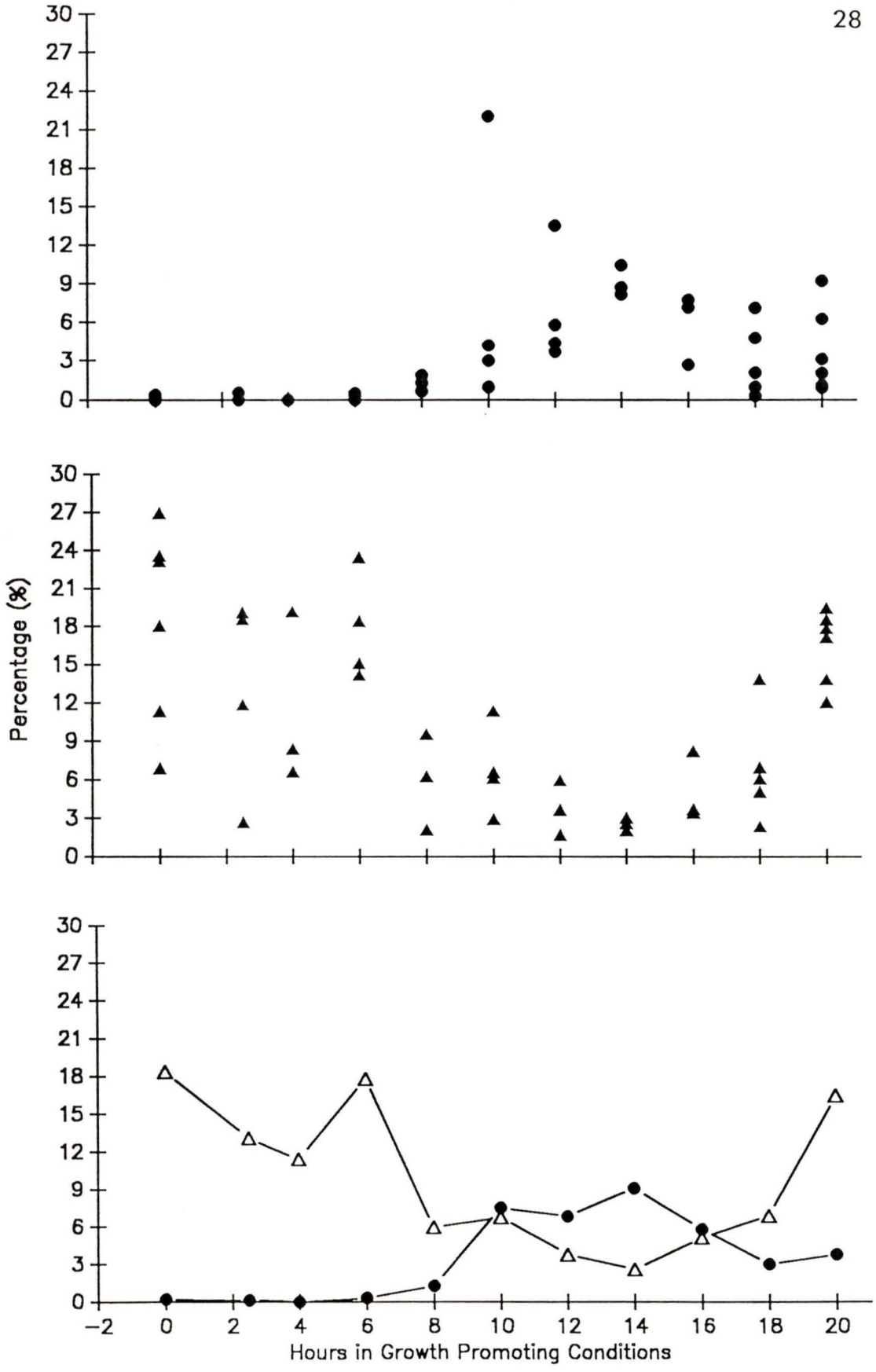

30
27
24
21
18
15
12
9
6
3
0
Percentage (%)
−2
0
2
4
6
8
10
12
14
16
18
20
Hours in Growth Promoting Conditions

Table 2. Changes in mitotic index (MI) and G2 index (G2I) in shoot apices placed in growth promoting conditions

Time(Hr)	N	Mean MI	Range of MI	Mean G2I	Range of G2I
0	6	.19	0.0 - 0.36	18.34	6.90 - 26.96
2.5	4	.13	0.0 - 0.52	13.05	2.69 - 19.09
4	3	.00	0.0 - 0.0	11.40	6.64 - 19.14
6	4	.31	0.0 - 0.50	17.79	14.19 - 23.46
8	3	1.25	0.63 - 1.87	5.98	2.10 - 9.56
10	4	7.53	0.97 - 22.00	6.76	2.92 - 11.39
12	4	6.84	3.69 - 13.50	3.75	1.70 - 5.98
14	3	9.09	8.16 - 10.40	2.56	2.04 - 3.04
16	3	5.84	2.65 - 7.73	5.13	3.43 - 8.24
18	5	3.03	0.28 - 7.12	6.88	2.36 - 13.88
20	6	3.77	0.92 - 9.21	16.47	12.08 - 19.43

with a decrease in mean G2I to a minimum of 2.6% (Table 2, Fig. 10). Mean MI decreased at 16, 18 and 20 hr., while G2I increased to 16% in the 20-hr collection. No external change in the appearance of buds was observed during the experiment, but seedlings placed in the greenhouse following the experiment flushed and their preformed shoots elongated normally.

3.4 Discussion

Squash preparations to assess MI of conifer shoot tips was initiated by Carlson *et al.* (1980). Since then several researchers have used squash preparations for this purpose (Anonymous 1985, Carlson 1985a, Colombo *et al.* 1988, O'Reilly *et al.* 1989), but procedures for the preparation and sampling of squashes were too varied to allow comparison among different studies. In order to understand the relationship between MI and the cell cycle in conifer shoots a staining protocol and sampling method were developed and tested. These procedures will increase the clarity and uniformity of squash preparations, and standardize sampling for MI determination.

3.4.1 Squash Preparation

The Feulgen method has long been used to quantitatively stain DNA and works well on shoot and root apices producing clear nuclei and chromosomes without cytoplasmic staining (Jenson 1962). In loblolly pine and other species (Greilhuber 1986) fixation at 0°C with 10% neutral formalin produced better preparations than FAA fixation. Hydrolysis with 5N HCl at 20°C is preferred since it is more easily controlled and produces more uniform staining than 60°C hydrolysis with 1N HCl (Fox 1969). The time of collection and fixation of apices should be standardized to minimize diurnal variability in MI (Denne 1966, Carlson

et al. 1980).

The anatomical components in the shoot tip must be recognized when preparing squash preparations. These include the shoot apex, foliar primordia, and the shoot axis. The shoot apex includes that portion of the shoot tip above the last foliar primordium (Parke 1959) and is comprised of 4 zones: apical, peripheral, rib meristem and pith (Owens and Molder 1973). These zones possess different cytological, biochemical and cell cycle characteristics (Fosket and Miksche 1966, Cecich et al 1972, Owens and Molder 1973, Riding and Gifford 1973, Cecich 1977, Gonthier *et al.* 1985). Some of these differences are observable under light microscopy and are useful when scanning squash preparations. Nuclei of the peripheral zone are spherical to oblong and stain intensely irrespective of cell cycle stage. Apical zone cells are large with large nuclei, which stain lightly due to dilution of DNA over a large nuclear area (Owens and Molder 1973). Rib meristem and pith cells are not easily differentiated from peripheral zone cells, except for pith cells which have cellular tannins. Squashing the apex in an upright position helps contain the pith cells in the center of the squash, making their identification easier. Pith cells from some species may contain large deposits of tannins which can obscure meristematic cells, making counts in the center region of the squash difficult (O'Reilly *et al.* 1989). Most squash preparations also contain cells from procambial strands which can be identified by their very elongate nuclei. Tannin filled pith cells (Fig. 7) and procambial cells (Fig. 6) should not be sampled for MI, and preparations with excessive contamination of procambial cells should be discarded.

Measurements of apical height and width from embedded and sectioned apices (Fielder 1986) or freshly dissected apices before fixation (O'Reilly *et al.* 1989) complement MI assessment. Apical size often correlates with apical function and stage of development (Gregory and Romberger 1972 a,b, Owens and Molder 1973,

Cannell 1978, Cannell and Cahalan 1979), and is useful in studies where dramatic developmental changes in apical size occur.

Foliar primordia increase in size basipetally and may also be used to assess MI since the leaves are the last portion of the bud to cease mitotic activity in the autumn and the first to resume mitotic activity in the spring (Fielder 1986, Owens and Simpson 1988, Fielder and Owens 1989). However, there would likely be high variability among primordia from the same apex due to differences in the developmental stage of primordia. The shoot axis also exhibits varied mitotic activity during the yearly growth cycle. Spring resumption of mitosis in the shoot axis precedes both cell elongation in the shoot axis and cell division in the terminal apex (Owens *et al.* 1985, Owens and Simpson 1988). However, as noted above, squash preparations of the shoot axis would be more difficult due to poor solution uptake, hardness of the more differentiated cells, and the high and variable frequency of tannin-filled cells. To avoid these difficulties longitudinal sections have been used to determine MI in this region (Owens and Simpson 1988).

3.4.2 Sampling to determine MI

A squash preparation of a conifer shoot apex is an assortment of cells from four cytological zones (Owens and Molder 1973). This produces regions in the preparation with variable MI. Sampling the most active mitotic region of the squash (Carlson *et al.* 1980) is subjective, but can be used to roughly estimate MI in different apices. This technique is most useful when apices become mitotically inactive in the fall and winter (Carlson *et al.* 1980, Colombo *et al.* 1988) or mitotically active after winter dormancy (Carlson 1985a). At this time the presence or absence of any mitotic figures is easily measured but quantitative differences may not be comparable. Dividing cells are principally in the peripheral zone and in

small primordia (Owens and Molder 1973). This would give an inflated measure of the MI for the entire apex. Also, the percentage of cells sampled in different apices would vary as apical size changed.

Recently, MI has been used to measure apical activity of seedlings during the summer and fall when differences are more likely to be subtle, involving changes in apical and zonal sizes, mitotic activity and cell cycle duration (Gregory and Romberger 1972b, Owens and Molder 1973, Colombo *et al.* 1988, Fielder and Owens 1989). This requires that MI be measured in the entire apex and all cells be sampled (O'Reilly and Owens 1989). While sampling cells from the entire apex gives an absolute measurement of cell number and MI, it is laborious for large apices which may consist of 3000 cells or more (pers. comm. C. O'Reilly).

The technique of horizontal scanning at fixed vertical intervals described in this report is a good compromise between sampling all cells in the apex and sampling the most mitotically active region. The numbers of cells counted is a fraction of the total, yet adequately samples the MI in the whole apex. Large apices will increase the number of scans required and cells counted per scan, but will sample an equivalent proportion of cells from each squash preparation.

The comparison made between the 200 μm and 40 μm sampling methods indicated that 200 μm was a sufficient vertical distance for sampling free growth and dormant apices of loblolly pine. The difference in the calculation of MI between the two sampling intervals was only 0.40% and was not significant. Standard errors of the mean were low for both methods, and was only halved by the 40 μm (5X) sampling interval. A scan interval of 200 μm should be adequate for most species and developmental stages. Due to the low percentages generated by horizontal scanning (0-15%), the arcsin transformation should be calculated on data for statistical analysis so that a normal distribution is maintained (Zar 1984).

During the winter when MI approaches zero, horizontal scanning will not be an accurate measure of the cessation of mitosis in all cells since scanning may miss mitotic figures. If the purpose of the study is to determine the dormancy status of the apex as indicated by the complete absence of mitotic activity, then the scanning method is not necessary, the presence or absence of mitosis is adequate. However, where relative changes in MI are required or in species such as loblolly pine which may not reach a MI of zero during winter (Carlson 1985a), the scanning method should be used. When needed, both MI, determined by horizontal scanning, and visual assessment of the absolute presence/absence of mitoses can be measured for each squash.

Mitotic index is a measure of the percentage of cells undergoing mitosis at the time of fixation. It has been reported to be proportional to the rate of cell division and inversely proportional to the length of the cell cycle (Lyndon 1973). Walker (1954) proposed that a change in MI results from either an alteration of the growth fraction (GF) (the percentage of cells actively traversing the cell cycle at any time), or a disproportional change in the duration of interphase or mitosis. However, no change in MI does not necessarily indicate that the cell cycle has not changed. Burholt and Van't Hof (1971) have shown experimentally in roots that MI can remain constant while cell cycle duration, the rate of cell division, and the growth fraction change. Therefore, interpretation of MI data requires an understanding of the factors responsible for changes in MI, and the realization that MI does not indicate changes in cell cycle parameters under all conditions.

3.4.3 Resumption of mitosis after cold storage

No information exists concerning the short term response of mitotically inactive cells from conifer shoot apices to growth promoting conditions. Owens and

Molder (1973), Owens and Simpson (1988), and Fielder and Owens (1989) have described the resumption of mitosis in the embryonic shoot and shoot apex of temperate conifers under natural conditions, but these observations were based only on weekly to biweekly collections. The purpose of this experiment was to determine if cells of the shoot apex would resume mitosis within a 24-hr period, and whether the scanning technique could detect these changes.

Studies of northern temperate conifers have shown that MI decreases to 0 during the winter months (Owens and Molder 1973, Carlson *et al.* 1980, Anonymous 1985, Fielder 1986, Colombo *et al.* 1988, O'Reilly *et al.* 1989), while a study conducted on loblolly pine showed that the lowest MI of shoot apices under ambient outdoor nursery conditions in Arkansas was 2.8% (Carlson 1985a). The results of this study indicate that a MI of zero in loblolly pine seedlings can be attained when a sufficient duration of growth limiting conditions (1°C and darkness) are imposed. Although some prophase and condensed metaphase figures were observed initially, the complete absence of anaphase and telophase figures indicated that mitosis was not proceeding in cold storage. This suggests that the continuous presence of MI during the winter observed by Carlson (1985a) may be due to both the comparatively mild and variable temperatures found in Arkansas and the lack of a stringent period of rest by this species (Garber 1983). Based on the studies of Garber (1983) and Carlson (1985b) the seedlings in my experiment received a full and possibly excessive amount of chilling and therefore were very receptive to growth promoting conditions.

The result was a very rapid resumption of mitoses between 10-14 hrs in shoot apices from a population of cells in G2 of the cell cycle (Fig. 8-10). Two results support this conclusion. The first is the inverse relationship between MI and G2I in the 14-hr collection (Fig. 10) and the expectation that G2 would be the first

population of cells to enter mitosis. The second is the peak in MI followed by a decrease which can be expected if a distinct, fairly synchronous population of cells had moved through mitosis (Van't Hof and McMillan 1969). Owens and Molder (1973) also found a G2 population in dormant apices of mature Douglas fir. While preliminary, my study demonstrates that the resumption of mitosis can be observed in conifer shoot apices using horizontal scanning even with small sample sizes, and that a variable pattern of MI should be expected during initial collections due to the synchronous movement of cell populations through mitosis.

Mitotic activity resumed from a G2 population of cells, but mitoses derived from cells which were in G1 in storage was not observed. Movement of G1 cells through the cell cycle is critical if mitotic activity is to be maintained. About 70-100% of the cells in the shoot apex are in G1 during the winter (Cottignies 1979, Owens and Molder 1973). In addition, G1 has been shown to be an important control point of the cell cycle under limiting conditions (Van't Hof 1968, Webster and Van't Hof 1969, Van't Hof and Rost 1972). Therefore, the time taken for G1 cells to enter S, and therefore re-enter the cell cycle, may be more indicative of the dormancy status of the shoot apex. Synchronous movement of G1 cells into S in the spring has been reported in ash (*Fraxinus excelsior*) (Cottignies 1979). Reactivation of G1 cells would be indicated if a second peak in MI occurred, or if apices were treated with tritiated thymidine to test for DNA synthesis. Changes in G2I at hour 20 in loblolly pine suggested that a new population of cells, possibly from G1 or S, had begun to enter G2. However, studies of longer duration are needed to confirm this speculation.

3.4.4 Resumption of Mitosis as a Research Tool

Date to bud burst (DBB) and dormancy release index (DRI) have been used to determine the growth potential of conifer seedlings during dormancy and cold storage (Garber 1983, Ritchie 1984, Carlson 1985b, Boyer and South 1989). The DBB test involves placing seedlings under promotive conditions and observing bud flushing over a 10-200 day period depending on the species, their dormancy status, and the promotive conditions used. Loblolly pine varies in DBB from 10-40 days during the winter (Carlson 1985b, Boyer and South 1989). These studies have shown that seedlings during early winter do not rapidly flush when placed in promotive conditions, whereas flushing greatly accelerates during January and February. However, bud flush results from a combination of the onset of development and the subsequent rate of development. Both cell division and cell elongation are involved (Fielder and Owens 1989). Therefore DBB is only a rough estimate of seedling dormancy status.

The resumption of mitosis is a more rapid and accurate method of determining seedling dormancy status than DBB since cellular changes in the shoot apex occur well before bud swell and indicate precisely when the bud is reactivated (Fielder and Owens 1989). In western redcedar the resumption of mitosis under promotive conditions has been observed to vary from several days to less than one day with increasing chilling, as in loblolly pine (pers. comm. M. Krasowski). Further work needs to be done to establish relationships between the time required for the resumption of mitosis, reactivation of G1 cells, bud flush, and seedling performance.

More rapid tests are needed to predict seedling performance. Cytological methods such as MI and the resumption of mitosis are more accurate and rapid than DBB since they measure one process, mitosis, which is more closely related to

biochemical and molecular processes than the more complex process resulting in DBB (Fielder and Owens 1989). This test will complement current tests and provide a cellular basis for future tests which examine biochemical and molecular processes.

CHAPTER 4

A VISUAL METHOD TO DETERMINE THE CELL CYCLE STAGE OF CONIFER NUCLEI

4.1 Introduction

The cell cycle is a fundamental concept in biology, and a basic unit of time at the cellular level (Mitchison 1971). The cell cycle is composed of 4 stages: a period of DNA synthesis (S), preceded by a G1 and followed by a G2 period, and mitosis (Howard and Pelc 1953). Few studies have examined the cell cycle in conifer shoot apices, although the measurement of mitotic index (MI), the percentage of cells in mitosis, is frequently used as indicator of cellular activity (Carlson 1985a) and anatomical dormancy (Fielder and Owens 1989). Studies of MI are useful, but provide little information about the cell cycle (Burholt and Van't Hof 1971, Carlson 1985a).

Microdensiometry has been used to determine the cell cycle stages of nuclei in conifer shoot apices (Cecich *et al.* 1972, Owens and Molder 1973) and has shown dramatic differences in interphase populations during the annual cycle of shoot growth. Further research in this area was hampered by the labor intensive nature of microdensiometry which severely limited the size of experiments and the ability to sample large populations of nuclei (pers. comm. J.N. Owens).

The cell cycle can also be studied by the incorporation of tritiated thymidine into nuclei (Quastler and Sherman 1959, Wimber 1966, Ormrod and Francis 1985). This has been successful only in a few angiosperms (Ormrod and Francis 1985, Gonthier *et al.* 1985), because apices are commonly protected by layers of leaf primordia or bud scales making solution penetration difficult (Gifford *et al.* 1963, Lyndon 1973). This is also true in conifers, many of which have suberized bud scales

protecting the shoot apex. There are no reports of successful incorporation into conifer shoot apices, except for a brief note by Taillander (1965) using *Pinus pinea*, but a description of the labelling procedure was not provided.

Several studies by Nagl (1970a,b, 1977) and Nagl and Scherthan (1985) using *Allium* root tips indicated that visual identification of G1 and G2 nuclei was possible after examination of chromatin structure, and measurement of nuclear volume and DNA content. Labelling with tritiated thymidine was required to identify nuclei in S stage (Nagl 1970b). In these studies chromatin structure referred to the abundance of euchromatin and heterochromatin, and the number and size of chromocenters. Light micrographs, written descriptions, and diagrammatic representations of nuclei by Nagl (1970 a,b, 1977) and Kupila-Ahvenniemi (1978) have also shown that G1 and G2 nuclei differ in staining intensity (SI). The objective of this study was to develop a technique to identify the cell cycle stage of nuclei in squash preparations using nuclear morphology and tritiated thymidine labelling. This technique would allow interphase populations to be identified without microdensiometry.

4.2 Materials and Methods

The loblolly pine (*P. taeda* L.) seedlings in these studies, unless otherwise stated, were in neoformed leaf initiation and had elongated 1-3 cm from cotyledon height. Seedlings were grown in growth chambers at 25°C day/night with 16 hour photoperiod (300 μmol) and were fertilized three times a week with 20-20-20 (N,P,K) at 100 ppm nitrogen. This will be referred to as experimental growth conditions.

4.2.1 Labelling of nuclei to determine cell cycle duration

Several preliminary studies were conducted to determine how to dissect and treat loblolly pine shoot apices with tritiated thymidine to determine the duration of the cell cycle. Shoot tips of 1-year-old seedlings in preformed leaf initiation had most foliar primordia dissected away exposing the apex. The entire seedling, which was grown in a plastic tube (Cone-tainer, Canby, Oregon) was then inverted in a rack and a 1.8 ml cryovial containing tritiated thymidine (2-5 μCi(74-185 kBq)/ml) and surfactant (.1% DMSO or tween 20) was attached to the shoot tip with a sponge. Shoot tips were immersed in the solution for 2 hours, then washed in distilled water for several minutes. Seedlings were returned to an upright position and apices were collected at intervals. Shoot tips were observed for visible damage when collected. Apices were fixed in FAA (formalin-acetic acid-alcohol), dehydrated in a tertiary butyl-alcohol series, embedded in tissueprep, longitudinally sectioned at 6 μm, and stained by the Feulgen technique (Jenson 1962). Slides were dipped in complete darkness in a 1:1 solution of NTB3 emulsion (Kodak, Rochester, NY) and distilled water at 42°C for 2-3 seconds, and then allowed to air dry in a upright position for several minutes. Slides were exposed in darkness for 6 weeks and then developed in dektol (Kodak, Rochester, N.Y.) diluted 1:1 with distilled water for 2 minutes at 10-15°C, rinsed for 10 seconds in distilled water, fixed for 8 minutes in Kodak fixer, washed in distilled water for 5 minutes, put through 95% and 100% alcohol for 3-5 minutes each and then mounted in histoclad.

4.2.2 Labelling of nuclei to determine cells in S

4.2.2.1 Experiment 1

Shoot tips of loblolly pine were excised from seedlings and dissected down to the last 2-3 foliar primordia covering the shoot apex. The shoot apex and subtending 3-4 cm of the shoot axis were then placed in distilled water with 1% DMSO for a minimum of 20 minutes at room temperature (approximately 29°C) as pretreatment, then immersed upside down in a vial containing the pretreatment solution plus 5.5 μCi (203.5 kBq)/mL tritiated thymidine (methyl-^{3}H in sterile aqueous solution, spec. act. 20Ci (740 GBq)/mmol, NEN, Mississauga, Ont.) for 2 hrs. Solution was in direct contact with both the apex and cut leaf traces, but not the cut vascular cylinder. Hereafter, treatment will refer to pretreatment in DMSO and subsequent exposure of tissue to DMSO and tritiated thymidine. Shoot tips were washed for 10 minutes following treatment in an excess of distilled water, and fixed, processed and squashed as described in chapter 3. After removal of the coverslip, slides were air dried and stored in the dark until prepared for autoradiography.

Slides and squashes were dipped in a 1:1 solution of NTB3 emulsion (Kodak, Rochester, NY) and distilled water at 42°C for 2-3 seconds in complete darkness, and then allowed to air dry in a upright position for several minutes. This produced a thin emulsion layer where the squash was located. Three slides were placed in each slide box with a desiccant, double wrapped in aluminum foil and exposed at -22°C for 7, 14, 21 and 40 days. Hereafter, exposure refers to autoradiographic exposure of emulsion in darkness at -22°C. An additional 14 treated shoot apices and one untreated apex were exposed for 8 days. The untreated apex served as a control to insure that grains were due to the presence of tritium in nuclei and not chemography, the creation of reduced crystals by chemicals present in the specimen

(Rogers 1979). Exposed slides were developed in complete darkness in dektol (Kodak, Rochester, N.Y.) diluted 1:1 with distilled water for 2 minutes at 10-15°C, rinsed for 10 seconds in distilled water, fixed for 8 minutes in Kodak fixer, washed in distilled water for 5 minutes, dehydrated in 95% and 100% alcohol for 3-5 minutes each and then mounted in euparal.

The preparations were exposed for variable periods of time then examined to determine the background level and grain density over labelled nuclei. Preparations exposed for 8 days were horizontally scanned using a 40X objective as in chapter 3. However, in addition to mitotic figures, nuclei in S (based on the presence of 3 grains within the circumference of a nucleus), and G1 and G2 (based on morphological criteria) were also identified. From these observations the percentage of cells in mitosis (MI), G1, S, and G2 were calculated. As a control, 14 apices were fixed without treatment and sampled for MI as in chapter 3. MI in treated and untreated apices was compared using a paired two-tailed t-test after arcsin transformation (Zar 1984)

4.2.2.2 Experiment 2

A second experiment was conducted using loblolly pine to determine whether the labelling observed in experiment 1 could be replicated, examine whether incorporation could be enhanced with higher concentrations of surfactant or tritiated thymidine, compare incorporation in shoot apices in preformed vs. neoformed growth, and determine if the technique was applicable to other conifers by applying a similar treatment to shoot apices of western redcedar (*Thuja plicata* D.).

Loblolly pine seedlings in neoformed leaf initiation, as previously described, and 2-year-old seedlings which had well formed terminal buds were used. In

January 1989, the 2-year-old seedlings which had been grown outdoors in Victoria, B.C. were placed under experimental growth conditions. This induced shoot elongation of the terminal bud and insured that cells of the shoot apex would be mitotically active. When the treatment was conducted, dwarf shoot elongation was nearly complete and a small bud with tightly appressed cataphylls enclosed the shoot apex. As described in experiment 1, shoot tips were excised from seedlings and dissected down to the last 2-3 primordia covering the apex. After dissection, shoot tips were floated in distilled water until all dissections were completed. Shoot tips were then pretreated in a 10% DMSO solution for 30 minutes followed by treatment in a solution consisting of 1% DMSO, and 10 μCi(370 kBq)/mL tritiated thymidine (from the same stock vial as experiment 1, approximately 5 months later) for 15, 30, 60 and 120 minutes. As in experiment 1, shoots were immersed upside down in the treatment solution exposing the apex and cut leaf traces to label. Five shoots were removed for each shoot type-collection period. Apices were treated thereafter as described in experiment 1. Two treated squash preparations from experiment 1 were dipped, exposed and developed with these slides to compare grain density from experiment 1 with that from experiment 2. Exposure was for 22 days.

Several apices from a western redcedar seedling were also treated. This seedling was resuming growth under 16 hr, 20°C conditions after removal from cold storage. During dissection to expose the shoot apex, shoot tips broke from the stem approximately 0.5-1.0 cm below the apex. Treatment was for 2 hours and differed from loblolly pine seedlings only in that the broken vascular cylinder near the shoot apex was also in contact with the treatment solution. Following treatment, redcedar apices were placed among loblolly pine apices for squash preparation and autoradiographic processing.

4.2.3 Microdensiometry

Shoot apices of loblolly pine seedlings in neoformed leaf initiation were fixed, stained and squashed as described in Chapter 3. One shoot apex which squashed well, and had no contamination of procambial or foliar primordia cells was selected for study and stored in the dark at 4°C. Only one preparation was used to eliminate variability in squashing pressure among preparations.

Peripheral zone nuclei were scanned from the margins of the squash where there was no contamination of pith or apical zone cells. Three horizontal scans were conducted and the first 100 nuclei or mitotic figures making contact with the sampling point were sampled for microdensiometric analysis. In addition, visual identification of interphase stage (G1, S or G2) was made.

Nuclei were measured using a 570-nm light source, a Zeiss SMP-05 scanning microphotometer (under a 100X oil immersion objective), and a Digital Equipment Corporation PDP 11/23+ microcomputer (Peet and Sahota 1984). Absorbance was calculated by computer measurement of the light intensity at .25 μm^2 pixels (squares) over the entire area of the nucleus. Total absorbance was calculated by summation of the absorbance of all the pixels located over the nucleus (Peet and Sahota 1984). This is theoretically more precise than the plug method using two wavelengths (Goldstein 1981). Total absorbance was used to determine cell cycle stage. In addition, nuclear area (NA) and the average absorbance of the nucleus which is a measure of its staining intensity (SI) were calculated (Peet and Sahota 1984) and compared in different cell cycle stages. To examine morphological variability, G1 nuclei were separated into 6 groups by increasing area. These groups were examined for differences in SI.

4.3 Results

4.3.1 Labelling of nuclei on intact seedlings to determine cell cycle duration

These preliminary studies indicated that partially dissected apices in pre-formed leaf initiation on intact conifer seedlings were damaged due to abundant sap flow from severed leaf traces and dehydration of the shoot tip following treatment. Autoradiographs showed no grains over nuclei. Based on these findings this method was abandoned.

4.3.2 Labelling of nuclei in isolated shoot tips to determine cells in S

4.3.2.1 Experiment 1

Pretreatment in 1% DMSO, followed by combined treatment in 1% DMSO and 5.5 μCi/ml of tritiated thymidine for 2 hrs resulted in incorporation of label into nuclei of loblolly pine shoot apices. Autoradiographic grains were present over nuclei after 1 week of exposure at -22°C (Fig. 11). The SI and NA of labelled peripheral zone nuclei were similar in appearance to S, G2 and large G1 nuclei as described in section 4.3.3. Nuclei were labelled in all apical zones, background levels were low, and grains were tightly grouped over nuclei. The untreated preparation exposed for 8 days showed no grains over nuclei which demonstrated that grains were not due to chemography. Longer autoradiographic exposure, up to 40 days, increased the number of grains over labelled nuclei (Fig. 12), but also increased background which made determination of lightly labelled nuclei difficult. Therefore, 8 days of exposure was used for identification of cell cycle stages in section 4.3.4.

With 8 days of exposure, both NA (as described in section 4.3.3) and the

Fig. 11. Treated loblolly pine squash preparation from experiment 1 exposed for 8 days, focused on grains above nuclei. X 757.

Fig. 12. Treated loblolly pine squash preparation from experiment 1 exposed for 40 days, focused on grains above nuclei. X 757.

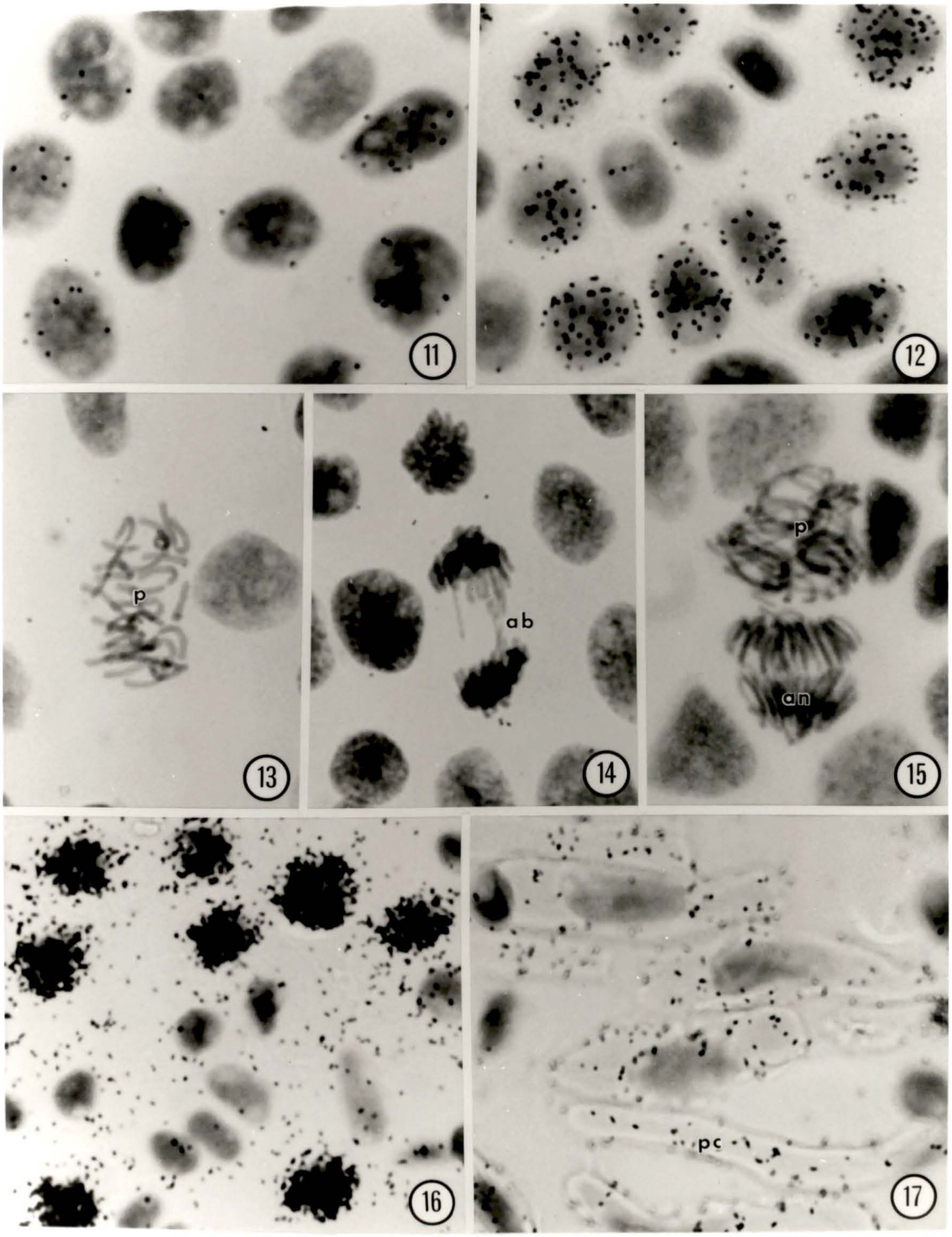
Fig. 13. Treated loblolly pine squash preparation from experiment 1, showing condensed prophase (p) chromosomes. X 757.

Fig. 14. Treated loblolly pine squash preparation from experiment 1, showing anaphase (ab) bridge. X 757.

Fig. 15. Loblolly pine squash preparation without treatment, showing thin, slender chromosomes of prophase (p) and anaphase (an) figures. X 757.

Fig. 16. Treated western redcedar squash preparation from experiment 2 exposed for 22 days, focused on grains above nuclei. X 757.

Fig. 17. Treated western redcedar squash preparation from experiment 2 exposed for 22 days, focused on grains. Note labelling of elongate procambial cells (pc). X 757.


11
12
p
13
ab
14
p
an
15
16
pc
17

number of grains per nucleus varied (Fig. 11). These factors made it difficult to determine the precise number of grains required to identify labelled nuclei. Based on the low background, absence of chemography, and close association of grains with nuclei, the presence of 3 grains over a nucleus was used to identify labelled nuclei. This was thought to be a conservative estimate of cells in S, since with only one week of exposure lightly labelled nuclei may not have had enough time to produce 3 grains.

Abnormalities in the form of enlarged and thickened prophase chromosomes (Fig. 13), and formation of anaphase bridges (Fig. 14) were observed in treated apices. These structures were not observed in untreated apices (Fig. 15). In addition, there was a significant ($p = .0008$) 26% loss in mean MI in treated versus untreated controls which indicated that treatment was affecting progression through the cell cycle (Table 3).

4.3.2.2 Experiment 2

Incorporation of tritiated thymidine into nuclei from the shoot apex of loblolly pine was confirmed by this experiment. The number of grains was somewhat lower and less uniform than in experiment 1, which indicated that increasing levels of surfactant or tritiated thymidine did not affect incorporation. However, this may also be due to the age (5 months) of the sterile aqueous stock solution. Consequently, a longer autoradiographic exposure was needed. Nuclei from apices in neoformed and preformed growth had only 1-2 nuclei labelled after 15 minutes treatment with tritiated thymidine, and increasing numbers of labelled nuclei through 1 hr of treatment. Treatment for 2 hours resulted in the most uniform distribution of labelled nuclei in squash preparations. No visual differences were observed in grain number over nuclei from apices in neoformed versus

Table 3. Comparison of mitotic index (MI) in treated and untreated apices

Apex Type	N	Mean MI	Standard Error	Decrease in MI in treated versus untreated control apices
Treated	14	4.61	.32	26.6 %
Untreated	14	6.28	.32	
Paired T-test		t= -3.78	DF= 26	p= .0008

preformed growth.

Incorporation in redcedar apices was the highest observed in experiments 1 or 2, obscuring the morphology of many nuclei in S (Fig. 16). Despite a high background level, labelled nuclei remained visually distinct. Cytoplasm of procambial cells in these squash preparations was also labelled (Fig. 17). This was not observed in preparations of loblolly pine shoot apices.

4.3.3 Microdensiometry

Scanning microdensiometry separated interphase nuclei into three groups by total absorbance (Table 4, Fig. 18). The lowest and highest absorbance groups coincided with measurement of anaphase-telophase and prophase-metaphase figures, respectively. This established the G1 and G2 level of DNA content. G2 nuclei had slightly higher total absorbances than prophase-metaphase figures (Table 4) but this was probably due to variation in total absorbance between interphase nuclei and chromosomes. Nuclei with an absorbance of 20-36 were considered G1, those between 36-52 in S, and those between 52-68 in G2. Separation of S and G2 nuclei was somewhat arbitrary since the total absorbance of S nuclei will overlap with G2 as DNA synthesis is completed (Ormrod and Francis 1985).

The majority of cells were in G1 stage (75%), and smaller percentages in S (5%), G2 (9%) and M (11%) (Table 4). Nuclei in G1 displayed considerable morphological variability but S and G2 nuclei were visually distinct from G1 nuclei. Visual discrimination between S and G2 nuclei was not possible.

The SI and NA increased from G1 to G2, but there was significant overlap between stages when the range of values were examined (Table 4). Individual measurements indicated that NA and SI were linked within cell cycle stages, so that

Fig. 18. Microdensiometric measurement of Feulgen-stained interphase nuclei from the peripheral zone of the shoot apex of loblolly pine.

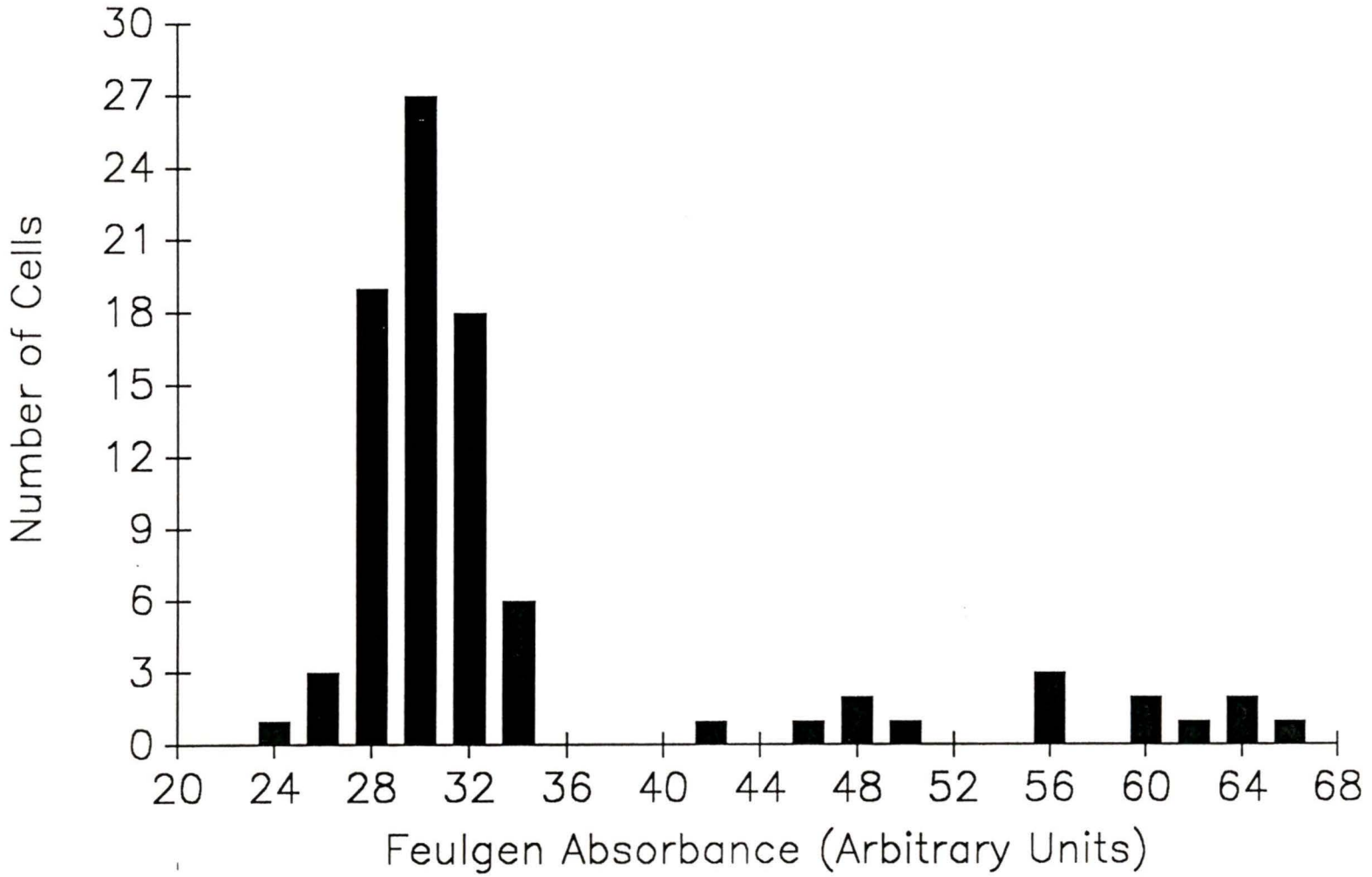

Number of Cells
30
27
24
21
18
15
12
9
6
3
0
20
24
28
32
36
40
44
48
52
56
60
64
68
Feulgen Absorbance (Arbitrary Units)

Table 4. Microdensiometry of peripheral zone nuclei

Stage	N	Mean Total Absorbance	Mean Stain Intensity (SI)	Range of SI	Mean Nuclear Area (μM^2) (NA)	Range of NA
G1	74	29.2 (0.23)	38.9	24.4 - 64.5	194	126 - 293
S	5	46.1 (1.24)	44.5	36.3 - 50.6	263	232 - 312
G2	9	59.5 (1.22)	49.6	42.6 - 54.3	301	274 - 341
M (A-T)	6	28.6 (0.56)	55.1	35.6 - 64.7	134	99 - 171
M (P-M)	5	55.6 (1.03)	42.2	29.3 - 58.6	337	256 - 392

Note: Figures in parentheses indicate ±1 standard error of the mean.

(A-T) anaphase-telophase, (P-M) prophase-metaphase

increases in NA decreased SI and vice versa. This interaction can be simulated by calculating an NA X SI factor, which is similar to the calculation of total absorbance using the plug method (Swift and Rasch 1956). When this is done (Table 5) the NA X SI factor is distinct for each cell cycle stage irrespective of differences in NA or SI of individual nuclei. In addition, when the morphologically variable G1 population of nuclei were separated into groups by increasing NA, the SI of the groups decreased proportionally (Table 6).

4.3.4 Visual identification of G1 and G2 Nuclei

Preparations which were treated with tritiated thymidine allowed identification of nuclei in S, and in addition mitotic figures, G1 and G2 nuclei could be identified based on visual morphology (Fig. 19, 20). G1 nuclei varied from small and darkly staining to large and lightly staining. The large sample of nuclei in S and G2 in this study were more variable in NA than the small sample observed in the microdensiometry study. However, the variation in NA of G2 nuclei affected SI proportionally (as described for G1 nuclei in section 4.3.3). The relationship between SI and NA made it possible to distinguish G1 and G2 nuclei. Mitotic figures which have a known DNA content (anaphase-telophase (2C), prophase-metaphase (4C)) assisted in these determinations.

Since horizontal scanning was made on the entire apex, nuclei from all zones of the apex were sampled. Rib meristem nuclei did not differ in morphology from peripheral zone nuclei (Fig. 19, 20). However, apical zone nuclei were larger (Fig. 21) and peripheral zone nuclei involved in early primordia formation (basal peripheral zone nuclei) were smaller (Fig. 22) at all stages of the cell cycle. Due to the compensating effect of SI with changes in NA, the large apical zone nuclei appeared very lightly stained (Fig. 21) and small basal peripheral zone nuclei

Table 5. Morphology of individual peripheral zone nuclei

Stage of Interphase	Nuclear Area (NA)	Staining Intensity (SI)	Total Absorbance	NA X SI ($X10^{-2}$)
G1	149.5	52.2	31.2	78.0
	202.8	33.4	27.1	67.7
	250.8	27.4	27.5	68.7
S	231.8	50.7	47.0	117.5
	285.0	36.7	41.8	104.6
	311.5	36.3	45.2	113.1
G2	274.3	53.6	58.8	147.0
	298.8	45.4	54.3	135.3
	340.8	42.6	58.1	145.2

Table 6. Differences in staining intensity (SI) of peripheral zone nuclei in G1 with different nuclear area

Range of Nuclear Area (μM^2)	N	Mean Total Absorbance	Mean Stain Intensity	Range of Stain Intensity (SI)
125-150	8	30.1	54.0	64.5 - 48.7
151-175	19	28.7	44.2	50.5 - 39.0
176-200	13	29.9	40.3	44.6 - 36.4
201-225	17	29.0	34.0	39.0 - 31.0
226-250	12	28.3	30.1	35.2 - 25.5
250-300	5	29.8	28.4	32.9 - 24.4

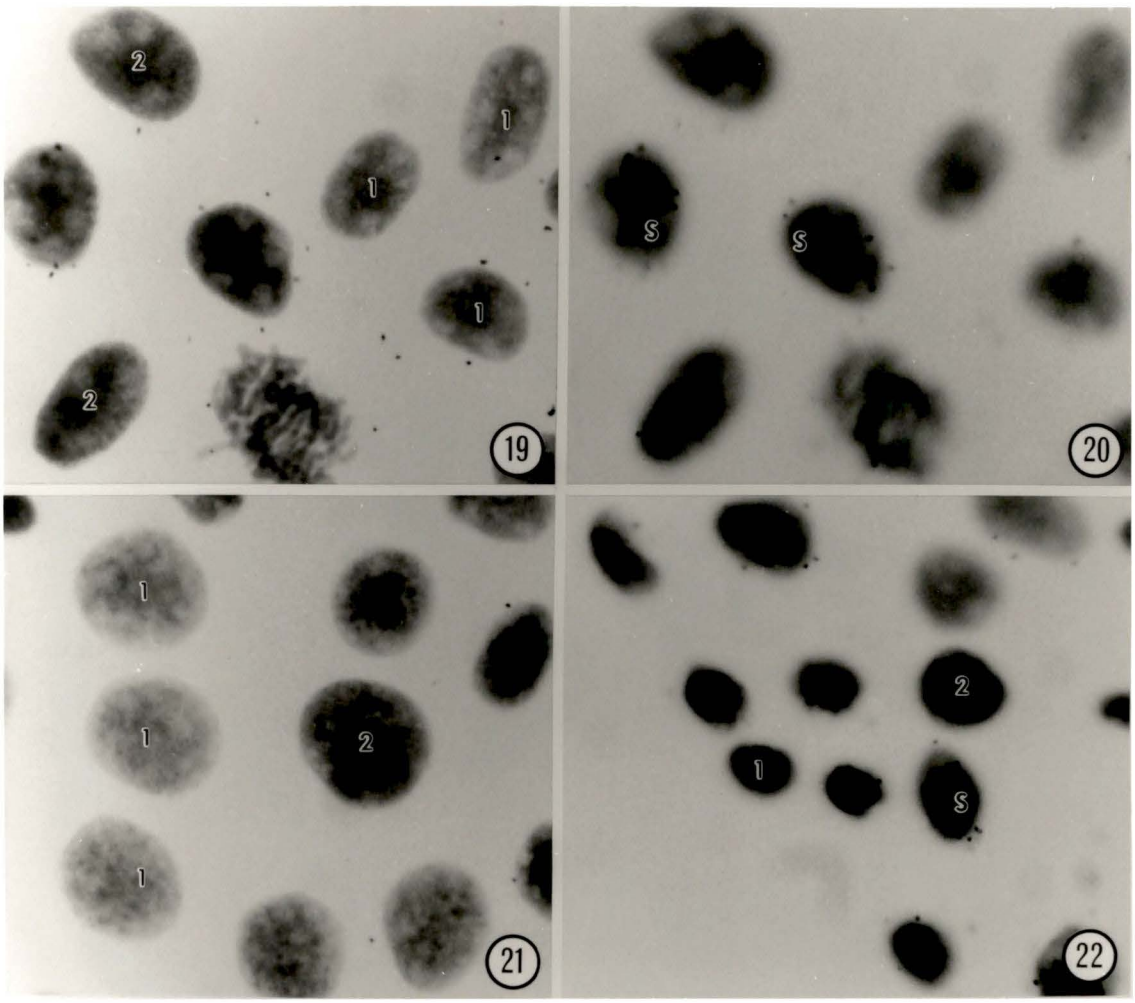
Fig. 19-22. Squash preparations of shoot apices of loblolly pine, showing nuclei in G1 (1), S (S), and G2 (2) stages of the cell cycle.

Fig. 19. Peripheral zone nuclei. X 757.

Fig. 20. Nuclei from Fig. 19, but focused on grains above nuclei. X 757.

Fig. 21. Apical zone nuclei. X 757.

Fig. 22. Basal peripheral zone nuclei, focused on grains above nuclei. X 757.


2
1
1
1
2
19
S
S
20
1
1
1
2
21
2
1
S
22

appeared very darkly stained (Fig. 22). This increased the variability of nuclear morphology in squash preparations but did not affect the ability to identify G1 and G2 nuclei.

Horizontal scanning of 14 treated apices showed 75% of the cells were in G1, 15% in S, 5% in G2, and 5% in M (Table 7). Standard errors for these determinations were low indicating that nuclei were being identified in a consistent manner. The percentage of cells in G1 determined by visual-autoradiographic identification was similar to the percentage determined by microdensiometry of the peripheral zone, but S was higher and G2 and M were lower using the visual-autoradiographic method (Fig. 23).

4.4 Discussion

4.4.1 Incorporation of tritiated thymidine

Incorporation of tritiated thymidine into nuclei of shoot apices is difficult (Gifford *et al.* 1963, Lyndon 1973), especially when attempting to label shoot apices of intact plants without significant disruption to the shoot tip. This was not possible in loblolly pine.

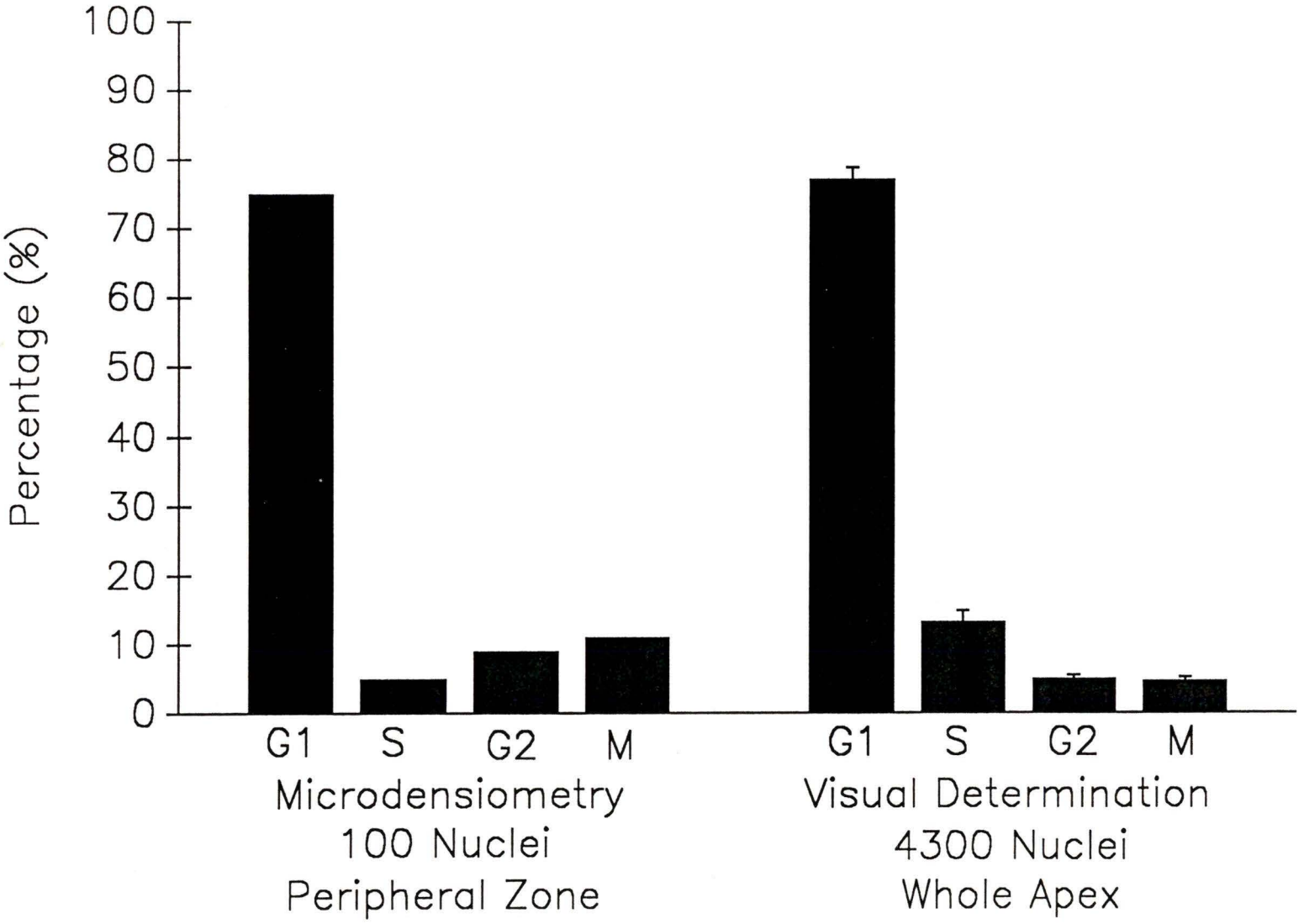
Later experiments were conducted to determine whether radioactive labelling of apical nuclei from isolated conifer shoot tips could be used to identify nuclei in S stage. These experiments demonstrated successful labelling of apices at two stages of development in loblolly pine, and in western redcedar. However, further study is required to increase the amount of label incorporated into apices, minimize the disturbance of apical cells prior to fixation, and develop less arbitrary criteria for identifying labelled nuclei in autoradiographs. Several observations from these experiments may aid in attaining these goals.

Table 7. The percentage of cells in cell cycle stages as determined by microdensiometry and the visual-autoradiographic method

Method	No. Nuclei Counted	G1	S	G2	Mitosis
Microdensiometry (PZ)	100	75.0	5.0	9.0	11.0
Visual (WA)	4971	75.9 (1.1)	14.8 (1.3)	4.7 (0.4)	4.6 (0.3)

Note: figures in parentheses indicate ±1 standard error of the mean.
(PZ) peripheral zone, (WA) whole apex.

Fig. 23. The percentage of cells in cell cycle stages as determined by microdensiometry and the visual-autoradiographic method.


Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
G1
S
G2
M
Microdensiometry
100 Nuclei
Peripheral Zone
G1
S
G2
M
Visual Determination
4300 Nuclei
Whole Apex

Loblolly pine shoot apices incorporated far less tritiated thymidine, based on grain number over nuclei, than western redcedar. The only experimental differences between these species was that the vascular cylinder was exposed in western redcedar and the length of the subtending shoot was shorter. Labelling of procambial cells in western redcedar suggests some movement of tritiated thymidine occurred through the vascular cylinder. Translocation of DMSO through the xylem has been reported previously (Nethery and Hurtt 1967) and may be more effective in the main vascular cylinder than in cut leaf traces. Therefore, exposing the vascular cylinder may be a method to enhance the amount of label entering the shoot apex.

Increasing the efficiency of labelling nuclei may allow a shorter treatment duration. Labelling of nuclei from conifer root tips, which are more absorbent than shoot apices, has been accomplished with as little as 30 minutes of treatment (Miksche 1967). Shorter treatment should reduce damage to mitotic figures and effects on mitotic activity. Both DMSO and tritiated thymidine have been observed to reduce MI and affect chromosome structure (Wimber 1959, Natarajan 1961, Herskovits 1962, Dille and King 1983, Liotti *et al.* 1989). Similar effects were observed in loblolly pine. Based on the low incorporation the affects observed here were likely due to DMSO treatment which has been shown to affect hydrophobic interactions in the DNA helix (Herskovits 1962), and reduce MI of root apices by inhibiting the entry of interphase cells into mitosis (Dille and King 1983). Anaerobic conditions in the treatment solution may also have an adverse effect (Bernier and Bronchart 1963, Webster and Van't Hof 1969). DMSO has been hypothesized to neutralize the active forms of oxygen in treatment solutions (Liotti *et al.* 1989). Until procedures are developed which do not affect MI, a population of untreated apices should be measured for MI as a control.

The criteria of three grains over a nucleus to identify labelled nuclei was based on the low background and labelling of nuclei which would be expected to be in S stage based on nuclear morphology. This is somewhat arbitrary but has been used in other studies (England *et al.* 1973). A more precise determination of labelled structures by calculation of grains over background levels is based on the assumption that nuclei are the same size (England *et al.* 1973). This was not possible due to the 4-5 fold variation in the size of nuclei in these preparations. However, England *et al.* (1973) have suggested that by increasing the grain density over nuclei, errors in identification using an arbitrary number of grains is substantially reduced. This appears to be the best approach given the variable morphology of nuclei from conifer shoot apices and the ease with which nuclei in S stage were identified in highly labelled redcedar preparations.

The duration of autoradiographic exposure is also important in interpreting autoradiographs. With only 8 days of exposure some lightly labelled nuclei may not have produced three grains. Conversely, with 40 days exposure, the presence of 3 grains over a nucleus might be due to either background or the nucleus being very lightly labelled. Therefore the estimate of cells in S in this study (with 8 days exposure) is a conservative estimate. If the concentration of label in cells can be increased, grain density over labelled nuclei should become more uniform as observed in redcedar preparations. This will increase the accuracy of identifying nuclei in S with a short duration of exposure.

Despite these problems, determination of the number of cells in S by labelling with tritiated thymidine is more accurate than microdensiometry because a larger sample of nuclei can be studied, and cells beginning and completing S are accurately identified (Ormrod and Francis 1985). The population of S cells in this study (14.8 %) is a conservative estimate due to the short period of exposure, but is

similar to labelling indices of 16-20 % measured in shoot apices of *Silene* (Ormrod and Francis 1985). With further improvements as outlined, the identification of conifer nuclei in S will become more accurate and quantitative.

4.4.2 Visual identification of G1 and G2 nuclei

The sample size of interphase nuclei in microdensiometry studies has been limited to 20-150 per collection (Cecich *et al.* 1972, Owens and Molder 1973, Cottignies 1979, Gonthier *et al.* 1985, Ormrod and Francis 1985) due to the labor intensive nature of this technique. For meaningful cell cycle studies it would be desirable to identify G1 and G2 nuclei nearly as easily as mitotic figures for MI determination. It was possible to identify the interphase stage of Feulgen-stained nuclei in squash preparations in the microdensiometry study, and the accuracy of this was later improved by using autoradiography to identify nuclei in S. This reduced the need for visual identification to only 2 stages, G1 and G2. These differ in DNA content by a factor of 2 and can be distinguished using the morphological characteristics of SI and NA. Changes in NA altered the SI within a cell cycle stage. Once the relationship between NA and SI was understood, nuclei could be placed into two groups, G1 and G2. Telophase and prophase which have a known G1 and G2 level of DNA content, respectively, served as visual references of NA and SI for G1 and G2 stages. Although nuclear morphology in different regions of the shoot apex varied considerably, it primarily resulted from differences in NA (Owens and Molder 1973). This did not affect the identification of cell cycle stages since SI was affected proportionally to NA (Fig. 19-22.).

Once the ability to visually identify G1 and G2 nuclei was learned and practiced the method was quick and accurate, requiring approximately 2-8 seconds per nucleus which was similar to the time needed to identify mitotic figures. This

was much more rapid than identification using microdensiometry which required approximately 5-8 minutes per nucleus. The visual method represented a 50-60 fold increase in sampling speed. However, an additional study is needed before the technique can be fully accepted for quantitative research purposes. This should involve paired observations of nuclei using the visual-autoradiographic method described here and microdensiometry to confirm the accuracy of the former. This approach has been used for angiosperm nuclei (Nagl 1970 a,b). My study supports these findings and suggests that visual-autoradiographic identification offers a more rapid method to routinely assess the cell cycle stage of conifer nuclei in developmental and physiological studies.

4.4.3 The cell cycle in the shoot apex of loblolly pine seedlings

The percentage of cells in a cell cycle stage is proportional to the time spent in that stage (Howard and Pelc 1953, Sparvolli *et al.* 1966). Walker (1954) criticized this relationship based on the assumption that cell populations increased in number due to cell division, which increased the number of younger versus older cells. This criticism does not apply to shoot apices in this study which continuously lose cells as new primordia are formed, thus maintaining roughly the same number of cells in the apex. Therefore, the positive correlation between the number of cells in a cycle stage and the relative duration of that stage is valid for the shoot apices in this study.

Cells in the shoot apex of young loblolly pine seedlings spend 75% of the cell cycle in G1 which suggests that the G1/S transition is the limiting factor for progression through the cell cycle in these apices. Under adverse conditions G1 has been shown to be a principal control point where cells accumulate (Van't Hof 1968, Webster and Van't Hof 1969, Van't Hof and Rost 1972, Van't Hof 1985). The

seedlings in this study were grown under favorable environmental conditions and were in a period of rapid growth following germination. This removed most external limitations on the cell cycle, but it did not remove internal factors. In *Helianthus* roots starved of carbohydrate most cells are held in G1 stage (Van't Hof and Rost 1972). With addition of carbohydrate, these cells exit G1 and enter S. Carbohydrate may also be the internal limiting factor for progression past G1 in the shoot apices of young conifer seedlings. During this early stage of seedling development the shoot apex is competing with elongating primary needles and stem and root tissue for carbohydrates which are primarily produced in the cotyledons (Kramer and Kozlowski 1979). Later in seedling development when carbohydrate production from primary needles and dwarf shoots increase, this situation may be less severe. The high percentage of G1 cells (55%) during early bud-scale initiation in mature trees has also been hypothesized to be caused by a low level of available carbohydrate (Owens and Molder 1973).

Little is known about the cause and functional significance of zonation in the shoot apex of conifers. Based on the microdensiometry study of peripheral zone nuclei, it initially appeared that a substantial increase in nuclear area was required for G1 nuclei to enter DNA synthesis (Mitchell *et al.* 1983). However, based on the very small S nuclei from the base of the peripheral zone and the very large G1 nuclei in the apical zone, this is obviously not a requirement. Instead variation in nuclear area may be primarily due to differences in cell size. Cavalier-Smith (1978) has suggested that cell volume determines the nuclear volume required to satisfy transcriptional requirements for progression through the cell cycle. However, there may be limitations to the ability of nuclei to increase in volume (Cavalier-Smith 1978). This was observed by Armstrong and Francis (1987) where increases in cell size in culture decreased the nuclear/cellular area ratio. This negatively affected

the concentrations of protein and RNA in the cytoplasm and adversely affected embryogenic potential.

The relationship between nuclear and cellular volume may be useful for examining the cell cycle in conifer shoot apices. Apical zone cells are large, vacuolate cells which exhibit little mitotic activity, while basal peripheral zone cells are small, dense, and mitotically active (Owens and Molder 1973, Cecich 1977). The difference between the zones may be in the ability of their nuclei to supply adequate RNA and protein to reach critical concentrations required in the cytoplasm for progression through the cell cycle.

This hypothesis involves the interaction of 2 independent components of the cell cycle: cell division (DNA synthesis-mitosis) and cell growth (Mitchison 1971, Cavalier-Smith 1985). These components are coordinated in cells growing in a steady state, but can act independently (Cavalier-Smith 1985). This can result in cell growth without division or division without cell growth. This view of the cell cycle is not widely used. Most studies of the cell cycle in shoot apices do not consider cell growth, but instead are more interested in factors related to cell division such as DNA synthesis, mitosis and the duration of the cell cycle (Gonthier *et al.* 1985, Ormrod and Francis 1985). An understanding of cell growth as an important cell cycle component may assist studies which examine zonation in the shoot apex.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

1. Squash preparations of shoot apices fixed in cold neutral formalin, hydrolyzed with 5N HCl at 20°C, and stained in Schiff's reagent produces excellent monolayer preparations of nuclei which can be made permanent and used for determination of mitotic index.

2. Regions of shoot tips removed for squash preparations must be specific (shoot apex, leaf primordia, or shoot axis) and not mixtures of these regions. Tannin filled pith cells and procambial cells should not be sampled for MI, and preparations with excessive contamination of procambial cells should be discarded.

3. Determination of MI may not be necessary in all situations, the presence or absence of mitosis is more rapid and is a satisfactory measurement if the research objective is to determine when mitotic activity begins or ceases.

6. Where relative changes in mitotic activity are desired MI can be accurately determined with a minimum of 10 apices by horizontal scanning at a vertical interval of 200 μM.

7. Interpretation of MI data should recognize that changes in MI may be caused by a disproportional change in the duration of either interphase or mitosis, or a change in the percentage of cells traversing the cell cycle (growth fraction). Conversely, MI may remain constant even though the duration of the cell cycle has been altered.

8. In loblolly pine seedlings removed from cold storage mitosis in shoot apices resumed from a G2 population of cells after 10-14 hrs in growth promoting conditions. The resumption of mitosis may be an indication of growth potential of the seedling and is a more rapid and precise measurement of this than is DBB. Further investigation is required to establish relationships between the reactivation of G1 cells, the resumption of mitosis, bud flush and seedling performance.

9. Partial dissection of shoot apices of intact loblolly pine seedlings and treatment of these shoot tips with tritiated thymidine damaged the shoot tip and was not a successful method for labelling nuclei.

10. Excised and carefully dissected conifer shoot apices can be labelled with tritiated thymidine allowing the identification of nuclei in S. Further experiments are necessary to increase the uniformity and intensity of labelling. This will allow treatment time to be shortened and increase the accuracy of identifying labelled nuclei.

11. Tritiated thymidine treatment resulted in abnormal mitotic figures and decreased MI by 26% compared to untreated controls. This may be due to the presence of DMSO and will require the use of controls without DMSO until a less damaging procedure is developed.

12. Cells in G1 and G2 can be visually identified using the morphological characteristics of NA and SI, in conjunction with comparison with mitotic figures which serve as reference points for the G1 and G2 level of DNA content.

13. Visual-autoradiographic identification of cell cycle stages is 50-60 times as rapid as microdensiometry, and is more accurate due to the increased sample size that is possible and the ability to precisely identify cells in early and late S.

14. Loblolly pine shoot apices in early neoformed leaf initiation had 75% of their cells in G1, 14.8% in S, 4.7% in G2, and 4.6% in M. These percentages correlate with the relative duration of each stage. The large proportion of cells in G1 suggests that the G1/S transition is limiting cell cycle progression in these shoot apices, possibly due to low levels of carbohydrate within the seedling at this stage of development.

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Techniques to study the cell cycle in the shoot apex of conifers

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