COMPARATIVE MORPHO-HISTOLOGICAL STUDIES ON THE SITES OF SHOOT INITIATION IN VARIOUS CONIFER EXPLANTS

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ABSTRACT

Various juvenile explants from conifers have been used to form shoot buds **in vitro**. The best explant must be selected empirically and the number of shoots formed varies with the explant. A comparative morpho-histological examination was made of the sites of shoot initiation arising from embryonic explants of **Pinus contorta** Loud. and **Pinus rigida** Mill., cotyledon explants of **Pinus radiata** D. Don and **P. contorta,** and epicotyl explants of **Picea glauca** Voss, **Picea mariana** B.S.P., and **Picea engelmannii** Parry. The study revealed that despite the different sites and timing of initiation of shoot formation, the patterns of shoot development were similar. In all species the formation of meristematic centres or meristemoids led to bud primordia and finally adventitious shoots with apical domes and needle primordia. This developmental sequence occurred in the absence of concomitant callus formation on the cytokinin-containing medium.

Keywords: bud induction; **in vitro** culture; meristematic centres; primordium formation; shoot initiation histology; **Pinus contorta**; **Pinus rigida**; **Pinus radiata**; **Picea glauca**; **Picea mariana**; **Picea engelmannii**.

INTRODUCTION

Various kinds of explants have been used for *in vitro* propagation of different conifer species (David 1982; Thorpe & Biondi 1984). However, in most of the conifers micropropagated *in vitro,* success has been achieved mainly with juvenile explants (Thorpe & Biondi 1984). The selection of different explants has been shown to have a significant bearing on shoot productivity (Aitken *et al.* 1981). However, the reason behind such a wide range of variation in morphogenic response of conifer cultures is not clear. Irrespective of the explant type or the species, in conifers in general, adventitious shoots form directly on the explants without any callus formation (Thorpe & Biondi 1984). Hence, we thought it of interest to compare the anatomy of the various conifer explants used in our laboratory, the sites and patterns of shoot initiation, and to attempt to correlate these to their productivity.

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MATERIALS AND METHODS

The embryonic and seedling explants chosen for the present investigation were (1) embryonic explants of *Pinus contorta* (Patel & Thorpe 1984a) and *P. rigida* (Patel *et al.* 1986), (2) cotyledon explants of *P. radiata* (Aitken *et al.* 1981) and *P. contorta* (Patel & Thorpe 1984a), and (3) epicotyl explants of *Picea glauca, P. mariana* (Rumary & Thorpe 1984), and *P. engelmannii* (Patel & Thorpe 1986).

The initial embryonic explants and those cultured for various lengths of time on shoot-forming medium were fixed in 1% glutaraldehyde in phosphate buffer (pH 7.2) and processed according to Botti & Vasil (1984) for JB-4 plastic embedding for light microscopy. The sections were stained with 0.05% toluidine blue 0 (pH 4.4) for 2 min (Yeung 1984). Localisation of DNA in paraffin sections was carried out as suggested by Berlyn & Miksche (1976). The cotyledonary explants at various stages of shoot formation were harvested and processed as reported by Patel & Thorpe (1984b) and Villalobos *et al.* (1985). Similarly the cultured epicotyl explants of the spruces were fixed in FPA (90 ml ethanol : 5 ml formaldehyde : 5 ml propionic acid) at various stages of shoot development (Rumary *et al.* 1986). They were then dehydrated in TBA series, embedded in Paraplast, and sectioned at 10 μM. The sections were stained with Azure B (Flax & Hines 1952) or Periodic acid-Schiff/Aniline blue-black (Jensen 1962; Jensen & Fisher 1968).

RESULTS

Patterns of Shoot Initiation in Various Explants

The morphological changes and patterns of bud initiation were identical in embryonic explants of *Pinus rigida* and *P. contorta.* After initial elongation on the cytokinin-containing medium, the cotyledons and hypocotyl region of the embryo showed considerable swelling when cultured on the shoot-forming medium. After about 10 days of culture their entire upper surfaces acquired a nodular appearance and in 2–3 weeks the complete upper surfaces of the cotyledons and hypocotyl were covered with distinct shoot and/or needle primordia. On the other hand, the surfaces in contact with the medium were virtually devoid of any shoots. Upon transfer to cytokinin-free medium the primordia developed into distinct shoots (Fig. 1). Under optimum conditions the embryonic explants of *P. contorta* and *P. rigida* produced on average 17 and 19 shoots respectively in 12–14 weeks (Table 1).

The cotyledon explants of *P. radiata* and *P. contorta* measured 3–5 mm (Yeung *et al.* 1981) and 5–7 mm (Patel & Thorpe 1984a) respectively, at the time of excision. They swelled and increased in length slightly during the first week of culture on BA (N^6-benzyladenine)-containing medium. After about 10–12 days of culture the lower cotyledon surface in contact with the medium appeared distinctly nodular. Within 3 weeks numerous shoot primordia became visible on the entire length of the cotyledonary explants of *P. contorta* (Fig. 2, at arrows) and *P. radiata* (Fig. 3, at arrows) on the side in contact with the medium. The cotyledon explants derived from each germinated seed of *P. radiata* produced on average 180 shoots in 12–13 weeks (Table 1). It should be noted that only 45–50% of the seeds produced suitable explants (Aitken *et al.* 1981).

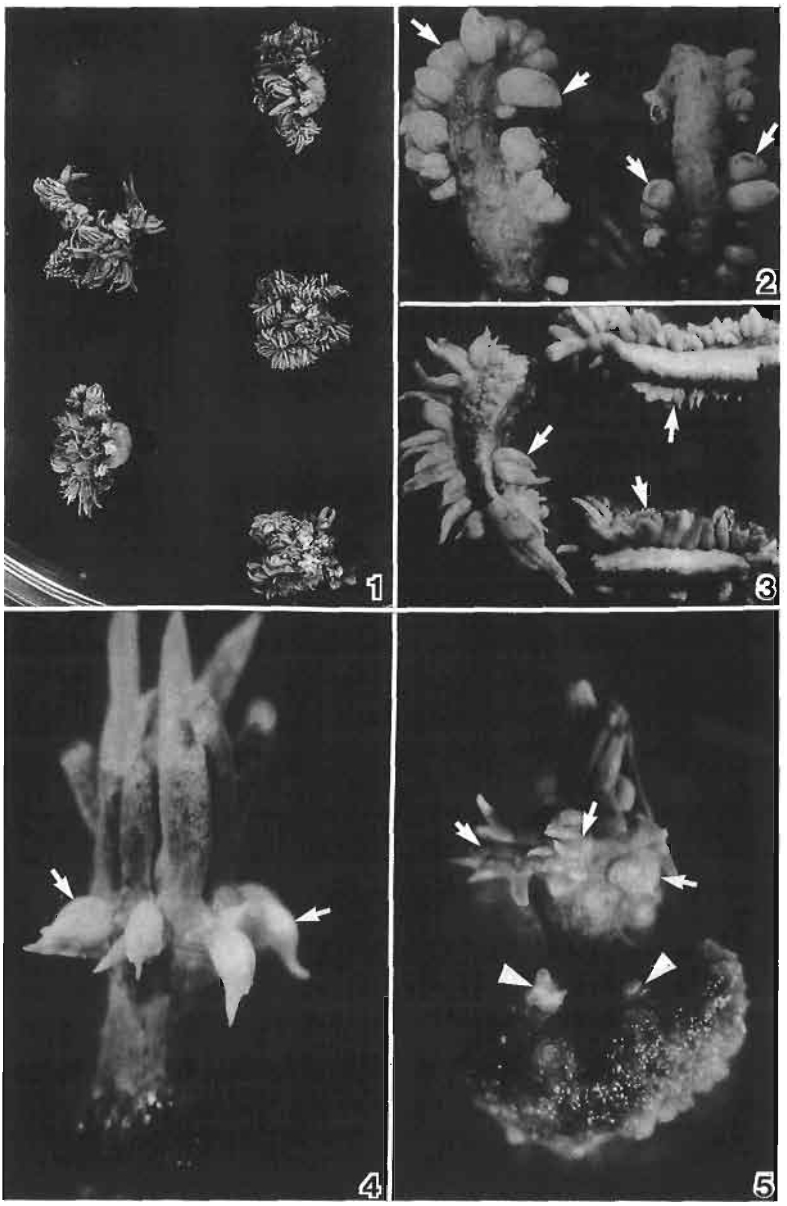

FIG. 1-5—Developmental patterns of adventitious bud formation in various conifer explants. Fig. 1 - Embryonic explants of **Pinus rigida** showing multiple shoot formation on their upper surfaces. Fig. 2 and 3 - Cotyledonary explants of **P. contorta** (Fig. 2) and **P. radiata** (Fig. 3) showing development of shoot buds (at arrows) along the entire length on the side in contact with the medium. Fig. 4 - Formation of inter-cotyledonary shoots (at arrows) in the epicotyl explant of **Picea engelmannii**. Fig. 5 - Development of secondary shoots (at arrows) on the epicotyl explant of **P. engelmannii** upon transfer to cytokinin-free medium.

In *Picea mariana* and *P. glauca* (Rumary & Thorpe 1984), as well as *P. engelmannii* (Fig. 4, 5), the initial epicotyl explant consisted of the shoot apex covered by epicotyledonary needles atop a swollen portion of the hypocotyl. Only 77 and 86% of the *P. glauca* and P. *mariana* seedlings respectively (Rumary & Thorpe 1984), and 85% of the *P. engelmannii* seedlings (Patel & Thorpe 1986) provided usable explants. A whorl of cotyledons was also found at the junction of the epicotyl and hypocotyl. After about a month of culture in shoot-forming medium containing cytokinins, inter-cotyledonary or primary shoots appeared inbetween the cotyledon bases (Fig. 4 at arrows). Transfer of the explants to a cytokinin-free medium allowed for more *de novo* shoot formation (secondary shoots) on the entire periphery of the explant (Fig. 5, at arrows). Monthly subcultures of these explants produced about 70 shoots within 128 days over several harvests (Table 1).

TABLE 1—Comparison of shoot-forming capacity among various conifer explants cultured **in vitro**

Species	Explant	Frequency of shoot formation (%)	Average No. of shoots/ explant	SFI*	Source
Pinus contorta	Embryo	92	16.8	15.4	Patel & Thorpe (1984a)
P. rigida	Embryo	89	19.2	17.0	Patel **et al.** (1986)
P. radiata	Embryo	87	9.0	7.8	Aitken **et al.** (1981)
P. radiata	Cotyledon	100	180.0	180.0	Aitken **et al.** (1981)
Picea mariana	Epicotyl	98	70.0	68.6	Rumary & Thorpe (1984)
P. glauca	Epicotyl	100	70.0	70.0	Rumary & Thorpe (1984)

* SFI (Shoot-forming index) = mean number of shoots per explant × percentage of seeds forming shoots/100.

Anatomy of Various Explants and Structural Changes During Shoot Initiation

The initial cotyledonary explants of *Pinus radiata* were meristematic with a fairly homogeneous population of cells, except for the vascular tissue (Yeung *et al.* 1981). The cells were isodiametric, with large centrally located nuclei (Fig. 6). Their cytoplasm contained prominent lipid and protein bodies as well as starch grains. These reserves were mobilised during the early period in culture (Douglas *et al.* 1982; Yeung *et al.* 1981). In the cotyledons cultured in the shoot-forming medium, the mitotic activity became confined to the epidermal and a few sub-epidermal cell layers from the surface in contact with the medium (Fig. 7, at arrows). Within 5 days of culture in cytokinin-containing medium, the smallest recognisable organogenic entities were visualised in the sub-epidermal layers (Fig. 8, at arrows). These structures, termed promeristemoids (Villalobos *et al.* 1985), developed further into meristematic domes in about 10–12 days of culture (Fig. 9). Shoots with well-organised apical domes and needle primordia protruded along the entire length of the cotyledon after about 3 weeks (Fig. 10).

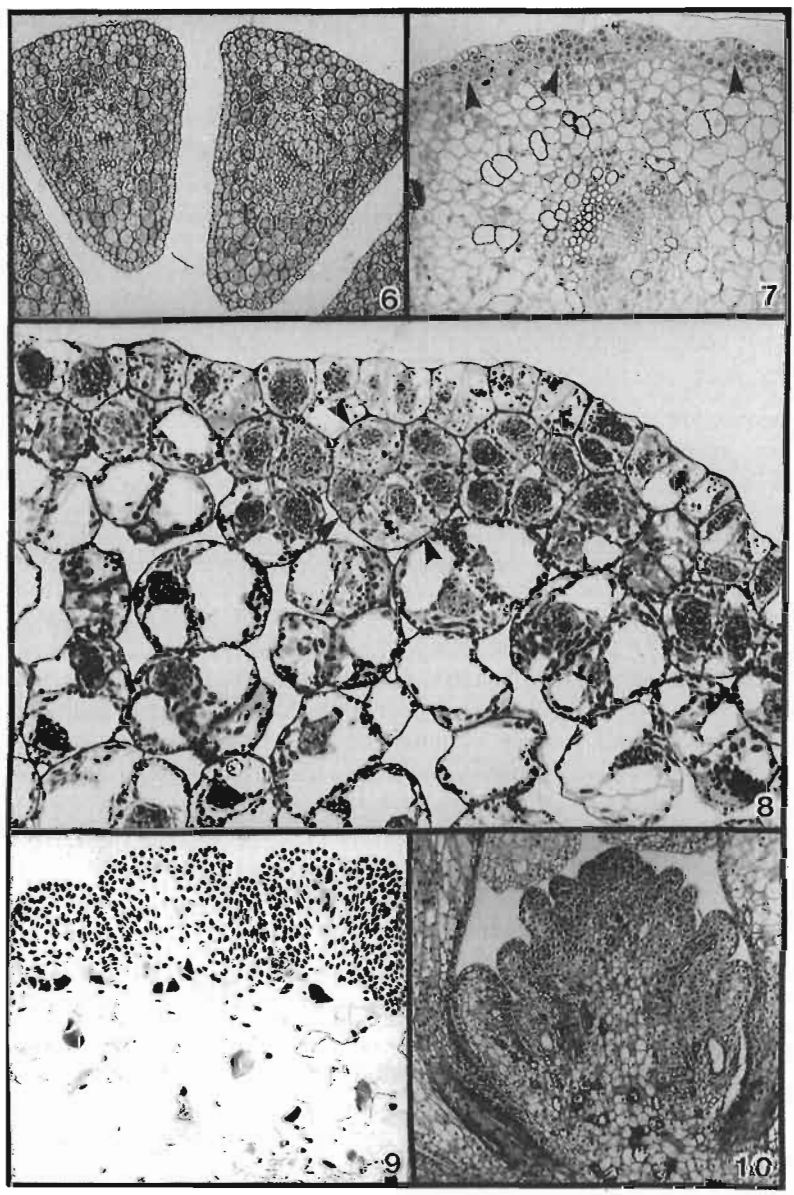

FIG. 6-10—Various stages of multiple shoot formation in cotyledon explants of **Pinus radiata**. Fig. 6 - Transverse section of the initial cotyledon explant showing pronounced reserves of starch (×210). Fig. 7 - Transverse section of explant cultured for 3 days under shoot-forming conditions (×210). Note active cell division in the epidermal and subepidermal cell layers on the cotyledonary face in contact with the medium. Fig. 8 - The earliest recognisable entity or the promeristemoid (at arrows) in the subepidermal cell layers of a 5-day-old explant (×1200). Fig. 9 - Localisation of DNA in developing shoot primordia (×130). Fig. 10 - A longitudinal section of a well-developed shoot showing the shoot apical meristem and needle primordia (×55).

The embryonic explants of *P. rigida* were quite undifferentiated. Their cells, like those of the cotyledon explants of *P. radiata*, were packed with nutrient reserves stored mainly in the form of lipids and protein bodies (Fig. 11, at arrows). The nuclei were condensed and of irregular shape and no mitotic activity was observed at this stage. Within 24 h of culture, small vacuoles started to appear in the cells as the reserves were mobilised; however, numerous lipid and protein bodies were still present (Fig. 12). After about 2 to 3 days in culture the reserves were mobilised completely and most of the cells in the explant started dividing. However, in contrast to the cotyledon explants of *P. radiata,* no preferential sites of cell division could be detected at this stage. The size of the cotyledons and hypocotyl region of the explant increased considerably because of periclinal and anticlinal division of the cells throughout the explant.

The target areas of preferential cell division became distinct after 5–7 days of culture. Unlike the cotyledon explants of *P. radiata,* the epidermal and subepidermal cells of the explant, which were in contact with the medium became highly vacuolated and filled with ergastic substances (Fig. 13, at arrows). These were mainly phenolics or tannins as indicated by development of a green colour with toluidine blue 0. On the other hand, cells from the layers of the cotyledons and hypocotyl which were not in contact with the medium contained densely staining cytoplasm and large nuclei (Fig. 14). Within these cell layers cell division was more pronounced, while cells underlying them gradually became vacuolated and non-meristematic. Equivalent cells from control embryonic explants which were cultured in the absence of cytokinin were also non-meristematic and vacuolated (Fig. 15). In the epidermal and subepidermal layers of the BA-treated explants, cell division activity continued in certain organogenic centres which led to the formation of meristemoids after about 10 days of culture (Fig. 16, at arrows). Further well-organised divisions in the meristemoids resulted in the differentiation of shoot primordia (Fig. 17). Upon transfer of the explants to a hormone-free medium the shoots and needle primordia elongated quickly and the entire upper surface of the embryo was covered with well-developed shoots. The apices of regenerated shoots showed very intense staining for DNA in the needle primordia and apical dome except in the quiescent zone (Fig. 18).

In contrast to the embryonic and cotyledonary explants of the pines, the epicotyl explants of the spruces were highly differentiated and contained various types of cells, tissues, and organs. In a longitudinal section, the initial explant showed an apical meristem and young needle primordia consisting of small meristematic cells, whereas the cells of the cotyledon bases and hypocotyl stub were highly vacuolated and non-meristematic (Fig. 19). After 2–3 days of culture in medium containing cytokinins, the target areas for primary shoot initiation became clear in the epicotyl explants. However, unlike the embryonic and cotyledon explants, the target cells appeared at regular intervals around the periphery of the main axis of the explant between the basal regions of the cotyledons (Fig.20, at arrows). The target cells continued to divide, and formed meristemoids which protruded as small domes between the cotyledons after 8 days of culture (Fig. 21, at arrow). Bud primordia developed from these meristemoids after 15 days of culture (Fig. 22). By this time the meristematic activity also extended into the cortical region of the main explant. After 25 days of culture, distinct shoot buds with small needle primordia were evident inbetween the cotyledons. These shoots

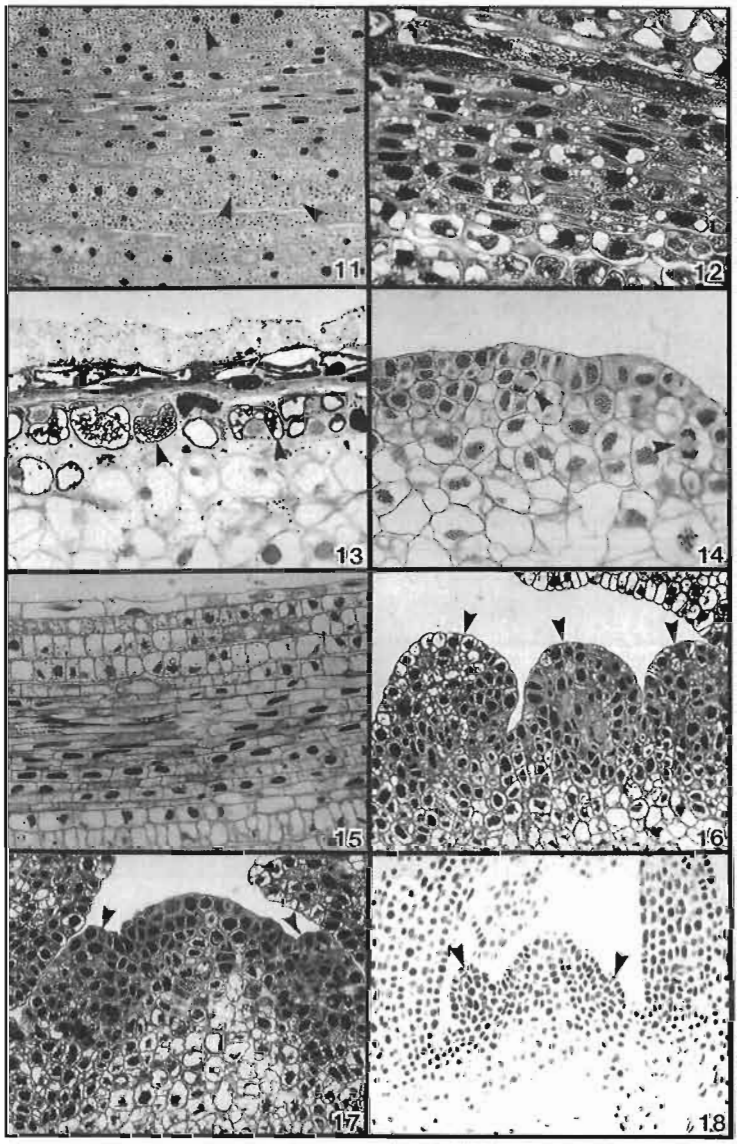

FIG. 11–18—Histology of bud initiation in **Pinus rigida** embryonic explants. Fig. 11 – Cells from a longitudinal section of the initial explant showing abundance of reserve substances (at arrows) (×260). Fig. 12 – Longitudinal section showing mobilisation of reserves during the initial period of culture (×260). Fig. 13 – Accumulation of ergastic substances in vacuoles of cells in contact with the medium (×260). Fig. 14 – Cell division (at arrows) in cell layers of the upper surface of explants cultured in cytokinin-containing medium (×260). Fig. 15 – Vacuolated and non-meristematic cells of the control explant (×260). Fig. 16 – Formation of organogenic centres (at arrows) after about 10 days of culture (×260). Fig. 17 and 18 – Development of a shoot bud (Fig. 17; ×260) and a well-organised shoot with needle primordia (Fig. 18, at arrows; ×110).

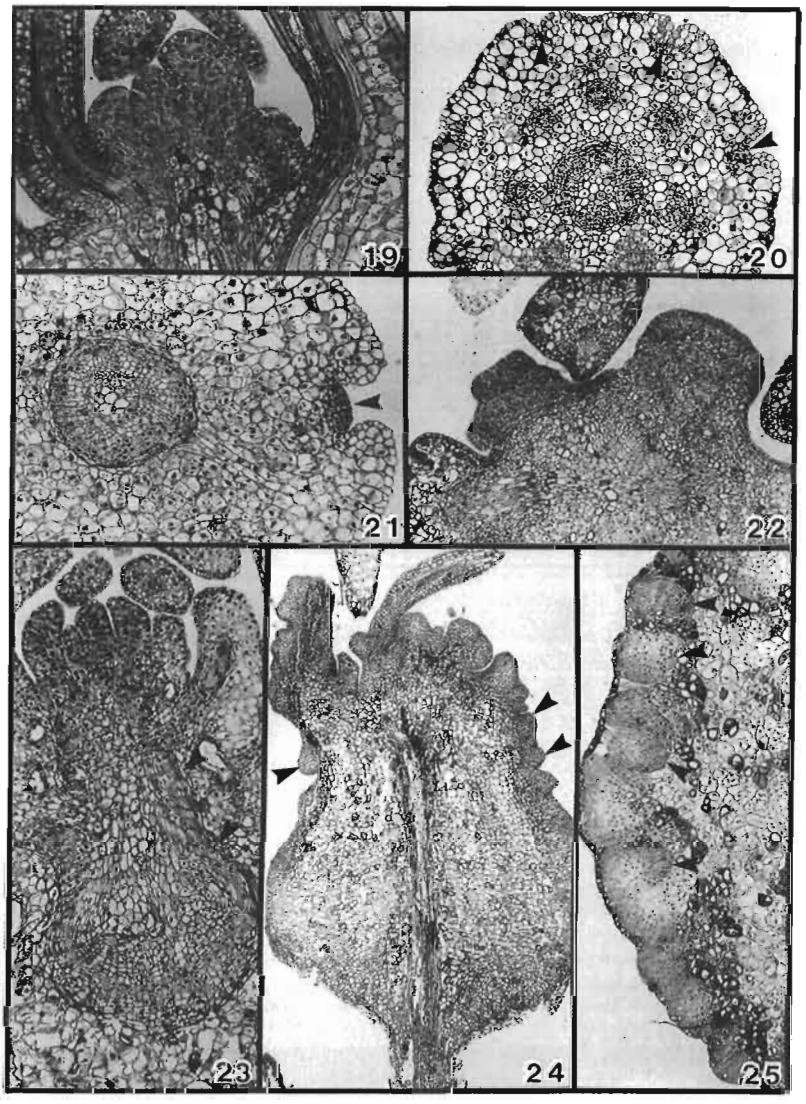

FIG. 19–25—Anatomy of primary and secondary shoot formation in **Picea glauca**. Fig. 19 – A longitudinal section of the apical portion of the initial explant (×125). Fig. 20 – Transverse section of a 2-day-old epicotyl explant taken from slightly below the origin of the cotyledons (×125). Note the target cells (at arrows) located at the periphery of the explants in between the basal regions of the cotyledons. Fig. 21 and 22 – Emergence of a meristemoid (Fig. 21 at arrow; ×125) and shoot primordia (Fig. 22; ×125) between the cotyledons. Fig. 23 – A well-developed primary shoot showing the vascular connection (at arrows) with the vascular cylinder of the explant (×52). Fig. 24 – A longitudinal section of the explant showing secondary shoot initiation (at arrows) (×52). Fig. 25 – A transverse section of a 40-day-old explant showing a ring of meristemoids at the periphery of the explant (at arrows) (×52). These meristemoids later develop into the secondary shoots.

developed their vascular systems which were then connected to the main vascular system of the explant (Fig. 23, at arrows). The explants were transferred to a hormone-free medium at this stage. Within a week of transfer, the entire explant became meristematic and showed evidence of secondary shoot development (Fig. 24, at arrows). The resulting mass of meristematic tissue contained numerous secondary shoot meristemoids at the periphery (Fig. 25). The meristemoids could easily be distinguished from the surrounding tissue because these cells possessed dense cytoplasm, large nuclei, and a distinct boundary (Fig. 25, at arrows). Further development of these meristemoids into shoot primordia and shoots was similar to that of the primary shoots, except that there was no continuity between their vascular systems and the main vascular system of the explant (Rumary *et al.* 1986).

DISCUSSION

The histological examination of the different cultured explants revealed various patterns of bud initiation and development in the diverse conifer explants. The induction of adventitious shoots occurred at different sites in the various explants, or at different times at different sites within the same explant, as in the epicotyl explants of the spruces. However, despite the variation in the sites of shoot initiation in the diverse explants studied, the developmental sequence leading to multiple shoot formation, i.e., the formation of (1) meristemoids, (2) bud primordia, and (3) adventitious shoots with well-organised apical domes and needle primordia, was almost identical in these species. The importance of meristemoid formation in the shoot initiation process in conifers has been recognised (e.g., Cheah & Cheng 1978; Coleman & Thorpe 1977). These structures have also been termed meristematic bud centres (Mott 1981), and meristematic tissue (Reilly & Brown 1976).

The selection of a suitable explant is a key to successful *in vitro* morphogenesis (Murashige 1974), e.g., the choice of the *P. radiata* explant and its developmental stage have been shown to dramatically alter the shoot-forming capacity of the system (Aitken *et al.* 1981; Aitken-Christie *et al.* 1985). Despite the wide variation in morphogenic response in conifer cultures, the fact remains that there is little or no callus formation (Thorpe & Biondi 1984). Under the culture conditions used, the target cells respond to the morphogenic signals, and undergo organised patterns of cell division leading to shoot formation rather than unorganised callus growth.

The embryonic explants of *P. contorta* and *P. rigida* were small, their cells were quite undifferentiated and less determined, and therefore presumably could be more easily rechanneled to organogenetic activity. However, with embryo cultures it has not been possible so far to increase the productivity above 20 shoots per explant. This is probably mainly because of the small explant size and the reduction in surface area capable of meristematic tissue formation, and also in part because of accumulation of ergastic substances in the cell layers in contact with the medium. In contrast to the embryonic explants, the cotyledon explants showed meristemoids and shoot formation in the cell layers in contact with the medium. The reason(s) behind the differential behaviour of cells in contact with the medium in the embryonic and cotyledonary explant is not clear. According to Aitken *et al.* (1981) the reason behind the high

productivity of the cotyledon explants, in comparison to isolated embryos, is the increased surface area in contact with the medium, although certain features of the *P. radiata* cotyledon explants may also play a role (Aitken-Christie *et al.* 1985). These cotyledons at the time of excision have less-developed stomatal complexes, thinner cell walls, lack epicuticular wax, and contain more protein and lipid reserves than older non-shoot-forming cotyledons.

In epicotyl explants of *Picea mariana* and *P. glauca,* the number of primary shoots formed was limited by the morphology of the explants. *Picea mariana* seedlings have three to five cotyledons with over 60% having four; *P. glauca* on the other hand, have five to eight cotyledons with over 55% bearing six cotyledons (Rumary & Thorpe 1984). Since primary shoot formation occurred between the cotyledons, *P. glauca* produced on average more inter-cotyledonary shoots than *P. mariana.* However, these primary shoots account for only a small number of total shoots produced by the epicotyl explant. The histological analysis showed that it was the secondary shoots that developed later in culture that made this explant highly productive, as several tiers of secondary shoot primordia developed at the periphery of the entire explant (see also Rumary *et al.* 1986).

According to Ross & Thorpe (1973), the region of the tissue in which the cells are activated is apparently influenced by physiological gradients of materials moving out of the medium into the tissue. The loci at which organised development is initiated in embryonic or cotyledon (as well as stem and hypocotyl) explants could be explained on the basis of this phenomenon, despite differences in location. However, shoot formation, at least the primary shoot formation, in epicotyl explants which occurs only between the cotyledons cannot be explained solely on the basis of physiological gradients of hormonal or nutritional factors from the medium. In these explants the inter-cotyledonary cells could also be activated in part by the movement of metabolites out of the cotyledons into the shoot axes.

As shown in Table 1, different explants lead to the production of different numbers of shoots. The comparative morpho-histological analysis of shoot formation carried out here allows for an interpretation of the patterns of shoot formation and an explanation of the efficacy of the various explants. These data, as well as those from other workers (e.g., Bornman 1983; von Arnold & Eriksson 1979), would indicate that the highest rates of bud induction can be obtained by judicious selection of the explant at the right physiological age and state, and exposure of the maximum surface area to the cytokinin-containing medium. However, unfortunately to date the best juvenile conifer explant must still be determined empirically, as we lack proven predictive methods for explant selection.

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REFERENCES

AITKEN, J.; HORGAN, K. J.; THORPE, T. A. 1981: Influence of explant selection on the shoot-forming capacity of juvenile tissue of **Pinus radiata**. **Canadian Journal of Forest Research 11:** 112–7.

AITKEN-CHRISTIE, J.; SINGH, A. P.; HORGAN, K. J.; THORPE, T. A. 1985: Explant developmental state and shoot formation in **Pinus radiata** cotyledons. **Botanical Gazette 146:** 196–203.

BERLYN, G. P.; MIKSCHE, J. P. 1976: "Botanical Microtechnique and Cytochemistry". Iowa State University Press, Ames.

BORNMAN, C. H. 1983: Possibilities and constraints in the regeneration of trees from cotyledonary needles of **Picea abies** in vitro. **Physiologia Plantarum 57:** 5–16.

BOTTI, C.; VASIL, I. K. 1984: Plastic embedding for light microscopy. Pp. 684–8 **in** Vasil, I. K. (Ed.) "Cell Culture and Somatic Cell Genetics of Plants" Vol. 1. Academic Press, New York.

CHEAH, K. T.; CHENG, T. Y. 1978: Histological analysis of adventitious bud formation in cultured Douglas fir cotyledon. **American Journal of Botany 65:** 845–9.

COLEMAN, W. K.; THORPE, T. A. 1977: **In vitro** culture of western red cedar (**Thuja plicata** Donn.). I. Plantlet formation. **Botanical Gazette 138:** 298–304.

DAVID, A. 1982: **In vitro** propagation of conifers. Pp. 72–108 **in** Bonga, J. M.; Durzan, D. J. (Ed.) "Tissue Culture in Forestry". Dr. W. Junk Publishers, The Hague.

DOUGLAS, T. J.; VILLALOBOS, V. M.; THOMPSON, M. R.; THORPE, T. A. 1982: Lipid and pigment changes during shoot initiation in cultured explants of **Pinus radiata**. **Physiologia Plantarum 55:** 470–7.

FLAX, M. H.; HIMES, M. H. 1952: Microspectrophotometric analysis of metachromatic staining of nucleic acids. **Physiological Zoology 25:** 294–311.

JENSEN, W. A. 1962: "Botanical Histochemistry, Principles and Practice". W. H. Freeman and Co., San Francisco and London.

JENSEN, W. A.; FISHER, D. B. 1968: Cotton embryogenesis: The entrance and discharge of the pollen tube in the embryo sac. **Planta 78:** 158–83.

MOTT, R. L. 1981: Trees. Pp. 217–54 **in** Conger B. V. (Ed.) "Cloning Agricultural Plants via **in Vitro** Techniques". CRC Press, Boca Raton, Florida.

MURASHIGE, T. 1974: Plant propagation through tissue cultures. **Annual Review of Plant Physiology 25:** 135–66.

PATEL, K. R.; THORPE, T. A. 1984a: **In vitro** differentiation of plantlets from embryonic explants of lodgepole pine (**Pinus contorta** Dougl. ex. Loud.). **Plant Cell Tissue and Organ Culture 3:** 131–42.

——— 1984b: Histochemical examination of shoot initiation in cultured cotyledon explants of radiata pine. **Botanical Gazette 145:** 312–22.

——— 1986: **In vitro** regeneration of plantlets from embryonic and seedling explants of Engelmann spruce (**Picea engelmannii** Parry). **Tree Physiology 1:** 289–301.

PATEL, K. R.; KIM, H. R.; THORPE, T. A. 1986: Plantlet formation in pitch pine (**Pinus rigida** Mill.) by tissue culture methods. **Forest Ecology and Management 15:** 147–60.

REILLY, K.; BROWN, C. L. 1976: **In vitro** studies of bud and shoot formation in **Pinus radiata** and **Pseudotsuga menziesii**. **Georgia Forest Research Paper 86:** 1–9.

ROSS, M. K.; THORPE, T. A. 1973: Physiological gradients and shoot initiation in tobacco callus cultures. **Plant and Cell Physiology 14:** 473–80.

RUMARY, C.; THORPE, T. A. 1984: Plantlet formation in black and white spruce. I. **In vitro** techniques. **Canadian Journal of Forest Research 14:** 10–16.

RUMARY, C.; PATEL, K. R.; THORPE, T. A. 1986: Plantlet formation in black and white spruce. II. Histological analysis of adventitious shoot formation **in vitro**. **Canadian Journal of Botany 64:** 997–1002.

THORPE, T. A.; BIONDI, S. 1984: Conifers. Pp. 435–70 **in** Sharp, W. R.; Evans, D. A.; Ammirato, P. V.; Yamada, Y. (Ed.) "Hand Book of Plant Cell Culture". Vol. 2. Macmillan Inc., New York, London.

VILLALOBOS, V. M.; YEUNG, E. C.; THORPE, T. A. 1985: Origin of adventitious shoots in excised radiata pine cotyledons cultured **in vitro**. **Canadian Journal of Botany 63:** 2172–6.

VON ARNOLD, S.; ERIKSSON, T. 1979: Bud induction on isolated needles of Norway spruce (**Picea abies** (L.) Karst.) grown in vitro. **Plant Science Letters 15:** 363–72.

YEUNG, E. C. 1984: Histological and histochemical staining procedures. Pp. 689–97 **in** Vasil, I. K. (Ed.) "Cell Culture and Somatic Cell Genetics of Plants". Vol. 1. Academic Press, New York.

YEUNG, E. C.; AITKEN, J.; BIONDI, S.; THORPE, T. A. 1981: Shoot histogenesis in cotyledon explants of radiata pine. **Botanical Gazette 142:** 494–501.