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In vitro Adventitious Bud Formation on Isolated Seedlings of *Pinus silvestris* L.

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Abstract. The formation of adventitious buds under the influence of 6BAP has been observed on various aerial organs of cultured *Pinus silvestris* seedlings. The inductive power of this cytokinin was found to depend on the concentration and the physiological age of the explant.

The different organogenetic potentialities of Gymnosperms *in vitro* have been demonstrated only recently. Organogenesis, as observed frequently, is characterized by the formation of adventitious buds which, after root regeneration, can produce new plantlets thus allowing a practical vegetative multiplication. Varying degrees of success were obtained depending on the species, namely *Cryptomeria japonica* (ISIKAWA 1974), *Pinus palustris* (SOMMER *et al.* 1975), *P. banksiana* (CAMPBELL and DURZAN 1975), *P. radiata* (REILLY and BROWN 1976), *P. contorta* (WEBB and STREET 1977), *P. pinaster* (DAVID and DAVID 1977), *Picea glauca* (CAMPBELL and DURZAN 1975, 1976), *P. sitchensis* (WEBB and STREET 1977), *Pseudotsuga menziesii* (REILLY and BROWN 1976, CHENG 1975, 1977, CHENG and VOQUI 1977, WINTON and VERHAGEN 1977), *Tsuga heterophylla* (CHENG 1976), *Biota orientalis* and various *Cupressus* (THOMAS *et al.* 1977), *Thuya plicata* (COLEMAN and THORPE 1977), (for review see DAVID and THOMAS 1978).

Our study on *Pinus silvestris* was pursued with a view to understanding the mechanism of adventitious bud formation on the aerial organs of the young plants. Moreover, we examined the cytological characteristics of the neoformed buds.

In this paper, we report the formation, localization of adventitious buds on seedlings and the initiation of cotyledon adventitious buds.

MATERIAL AND METHODS

The seedlings of *Pinus silvestris* L. used came from the USSR (Riga), and were supplied by VILMORIN (France). For all the experiments, the seeds were sterilized on the surface with calcium hypochlorite (80 g l^{-1}) for 20 min and then rinsed three times in sterile distilled water. Their culture was undertaken in one of two ways: either the embryos excised from sterilized seeds were

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TABLE 1

Numbers of explants forming adventitious buds in culture of seed embryos, of 2- and 5-day old seedlings depending on the concentrations of cytokinin 6BAP

Concentration of 6BAP [M l^{-1}]	Seed embryos	2-day seedlings	5-day seedlings
0	0	0	0
5.0×10^{-7}	7	0	0
2.5×10^{-6}	35	15	0
5.0×10^{-6}	6	45	7
2.5×10^{-5}	3	5	25
5.0×10^{-5}	0	0	5
1.0×10^{-4}	0	0	0

cultured directly or the sterilized seeds were germinated aseptically on filter paper and, after germination, were resterilized as earlier and then cultured.

Explants (whole seed embryos and young seedlings) were cultured on agar medium in plastic Petri dishes (5 cm in diameter) containing 9 ml of medium. The basal medium contained macro-and oligoelements of MURASHIGE and SKOOG modified according to LIN and STABA (1961), with FeEDTA (5 ml l^{-1}), vitamins according to NITSCH and NITSCH (1965), 20 g l^{-1} of sucrose and 0.8% of agar. The cytokinin 6BAP (6-benzylaminopurine) was used alone at various concentrations. The pH of this medium was adjusted to 5.6 before autoclaving (120 °C, 20 min).

Seedlings were cultured in such a way that the cotyledons remained above the surface and the radicles and the hypocotyls were immersed in the medium. Four explants were cultured per Petri dish. The cultures were maintained at a temperature of 25 °C under 16 h lighting a day.

For histological purposes, the explants were fixed in FAA or modified Navachine by RANDOLPH, dehydrated by way of ethyl alcohol and embedded in paraffin. Serial sections (5μm) were prepared on a rotatory microtome, and were double stained with Gallocyanine and Red Ruthenium, and mounted in Canada balsam.

RESULTS AND DISCUSSION

A. Effects of 6BAP Concentration and Explant Age on Bud Induction

The inductive effect of 6BAP has been tested with six concentrations ranging from 5×10^{-7} to 10^{-4} M l^{-1} on seed embryos and on 2- and 5-day old seedlings, respectively.

In Table 1, we report the numbers of explants producing adventitious buds after 4 weeks in culture. Each treatment consisted of 120 replicates.

It appears from Table 1 that the highest percentage of bud induction (37.5% ± 8.6%) was observed in group 2 where the culture medium contained 5×10^{-6} M l^{-1} of 6BAP. Interestingly, a greater increase in cytokinin concentration becomes necessary as the seedling progresses in its development.

B. Morphogenesis of Neoformed Buds

Bud neoformation can take place in two different sites and often simultaneously on the same explant (Fig. 1B).

1) Shoot adventitious buds (Figs. 1A, B, 2)

Adventitious buds appeared in a variable number near the shoot apical meristem (SB, Fig. 1A, B) which gave rise to a plantlet in which the shoot apex had many vegetative points side by side and produced multiple leaf primordia.

In this case, the hypocotyl lengthened a little whereas the growth of the epicotyl axis was inhibited. However, an extension of the apical meristem was observed. Transversal and longitudinal histological sections enable us to observe the vegetative points at various stages of development. We found up to 6 vegetative points at one apex (Fig. 2) in a transversal section where the two youngest vegetative points 1 and 2 are situated at a level more proximal than that of the section shown in this figure.

Thus, the origin of these supernumerary apices may be interpreted by three different hypotheses. They can result from the partition of the initial apical meristem, or from the development of axillary buds of the cotyledons, or from the neoformation of vegetative points following the dedifferentiation of internodal groups of cells.

2) Cotyledon adventitious buds (Figs. 1B, C, D, 3A, B, C)

A single adventitious bud apparently located at the tip of the cotyledon was initiated. Several of the early stages (starting from the first week in culture) of the development of neoformed buds have been studied by cytohistological sections. The bud arises from a dedifferentiation zone under an epidermis situated near the apical side of the cotyledon procambium (I, pc, Fig. 3A). It forms progressively a group of cells on which the adventitious bud will organize (vp, Fig. 3B) and develop itself leaving behind the cotyledon tip. Its vascular system is in continuity with that of the cotyledon (arrows, Fig. 3C).

Therefore, it is clear that the adventitious bud is subterminal.

Moreover, the cotyledons of the same seedling do not represent the adventitious buds at the same stages of organogenesis. Their induction therefore appears in an asynchronic way, probably depending upon the vigor of each cotyledon.

Our cultures of whole seedlings of *Pinus silvestris* have produced cytokinin 6BAP-induced adventitious buds. The results of the present investigation suggest the following remarks:

1) The presence of 6BAP alone in the culture medium induces bud formation as has been shown by many authors for other species of Gymnosperm.

2) The 6BAP concentration necessary for bud induction depends upon the physiological age of the explant. The induction of adventitious buds on the aged tissue requires a higher concentration of cytokinin than does that of less differentiated tissue.

3) Under our experimental conditions, the neoformed buds appeared at specific sites which will facilitate a detailed experimental study of the mechanism of *in vitro* bud differentiation.

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Figures at the end of the issue.

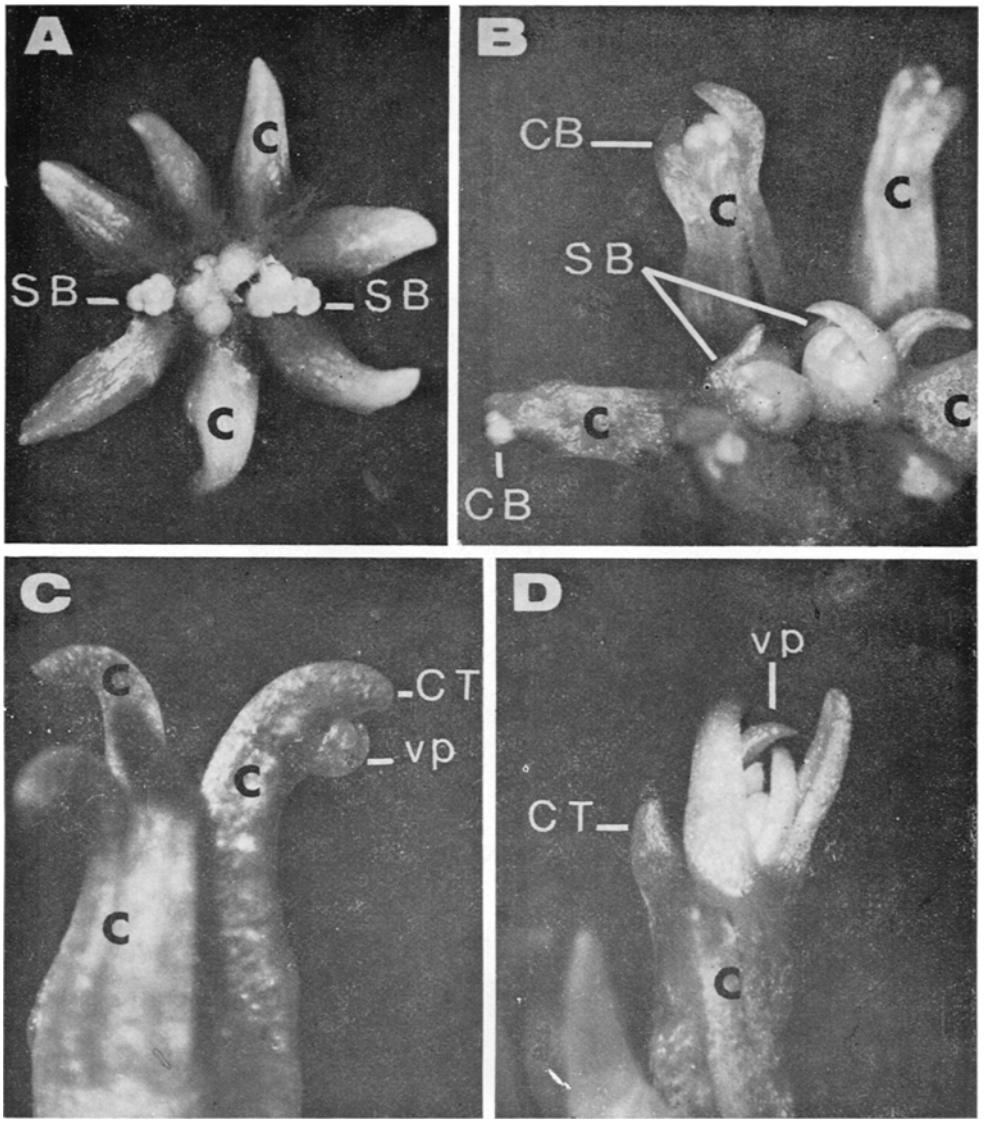

Fig. 1. Morphogenesis of adventitious buds in culture of 2-day old seedlings on LIN and STABA basal medium containing 5×10^{-6} M l^{-1} of 6BAP. – (A) Adventitious buds induced on shoot tip (SB) after 3 weeks in culture ($\times 10$). (B) Adventitious buds induced on the shoot tip (SB) and on the cotyledon tip (CB) after 4 weeks in culture ($\times 10$). (C) Young adventitious bud on the cotyledon after 2 weeks in culture ($\times 15$). (D) Aged adventitious bud on the cotyledon after 4 weeks in culture ($\times 15$) – CT: cotyledon tip; vp: vegetative point of the adventitious bud; C: cotyledon.

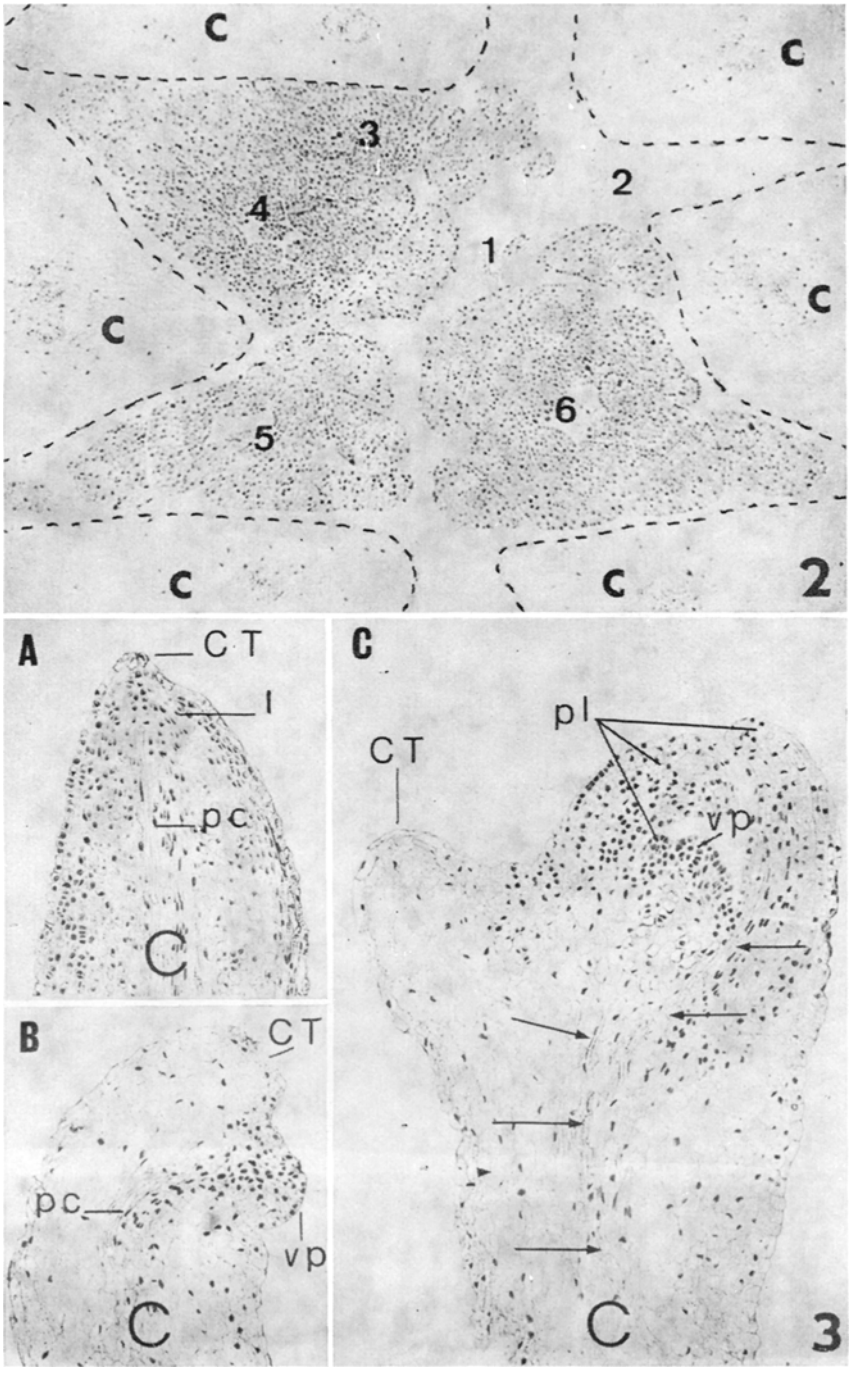
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Fig. 2. Histological sections stained with Gallocyanine and Red Ruthenium (culture of 2-day old seedlings on LIN and STABA basal medium containing 5×10^{-6} M l^{-1} of 6BAP, fixation in FAA). — Transversal section of a shoot after 4 weeks in culture showing multiple vegetative points side by side (1 to 6). Youngest apices 1 and 2 are situated below this section ($\times 60$). The dotted line indicates the cotyledon sections (C).

Fig. 3. Histological section as in Fig. 2. — (A) Longitudinal section of an adventitious bud induced on the cotyledon after 1 week in culture: initiation of the bud on a zone of dedifferentiation (I) near the end of the cotyledon procambium (pc) ($\times 40$). (B) Longitudinal section of an adventitious bud induced on the cotyledon after 2 weeks in culture: formation of the bud on a cellular clump organizing into a vegetative point (vp) ($\times 40$). (C) Longitudinal section of an adventitious bud induced on the cotyledon after 4 weeks in culture: the active vegetative point (vp) has initiated primary leaves (pl), the vascular system of the bud is in continuity with the vascular bundle of the cotyledon (arrows) ($\times 50$) — CT: cotyledon tip; C: cotyledon.