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Hormonal Control of Growth and Differentiation in Conifer Tissues *in vitro*

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Abstract. The mechanisms by which exogenously applied plant growth regulators act to express those genes that are selectively involved in cell and tissue differentiation are not at all well comprehended. However, the ontogenetic sequences of events that enable receptor or target cells to be activated and to undergo dedifferentiation and redifferentiation, can often be followed experimentally and can lead to a better understanding of the causal relations and control mechanisms in coordinated cell growth and development.

Cytokinins, applied either in an agar medium or as high-concentration, short-duration pulses to explants or as high-concentration, intermittent sprays to intact plants, induce switches in the normal developmental pattern of cells of certain explanted tissues. Examples of cells that are particularly receptive are the subsidiary cells of stomatal complexes and the differentiating cells of very young presumptive leaf primordia, that is before the latter have become irreversibly determined as leaves.

In an attempt to understand better the regulation of development, that is of growth, differentiation and morphogenesis, increasing use is being made of *in vitro* culture techniques. However, it may not often be appreciated that when culturing explanted tissues, cells and protoplasts *in vitro*, the normal synchronized qualitative and quantitative sequences of events that comprise an organism's vegetative and reproductive life cycles have been dramatically disturbed. In efforts to restore the interrupted developmental process or to achieve the desired response, many plant physiologists rely almost solely on manipulation of plant growth regulators (CASSELLS *et al.* 1982), ignoring other important component factors such as genotype, physical and chemical environments, and stage of development and pretreatment of the donor plant.

If, for argument's sake, it is assumed that morphogenesis in tissues cultured *in vitro* is best achieved by appropriate control of growth regulator levels in the culture medium, the investigator should at least be aware of the negative effects that may be exerted by the latter (NASH and BORNMAN 1973, DEBERGH *et al.* 1981, DEBERGH 1983, BORNMAN and VOGELMANN 1984). In some explant systems development seemingly cannot be controlled *via* the quantitative interaction between exogenously applied auxin and cytokinin. This then necessitates information not only on endogenous plant

growth regulator levels, but also on how these levels change in specific explant sites during specific differentiation responses. At the present time this information, if not unattainable, is very difficult to acquire. Then there is also the possibility that having such information will not necessarily guarantee adequate explanations of developmental phenomena (FOSKET 1979).

Nevertheless, even in the absence of analytical data on changing endogenous plant growth regulator levels, observations on the rate of macromolecular synthesis (VILLALOBOS *et al.* 1984a), carbon metabolism (OBATA-SASAMOTO *et al.* 1984), uptake kinetics (MINOCHA and NISSEN 1982, VOGELMANN *et al.* 1984) as well as on cell and tissue sites and cytological response (YEUNG *et al.* 1981, JANSSON and BORNMAN 1981, BORNMAN and VOGELMANN 1984) do allow certain deductions to be made with regard to the growth regulator-induced control of development.

PLANT MATERIALS

Picea abies (L.) KARST. (Norway spruce) is a convenient species for *in vitro* culture studies: firstly, the number of reports on the culture of its tissues *in vitro* is accumulating (CHALUPA and DURZAN 1973a, ARNOLD and ERIKSSON 1978, 1979, JANSSON and BORNMAN 1980, 1981, ARNOLD 1982, BORNMAN and JANSSON 1982, BORNMAN 1983, BORNMAN and VOGELMANN 1984, VOGELMANN *et al.* 1984); secondly, cotyledons, leaves of flushing buds, and resting buds are among the variety of organs and tissues that can be used; and thirdly, detailed morphological and physiological information is available on its embryonic shoot and apical meristem (ROMBERGER 1966, ROMBERGER *et al.* 1970, CHALUPA and DURZAN 1973b, JANSSON and BORNMAN 1983, JANSSON *et al.* 1983).

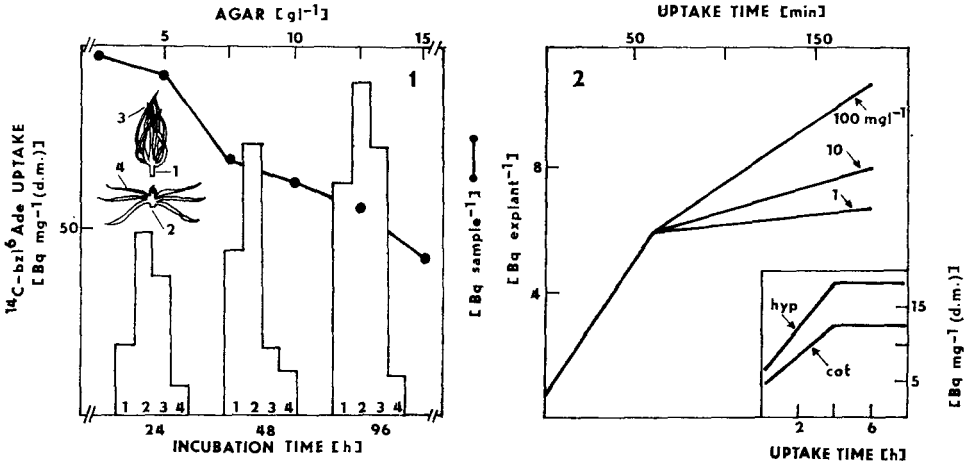

Fig. 1. Effects of rigidity of Tayio agar and positioning of primary explant of 14-day-old *Picae abies* on accumulation of ^{14}C-bzl^{6}Ade (BA) in the explant (●) as well as in hypocotyls (1, 2) and cotyledons (3, 4).

Fig. 2. Time course of bzl^{6}Ade (BA) uptake by *Picea abies* explants incubated in labelled solutions containing 1, 10 and 100 mg l^{-1} bzl^{6}Ade. - Inset: distribution of radioactivity of 25 mg l^{-1} bzl^{6}Ade between hypocotyls (hyp) and cotyledons (cot).

EXPERIMENTAL PROCEDURES

For the induction of adventitious and lateral buds, benzyladenine (bzl^{6}Ade) can be applied (1) by incorporation in an agarified medium (1—3 mg l^{-1}); (2) as a high concentration (25 mg l^{-1}), short duration (2 h) pulse to the explants; or (3) as a very high concentration (250 mg l^{-1}) intermittent (every other day for 10 days) spray to 5-week-old greenhouse-grown seedlings. Account should be taken of the effect of the gelling agent, especially its matrix potential, on the uptake by explanted tissues of cytokinin (DEBERGH *et al.* 1981, DEBERGH 1983, BORNMAN and VOGELMANN 1984). Auxins (naphthaleneacetic acid, NAA, and indolebutyric acid, IBA) can also be applied as very high concentration (125 mg l^{-1}), medium duration pulses (12 h) for root initiation *in vivo* of shoots developed *in vitro*.

Positioning of explants affects induction of adventitious buds both in terms of distribution and number. For example, a primary explant of a 14-day-old spruce seedling consists of the cotyledonary node with *ca.* 7 cotyledonary needles, an emerging plumule and a hypocotyl stub. This explant can be placed upright in an agar-based medium with its cotyledons erect or the cotyledons can be flattened horizontally so that full surface contact is made with the medium (Fig. 1). Alternatively, either before or after pulse treatment of the primary explant, the cotyledons can be removed as secondary explants and placed on an agarified medium containing or lacking plant growth regulators, as the case may be.

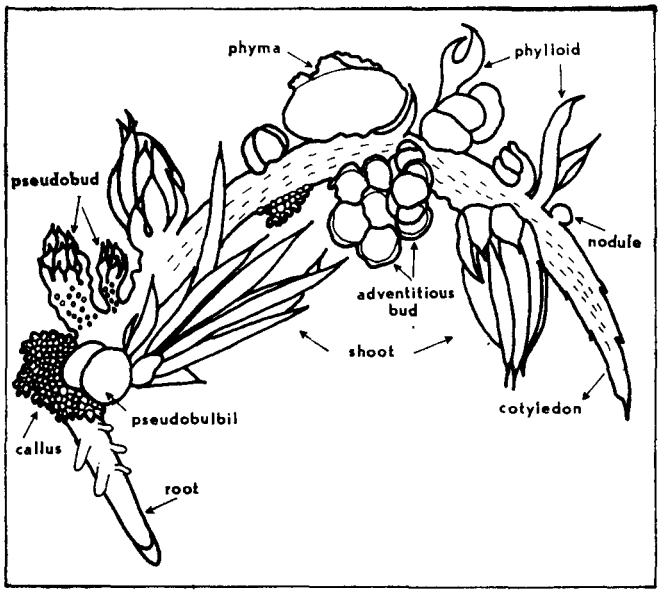

Fig. 3. Composite sketch of a 14-day-old *Picea abies* cotyledon after 3—6 weeks in culture showing various types of adventitious structures that may be induced depending on the auxin — cytokinin — genotype interaction.

In order to monitor uptake, radiolabelled N^6-benzyl[8-^{14}C]adenine was added to unlabelled bzl^{6}Ade for an absolute activity of 1.67 kBq ml^{-1} and radioactivity determined by scintillation spectrometry.

OBSERVATIONS

Effects of rigidity of agar (Tayio) and of positioning of the primary explant on accumulation of ^{14}C-bzl^{6}Ade are shown in Fig. 1. An inverse correlation was found between bzl^{6}Ade uptake and stiffness of the gel. The ratio of distribution of the label between hypocotyl and cotyledon was found to be 1 : 1 when explants were positioned with their cotyledons elevated, as compared with 15 : 1 when the cotyledons were placed flush with the gel. Significantly larger numbers of adventitious buds were produced in the latter case (see BORNMAN and VOGELMANN 1984). The time course of bzl^{6}Ade uptake by explants incubated in labelled solutions containing 1, 10 and 100 mg l^{-1} of the growth regulator and the distribution of radioactivity between hypocotyls and cotyledons are shown in Fig. 2. Uptake was linear for about one hour at each concentration. Extrapolation showed that independent of washing some bzl^{6}Ade was initially bound to the explants. At a fixed concentration of 25 mg l^{-1} uptake of bzl^{6}Ade by hypocotyls and cotyledons was linear over the first 4 h. The largest number of adventitious buds was produced by explants pulsed for 2 h (data not shown). Primary explants of 5-week-old greenhouse-grown seedlings pretreated by five soaking sprays (over a 10-day period) with bzl^{6}Ade, respond within 14—21 days after transfer to a growth regulator-free medium by the development of axillary and adventitious buds. Axillary buds arise in the leaf axils whereas adventitious buds develop from the lower abaxial surface of the cotyledons as well as elsewhere on the developing shoot, including its apex.

Fig. 3 is a composite of the variety of adventitious structures that have been observed on 14-day-old cotyledons cultured separately on media containing either a cytokinin or an auxin, or a combination of both of these hormones. In the absence of applied cytokinin, auxin (IBA 1—3 mg l^{-1}) invariably induces roots, the initiation of which is traceable to meristemoids

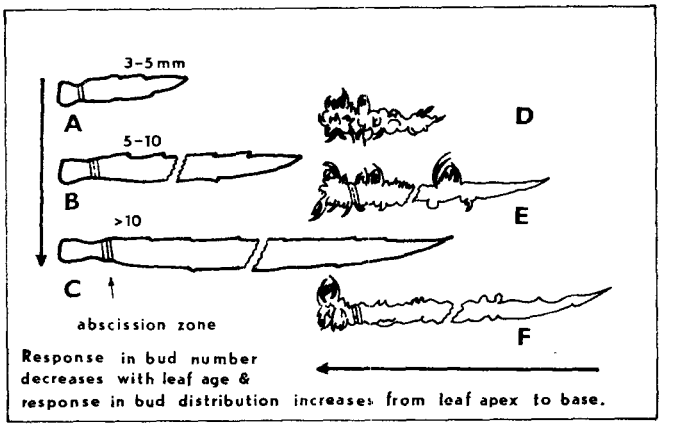

Fig. 4. Effect of age and position on induction of adventitious buds in leaves from flushing buds of *Picea abies*.

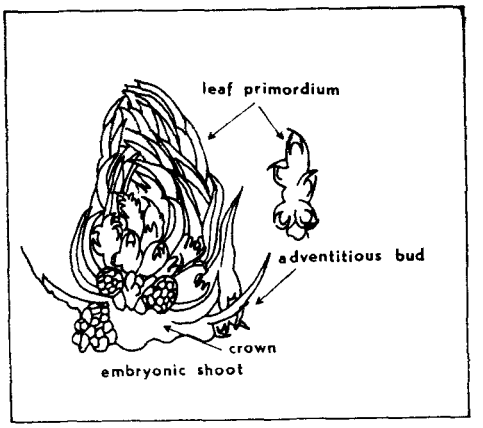

Fig. 5. Response of the embryonic shoot (plus crown) of *Picea abies* to a 2-h pulse treatment with 25 mg l^{-1} bzl^{6}Ade; after 4—6 weeks.

that arise in newly-formed parenchyma around the vascular cylinder at the cotyledon's base (in *Pinus sylvestris* roots are also initiated acropetally along the cotyledon). Callusing usually accompanies root production. In the absence of applied auxin, cytokinin (bzl^{6}Ade) incorporated in the medium or administered as a pulse results in the initiation of adventitious buds, as well as of bud-like structures identified as pseudobulbils, pseudobuds, phymas, phylloids and nodules. However, the proportion of the bud-like structures, especially of pseudobuds and phylloids, has been observed to increase with an increase in concentration of applied cytokinin or with a decrease in the matrix potential of the gel (resulting not only in increased uptake of cytokinin but also in hyperhydration or vitrification of tissues). Combined with cytokinin, auxin even at nanomolar concentrations edges differentiation towards histo- rather than organogenesis.

The term pseudobulbil (compare "meristemoidal dome", COLEMAN and THORPE 1977) is used to denote adventitious structures that are not visually identifiable as adventitious buds, but which eventually may give rise to shoot buds or become necrotic and cease to develop. Pseudobuds are extended, usually vitrified, bud-like structures, but often have damaged or retarded apical meristems and short, distended leaf-like appendages. Phymas, upon sectioning, are seen to consist of pustular nodules of undifferentiated cells, and are up to 3 mm and more in diameter. Phylloids are leaf-like structures with unusually large parenchyma cells, either with only weakly developed vascular tissue or lacking it altogether.

Response of leaves from flushing buds has been found to depend mainly on four factors (JANSSON and BORNMAN 1980, 1981): (1) method of explanting, (2) age of the leaf, (3) position on the leaf axis, and (4) ratio of cytokinin to auxin. Careful excision of the leaf explant ensures that the basal cushion of presumptive meristematic tissue proximal to the abscission zone is included. The number of adventitious bud primordia (Fig. 4) that can be induced decreases with leaf age (that is, length) as does their distribution from leaf base to leaf apex (that is, in an acropetal direction distal to the abscission zone). Based on observations of the morphogenic effects of different ratios of bzl^{6}Ade to NAA, from 10 000 : 1 to 1 : 1, it was concluded that the level of endogenous auxin decreases progressively from the leaf base to its tip,

so that the particular concentration of exogenously applied auxin that promotes shoot-bud initiation midway along the leaf, simultaneously inhibits it at the leaf base.

It was shown earlier (JANSSON and BORNMAN 1983) that retention of the collenchyma plate or "crown" at the base of the embryonic shoot greatly increased the induction of adventitious buds in the leaf primordia following a pulse treatment with 25 mg l^{-1} bzl^{6}Ade. Transport studies (JANSSON *et al.* 1983) confirmed that the crown in its collenchymatous state acts as a selective barrier for the transport of radiolabelled indoleacetic acid and phosphate. Subsequently, it has been found that in some 18-year-old clones, following intermittent spraying of intact plants or pulse treatment of embryonic shoots with bzl^{6}Ade (as described above), as many as 50 adventitious buds can be induced per embryonic shoot, with up to 5 per primordium (Fig. 5). Furthermore, in some cases it has been possible to root the shoot buds *in vivo* following their elongation *in vitro*. The redetermination of leaf primordia into shoot primordia is perhaps an indication that the former were not yet reversibly determined. According to ROMBERGER (1963), apical growth in the conifer's leaf primordium begins to be replaced by intercalary growth when its length is less than *ca.* 400 μm. However, intercalary growth is probably not a reliable parameter of determination since initiation of bud meristemoids also occurred in primordia less than 400 μm long. (Is differentiation of procambial tissues in the leaf primordium a probable parameter?) With the meager evidence at hand at present, it appears that adventitious bud initiation is determined *de novo* by the cytokinin-controlled induction of meristemoids rather than *via* a reversal of the determined state.

DISCUSSION

Notwithstanding the lack of quantitative data of both endogenous hormone levels and sensitivity of specific target cells and tissues in terms of their ability to respond to triggers of differentiation, the work on conifer tissues *in vitro* is contributing to a better understanding of the role of the two major morphogens, particularly that of cytokinin.

Investigations on *ca.* 7-day-old cotyledons of *Pinus radiata* (AITKEN *et al.* 1981, YEUNG *et al.* 1981, BIONDI and THORPE 1982, OBATA-SASAMOTO *et al.* 1984, VILLALOBOS *et al.* 1984a, b) in T. A. Thorpe's laboratory has shown that:

(1) cytokinin must be present in the medium during the first three days in culture, although switches in the distribution and rate of macromolecular synthesis, developmental pathways and mitotic activity are induced within 24 h in culture; (2) cytokinin is directly involved in the induction of adventitious buds; (3) cytokinin and photomorphogenic light are required for the development of meristematic tissue and shoots, although initially light can be withheld but then only for up to 10 days; and (4) cytokinin acts primarily on cells of the cotyledons' stomatal complexes in surface contact with the medium.

Work in our laboratory with 14-day-old cotyledons, leaves of flushing buds and embryonic shoots has led us to conclude that:

(1) gradients exist within a single cotyledon or leaf and that the levels of endogenous auxin in conjunction with those of cytokinin applied exo-

genously, control not only qualitatively the type of adventitious structures induced, but also quantitatively the numbers of adventitious shoot buds, and additionally, also determine their positional development; (2) in terms of morphogenesis auxin and cytokinin each display both a positive (organogenic) and a negative (histogenic) effect since individually at a given level each may induce either adventitious buds (cytokinin) or roots (auxin), whereas increasing the level of auxin in a bud-induction programme or that of cytokinin in a root-induction programme results in the predominance of callus; (3) account should be taken of the physiological conditon of the cotyledon and leaf explants and the degree of anatomical specialization of their epidermal, hypodermal and mesophyll tissues, since these vary according to the chronological age of seedling and plant and the ontogenetic development within the foliar organ from its apex to its base; and (4) the subsidiary cells of the stomatal complexes and differentiating cells of non-determined presumptive leaf primordia appear to be primary promeristemoids that are particularly receptive to applied cytokinin.

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