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BUD AND ROOT DIFFERENTIATION IN CONIFER CULTURES

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ABSTRACT

Following the induction of buds and subsequent plantlet formation from the cotyledons of longleaf pine (Pinus palustris) in vitro, similar techniques were used to obtain plantlets from the embryos of P. taeda, P. elliottii, P. rigida, P. radiata, P. sabiniana, P. virginiana, and Pseudotsuga menziesii. Bud differentiation was achieved from the cotyledons on a modified basal media of Gresshoff and Doy and/or Schenk and Hildebrandt using different concentrations of benzyladenine (BA) and auxin (NAA or IBA). The rooting response of most pines and Douglas-fir is sporadic and unpredictable, and only a few plantlets have been obtained in any of these species. More recently, controlled differentiation of buds has been extended to primary needles of Monterey pine and Douglas-fir, and less frequently to secondary needles of young seedlings in the former. To date, root initiation has been nil following bud formation on older leaves. Highly variable genotypic responses have been observed in all species on the media tested.

Conifers have long occupied a significant place in tissue culture research. Over 50 years ago Schmidt (1) attempted to grow the embryos of several conifers free of the surrounding nutritive female gametophyte with little success. Since that time over 95 species or cultivars of gymnosperms in 25 genera and 9 families have been placed in sterile culture. Of these 95 species, 87 are members of the Coniferae, and almost half of these belong to the genus Pinus (2).

The many potentialities offered by the application of tissue culture techniques to problems in forest tree improvement are now widely recognized and this topic has been the subject of several recent reviews (3,4,5). Concomitant with this upsurge in interest, many workers in laboratories around the world are now engaged in problems relating to organogenesis and cloning of economically important forest trees. Although one can find numerous reports of organ differentiation in gymnosperms in vitro, only in a few instances has it been possible to produce plantlets capable of being transferred to soil for sustained autotropic development (6). Obviously, the future success of parasexual manipulation and genome modification of forest trees depends upon the ability of researchers to optimize cloning procedures for different species.

EXPERIMENTAL OBSERVATIONS

Physiologists have long recognized the deleterious role exerted by aging of cells and tissues in the vegetative propagation of long-lived woody plants. Considerable optimism in overcoming these difficulties was expressed when we were able to establish and maintain cambial cultures from older trees of several pines and other gymnosperms on chemically defined media for the first time (7). Unfortunately, many subsequent attempts in our laboratory to induce bud and/or root differentiation in callus cultures of the southern pines, notably Pinus elliottii Engelm. and P. palustris Mill., on a wide variety of nutrient media containing varying concentrations of auxins, cytokinins, and gibberellins repeatedly failed. Adventitious bud differentiation followed by root and plantlet formation was finally first achieved from the cotyledons of mature embryos in Pinus by Sommer in 1973 (8). Since this time bud differentiation has been obtained from the cotyledons of several pine species, followed by plantlet formation in 6 of the 10 species cultured (Table 1).

SPECIES	MEDIUM	PER CENT CULTURES FORMING BUDS
Pinus palustris**	1*	79
	2	100
P. taeda**	1	--
	2	26
P. elliottii**	1	50
	2	17
P. echinata	1	8
	2	25
P. virginiana**	1	7
	2	21
P. rigida**	1	67
	2	50
P. strobus	1	41
	2	52
P. ponderosa	1	12
	2	--
P. sabiniana**	1	--
	2	82

TABLE 1. Influence of inorganic nutrients on bud differentiation in several pine species.

* Modified Gresshoff and Doy (Sommer et. al., 1975)

** Plantlets formed when transferred to basal medium, with or without auxin.

In these and subsequent experiments using mature embryos of seed from wild populations, or from half-sib families, we have observed much genotypic variation in the ability to induce adventitious buds and/or roots on identical nutrient media under essentially uniform cultural conditions (vis., cultures maintained at 25° ± 3°C under a 15-hr. photoperiod, illuminated with approximately 500 ft-c cool white, fluorescent light).

Another important source of variation in bud differentiation among species appears to lie in inherent differences in the aging of seed. This appears to be especially important in larger seeded species such as longleaf pine which ungoes rapid deterioration with storage time (9). Many deterious cytological and genetic effects are known to occur with aging of seed (10).

In addition to these genotypic and aging responses within species, most researchers have encountered highly variable genetic and species differences to organogenesis on a variety of chemically defined media. Such widespread differences in response often result from presently ill-defined differences in the inorganic composition of the basal medium, interacting with varying levels of growth factors such as cytokinins and auxins. For example, the basal media used for successful bud differentiation in longleaf pine (a modified Gresshoff and Doy medium containing 6-benzyladenine (BA) at 1 or 5 mg/l and napthalene acetic acid (NAA) at 2 mg/l) was only sporadically effective in inducing bud formation in Douglas-fir (Pseudotsuga menzieii (Mirb.) Franco). In one series of controlled experiments where NAA and BA were varied to give 35 different concentration combinations using 10 embryos of Douglas-fir in each combination, bud differentiation varied from 0 to 100% on the same basal medium. No buds were formed when NAA was used at 2 mg/l and BA at 1, 2, or 8 mg/l; whereas, 80 to 100% of the cotyledons produced buds when BA was supplied at 0.1 mg/l (100%) to 16 mg/l (80%). Likewise, in another series of experiments in which the levels of nitrogen, magnesium and phosphorus were varied in two basal media (media I and II) along with different combinations of NAA and BA, significant differences in response were observed between the two media. Medium I which contained lower levels of nitrogen and phosphorous routinely produced a higher frequency of buds than medium II at almost all combinations of NAA and BA in the case of Douglas-fir (11). However, for longleaf pine and some of the other southern pines, bud differentiation occurred more frequently on media II (see Table I). When the buds of Douglas-fir were excised and transferred to the same medium used for root initiation in longleaf pine, less than 1% of the buds rooted to form plantlets. Eventhough 100% bud formation was achieved for Douglas-fir, thus far the production of plantlets for this species has been highly sporadic.

In more recent studies with Monterey pine (P. radiata, D. Don) a modified Schenk and Hildebrandt basal medium supplied with 5 mg/l BA and 0-0.5 mg/l NAA was significantly more effective in inducing buds on the cotyledons or hypocotyls of mature embryos than the basal medium used by Sommer et al for longleaf pine and the other pines previously reported (12). In this species buds were formed within 3-7 wks. on 53-85% of the explanted embryos, however, when they were excised and transferred to various types of rooting media without auxin or with low concentrations of indolebutyric acid (IBA) or NAA (0.01-1 mg/l) considerably less than 1% of the developing shoots produced roots.

In subsequent attempts to extend adventitious bud formation in P. radiata to chronologically older organs, i.e., to primary and secondary needle segments (1 cm in length) from sterile-growth seedlings, some success was obtained by the use of a liquid pretreatment technique using 1/2 concentration of the Schenk and Hilderbrandt basal media containing BA or isopentyladenine (iP) at 5 mg/l for varying periods of 1-4 wks. In some experiments conducted with primary leaves using a pretreatment time of 3 wks followed by transfer to a basal agar medium without cytolkinin or auxin, adventitious buds were formed in 80% of the cultures. In contrast, adventitious buds were produced from the immature basal portion of secondary needles in only a few cultures (12).

Many of the buds produced from primary leaf segments of Monterey pine failed to undergo rapid internodal elongation and continued to produce thick clusters of numerous second generation primary leaves averaging 0.5-2 cm in length after 30-60 days on the agar medium. These leaves, in turn, were excised and pretreated in liquid culture as previously described for 9 or 21 day periods prior to transferring to the basal agar medium. In these experiments even the 9 day liquid pretreatment time

proved deleterious to the young succulent needles and less than 5% of the culture produced any third generation buds.

CONCLUSIONS AND FUTURE OUTLOOK

From these and other studies of organogenesis in conifers it becomes increasingly more obvious that numerous, specific cultural requirements will have to be developed for most species, frequently even among closely related members of the same genus.

In addition to differences in species responses, one must also fully understand or attempt to understand the nature of the material being cultured. In many instances organs or tissues excised from young, juvenile plants have already aged to the extent of being refractive to treatment. The objective of most researchers is to develop procedures for reversing cells and tissues to an "embryonic" condition leading to controlled organogenesis. Progress in this respect with most coniferous species has thus far been slow, but nevertheless encouraging. Even at the present time procedures for optimizing the commercial cloning of horticultural or agronomic plants are limited to a rather small number of species. To develop comparable systems for practical use in tree improvement programs several criteria appear essential, namely: (1) the cultured material should be easily established and maintained, preferably on a chemically defined medium; (2) consistent differentiation of buds and roots, embryoids, or plantlets must be achieved with a minimum of time to reduce the incidence of genetic change under artificial cultural conditions; (3) subsequent generations of plantlets or propagules must be easily attained for mass production of desirable genotypes; (4) newly formed plantlets must survive transfer to semi-controlled conditions for antotropic development, followed by successful transfer to the field; and (5) newly formed individual plants must develop true to type as witnessed by phenotypic responses and field performance.

Although some of our original longleaf and loblolly (P. taeda L.) pine plantlets have developed into autotropic seedlings for outplanting to the field, all of the above criteria (especially numbers 2 and 3) have not yet been met for optimizing commercial production.

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INFLUENCE OF PARENTAL TREE GENOTYPE ON THE POTENTIAL FOR IN VITRO CLONAL PROPAGATION FROM LOBLOLLY PINE EMBRYOS

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ABSTRACT

Embryos from ten loblolly pine (Pinus taeda L.) seed families, the progeny of trees selected as breeding stock in an industrial tree improvement program, were tested for bud induction response in vitro. Two of the ten families were cultured under conditions in which almost all embryos produced buds, demonstrating that buds can be induced from improved loblolly pine seed without imposing selections. Thus, the potential exists for producing clones from virtually every embryo cultured. When growth regulators were supplied at concentrations which were marginal for bud induction (2 mg/l NAA and 5 mg/l BAP), the culture environment had a profound influence on the performance of the various seed families. Embryos from families with the highest 4 to 8 year field growth ratings tended to be the better bud producers under marginal tissue culture conditions.

INTRODUCTION

In the past few years, important advances have been made in developing methods for clonal propagation of coniferous tree species in vitro[1,3,5,6,7]. These methods are applicable to embryonic tissues, tissues from young seedlings, and, to a lesser extent, callus derived from these sources. Explants from different individuals have varied in their responses to organ inducing stimuli[4,5,7]. The most recent reports indicate that with some species, cultural conditions can be altered such that virtually every explant will form some adventitious buds[2,4]. In these reports, the inocula were identified by tree species and tissue types, but not further defined. Thus, two important questions related to the application of current tissue culture technology in forest tree improvement programs remain unanswered. First, how will the progeny of trees selected as genetically improved breeding stock perform in tissue culture? Second, since tissue culture can be applied such that not all individuals yield propagules, what genetic traits are being selected for in the survivors? The latter consideration is of particular interest in that early selection criteria for desirable traits will be needed to efficiently reduce the time required between successive advanced generations of improved trees.

This paper reports the bud induction response of embryos from ten seed families collected from individual loblolly pine (Pinus taeda, L.) trees selected for breeding stock in the North Carolina State University Industry Tree Improvement Cooperative. The bud induction response is reported as the frequency of embryos which produce buds and the number of buds per embryo. Average bud induction responses of the seed families were examined for possible correlations with field-tested traits of economic importance.

METHODS AND MATERIALS

All seeds used in this study were from open-pollinated loblolly pine trees rated as good general combiners. Seeds were obtained from five trees in a coastal plain seed orchard and five in a piedmont orchard, both located in South Carolina. The qualitative growth ratings of the seed families was provided by the North Carolina State University

Industry Tree Improvement Cooperative. These evaluations were based on 4 and 8 year height and diameter growth of the progeny from numerous specific crosses involving each mother tree. The progeny tests were carried out on a number of different sites. Qualitative growth ratings are relative only to seed produced in the same orchard[9].

Mature seeds were surface-sterilized by immersion in 50% Clorox® for 10 minutes, then rinsed three times with sterile water. Surface-sterilized seeds were cold-stratified at 4°C, by immersion in sterile water for two days and covered with moist cheesecloth for five days[8]. These seeds were placed in sterile water at ambient temperature for 36 to 40 hours; seed coats were removed; nucellar tissue was surface-sterilized and rinsed as previously described and stored overnight in sterile water prior to excising embryos.

Inocula were either excised embryos planted vertically or excised cotyledons laid horizontally on the bud induction medium. This medium was medium No. 1 developed by Sommer et. al. primarily for longleaf pine[6]. The growth regulators in this medium [2 mg/l naphthalene acetic acid (NAA) and 5 mg/l 6-benzylaminopurine (BAP)] were marginal for bud induction with excised loblolly pine embryos. In some instances, the growth regulators were altered to improve the bud induction response. Cultures were maintained in one-ounce glass bottles with screw caps, each containing 10 ml. of medium. Culture Environment A was constant illumination with cool white fluorescent light at 900 ft. cdl. at 21±0.5°C for 16 hr. and 7±0.5°C for 8 hr. each day. Environment B was 16 hr. cool white fluorescent light per day at 900 ft. cdl., and the temperature was 25±0.5°C. The bud induction response was quantified at low magnification after six weeks. Bud clusters were scored as three buds.

RESULTS

Isolated individuals or clusters of adventitious buds formed on cotyledons and were easily observed after 5 to 6 weeks in culture. Cotyledon excision and/or changes in growth regulators did not influence the time required for bud formation. Bud induction was complete at six weeks for all seed families as no average increase in buds occurred when embryos were extended to nine weeks in culture. Buds and bud clusters were isolated and transferred to one-half strength basal medium without growth regulators for further growth. On this medium, bud clusters produced no more than three leafy shoots.

Table I shows the bud induction response of excised cotyledons from ten embryos each of two coastal plain seed families (CA and CE). With this culture regime, adventitious buds were produced from cotyledons of almost all embryos cultured and the response of the two seed families was approximately equal. Plate 1 shows a representative sample of shoots produced from one seed of Family CA.

TABLE I

BUD INDUCTION RESPONSE
OF TWO COASTAL PLAIN SEED FAMILIES

SEED LOT	PERCENT OF EMBRYOS FORMING BUDS	AVERAGE NUMBER OF BUDS PER EMBRYO WITH BUDS	MAXIMUM NUMBER OF BUDS PER EMBRYO
CA	90	16.6	38
CE	80	16.0	41

Note: Growth regulators were 3-indoleacetic acid (IAA) (2.5 mg/l) and Zeatin (2.0 mg/l). The culture environment was A = constant illumination with 900 ft. cdl. cool white fluorescent light and a temperature of 21±0.5°C (16 hr.) and 7±0.5°C (8 hr.) per day.

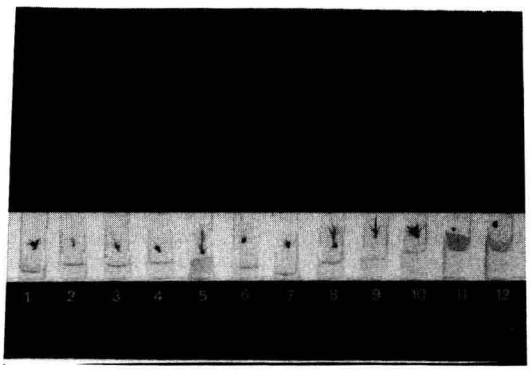


PLATE I

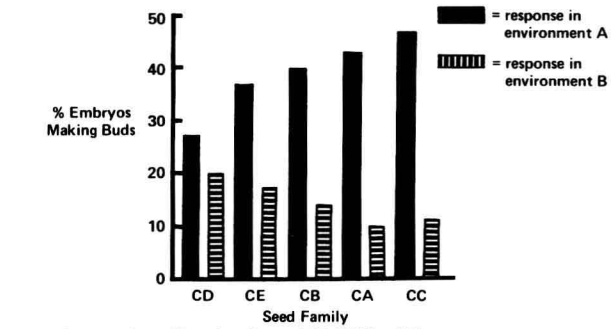
Representative group of shoots from one seed of family CA.

These results show that currently available tissue culture techniques can be applied to the progeny of loblolly pine trees selected as genetically improved breeding stock. Modest numbers of shoots (average 16) were produced from individual embryos which suggests the potential clone size obtainable from individual embryos in a single induction trial. Even larger numbers of shoots from one seed might be obtained by inducing additional buds from tissues of adventitious shoots.

The effect of culture environment on bud induction from excised embryos was tested using culture medium with the growth regulators at marginal concentrations for bud formation (2 mg/l NAA and 5 mg/l BAP). Figures 1 and 2 show the percentage of intact embryos forming buds from coastal plain and piedmont families respectively. The culture environment did influence the bud induction response of both groups of seed families under this growth regulator regime. In Environment B, the percentage of embryos producing buds was low (7% to 28%). Only families PB (7%) and PE (28%) responded differently (LSD = 95%). When embryos were cultured in Envi-

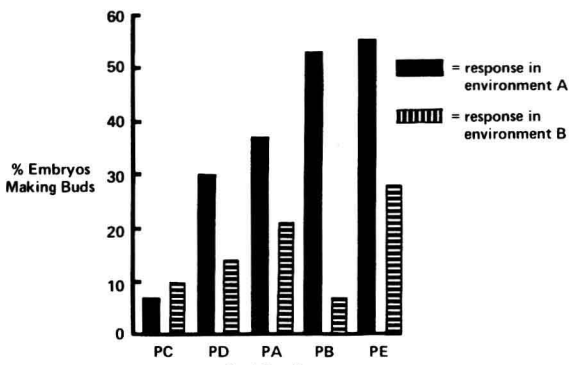
ronment A, the percentage of embryos producing buds increased significantly in many families. In Environment A, a number of significant differences between families (LSD = 95%) were observed (Figure 1, CC>CD; Figure 2, PB=PE>PA=PD>PC).

FIGURE 1 – BUD INDUCTION RESPONSE AMONG COASTAL PLAIN SEED FAMILIES IN TWO CULTURE ENVIRONMENTS



Note: Average of two 15 seed replicates, LSD (95%) = 19%.

FIGURE 2 – BUD INDUCTION RESPONSE AMONG PIEDMONT SEED FAMILIES IN TWO CULTURE ENVIRONMENTS



Note: Average of two 15 seed replicates, LSD (95%) = 19%.

Table II shows field growth evaluations and adventitious bud production response in tissue culture for the ten seed families using marginal growth regulator concentrations and the two culture environments. Bud production in the more favorable environment (A) suggests that families with better growth ratings may also be better bud producers under these conditions. Of the five coastal plain families tested, two (CA and CC) were the better bud producers. These families received excellent growth ratings in field tests. Although Family CB was also rated excellent for growth, it was similar in bud production to families with average growth ratings. The piedmont seed family with the poorest field growth rating (PC) was also the poorest bud producer. Family PD received the highest growth rating of the piedmont families tested (above average); however, its bud production response was lower than some piedmont families.

TABLE II

COMPARISON OF TISSUE CULTURE BUD PRODUCTION AND GROWTH CHARACTERISTICS FOR TEN SEED FAMILIES

FAMILY	GROWTH RATING[a]	BUDS PER EMBRYO CULTURED[b] ENVIRONMENT A	ENVIRONMENT B
CA	E	5.4	0.6
CC	E	4.8	0.8
CB	E	1.9	0.9
CD	A	1.2	0.8
CE	A	1.1	0.6
PE	A	4.4	2.6
PB	A	4.0	0.2
PA	A	2.8	1.2
PD	A+	2.1	1.1
PC	A-	0.1	0.4
		LSD 95% = 1.7	LSD 95% = 1.9

[a] Qualitative evaluation (A = average, E = excellent) of the seed families based on 4-8 year growth in the field as compared to other progeny produced in the same seed orchard.

[b] The unit, buds per embryo cultured, combines components of frequency and intensity of bud production on intact embryos cultured for six weeks on a medium with growth regulators BAP at 5 mg/l and NAA at 2 mg/l. Environment A = continuous light 900 ft. cdl. and 21°C for 16 hr., 7°C for 8 hr. Environment B = 16 hr. light 900 ft. cdl. per day and constant temperature at 25±0.5°C. Data are averages based on two replicate groups of 15 embryos each from cold-stratified seed.

The correlation between tissue culture bud production and field growth ratings of the families is weak. It is obvious that bud induction under marginal conditions is selective amongst these families. Some seed families with either very high or very low 4 to 8 year field growth ratings can be distinguished from the others on the basis of bud production. Using two of the families, we have shown that culture conditions can be altered such that buds are produced from almost every embryo cultured. Therefore, tissue culture techniques offer the potential for producing clones from improved seed without introducing unwanted selections. With the advent of a reliable rooting technique, propagules produced under marginal, therefore selective tissue culture regimes, could be evaluated for growth in the field. Assured selections of young trees with desirable growth rates can be confirmed only after 15 to 20 years in the field. Since growth evaluations must be long-term, early selection methods with potential, although unproven, should be used and tested.

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AN ANATOMICAL AND CYTOLOGICAL PRESPECTIVE ON PINE ORGANOGENESIS *IN VITRO*

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ABSTRACT

The anatomical and cytological similarities surrounding bud and root initiation from several cultured pine tissues and organs are described. These events in pine are compared and contrasted with similar events as they occur in other selected plant species. The fundamental aspects drawn from the anatomical comparisons are evaluated for their impact on clonal propagation of pines via tissue culture.

INTRODUCTION

Most reports of multiple shoot regeneration from conifer species are similar in that the adventitious buds arise from cultured embryo or excised embryo parts.[3,4,5,20] Only a few workers have reported shoot regeneration from more disorganized callus cultures of conifers[1,25] or in other woody species[9,10,12,13,24]. The relative ease of shoot regeneration from organized tissues of plant organs suggests that the anatomical location of cells may play a decisive role in their ability to generate organized meristems. This suggestion is supported by results obtained with many herbaceous species which easily regenerate shoots from excised leaves in culture.[2] Anatomical studies of such leaves indicate that specific tissue areas participate in shoot initiation for each species. Shoots may come exclusively from epidermal cells or from cells near[23] or below the epidermis.[11] In general, shoots and even somatic embryos arise from the peripheral cell layers of plant organs and in the case of less organized cell masses they also arise near or within the exterior cell layers.[21] Roots, on the other hand, originate from internal cells of *in vitro* cultures usually associated with any vascular tissues as is common *in vivo*.[7,20]

It is evident from the literature of organogenesis in culture that only certain cells in an organ or callus explant respond by forming buds or roots. The cells which respond to a given growth regulator stimulus seem to be quite localized with respect to the existing anatomy of the explant even in the case of callus.[17] To the extent that one might recognize these anatomical relationships for a particular plant, the anatomical events may serve as guide posts in perfecting methods for more reliable shoot/root regeneration from different organs. Anatomical guidelines may also be a valuable aid in selecting appropriate initial explants for culture. This paper will explore the influence of anatomy on *in vitro* shoot and root formation from parts of embryos, seedlings and trees of loblolly pine (*Pinus taeda* L.) in comparison with similar events in other plants.

RESULTS AND DISCUSSION

Excised embryo parts of loblolly pine regenerate adventitious shoots on their surfaces when placed on a nutrient medium (GD1) with appropriate concentrations of the growth regulators 6-benzylaminopurine (BAP) and α-napthaleneacetic acid (NAA).[14,15] This response is more frequent with excised cotyledons than with hypocotyls or shoot tips but all three are similarly affected by exogenous cytokinin concentrations (Figure 1). The initial explants which will generate buds each have in common an obvious epidermal layer subtended by several layers of relatively undifferentiated cells (Plate 1). Cotyledons will serve as a representative example to demonstrate the observed influence of anatomy on adventitious bud generation in culture. Exposed to excess auxin, cell divisions begin at the cut ends of the cotyledon and eventually the entire cotyledon forms callus without initiation of adventitious buds. Exposed to excess cytokinin, division is focused in the epidermal and subepidermal region and these cells divide periclinally to generate additional layers of cells but shoot formation is rare (Plate 2). When supplied with adequate exogenous

cytokinin and little or no auxin, division is restricted to a few localized areas near the epidermis. These cells continue to divide locally to form organized protruding cell masses (Plate 3) which will later develop into shoots (Plate 4). The shoots continue to grow and can later be excised for propagation. The response pattern observed for cotyledons is also evident in hypocotyl (Plate 5) and in shoot tips although less clearly demonstrated in the latter due to their much more complex organization.

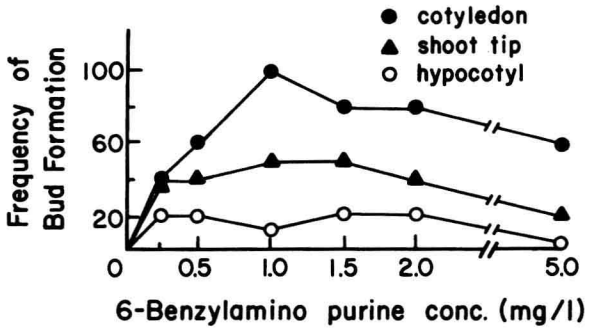

Figure 1. Adventitious bud induction from excised embryo parts.

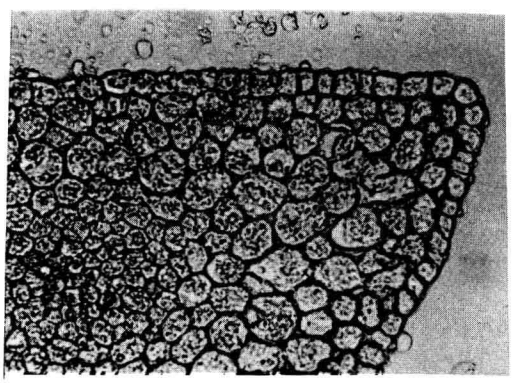

Plate 1. Part of a transverse section of loblolly pine cotyledon after exposure of the seed for 7 days in 1% H_2O_2 solution. 320 X.

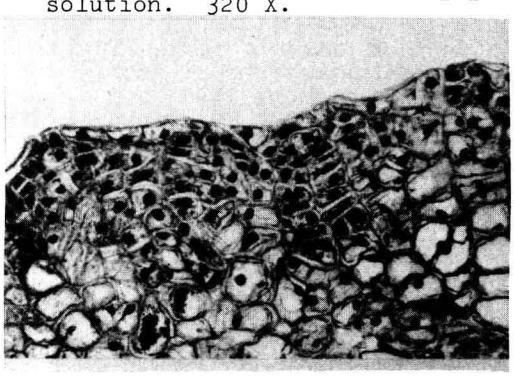

Plate 2. Part of a transverse section showing much periclinal cell division in the surface region of an excised loblolly pine cotyledon after 6 weeks culture on a medium supplying an excessively high cytokinin concentration, BAP (5.0 mg/l). 200 X.

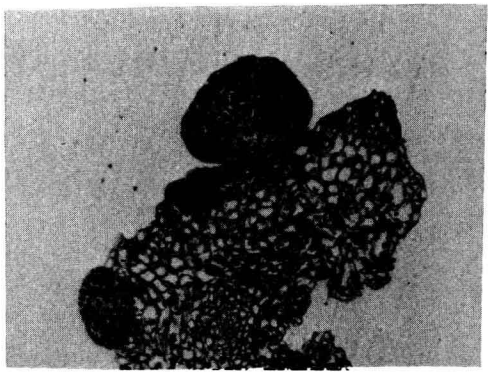

Plate 3. A transverse section of a cultured loblolly pine cotyledon showing an early stage (4-6 weeks) of adventitious shoot formation from the peripheral region of the cotyledon. 60 X.

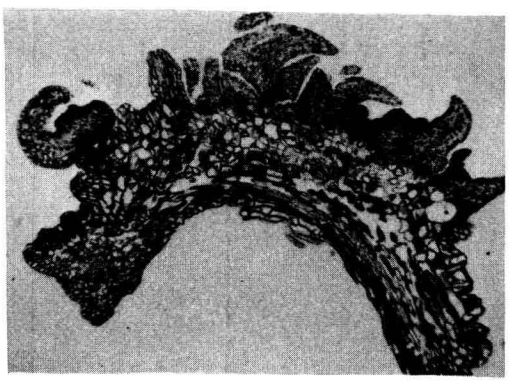

Plate 4. A longitudinal section of a cultured loblolly pine cotyledon at a later stage. (12 weeks) of adventitious shoot formation from the peripheral region showing very little involvement of subtending tissues. 30 X.

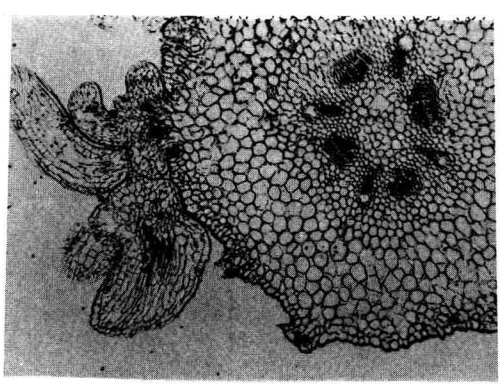

Plate 5. A transverse section of a cultured hypocotyl segment of loblolly pine (12 weeks) showing the peripheral origin of adventitious shoots with very little activity in the subtending tissues. 50 X.

Shoots originate from cells near the periphery of excised loblolly pine embryo parts with little involvement of subtending parenchymatous cells (Plates 3, 4 and 5). The lack of internal connection is further supported by

the ease with which these adventitious buds of pine may be detached from the parent tissue with forceps. We have not observed extension of the newly formed shoot axis of individual buds into the interior explant tissues or any association with explant vascular tissues as was suggested for longleaf pine.[20] However, several buds may arise close to one another and their combined influence leads to a general proliferation of epidermal cells in the immediate area. Buds then can appear to originate from internal cells since adjacent cell proliferation tends to cover them up. These proliferating cell masses can subsequently generate new buds at their surfaces but the exact point of origin is again obscured by continued adjacent cell division.

Whether shoots originate from one or several epidermal cells and whether cells immediately subtending the epidermis are also involved has not been determined with certainty. However, shoot generation in excised loblolly pine embryo parts does bear a striking resemblance to multiple shoot generation from leaf pieces of gesneriads and this resemblance may extend to the actual cells involved. Studies in this laboratory using Streptocarpus hybridus 'constant nymph'[6] will serve to demonstrate the exclusive localization of this response in the leaves. Surface sterilized leaves were cut into small pieces and placed adaxial side up on a nutrient agar medium. In about ten days, cell divisions occurred in the upper epidermis which produced buds and ultimately shoots which could then be harvested for propagation within 30 days. Shoot initiation is restricted solely to one or two epidermal cells (Plate 6). Cell divisions were rare in other parts of the leaf until a more generalized cell division set in near 30 days after which new shoots were not generated. Under stronger growth regulator stimulus, cells in other parts of the leaf also divided to form callus as in pine. Shoots are not immediately forthcoming from this callus growth.

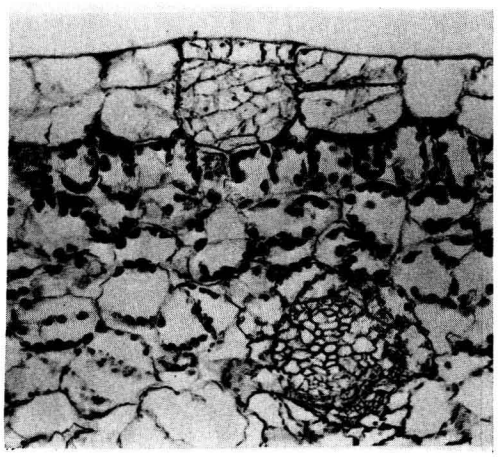

Plate 6. A cross section of a cultured leaf of Streptocarpus hybridus 'constant nymph' showing the localized divisions in a single epidermal cell which will ultimately produce an adventitious shoot. Note the lack of activity in subtending tissues.

Several generalizations concerning bud initiation emerged from the anatomical study of cultured cotyledons in loblolly pine. These generalizations draw support through similarities with bud induction in other species. First the initial explant material had a defined but not strongly differentiated epidermis subtended by relatively homogenous parenchymatous cells. Second, only the cells in the epidermal region respond to exogenous cytokinin while cells of the other tissues do not. Third, the interior parenchymatous cells either by virtue or position or physiological state do not divide under the stimulus of low exogenous auxin or cytokinin concentrations. These generalizations, related to the anatomy, can be useful in a search for optimal growth regulator stimuli to foster localized epidermal divisions while preventing divisions in the underlying parenchymatous cells. The localized epidermal divisions, situated as they are at the periphery of a mass of nondividing parenchyma cells, do form buds. Culture conditions which stimulate more general divisions in the epidermis or divisions and cell enlargement in the subtending cells, disrupt anatomical organization and also inhibit bud formation.

The initial anatomy of the excised organ is important. Even young scale leaves and needles (less than 5 mm in length) have well differentiated epidermis and subepidermal layers and the subtending cells are already differentiated into the spongy mesophyll cells characteristic of pine. Excised young needles or scale leaves of loblolly pine do not produce buds on their surfaces in culture. When cultured on a cytokinin/auxin ratio favoring bud induction on excised cotyledons, they show no cell division whatsoever, but when planted on a medium favoring callus formation, cell divisions occur but they are internal and restricted to the transfusion tissue of the vascular trace (Plate 7). The early stage of callus formation occurs near the cut ends and progresses inward to involve the transfusion tissue throughout the leaf. Thus, the anatomy and degree of cell differentiation do not favor divisions in peripheral cells and the favored internal cell divisions are not localized but continue throughout a disorganized callus. The advantage toward localized peripheral divisions apparent in the anatomy of cotyledons is not available in scale leaves and needles. However, the advantage can be realized in the less completely differentiated area between the base of the needles and the emerging shoot tip of needle fascicles which can produce buds from peripheral tissues similarly to cotyledons. The procedures reported for bud generation directly from excised scale leaves of Pinus radiata[18] would appear to work with the anatomy of those leaves rather than against it as we observed with loblolly pine.

The tissue culture responses of cotyledons, scale leaves and needles of loblolly pine are correlated strongly with their anatomy. Thus it would appear that on the basis of anatomy some organs can be eliminated as source material for direct de novo bud formation. On the other hand, it does not follow that appropriate anatomy guarantees success in bud generation. The capacity for bud generation from excised cotyledons can be greatly affected by the prior treatment of the embryo before the cotyledons are excised (Table 1). These treatments do not affect the general anatomy of the cotyledons to any significant degree. At the time of excision, the appearance of all cotyledons in these treatments resembled

that of Treatment No. 6 shown earlier in Plate 1. Yet from this common anatomy, the frequency and intensity of bud production can be greatly modified according to prior treatment. The trend that emerges from these experiments is that some stimulation of embryo germination is beneficial (compare treatments 3 through 6 with 1 and 2) but if the germination process is allowed to progress too far toward embryo growth (treatments 7 and 8), bud induction is inhibited and finally eliminated. Cotyledons inhibited in bud production in this way simply grow in culture as cotyledons and do not form callus. Thus the time element in cell differentiation leading to organ anatomy in cotyledons apparently aids adventitious shoot production in that the peripheral cells are among the last to remain sensitive to low levels of exogenous growth regulators. That these cells are situated peripheral to the cell mass of the organ may itself dictate that localized cell divisions will organize to form shoots.

Root initiation in culture can be observed directly from excised cotyledons of loblolly pine (Plate 8) as well as from excised adventitious buds (Plate 9). Anatomical study of these cases shows new roots originated from localized divisions in close association with existing vascular tissues. It appears that localized, as opposed to general, cell division is a prerequisite for both shoot and root initiation. The contrast between peripheral shoot origin and internal root origin would suggest that when localized divisions are stimulated, the subsequent interaction with adjacent cells determines whether a shoot or root will be formed. Dividing centers adjacent to vascular tissue and surrounded by non-dividing cells as in the interior of the explant become roots. Centers situated at the periphery of a mass of non-dividing cells form shoots.

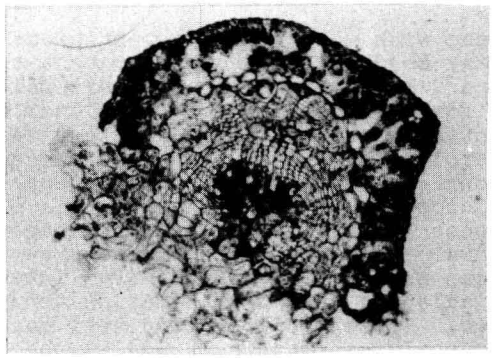

Plate 7. A transverse section of a loblolly pine needle cultured on NAA (0.1 mg/l) for 10 weeks. Note the focus of cell division leading to callus in the transfusion tissue interior to the endodermis and the lack of activity in the peripheral mesophyll and epidermal cells. 100 X.

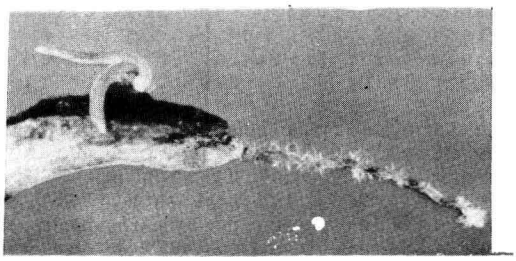

Plate 8. Loblolly pine cotyledon cultured 11 weeks on zeatin (1.0 mg/l) + NAA (0.01 mg/l). Note the peripheral origin of the adventitious shoot and the interior, vascular origin of the root extending to the right (Mott and Palta, unpublished).

Table 1. Effects of pre-treatments of intact embryos upon subsequent adventitious bud induction on excised cotyledons of Pinus taeda L.

Pre-treatment of embryos	% embryos forming buds on excised cotyledons	Average number of buds/embryo with buds
1. Nonstratified seed + water soak (48 hrs.)	20	3.0
2. Cold stratified (7 days) + water soak	20	4.0
3. 1% H_2O_2 (1 day)	20	2.0
4. 1% H_2O_2 (3 days)	80	14.6
5. 1% H_2O_2 (5 days)	100	20.8
6. 1% H_2O_2 (7 days)	100	17.8
7. 1% H_2O_2 (7 days) + 1 week on seedling growth medium (GD1 x 1/2)	30	5.0
8. H_2O_2 (7 days) + 2 weeks on seedling growth medium (GD1 x 1/2)	0	0