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THE DEVELOPMENT OF ADVENTITIOUS BUDS IN *PINUS OOCARPA*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Robert M. Beaty

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ABSTRACT

When 10-day/12-16 mm cotyledons of axenically grown seedlings of Pinus oocarpa are excised and placed on a modified MS medium with appropriate concentrations of growth regulators (25 uM BAP and 25 nM NAA) for 14 days then transferred to a basal medium (same medium without growth regulators) adventitious buds are induced. These buds will elongate and root under conducive conditions. The purpose of this research was to document the morphological events that lead to adventitious bud formation. The initial cotyledonary explant has an even and ordered epidermis on all three surfaces. After 3 days in culture, the two epistomatic surfaces become irregular and uneven. The abaxial surface remains ordered. The first evidence of meristemoid formation occurs after 15-19 days in culture. The meristematic area increases in size rapidly and soon forms a dome which gives rise to leaf primordia. Fully developed adventitious shoots are present at day 40 showing the organization typically associated with pine apical meristems. When 8-day old explants 3-7 mm in length are cultured under the same conditions as described above, the most prominent structure observed is an aberrant bud. These buds are asymmetrical and poorly organized.

Their leaf primordia have unusually large cells. The ontogenetic events that lead to aberrant buds are indistinguishable from those that lead to the formation of viable adventitious buds up to the point when domes are formed and leaf primordia arise. Other aberrant structures such as phylloids were observed. When 10-day old explants 4-24 mm were cultured under the same regime, viable adventitious buds were produced over the entire range of sizes. Since this range of sizes includes the same as those of the 8-day old explants (3-7 mm), we are able to state that the size of the explant does not effect its ability to produce viable adventitious buds.

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog Medium
GD	Greshoff and Doy Medium
BAP	6-Benzylaminopurine
NAA	Naphthaleneacetic acid
C	Degrees Celcius
uM	Micron

CHAPTER I

LITERATURE REVIEW

Micropropagation of Conifers

The first adventitious buds produced in a conifer system were reported by Ball (1950). Using green shoots from a burl of Sequoia sempervirens, Ball was able to induce callus growth and subsequently bud formation. It was not until 1975, however, that complete plantlet regeneration was reported by Sommer et al. with Pinus palustris. Since that time, adventitious bud formation has been achieved for many conifers using various explant material (Table 1).

Histological Work

Histological studies describing the organogenic changes that occur in explants generally describe several points over the developmental period which document the major events in the process. Several researchers have listed the events that lead to organogenesis and plantlet regeneration in conifers (Coleman and Thorpe, 1977; Cheah and Cheng, 1978; Aitken, Horgan, and Thorpe, 1981; Yeung et al., 1981).

Table 1

Adventitious Bud Formation in

Selected Conifer Species

Species	Explant	Senior Author/Yr.
Sequoia sempervirens	green shoots from burl	Ball/1950
Pinus palustris	embryo	Sommer/1975
Pseudotsuga menzeissi	cotyledon	Cheng/1975
Picea glauca	hypocotyl	Campbell/1975
Pseudotsuga menzeisii	embryo	Sommer/1975
Tsuga heterophylla	cotyledon	Cheng/1976
Picea glauca	hypocotyl	Campbell/1976
Pinus radiata	embryo/ needle	Reilly/1976
Pseudotsuga menzeisii	embryo/	Reilly/1976
Thuja plicata	cotyledons/ shoot apex	Coleman/1977
Pinus pinaster	cotyledon/ shoot apex	David/1977
Abies balsemea	embryo	Bonga/1977
Picea abies	embryo/shoot apex/needle/ seedling*	Webb/1977
Pinus contorta	embryo/shoot apex/needle/ seedling*	Webb/1977

Table 1 (continued)

Species	Explant	Senior Author/Yr.
Picea abies	embryo	von Arnold/1978
Pinus sylvestris	embryo/ seedling*	Tranvan/1979
Picea abies	needles	von Arnold/1979
Picea abies	buds	von Arnold/1979
Pinus strobus	embryo	Minocha/1980
Pinus sylvestris	shoot apex	Bornman/1980
Picea abies	needles	Jansson/1980
Pinus wallichiana	hypocotyl	Konar/1980
Picea abies	mature needle	Jansson/1981
Pinus monticola	embryo/ cotyledon	Mott/1981
Pinus radiata	embryo/ shoot apex	Horgan/1981
Pinus contorta	embryo	von Arnold/1981
Pinus pinaster	cotyedon/ needle	David/1982
Picea abies	apical/ lateral buds	Jansson/1983
Pinus coulteri	embryo/ cotyledon/ hypocotyl	Patel/1983
Pinus caribaea	embryo	Webb/1983

Table 1 (continued)

Species	Explant	Senior Author/Yr.
Pinus nigra	embryo	Kolevska-Pletikapic 1983
Pinus contorta	embryo/ cotyledon/ hypocotyl	Patel/1984
Pinus ponderosa	embryo	Ellis/1984
Pinus oocarpa	embryo/ cotyledon	Franco/1985
Pinus brutia	embryo/ cotyledon	Abdullah/1985
Pinus strobus	cotyledon/ hypocotyl	Kaul/1985
Pinus strobus	embryo	Flinn/1986

* "Seedling" is used here to denote an explant consisting of hypocotyl segment, cotyledons, and apical meristem.

Their descriptions follow the sequence of organogenic events starting with initiation of the primordia to the rooted plantlet. Although the timing of each an event may differ among species, the sequence remains the same.

Initial Explant

Limited attention has been given to the anatomy of the initial cotyledonary explant. Yeung et al. (1981), however, gave a thorough description of Pinus radiata cotyledons used as explant material. A prominent feature of the explant was the presence of numerous densely stained nuceli throughout the cotyledon, but especially in the epidermis. The cell divisions throughout the cotyledon were anticlinal and the epidermal regions that were highly mitotic were believed to be the areas from which stomatal complexes would arise. Reserve substances, namely starch and protein, were present in significant quantities. Another feature observed by Riding (1982) in P. radiata was the lack of stomata on the abaxial surface. Patel and Thorpe (1984) displayed transverse sections of P. radiata initial explant material (day 0). The cotyledons consist of a very ordered epidermis. The subepidermal

tissue consists of a layer of 1-3 cells. These cells are, in general, larger than the epidermal cells, but smaller than the cells of the mesophyll layer lying directly beneath.

Initial Anatomical Changes in the Cotyledon as a Result of Hormonal Treatment

Transverse sections of cultured cotyledons of P. radiata show a few small, but significant, changes in the cotyledon after 3 days in culture. In micrographs of P. radiata (Patel and Thorpe, 1984) one can observe the early divisions in the subepidermal region as evidenced by the fact that the cells of the epidermal and subepidermal layers stain densely. It is also apparent that the cotyledon's characteristic triangular shape has begun to change due to the cell divisions occurring below the surface.

Origin of Meristematic Zone

There has been much discussion and debate in the literature over the tissue or cellular origin of the meristem. Most investigators who use a similar culture methodology in which the growth regulators are included in the medium and the explant is placed on agar report that the meristematic activity was first observed in the

epidermal or subepidermal layer (Sommer et al., 1975; Cheah and Cheng, 1978; Tranvan, 1979; Yeung et al., 1981; Jansson and Bornman, 1981; Mott and Amerson, 1982; and David et al., 1982). Using histological methods, Villalobos et al. (1985) were able to identify cells believed to be involved in meristematic activity by observing thier shape and noting the two and four cell clusters in the subepidermal layer before they were at a stage at which they would stain darkly. He termed these structures "promeristemoids." David et al. (1982) also using a solidified agar medium to culture cotyledons of Pinus pinaster reported that "buds were induced from outer mesophyll layers."

Bornman (1983) using a culturing method in which a pulse treatment of growth regulators was applied either by a short (3 hr) treatment at a high concentration or by vacuum infiltration, noted that meristemoids originated in the subepidermal layer, but also in the adjacent mesophyll cells.

Bud Development

Yeung et al. (1981) presented pictures of early bud development in P. radiata in which the meristematic

region has taken on a nodular appearance. These regions soon become dome shaped and initiate needle primordia as similarily shown by Cheah and Cheng (1978) for bud development in Pseudotsuga menzeisii. Many researchers have described fully formed adventitious buds which were derived from various plant parts such as cotyledons (Cheah and Cheng, 1978; Tranvan, 1979; von Arnold, 1979; Mott and Amerson, 1982; Bornman, 1983; Bornman and Vogelman, 1984; Franco and Schwarz, 1985), embryos (Sommer et al., 1975; Reilly and Brown, 1976; von Arnold and Eriksson, 1978; Tranvan, 1979; Minocha, 1980; Patel and Berlyn, 1983), hypocotyls (Campbell and Durzan, 1975; Franco and Schwarz, 1985), primary and secondary needles (Jansson and Bornman, 1981), on needle primordia of apical and lateral buds (von Arnold, 1979; Jansson and Bornman, 1983), and from callus (Ball, 1950; Kaul, 1985). Even though the adventitious buds are derived from different sources, they all exhibit the same general form and stucture in longitudinal section. They also bear a strong resemblance to the developing shoot apical dome in embryos of several pine species: Pinus strobus (Spurr, 1949), Pinus ponderosa (Tepper, 1964), Pinus lambertiana (Fosket and Miksche, 1966), and Pinus radiata (Riding, 1972).

Aberrant Structures

Bornman (1984) observed aberrant and other unique structures while studying the effects of gel concentrations on organogenesis in Picea abies. He found that more buds were produced at medium (2.5-10g Tayio agar l^{-1}, 1-5g Gelrite l^{-1}) to low (2.5 and 5.0g Tayio l^{-1}, 1.5g Gelrite l^{-1}) gel concentrations, but at the lower levels the buds were most often damaged by the effects of vitrification. The most distinctive features of these vitrified "pseudobuds" was the lack of small densely stained cells in the region of the apical meristem, the unusually large size of the cells in the leaf primordia, and the misshapen apical dome and leaf primordia. Bornman (1985) described and defined various aberrant structures present on cotyledons of Picea abies after 14 days of culture. They are:

pseudobulbil - "...not usually identifiable as adventitious buds, but which eventually may give rise to shoot buds or become necrotic and cease to develop."

pseudobuds - "...extended, usually vitrified , bud-like structures, but often have damaged or retarded apical meristems and short, distended leaf-like appendages."

phyma -"... pustular nodules of undifferentiated cells in cross section up to 3 mm or more in diameter."

phylloids - "...leaf-like structures, with unusually large parenchyma cells either with weakly developed vascular tissue or lacking it altogether."

Coleman and Thorpe (1977) working with cotyledons of Thuja plicata, western red cedar, described "meristemoidal domes" that could not be clearly identified as adventitious buds. These structures were observed to differentiate into either adventitious buds or roots. From this, Coleman and Thorpe concluded that these adventitious meristems were not fully differentiated and possessed the capability of becoming as either shoots or roots. The description of these "meristemoidal domes" bares a strong resemblance to Bornman's (1985) description of "pseudobulbils" even though the "pseudobulbils" were never observed to produce roots.

Scanning Electron Microscopy

Kirby and Schalk (1982) published a detailed SEM study of developing Douglas-fir adventitious shoots. The surface of the initial explant was made up of even ranks of long smooth surfaced rectangular cells lying

between irregular double and triple rows of stomata. The subsidiary cells surrounding the stomata are noticeably smaller in surface area than the long ranks of cells between the double and triple rows. The epicuticular wax was found to be heavier in the region of the stomata than in the other areas of the explant. The rupturing of the epidermal surface by a proliferation of subsurface callus was first observed 7 days after culture initiation. The first sign of meristematic dome formation occurred at 2 weeks when "bump-like projections" appeared on the surface of the cotyledon. These domes were observed to rupture through the epidermis in the areas of callus proliferation. Leaf primordia formed on the domes at about 3 weeks.

Renfroe and Berlyn (1985) also presented an SEM micrograph of a cultured Pinus taeda embryo which had an area of actively dividing cells. Villalobos et al. (1985) published SEM micrographs of cultured Pinus radiata cotyledons after 10-days in culture showing dome shaped structures that represent early meristemoid formation. Also shown were early buds with developing leaf primordia. These buds were shown tobe continuous with the epidermis, unlike those described by Kirby and Shalk (1982).

The Effect of Size, Age, and Physiological State of the Explant on Adventitious Bud Formation

Numerous researchers have mentioned how the size, age, and physiological state of the explant effects the number of adventitious buds produced. Jansson and Bornman (1981) stated that there was a "critical needle length" that should be considered when culturing needles of Picea abies. Von Arnold and Eriksson (1979) also working with needles of Picea abies observed that more adventitious bud primordia were formed on short needles (1-3 mm) than on long needles (3-5 mm). Bornman (1985) stated that the "number of adventitious bud primordia that can be induced decreases with leaf age (that is, length)" thus correlating leaf age with the length of the explant. He also noted that the "physiological condition" of the explant was a factor and may "vary according to the chonological age of the explant." Yeung et al. (1981) found that in Pinus radiata cotyledons undetermined and undifferentiated cells were more responsive to the treatments. For this reason, the time at which the explant was selected was deemed important. David et al. (1982) also found that adventitous bud production was not so much dependent on the type of

organ explant used as the extent of the cell differentiation in cotyledons of Pinus pinaster. Cheng (1977), working with cotyledons of Pseudotsuga menzeisii stated that the age of the explant, from 2 weeks to 2 .pa months old, had no influence on the adventitious bud production. She did, however, attribute the differences in response to the "physiological condition and genomic composition of the plant material." Aitken et al. (1981) found that when embryos were used as explants and cultured on a defined agar medium a low number of buds (9) resulted. When embryos were grown on the same agar medium for 7 days and the cotyledons were then excised and cultured further a slightly higher number (18) was obtained. When cotyledons from germinated seeds were used an even higher number (180) was obtained. Therefore, it is likely that all three factors (size, age, and physiological state) had changed in this study. In other work with cotyledonary explants, Aitken (1985) found that certain morphological and physiological characteristics of the cotyledons changed following germination. Cotyledons 1 day following germination produced the largest number of adventitious buds and had less developed stomatal complexes, thinner cell walls, no epicuticular wax, depleted protein reserves, and

unhydrolyzed lipid reserves, whereas cotyledons from seedlings 5 days following germination had entirely opposite features including no bud production. Cotyledons from seedlings 2-3 days following germination had features that were intermediate and only produced meristematic tissue at the apical tip of the cotyledon. Aitken concluded that the features exhibited by the seedlings 5 days old were an indication that the tissue was past its physiological prime for the induction of adventitious buds.

CHAPTER II

TEN-DAY OLD EXPLANTS

Introduction

Pinus oocarpa is one of two pine species used most in Central and South America for pulp and timber (*P. caribaea* being the other). For this reason, an understanding of its growth habit and its potential for improvement is important. Franco and Schwarz (1985), using cotyledons, reported the first micropropagation of *P. oocarpa*. Since the yield of this propagule depends primarily on the production and quality of adventitious buds, we began an investigation into the origin of the adventitious buds and their development. By understanding the ontogeny of the buds, we hope to be able to better manipulate this propagation method.

Numerous reports have shown histological sections of adventitious buds in conifers, but few offer a complete developmental description. The most complete histological studies to date are by Cheah and Cheng (1978) using *Pseudotsuga menzeisii* and Yeung et al. (1981) with *Pinus radiata*. Kirby and Shalk (1982) reported the developmental sequence of adventitous buds in a conifer (*Pseudotsuga menzeisii*) using SEM

techniques. We present here for the first time both SEM and histological micrographs describing adventitious bud formation in P. oocarpa.

Materials and Methods

Culture Conditions

The seed source and culture conditions were nearly identical to those used by Franco and Schwarz (1985). Open pollinated seed was collected in Baja Verapaz, El Chol, Guatemala (alt. 3000') by International Forest Seed Co., of Odenville, AL. Once received by our lab, the seed was stored at 0-4 C. The seed were surface sterilized in 20% Clorox (2.5% sodium hypochlorite) for 30 minutes by shaking the solution and seed gently in a rotary shaker. The seed were rinsed once with distilled water and then with a dilute HCl solution (0.1 ml of 1N HCl per 100 ml H_2O) followed by two additional rinses with sterile distilled water. Following this they were were then planted under axenic conditions in storage dishes (80 x 100 mm) containing an potting soil mixture (1:1 vermiculite: sand), watered with sterile water and placed in a growth chamber under a 16 hr. light regime at 25 +/- 1.5 C. Ten days after planting, the seedlings

were removed from the soil and the hypocotyl was cut to remove the root. The remaining hypocotyl and intact cotyledons were sterilized in a 20% Clorox wash and rinsed twice with sterile distilled water. The cotyledons were excised and placed on the bud initiation medium: an MS medium (MSM) as modified by Cheng (1977) with 25 uM BAP and 25 nM NAA, 3% sucrose, and 1% agar (Franco and Schwarz 1985). About 25 ml of medium were used in each plastic Petri dish (15 x 100 mm). Explant cultures were maintained in a growth room under a photoperiod of 16 hr. light at 25 C +/- 2.0 C and 8 hr. darkness at 20 +/- 1 C. Illumination was provided by fluorescent light (Gro-lux) at 10 W/M^2. After 14 days the cotyledons were transferred to the basal nutrient medium (same as above but without growth regulators). A representative tissue sample was taken every third day over a 40 day period beginning on the day of excision (day 0). At least two replicates of each experiment were performed with each consisting of at least 80 seeds. The cotyledonary explants of one seed were all placed in a single petri dish. Sections were made of at least three tissue samples from each sample day (every third day 0-30 and then day 40) in each experiment with growth regulators (avg. 18

cotyledonary explants per sample day). Sections of tissue were made from sample days 0, 12, 21, 30, and 40 in the control experiments.

Tissue for histological observations was killed and fixed in CRAF III according to Sass (1958), dehydrated in an ethanol-tertiary butyl alcohol series, embedded in paraffin, and sectioned (10 uM) on a rotary microtome. Sections were stained with Gill's hematoxylin (1974) and were made permanent with Histomount histological mounting medium. Sections were photographed on 35mm Olympus Vanox microscope and an Olympus PM-10-A camera Kodak Pan-x black and white film.

The material for scanning electron microscopic observations was first fixed in CRAF III fixative and then postfixed overnight in 2% OsO4 in a 0.05M phosphate buffer, pH 7.2, with 0.1M (w/v) sucrose (modified from Kirby and Shalk, 1982). The tissue was then dehydrated in an acetone series (25,50,75,100%) and dried with a Ladd critical point dryer using liquid CO_2 as the intermediate fluid. The specimens were coated with gold-paladium using a Denton DV-515 vacuum sputter coater. Observations were made and recorded using an ETEC Autoscan microscope and Polaroid 55 land film.

Results and Discussion

In this work, we present both SEM and histological micrographs in a detailed description of the changes that occur in cultured pine cotyledons leading to the formation of adventitious buds. We have established a sequence of events that illustrates the changes that occur in both the surface features and in the various tissues of the explant over a 40 day period. We have also established a time frame in which these changes occur and we are able to correlate the changes in the surface features with changes observed in the histological sections.

Several works have described the sequence of events that lead to adventitious bud formation in conifers such as Thuja plicata (Coleman and Thorpe, 1977), Pseudotsuga menzeisii (Cheah and Cheng, 1978), and Pinus radiata (Yeung et al., 1981). The sequence of events listed for each is similar, and, in fact, is probably applicable to most species producing adventitious buds as Yeung et al. (1981) and Thorpe (1980) have stated. The sequential changes observed in adventitious bud formation of Pinus oocarpa from the initial explant are as follows: surface changes, early merstemoid, meristematic region,

orgainzed meristem with leaf primordia, and adventitious bud.

Histological sections of day 0 tissue illustrate the anatomy of the untreated axenically grown cotyledon. In transverse sections, the cotyledons are triangular in outline and the cells of the epidermis are even and regular on all three sides (plate 1, fig. 1). The round mesophyll cells surrounding the central vascular tissue are compact and exhibit very little intercellular air spaces. Picea abies (von Arnold, 1979) and P. radiata (Patel and Thorpe, 1984) show a similar anatomy to P. oocarpa for cotyledon explants on the day of excision. In contrast, longitudinal sections of a day 0 P. menziesii cotyledon by Cheah and Cheng (1978) showed a considerable amount of intercellular air spaces. Although we occasionally noticed large intercellular air spaces in the cotyledons, the anatomy most commonly observed was closer to that demonstrated by Yeung et al. (1981) in P. radiata.

SEM micrographs of the day 0 tissue (plate 2, fig.10) demonstrate many of the features described by Riding and Aitken (1982) from an epidermal peel of P. radiata and Kirby and Shalk (1982) from SEM micrographs

Plate 1. Histological micrographs of adventitious bud development in 10-day old explants of Pinus oocarpa.

Figure 1. Transverse section of the initial cotyledonary explant (day 0). The epidermis is ordered even on all three sides. The mesophyll tissue is compact and allows little intercellular space. The bar represents 46 uM.

Figure 2. After three days on hormonal medium, the epidermal cells of the epistomatic surfaces have become irregular and the surface has become uneven. The bar represents 46 uM.

Figure 3. Between 15-18 days in culture, meristemoid formation first occurs. The bar represents 28.5 uM.

Figure 4. The meristematic region increases in size extending down into the mesophyll tissue. The bar represents 28 uM.

Figure 5. The meristematic region may first grow in size vertically as in fig. 4 or laterally as shown here. The bar represents 28.5 uM.

Figure 6. The meristematic region begins to protrude above the surrounding epidermis. The first signs of leaf differentiation the meristematic area may be evident. The bar represents 46 uM.

Figure 7. The meristematic area may not include the epidermis at first, but must eventually include epidermal cell divisions if it is to expand upward and form an adventitiuos bud. The bar represents 43 uM.

Figure 8. Meristematic dome with two leaf primordia. Note the numerous anticlinal divisions in the epidermis just to the left of the center of the dome and the periclinal divisions just to the right of the center of the dome. The bar represents 25 uM.

Figure 9. Well developed adventitious bud. Note the procambial strands in the area of the leaf primordia (arrows). The cytohistological zones associated with pine apical meristems are represented as follows I. apical zone initial, II. central mother cell zone, III. peripheral tissue zone, IV. rib meristem. The bar represents 28 uM.

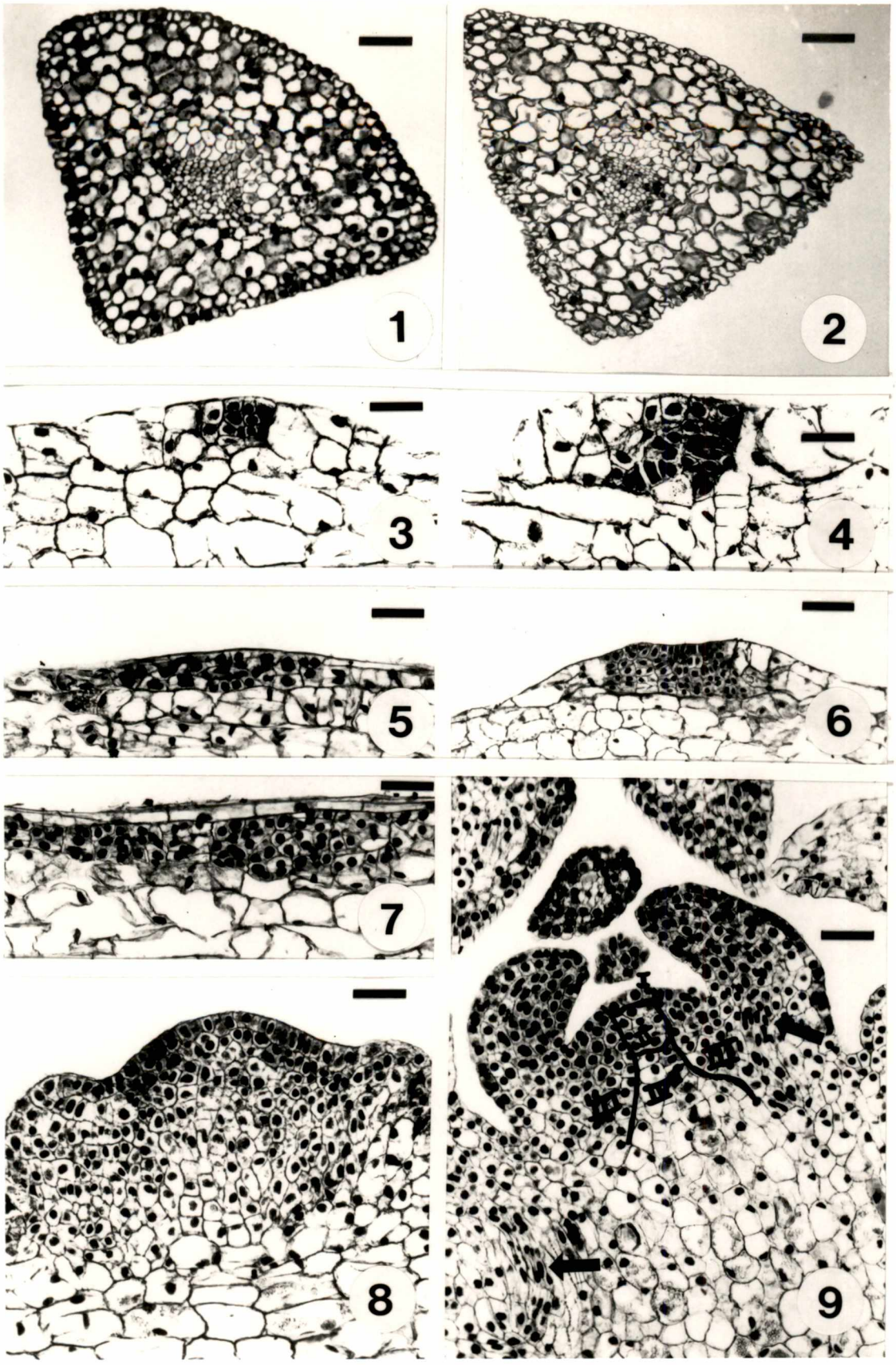

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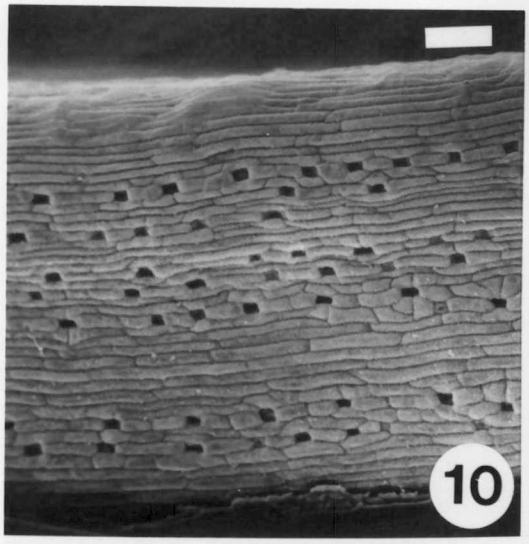
Plate 2. Scanning electron micrographs of adventitious bud development in 10-day old explants of Pinus oocarpa. The initial explant and early surface changes in the cotyledon.

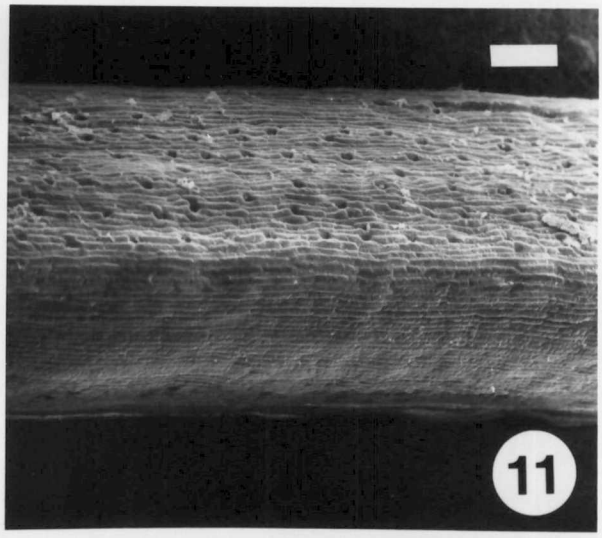
Figure 10. Initial explant (day 0). Stomata occur in rows of two or three surrounded by small subsidary cells. Rows of elongated rectangular cells are interspersed between the rows of stomata. The bar represents 83 uM.

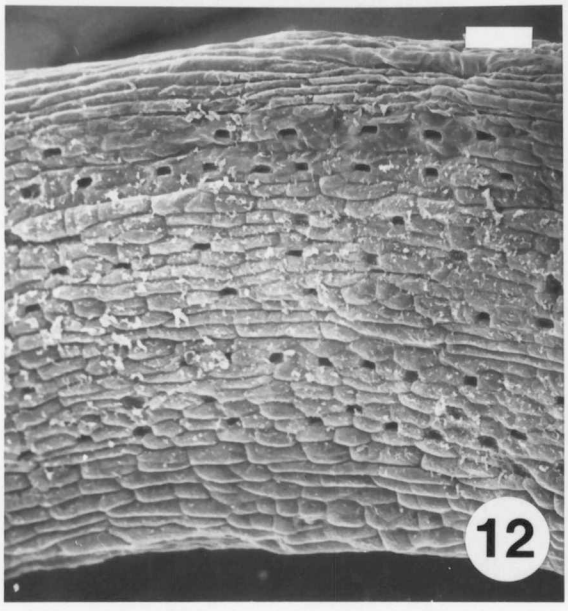
Figure 11. Explant after three days in culture showing the abaxial side with almost no stomata and one of the epistomatic surfaces with numerous stomata. The bar represents 114 uM.

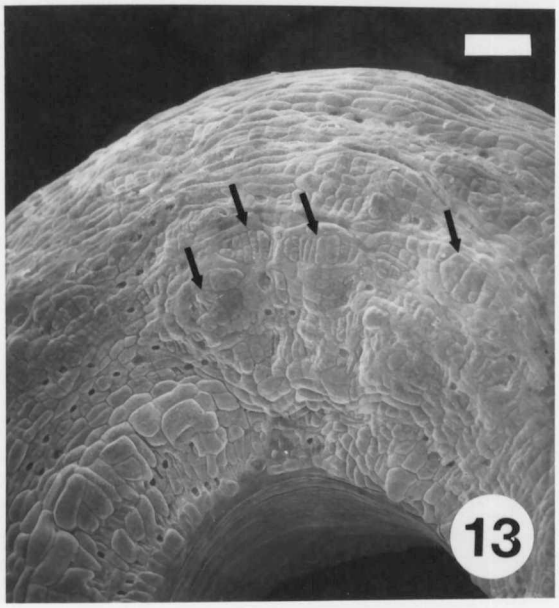
Figure 12. After six days on the hormonal medium, the cells of the epidermis have begun to divide The bar represents 96 uM.

Figure 13. Disruption of the regular rows of cells increases as the meristematic areas become nodular and small clusters of cells appear within these areas (arrows, day 12). The bar represents 192 uM.


10


11


12


13

oocarpa and Kirby and Shalk (1982) from SEM micrographs of P. menziesii. Even ranks of long smooth surfaced rectangular cells lie between irregular double and triple rows of stomata. The subsidiary cells surrounding the stomata are noticeably smaller in surface area than the long ranks of cells between the double and triple rows. Most of the stomata occur on the two epistomatic surfaces while the abaxial surface generally has few, if any, stomata.

By day 3 the cotyledon explants have curled. This curling was observed to always occur in the direction of the abaxial side of the cotyledon. The inner portion of the curled cotyledon (i.e., the portion towards the curl) displays cells that are noticeably smaller than those on the outer portion of the same side. The day 3 transverse sections reveal that changes have taken place in the epidermal layers of the two epistomatic surfaces (fig. 2) as evidenced by the uneven appearance of the epidermis and te irregular shape of the individual cells. The abaxial surface of the cotyledon remains relatively smooth and the epidermal cells have retained their original shape. At this stage, intercellular air spaces in the mesophyll region have begun to appear. In P. radiata (Patel and Thorpe, 1984), the initial

cotyledon explant has a similar appearance in transverse section to our day 0 material. Day 6 SEM micrographs (fig. 12) show that the epistomatic surfaces have very few of the rows of long rectangular cells still intact. Most of the cells of the epistomatic surfaces have become noticeably smaller as a result of cell divisions in the epidermis.

By day 12 to 15 groups of small cells (i.e. 8-10 cells) containing darkly stained nuclei which occupy the majority of the cell occur in or near the epidermis of the cotyledon on the two epistomatic surfaces. Meristematic areas may include epidermal cells (fig. 3, 4, 5, and 6) or they may be restricted to the subepidermal layers (fig. 7). Both periclinal and anticlinal divisions occur in both types of development. The cells of the meristematic region are small relative to the surrounding cells of the epidermis and mesophyll. Villalobos et al. (1985) demonstrated with cultured cotyledons of P. radiata that "promeristemoids" originate from a single cell in the subepidermal layer of the explant. As pointed out by Villalobos, this type

of structure may also be present in P. abies (Bornman, 1983). Other work with cultured conifer cotyledons report that meristematic areas can arise in either the epidermal or subepidermal layers (Mott and Amerson, 1982, Yeung et al. 1981, Sommer et al., 1975). Some researchers, however, suggest that the hypodermal layer (Cheah and Cheng, 1978), and in some cases even the hypodermal-mesophyll interface (Bornman, 1983) might be the site of initiation of meristem formation.

Meristematic domes (Kirby and Schalk, 1982) begin to appear between day 12 and 15 as a result of the localized cell divisions at or near the surface (fig. 13). SEM micrographs (day 12) document the disruption of the regular rows of stomata separated by the even rows of rectangular cells. Raised areas of cells and newly formed domes appear in clusters first near the tip of the cotyledon. The type of dome development in plate 3, figure 14 is similar to that presented by Villalobos et al. (1985) in P. radiata cotyledons. The ontogeny of the meristematic dome development presented here differs from the work of Kirby and Shalk (1982) with cultured P. menziesii cotyledons in which the meristematic domes were observed to protrude through the surface layer rupturing the epidermis.

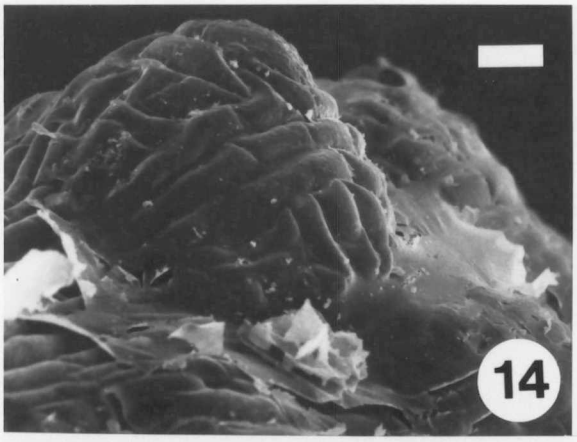
Plate 3. Scanning electron micrographs of adventitious bud development in 10 day old explants of Pinus oocarpa. Development and differentiation of the apical dome.

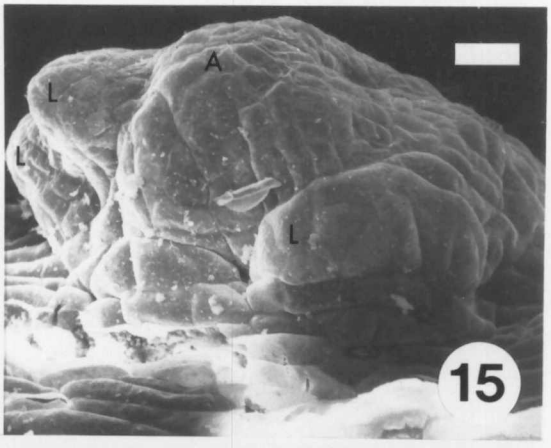
Figure 14. Meristematic dome protruding above the epidermis. Note the continuity of the epidermal surface over the entire dome (day 15). The bar represents 83 uM.

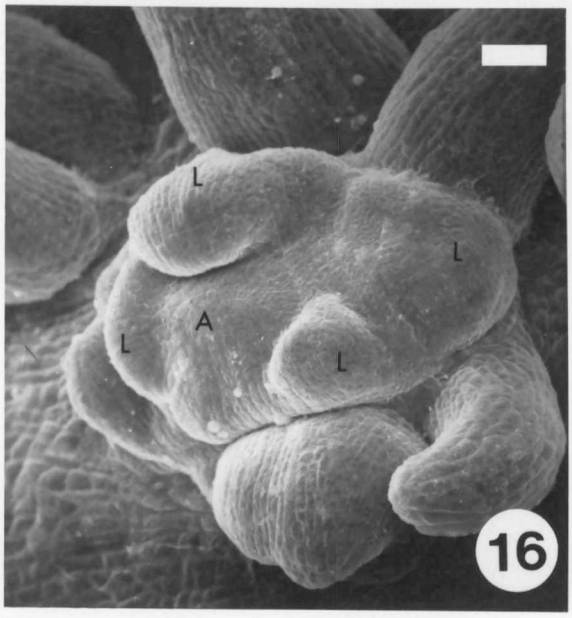
Figure 15. Meristematic dome with leaf primordia (day 19). "L" indicates leaf primordia. "A" indicates the shoot apex. The bar represents 71 uM.

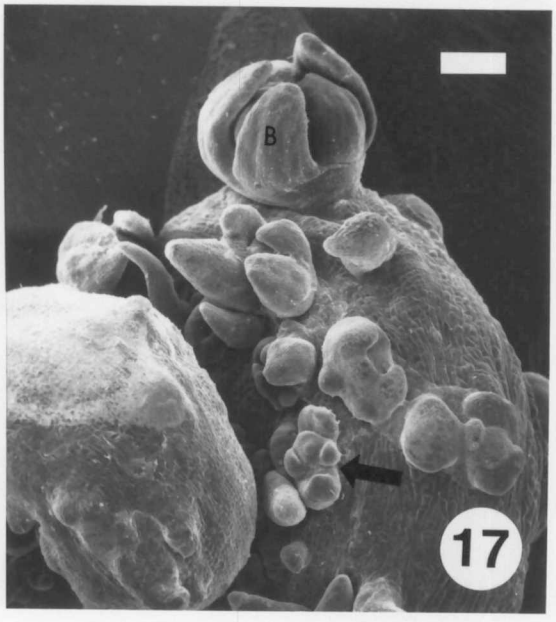
Figure 16. Adventitious bud with numerous leaf primordia and elongating leaves (40). "L" indicates leaf primordia. "A" indicates the shoot apex. The bar represents 156 uM.

Figure 17. Adventitious bud with many well developed leaves has begun to elongate (day 40). "B" indicates a well developed adventitious bud. Arrow indicates another adventitious bud early in its development. The bar represents 714 uM.


14


A
L
L
L
15


L
L
A
L
L
16


B
17

The dome soon achieves a higher degree of organization and begins to produce leaf primordia (fig. 15). Longitudinal sections demonstrate the organizationof the newly formed dome and leaf primordia. Both periclinal and anticlinal divisions occur in the epidermis to the right and left respectively of the apex allowing for the successive production of leaf primordia. Cheah and Cheng (1978) using P. menziesii cotyledons presented a longitudinal section of a developing bud primordium with newly formed leaf primordia that is very similar to figure 8 in its overall appearance. The apex continues to grow giving rise to numerous leaf primordia until a well developed adventitious bud is formed. The longitudinal section in figure 9 illustrates the typical orginization of an adventitous bud of P. oocarpa. Note the periclinal divisions in the epidermis of the dome and the procambium initials (arrows) in the tissue immediately below the newly formed leaves. SEM micrographs of the early buds (day 40, fig. 16) show several leaf primordia (L) forming with the apical dome (A) exposed. More developed shoots have the dome enclosed by the primordial leaves (fig. 17) in a manner resembling the appearance of the first leaf primordia in

a developing embryo of Pinus banksiana (Cecich and Horner, 1977). Numerous researchers have presented longitudinal sections of adventitious shoots grown from cotyledons (Bornman, 1983; Mott and Amerson, 1982; Tranvan, 1979; von Arnold, 1979; Cheah and Cheng, 1978; Bornman and Vogelman, 1984), embryos (Minocha, 1980, Sommer et al., 1975; Patel and Berlyn, 1983; von Aronld and Eriksson, 1978; Reilly and Brown, 1976; Tranvan, 1979), hypocotyls (Campbell and Durzan, 1975), callus (Kaul, 1985; Ball, 1950) primary and secondary needles (Reilly and Brown, 1976), mature needles (Jansson and Bornman, 1981), and on needle primordia of apical and lateral buds (Jansson and Bornman, 1983). All of these histological sections generally display a similar shape and appearance in overall anatomy and organization. Features of figure 9 bare a strong resemblance to the developing apical dome in embryos of several pine species: Pinus strobus Spurr (1949), Pinus ponderosa Tepper (1964), Pinus lambertiana Fosket and Miksche (1966), and P. radiata Riding (1972). The four zones associated with the cytohistological zonation of conifer apical domes were described in by Sacher (1954) with P. lambertiana seedlings and by Fosket and Miksche (1966) with developing embryos of P. lambertiana. (I. apical

initial zone; II. central mother cell zone; III. peripheral tissue zone; IV. rib meristem). In P. lambertiana the apical initial zone is characterized by being a region at the top of the developing apex with cells not staining as intensely as the surrounding cells. Located below the apical initial zone are the central mother cells which give rise laterally to the peripheral tissue zone and to the rib meristem zone by way of divisions from the basal region of the central mother cell zone. Zones with these same characteristics can be found in a day 40 longitudinal section of an apical dome of P. oocarpa (fig. 9).

Appropriately cultured and manipulated explant tissues of P. oocarpa produce morphologically complete apical meristems via organogenesis. The sequence of ontogenetic events, as described by histological and SEM investigation, leading to complete bud formation is similar to those found in other Pinus species. Interestingly, no evidence for mesophyll tissue development of meristemoids was found. The cell and tissue organization of the hormonally induced apical meristems was found to be similar to that described for pine embryo apices.

CHAPTER III

EIGHT-DAY OLD EXPLANTS

Introduction

There was evidence in the work of Franco and Schwarz (1985) that explants 8-days old and 3-7 mm in length (avg. 5.0 mm) gave very different morphogenetic results when cultured under the same protocol as the 10-day old explants 12-16 mm in length (avg. 14.0 mm). Thus, an experiment was employed to determine if there were any anatomical differences in the initial explants and/or in the formation and development of meristematic centers that lead to adventitious buds.

An additional experiment was designed to determine the relationship between the size of the explant and its ability to produce viable buds. Henceforth, the term "viable bud" will mean an adventitious shoot or bud that is capable of elongating and being rooted while the term "non-viable bud" will mean a shoot or bud that will not elongate or root.

Materials and Methods

Culture Conditions

The seed source and culture conditions were nearly

identical to those used by Franco and Schwarz (1985). Open pollinated seed was collected in Baja Verapaz, El Chol, Guatemala (alt. 3000') by International Forest Seed Co., Odenville, AL. Once received by our lab, the seed was stored at 0-4 C. The seed was surface sterilized in 20% Clorox (2.5% sodium hypochlorite) for 30 minutes by shaking the solution and seed gently in a rotary shaker. The seed were rinsed once with distilled water and then with a dilute HCl solution (0.1 ml of 1N HCl per 100 ml H_2O) followed by two additional rinses with sterile distilled water. The seed were then planted under axenic conditions in storage dishes (80 x 100 mm) containing an artificial soil mixture (1:1 vermiculite: sand), watered with sterile water and placed in a growth chamber under a 16 hr. light regime at 25 +/- 1.5 C. At the appropriate time, either 8 or 10 days after sowing, the seedlings were removed from the soil and the hypocotyl was cut to remove the root. The remaining hypocotyl and intact cotyledons were sterilized in a 20% Clorox wash and rinsed twice with sterile distilled water. The cotyledons were excise and placed on the bud initiation medium: an MS medium (MSM) as modified by Cheng (1977) with 25 uM BAP and 25

nM NAA, 3% sucrose, and 1% agar (Franco and Schwarz 1985). About 25 ml of medium were used in each plastic Petri dish (15 x 100 mm). Explant cultures were maintained in a growth room under a photoperiod of 16 hr. light at 25 C +/- 2.0 C and 8 hr. darkness at 20 +/- 1 C. Illumination was provided by fluorescent light (Gro-lux) at 10 W/M^2. After 14 days the cotyledons were transferred to the basal nutrient medium (same as above without growth regulators). A representative tissue sample was taken every third day over a 40 day period beginning on the day of excision (day 0). At least two replicates of each experiment were performed with each consisting of at least 80 seeds. The cotyledonary explants of one seed were all grown in one petri dish. Sections were made of at least three tissue samples from each sample day (day 0, 3, 6, etc.) in each experiment using growth regulators (avg. 18 cotyledonary explants per sample day). Sections of tissue from at least three experiments were made from sample days 0, 12, 21, 30, and 40 in the control experiments. Representative samples were taken for the SEM analysis.

Tissue for histological observations was killed and fixed in CRAF III according to Sass (1958), dehydrated

in an ethanol-tertiary butyl alcohol series, embedded in paraffin, and sectioned (10 uM) on a rotary microtome. Sections were stained with Gill's hematoxylin (1974). Specimens were made permanent with Histomount histological mounting medium. Sections were veiwed on an Olympus Vanox microscope and an Olympus PM-10-A camera with Kodak 125 Pan-x black and white film to record observations.

The material for scanning electron microscopic observations was first fixed in CRAF III fixative and then postfixed overnight in 2% OsO4 in a 0.05M phosphate buffer, pH 7.2, with 0.1M (w/v) sucrose (modified from Kirby and Shalk, 1982). The tissue was then dehydrated in an acetone series (25,50,75,100%) and dried with a Ladd critical point dryer using liquid CO_2 as the intermediate fluid. The specimens were coated with gold-paladium using a Denton DV-515 vacuum sputter coater. Observations were made and recorded using an ETEC Autoscan scanning electron microscope at 20kV with Polaroid 55 land type film.

Results and Discussion

In this work, we have established a developmental sequence for the aberrant buds that occur on the treated

8 day old cotyledonary explants 3-7 mm in initial length. We have also shown the anatomy of several other aberrant structures such as phylloids and phymas. By producing viable buds on explants that are 4-24 mm in length and 10 days old, we have shown that the ability of the explant to produce viable buds is not dependent on the initial size of the explant, but rather on its age and resultant physiological state. Thus, the physiological changes that occur in the explant over the 48 hour period (8-10 days) effect whether or not the explant is able to produce viable buds. This finding is supported by the fact that 10-day old explants 4-7 mm in length produce viable buds whereas 8 day old explants 3-7 mm in length do not.

Histological sections of the initial 8-day old cotyledonary explant (day 0) tissue demonstrates that its anatomy is very similar to the day 0 tissue of the cotyledons grown axenically for 10 days (chapter II, plate 1, fig. 1, p. 23). In transverse section the epidermis appears relatively even and ordered and nearly all the divisions are anticlinal. A few of the cells stain darkly indicating high metabolic activity.

The mesophyll cells are compact resulting in little intercellular space. There is one central vascular bundle surrounded by a poorly defined endodermis. The structure of P. radiata (Yeung et al., 1981; and Patel and Thorpe, 1984) cotyledonary explants are similar to the 8 day old explant of P. oocarpa .pa in that both species have an ordered epidermis and a mesophyll region with compact cells. The surface features of the 8-day old initial explant (plate 4, fig. 18, day 0) as seen by SEM micrographs are essentially identical to those of the 10-day old initial explant of P. oocarpa (day 0). The 8-day old explants of P. oocarpa also share similar surface features with SEM micrographs of initial cotyledonary explants of Pseudotsuga menzeisii (Kirby and Schalk, 1982) in that they both have double and triple rows of stomata, small subsidiary cells around the stomata, and rows of elongated cells between rows of stomata.

Numerous aberrant structures were observed over a 40 day period in the 8-day old explants of P. oocarpa. In one type of early development, a small cluster of densely stained cells was observed near the epidermis. These small groups of cells sometimes included the epidermis or were restricted to the subepidermal layers.

Plate 4. Scanning electron micrographs of aberrant bud development in the 8 day old cotyledon explants.

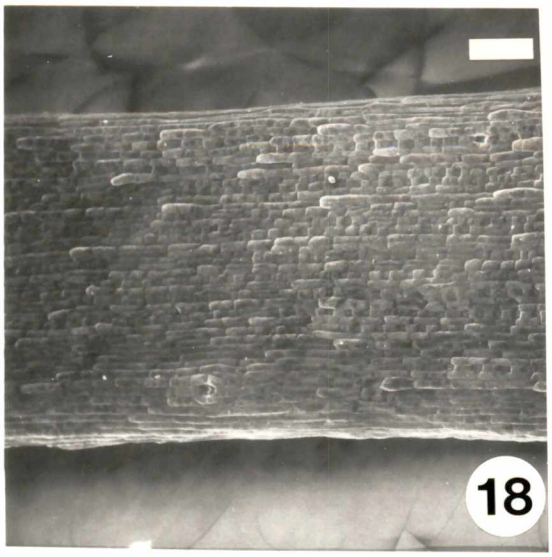
Figure 18. Day 0 explant. The double and triple rows of stomata are surrounded by small subsidiary cells. Between these rows of stomata lie elongated rectangular cells. The bar represents 111 uM.

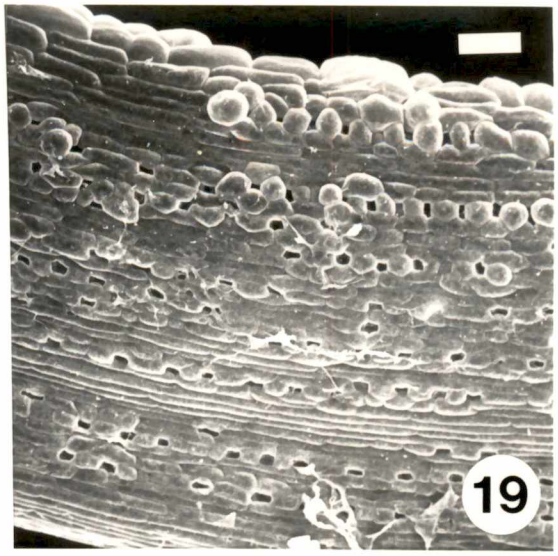
Figure 19. By day 3, the cells surrounding and particularly those between the stomata begin to swell. The bar represents 91 uM.

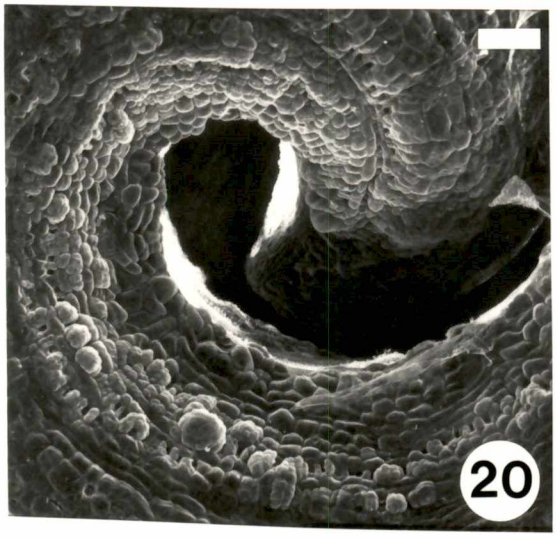
Figure 20. The epidermis becomes very rough and irregular after 15 days in culture. Domes begin to form from the cells surrounding the stomata. The bar represents 208 uM.

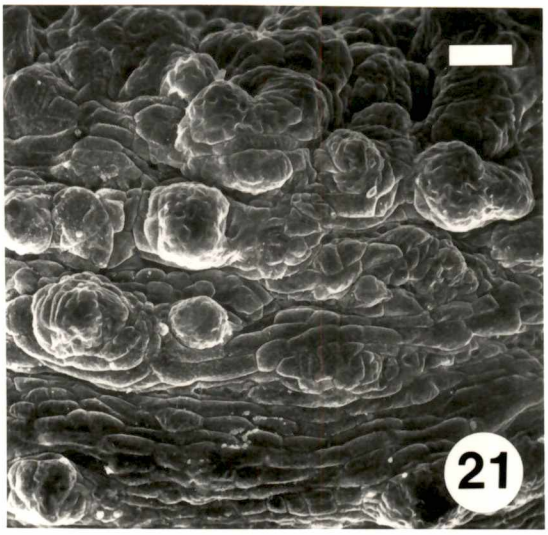
Figure 21. Irregularly shaped meristematic domes. Note the dimpled and wrinkled appearance of the domes and the folding around the perimeter. The bar represents 166 uM.

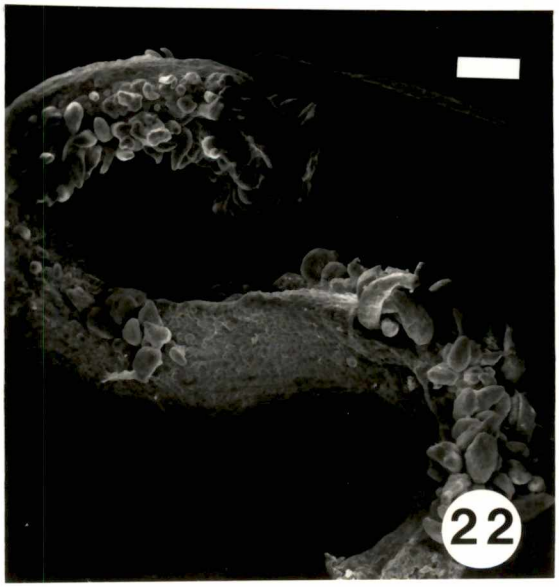
Figure 22. Cotyledon with mature structures at day 40. Numerous aberrant structures are present with many leaf-like structures. The bar represents 1000 uM (1 mm).


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Cell divisions were observed to occur in both periclinal and anticlinal planes within the clusters. The cells in these clusters were generally smaller than the surrounding cells and a large portion of their intracellular space was occupied by a large densely staining nucleus. These early meristemoidal regions closely resembled those observed in the development of the 10-day tissue that produced normally structured adventitious buds. No further histological changes were occured until about the time the meristematic domes began producing leaf primordia.

In SEM micrographs, changes in the epidermis can be seen as early as day 3 (fig. 19) when the small subsidary cells directly adjacent to and particularly between the stomata become enlarged. As these cells enlarge, they also begin to divide and by day 15 (fig. 20) this development results in a very uneven surface which consists of small clusters of cells and domes. As this development progresses, many domes appear (fig. 21). These domes can be distinguished from the domes in the 10-day old explants by several features.
The surface of the 10-day domes show no folding of

tissue (chapter II, fig. 14, p. 30). In contrast the cells of the dome of the 8-day old explants appear dimpled and wrinkled (fig. 21). It is also quite common for there to be a good deal of folding between the edges of the dome and the surrounding epidermis in the 8-day tissue (fig. 21).

The most commonly observed structure in the 40 day old tissue is that of a mishapen, poorly organized bud-like organ. A median longitudinal section of these structures (plate 5, fig. 23, 24, and 25) shows that the domed area is lacking in the apical organization associated with pine adventitious bud formation and pine apical meristems in general (Sacher, 1954; Fosket and Miksche, 1966). The dome does not exhibit the symmetry and organization of the meristematic domes of the 10-day explants. Although the aberrant domes posses an area of small, darkly staining cells in the apical region, what should have been the central mother cell zone of the apex is often narrow and asymmetrical. Figure 24 is representative of the aberrant domes in that it is difficult to discern the region of the apex that is associated with organized development and production of the next succession of leaf primordium. The area that gives rise to the next leaf primordium is typified by

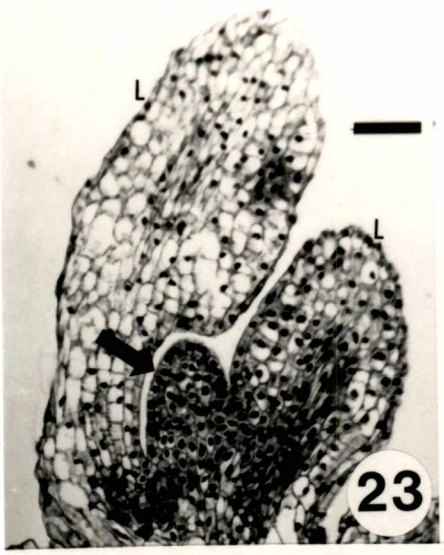
Plate 5. Histological micrographs of various types of aberrant structures derived from the 8-day old explants of Pinus oocarpa.

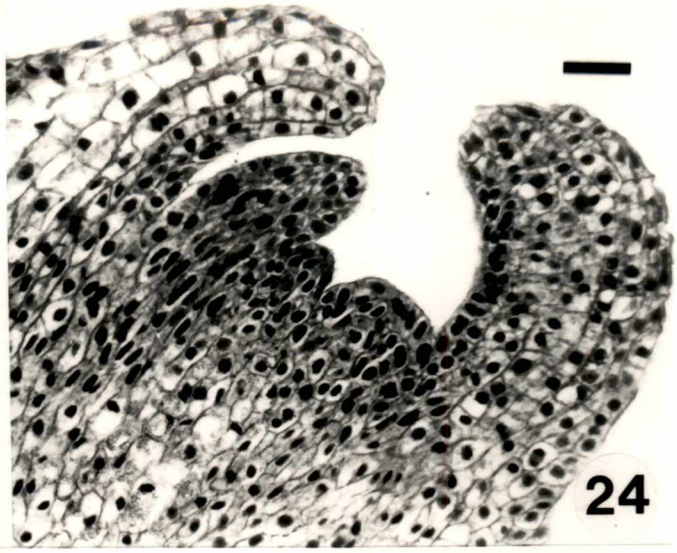
Figure 23. Aberrant bud with asymmetrical dome and aberrant leaf primordia. The leaf primordia have unusually large cells throughout the tissue. The bar represents 69 uM.

Figure 24. Aberrant bud with several leaf primordia. The meristematic center does not exhibit the organization usually associated with apical domes. The bar represents 28 uM.

Figure 25. Another aberrant structure, not associated with the aberrant bud, is shown. This structure has an intact epidermis and has vascular tissue. The bar represents 40 uM.

Figure 26. Aberrant structure known as a phylloid showing vascular tissue. The bar represents 69 uM.


L
L
23


24


L
L
P
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26

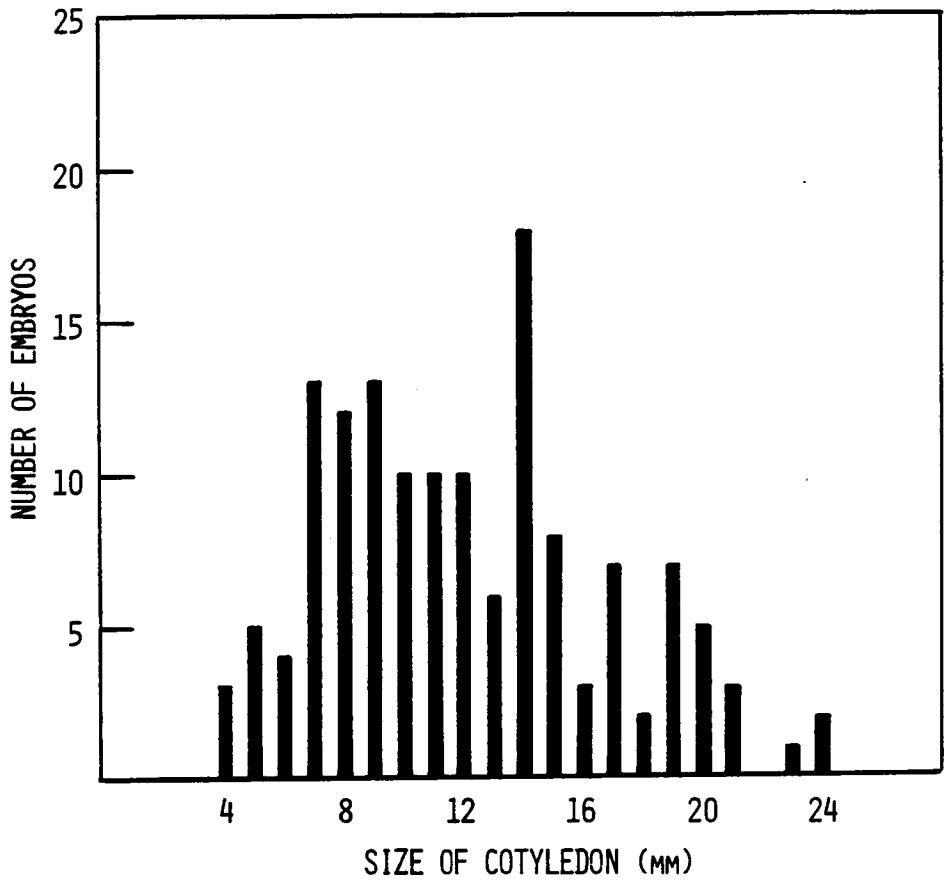
actively dividing cells just to the right and left of the center of the apex. At 40 days there are usually no more than 4 leaf primordia present on the aberrant buds and quite often an apex will produce only two leaf primordia. The leaf primordia become enlarged and mishapen as a result of random cell enlargement (fig. 23). Although the leaf primordia are aberrant, vascular tissue soon develops. SEM micrographs of day 40 tissue (fig. 22) show many areas with leaf-like sructures. Histological observations show that many, but not all, are associated with a meristematic dome.

Bornman (1984), using 14 day old seedlings of Picea abies with intact cotyledons of as explants, published micrographs of normal and aberrant vitrified buds. The normal bud was characterized by a well organized apical region that stained densely and produced numerous sets of leaf primordia. The vitrified "pseudobuds" were characterized by unusually large cells throughout the domed area and leaf primordia. There was no clearly defined area of meristematic activity in the "pseudobuds." Bornman attributed the vitrified buds and their aberrant structure to the concentration of the gel used to solidify the medium. At low gel concentrations (2.5 and 5.0g Tayio l^{-1}, and 1 and 1.5g Gelrite l^{-1}) a

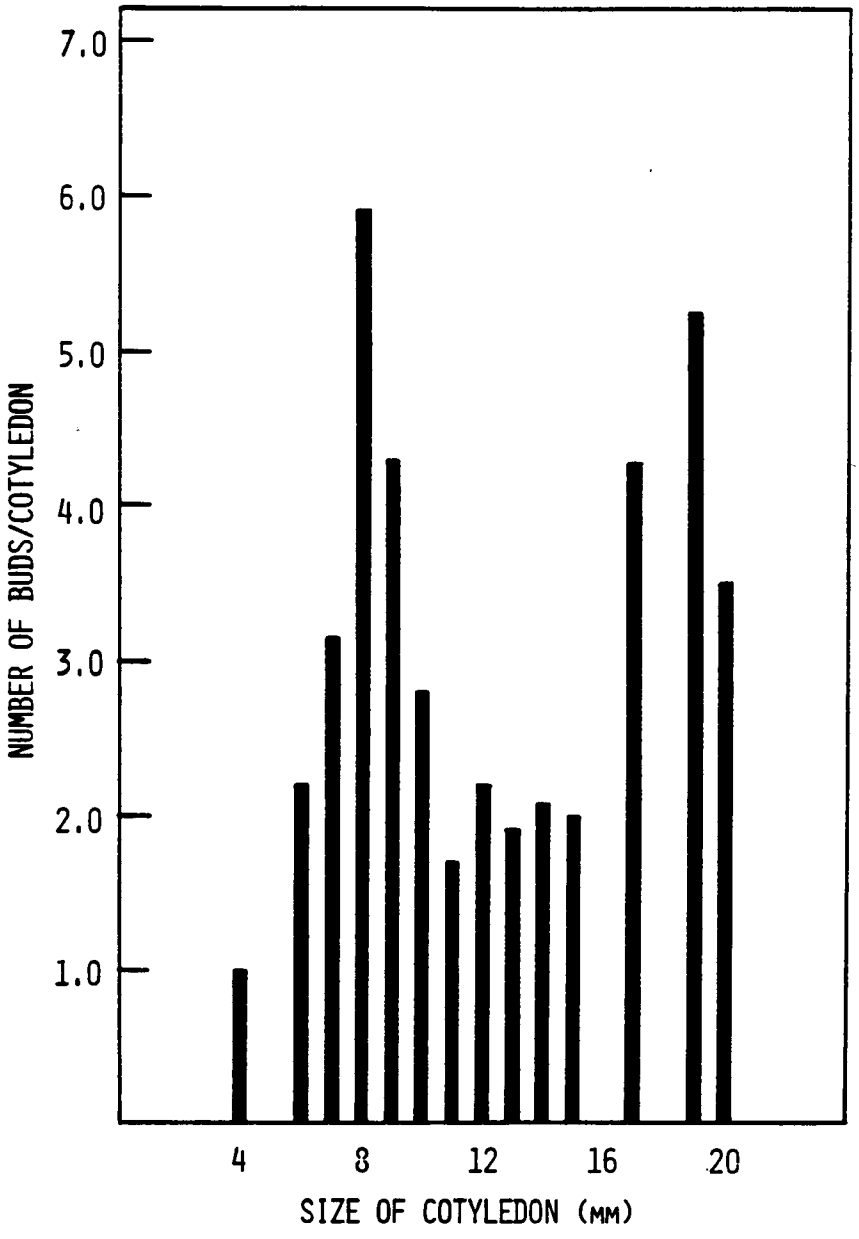
higher number of buds were produced per explant, but many of these buds were vitrified substantially reducing the number of viable buds. Von Arnold (1984) reported abnormal shoots that developed on embryonic explants of Picea abies. These shoots were "dark green and translucent." They were capable of elongating and growing further after spontaeously forming resting buds or after being subjected to several weeks of short day conditions. Another type of structure that developed was a phylloid (fig. 26). Phylloids, as defined by Bornman (1985), are "leaf-like with unsually large parenchyma cells with either weakly developed vascular tissue or lacking it altogether." Those seen in P. oocarpa quite often had one or two areas of highly mitotic cells at or near the base of the structure and in close proximity to the vascular tissue. Along with the aberrant bud in figure 25 is a structure that resembles Bornman's (1985) description of a phyma (P), a "pustular nodule of undifferentiated cells in cross section up to 3 mm or more in diameter", except that this structure has differentiated vascular tissue. Although not in a conifer, a similar aberrant structure referred to as an

"organoid" was described by Seckinger (1979) in Quercus rubra. This structure was a large parenchymatous growth with a well defined epidermis and vascular tissue.

Many works have mentioned the effects of the age, physiological state, and length of the explant on the ability of the explant to produce adventitious buds. In our second experiment, all sizes of cotyledons available at 10 days were excised and cultured. Since the range of sizes was from 4-24 mm, we were able to make two comparisons: First we compared the number of viable buds produced per explant in the 10-day old/4-7 mm tissue with the number of viable buds produced in the 8-day/3-7 mm tissue (graph 1). This allowed us to investigate the relationship between the size of the explant and its ability to produce viable buds. Secondly, we compared the number of buds produced per explant over the entire range of sizes to determine the sizes of explants at 10 days which were the best producers of viable buds (graph 2).



Graph 1. Histogram showing number of embryos vs. length of cotyledon in mm.



Graph 2. Histogram showing number of buds/cotyledon vs. the size of the cotyledon in mm.

Although the 8-day/3-7 mm explant material produced no viable buds after 40 days in culture, the 10-day/4-7 mm tissue produced viable buds. These buds elongated and rooted readily when treated as previously described by Franco and Schwarz (1985). Because the 10-day explants include the range of sizes found in the 8-day material, this demonstrates that the length of the explant is not a factor in the production of viable buds. The only difference in the two culture methods was the length of time the seedlings were grown, 8 or 10 days, before the explants were placed in culture. The second graph (graph 2) shows the distribution of lengths obtained at 10 days vs. the number of embryos at each length. Until this time, we have used an explant in the size range of 12-16 mm because this was the most abundant size at 10 days and thus the most representative explant. We now realize that although the other lengths are not present in as large a quantity, some may be better bud producers on a per explant basis (graph 2).

In early work with Pseudotsuga menzeisii cotyledonary explants, Cheng (1977) determined that age

was not a factor in the ability of that explant to produce bud primordia. No difference was found in the regenerative potential of explants when the age of the seedling from which the explant was taken was between 2 weeks and 2 months old. Various responses to the culture methods were observed and Cheng inferred that these differences in response to a defined medium and given amount of growth regulators were the result of the "variations in physiological condition and genomic composition " of the explants.

Von Arnold and Eriksson (1979) found that the short cultured needles (1-3 mm in length) of Picea abies were more easily indeced to form bud primordia than the longer needles (3-5 mm in length). Working with the same species, Jansson and Bornman (1981) noted that "there exsists a critical needle length which should not be exceeded when attempting in vitro induction of organ primordia."

Bornman (1985) found that the potential for inducing bud primordia in Picea abies decreased "with the leaf age (that is, length) " and also noted that the number of primordia induced decreased acropetally along the needle. Bornman further stated that the physiological condition of the explant should be

considered since it may vary with the chronological age of the explant.

In Pinus sylvestris, Tranvan (1979) observed that more cytokinin was needed with older embryo and cotyledonary explants and stated that "bud induction depends on the physiological age of the explant."

Aitken et al. (1981) found that the cotyledons from axenically germinated seeds of P. radiata produced the greatest number of rootable adventitious shoots (180). More specifically, a certain early stage of the germinated seed was selected because of its superior ability over a later, more developed, stage to produce rootable adventitious shoots. Thus, explants were selected by a physical charateristic (appearance of the radicle) over several days and not by time (number of days post germination). Cotyledons taken from embryos germinated on an agar medium were smaller and produced fewer rootable adventitious shoots. Cultured whole embryos produced even fewer rootable adventitious shoots.

In these experiments, we have shown that 8-day old explants of P. oocarpa 3-7 mm in initial length produce

numerous aberrant structures when cultured under the described condtions. The most prominent structure after 40 days in culture is an aberrant bud which has a poorly organized and asymmetrical apical dome. The leaf primordia that arise from this dome are also aberrant and are charaterized by unusually large and irregular cells throughout the primordia. Other structures such as phylloids were also present. We have also shown that the ability of the explant to produce viable adventitious buds is not dependent on the initial size of the explant, but instead on its age. Eight day/3-7 mm explants produced no viable buds while 10-day/4-20 mm tissue produced viable buds.

CHAPTER IV.

SUMMARY

When cultured on a modified MS medium with growth regulators, 10-day old cotyledonary explants of Pinus oocarpa 12-16 mm in length produce adventitious buds that will elongate and root. We have described the morphological events that lead to adventitious bud formation. The epidermis of the cultured explants undergo changes after 3 days and the first evidence of meristemoid formation was observed in day 15 tissue. Meristematic domes appeared between 15-19 days followed closely by leaf primordia formation. Adventitious shoots were present by day 40. This sequence of events was typical of other conifer species (Yeung, et al., 1981; Thorpe, 1980).

Eight-day old explants 3-7 mm in initial length cultured on a modified MS medium with growth regulators produce aberrant buds that do not elongate or root. The morphological events that lead to the formation of aberrant buds are indistinguishable from events that lead to viable bud production in the 10-day/12-16 mm explants up to the point of dome formation and leaf initiation. The aberrant apical domes are asymmetrical

and unorganized and the leaf primordia are comprised of irregular, unusually large cells. Other aberrant structures such as phylloids were also observed. When a range of 10 day cotyledons 4-24 mm in length are cultured, viable adventitious buds are produced over the entire range. Since 8-day/3-7 mm cotyledons do not produce viable buds, the size of the explant was determined not to be a factor controlling bud production.

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