PHYSIOL. PLANT. 56: 102–107. Copenhagen 1982

In vitro adventitious budding on *Pinus pinaster* cotyledons and needles

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David, A., David, H. and Mateille, T. 1982. In vitro adventitious budding on *Pinus pinaster* cotyledons and needles. – Physiol. Plant. 56: 102–107.

Adventitious budding can be induced on two types of *Pinus pinaster* Ait. organs. Cotyledons (10-mm-long), derived from 8 to 10-day-old seedlings, show morphogenetic response when an appropriate mineral solution is used ($NH_4^+/K^+ = 1$). Of the various cytokinin concentrations added to this optimal mineral medium, 0.8 μ*M* BAP (6-benzylaminopurine), with 5 n*M* NAA (1-naphtaleneacetic acid), promoted organogenesis best. Buds were induced from outer mesophyll layers.
Short shoots and the elongating needles (70-mm-long) were collected from cuttings of a mature tree (10-years-old). These cuttings benefited from physiological advantages of a well-developed root system (by heating the substrate). In order to stimulate in vitro organogenesis, they had been sprayed every week from March to May with 10 μ*M* BAP.
When cultivated in the presence of 10 μ*M* BAP and 25 n*M* NAA, 79% of the explants produced buds from dome-shaped meristematic cell clusters that pre-existed at the top of the short shoots. Moreover, among these, 42% gave rise to adventitious buds induced from proliferating mesophyll cells at the needle base. The morphologies of the two kinds of shoots were similar. Adventitious budding on these two different explants should allow vegetative multiplication of selected seedlings and elite trees.

Additional key word – Organogenesis.

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This paper represents part of the contributions to the 1st Workshop of the International Conifer Tissue Culture Work Group, held in Landskrona, Sweden in September 1981.

Introduction

Some studies concerning adventitious bud production from coniferous tissues have already been reported. David and Thomas recently (1979) reviewed this topic. Since then, new results have been published: Von Arnold and Eriksson (1978, 1979, 1979), Tranvan (1979), Minocha (1980), Bornman and Jansson (1980), Jansson and Bornman (1980), Rancillac (1979, 1981). In addition, autotrophic new plant regeneration has been reported on and discussed by Chalupa (1977), David (1979) and Horgan and Aitken (1981). In general, most of the research was performed with young starting material. However, Von Arnold and Eriksson (1979) were able to induce adventitious budding from *Picea abies* tissues which consisted of needle primordia collected from adult trees, 5 to 50 years old, and 1 to 5 mm needles collected from buds that were isolated just after flushing. Needle explants (10 to 15-mm. long), associated with short shoots, have been used by Bornman and Jansson (1980). The material was collected from 20-week-old plants. A frost hardening treatment had been applied so that the physiological development would be equivalent to that of open cultivated one-year old plants.

In this paper we report on adventitious budding involving two types of *Pinus pinaster* explants, which are different from one another in terms of anatomical structure and physiological development: cotyledons chosen as a model system in order to study the effect of various nutrients on morphogenetic response and short shoots with their needles (Fig. 3B), derived from cuttings of a 10-year-old tree, were also used.

Abbrevations – AC, activated charcoal; BAP, 6-benzylaminopurine; CK, cytokinin; MS, Murashige and Skoog; NAA, 1-naphtaleneacetic acid.

Received 10 December, 1981; revised 22 May, 1982

/0900102-06 $03.00/0

Materials and methods

Ten-mm-long cotyledons were collected from 8 to 10-day-old disinfested seedlings. Explants were placed either flat upon the surface of the medium, or their basal end embedded in the agar.

Two different mineral solutions were compared; their concentration (m*M*) in each element is presented in Tab. 1. In solution I, the macronutrients used were those of Cheng (1977) for in vitro adventitious budding of *Pseudotsuga menziesii* cotyledons, supplemented with Murashige and Skoog (1962) micronutrients (half-strength) except for Fe EDTA (which was included at one-fifth strength). Solution II was that of Reilly and Washer (1977) for bud induction on *Pinus radiata*. Following organic substances were added: 7 μ*M* thiamine-HCl, 1.4 m*M* Inositol, 88 m*M* sucrose, 0.7% Bacto agar (Difco). The plant growth regulators used were BAP, at 0.8, 1.5, 5 and 10 μ*M*, and NAA at 5, 10 and 50 n*M*. The pH values ranged from 5.5 to 5.7. The cultures were maintained under a continuous light (Sylvania, F40 T 12 Gro-lux, 11.6 W m^{-2} at 25 ± 1°C).

Short shoots were derived from cuttings of a 10-year-old tree, cultivated in a greenhouse, and grown under a nutrient regime. The development of the root system was stimulated by warming the substrate (25°C). These cuttings were sprayed every week from March to May, with BAP (10 μ*M*) and a fungicide (50% Benomyl). The experiments were performed in May according to the standard procedure previously published by David et al. (1978). At that time, the needles were 70-mm-long (the average in situ length of a needle at the end of the growth period is 150 mm). The mineral solution was previously documented for the culture of a 2nd generation of short shoots (David et al. 1978). MS micronutrients were used (half-strength) and organic components added were: 1.1 μ*M* thiamine HCl, 0.55 m*M* Inositol, 117 m*M* sucrose, 1% Bacto agar. Plant growth subtances used in these experiments were BAP (10 μ*M*) and NAA (25 n*M*). This induction medium is designated IIIa. The explants were maintained in similar physical conditions to those of cotyledons. The shoot elongation of induced buds occurred on media differing from medium IIIa in the following modifications:

medium IIIb = 0 hormone + 58 m*M* sucrose + 1% Bacto agar;
medium IIIc = 0 hormone + 58 m*M* sucrose + 0.8% Bacto agar + 0.5% activated charcoal (AC);
medium IIId = 20 μ*M* BAP + 50 n*M* NAA + 58 m*M* sucrose + 0.8% Bacto agar + 2% AC.

Buds were grown under a 16-h light period (20 W m^{-2}) at 25 ± 1°C and an 8-h dark period at 18 ± 1°C. Anatomical studies were performed on tissues fixed in Nawashine solution, dehydrated in ethanol-toluene series, embedded in paraffin, and stained in gallocyanin and ruthenium red solutions (Gabe 1968).

Results

Initiation and development of adventitious buds on cotyledons

Preliminary experiments have shown that the most intense organogenic activity occurred when the cotyledons were placed flat upon the surface of the medium (62 instead of 32%, which was obtained when the basal end of the cotyledons was embedded in the agar). All the following experiments were performed using the first type of culture.

Response to 2 different mineral solutions. Two different mineral solutions were compared (solutions I and II); they were supplemented with several previously listed organic elements (see Materials and methods) plus the following hormonal balance: 0.8 μ*M* BAP and 5 n*M* NAA. Figure 1 summarizes the influence of the two media used in this study. Medium I stimulated best the adventitious budding (Fig. 3A), while total inhibition of the morphogenetic response occurred with medium II. Moreover, medium II, highly concentrated in K^+, allowed elongation of the cotyledons with maintenance of their morphology in spite of the lack of organogenetic response.

Influence on organogenesis of the hormonal level in the medium. Several BAP and NAA balances were added

Tab. 1. Mineral composition of media I and II (m*M*).

	K^+	NH_4^+	Ca^{2+}	Mg^{2+}	NO_3^-	Cl^-	SO_4^{2-}	$H_2PO_4^-$
I	10	10.3	1.5	0.75	20.6	3	0.75	0.6
II	25	2.6	1.35	1.6	25	2.7	1.6	2.6

	Fe^{2+} EDTA	Mn^{2+}	Zn^{2+}	Na^+	Cu^{2+}	Co^{2+}	Bo_3^-	I^-	MoO_4^-
I	2×10^{-2}	5×10^{-2}	18×10^{-3}	10^{-3}	5×10^{-5}	5×10^{-5}	25×10^{-2}	2.5×10^{-3}	10^{-3}
II	6×10^{-2}	2.5×10^{-2}	5×10^{-3}	10^{-3}	10^{-3}	5×10^{-3}	30×10^{-2}	5×10^{-3}	10^{-3}

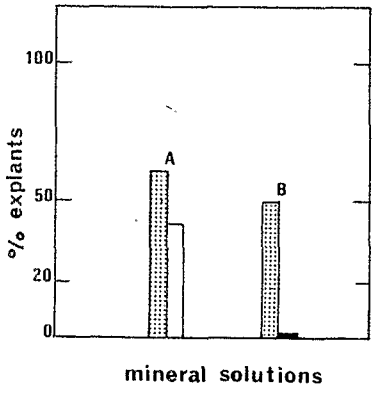

Fig. 1. The effect of two different mineral solutions on adventitious budding of cotyledons: The histograms indicate the average of percentages obtained during 2 experiments. A, percentage of explants still alive after 25 days of culture and B, percentage of explants from which new buds arose (number of explants tested: 150). Solid column, medium I; open column, medium II.

to mineral solution I, supplemented with organic compounds. Figure 2 indicates that 0.8 μ*M* BAP (with 5 n*M* NAA) induced the most intensive adventitious budding. Indeed, 83% of the cotyledons still alive after 25 days of culture formed buds, while 14% produced a surface spread callus. When the CK level was increased, the number of explants expressing a positive morphogenetic response decreased (65–60%). In respect of the buds, the higher hormone concentrations militated against an ordered development, whereas the percentage of cotyledons over which callus appeared increased (17 to 40%). A relatively high CK concentration (10 μ*M*), even if it permitted the cells to dedifferentiate and divide, prevented the development of organized structures; the unorganized proliferations were visible after less than 15 days of culture. The use of an hormone-free medium did not permit any response.

Histological origin of adventitious buds. The study of longitudinal sections, made from cotyledon explants during their culture, showed that only the cells of the superficial layers of the mesophyll tissue dedifferentiated in several sites, forming areas of primary meristematic cells from which bud primordium arose. Moreover, the whole explant kept its initial aspect because the internal layers of the cotyledon were not under the control of BAP.

Initiation and development of adventitious buds on needles

Induction stage of adventitious budding. In a previous experiment, we noticed that it was difficult to control efficiency of disinfestation of the short shoots when the explants were collected from open-cultivated trees. When the cuttings, grown in a greenhouse, were sprayed weekly with a fungicide, the infection rate of the explants was minimal (4%).

At the time of collecting, we observed dome-shaped meristematic cell clusters at the top of the short shoots and, very rarely, the presence of a tiny bud. After 3 weeks of culture, 79% of the explants budded at the apex of the short shoots. Moreover, among these, 42% showed small excresences at the basal surface of the needles that subsequently developed into buds. It should be pointed out that at the period of collecting the needles were elongating.

Histological origin of adventitious budding. Anatomical observations showed that superficial layers of short shoot tissues underwent non-organized proliferations. The vacuolated cells synthesized phenolic compounds. On the other hand, the cells of the outer layers of mesophyll located at the needle base proliferated and gave rise to a mass of meristematic cells protruding through the explant and later on, differentiating into a bud. It is known that *Pinus pinaster* needles elongate because of the division of their basal cells. Thus, organogenesis, due to the influence of growth regulators present in the medium, is the result of dedifferentiation of certain cells showing a mitotic activity.

Elongation stage of the buds. After 3 weeks of culture on inducing medium IIIa, the explants were divided into 2 groups. The first group was sub-cultured for 20 days in a medium devoid of definite growth regulators (IIIb), then in a medium containing AC (IIIc). The second group was directly cultured in the medium supplemented with AC (IIIc). A direct transfer from an

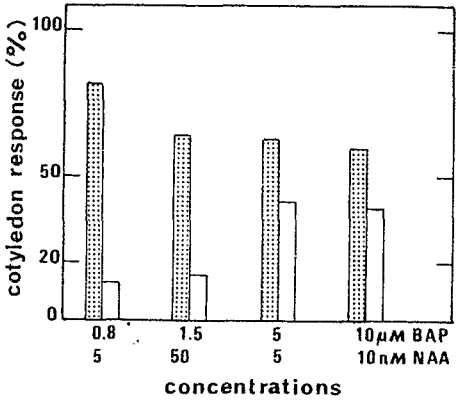

Fig. 2. The effect of BAP concentrations on cotyledon response. Solid column, budding; open column, callusing. For each hormonal balance 100 explants were tested.

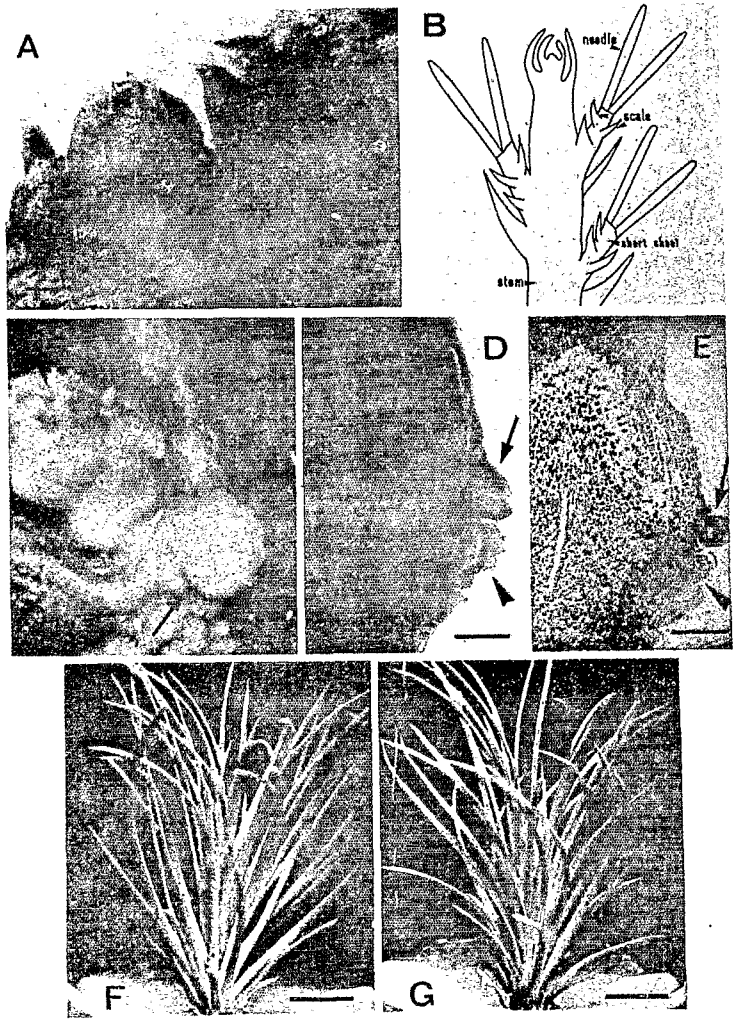

Fig. 3(A–G). Adventitious budding on *Pinus pinaster* cotyledons and needles. A, adventitious budding on isolated cotyledons lying flat on medium I + BAP (0.8 μ*M*) + NAA (5 n*M*); B, diagram of a *Pinus pinaster* shoot; C, organization of an apical short shoot bud (▶) and adventitious budding on a needle (→); D, emergence (→) at the base of a needle from which bud will be formed, (▶) proliferation of the short shoot; E, longitudinal section through needle and short shoot. Note the formation of a bud primordium (→) at the needle base and a callus (▶) along the surface of the short shoot; F, shoot originating from short shoot's apex; G, adventitious shoot from needle. Scale bars on A, C represent 1 mm, D, E 0.5 mm, and F, G 5 mm.

inducing medium to an elongating medium supplied with AC, promoted healthy apical and adventitious bud development. This development was more definite than that of buds having undergone a culture period in medium IIIb. When the elongation was adequately advanced (10-to 15-mm-long axis), the shoots were excised from the initial explants and transferred to medium IIId (provided with 2% AC, 20 μ*M* BAP and 50 n*M* NAA). The effectiveness of this medium was established after different experiments on bud elongation, performed with material of various origins. Consequently, we noticed that the simultaneous presence of AC and a hormonal balance accelerated shoot growth and avoided the establishment of apical dominance in a bud clump.

Our experimentation with needles from cuttings of a 10-year-old tree has allowed us to obtain two different groups of morphologically similar, 30-mm-long shoots. One group originates from apical meristematic cells of short shoots, whereas the other comes from adventitious buds of their needles.

Discussion

Induction of adventitious budding on *Pinus pinaster* cotyledons may be adequate for rapid micropropagation via seedlings originating from controlled pollination. Use of a physiologically more advanced experimental material (bifoliar short shoot) could lead to large scale clonal micropropagation of elite trees.

Various authors have reported results concerning adventitious bud formation on isolated cotyledons (Cheng 1976, Chalupa 1977, Tranvan 1979, Rancillac 1979, 1981). The cytokinin used regularly was BAP, combined with NAA. Hormonal concentrations depended on the species and, particularly, on the age of the seedlings from which the organs were collected (Tranvan 1979). As a matter of fact, it was necessary to increase amounts of cytokinin when more differentiated organs were utilized.

Even if we now have a better understanding of hormonal requirements, no detailed studies with gymnosperms have as yet been performed that deal with the influence of mineral nutrients on adventitious bud formation. The various media that we compared differed in the concentrations of various components. One of the main differences was NH_4^+/K^+ ratio. Its value was 1 in the case of medium I and 0.1 in medium II. Our results concerning morphogenetic activity of *Pinus pinaster* cotyledons revealed a positive effect on adventitious budding when the ratio was 1. This could explain the effect of medium I and could be closely connected with the results of A. David (1973, Thesis, University of Paris VI, Paris, France) who, studying sub-culture requirements of the same species, demonstrated that NH_4^+ favoured tissue proliferation. Consequently, the organogenic influence of CK would appear mainly in cells with mitotic activity.

Working with bifoliar short shoot explants of *Pinus sylvestris* frost hardened plants, whose physiological age was equivalent to one year, Bornman and Jansson (1980) observed adventitious budding on a medium supplemented with CK. The retention of the short shoot apex seemed to stimulate in a marked manner bud differentiation in the surrounding apical cushion. In this study, we reveal needle capacity to produce buds and emphasize the influence of a hormonal pretreatment of the experimental material, before the in vitro step, on the occurrence of a morphogenetic capacity. The histological observations of Cheath and Cheng (1978) showed that adventitious budding on cotyledons originates from the outer layers. With regard to bifoliar short shoot explants, we observed that the emergence of buds was restricted to the needle base as a result of the proliferation of its mesophyll cells that were still manifesting a mitotic activity (needle elongation area).

Moreover, Vazart et al. (1979) noted that the cells involved in adventitious bud formation on hypocotyl explants of *Biota orientalis* were located in the outer cortical layers. These cells retained a slight mitotic activity. Thus, these various results demonstrate that adventitious budding does not depend on the kind of organ but on the degree of cell differentiation. CK would act on cellular layers in which a mitotic activity still exists.

In many cases, bud development is proving difficult. The use of AC supplemented medium promotes growth stimulation. AC adsorbs fairly large amounts of hormones (Weatherhead et al. 1978, 1979); its stimulating effect on elongation, observable when hormones are supplied, causes us to question the manner in which this interference arises. A systematic study should be carried out on genetically identical groups of shoots for a better understanding of the effect of the composition of an elongating medium.

Various species have been regenerated, in particular: *Pinus palustris* (Sommer et al. 1975), *Picea glauca* (Campbell and Durzan 1976), *Pinus radiata* (Horgan and Aitken 1981), *Sequoia sempervirens* (Boulay 1979), *Pinus pinaster* (David et al. 1978, David 1979, Rancillac 1979, 1981), *Pinus sylvestris* (Bornman and Jansson 1980). Wochok and Aboel-Nil (1977), studying root tips from plantlets differentiated from somatic cells, observed the chromosomal stability of these cells.

In our future studies, we intend to compare the ontogenesis and the rooting capacity of shoots possessing similar genetical inheritance, but derived either from reactivated meristematic cells (apical shoots) or from the dedifferentiation of mesophyll cells (adventitious shoots).

Acknowledgements – The research dealing with adventitious budding on needles was supported by a grant from AFOCEL, 77370 Nangis, France. We express our sincere thanks to Dr. C. H. Bornman for his critical reading of this manuscript.

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Edited by C. H. B.