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A System for Repeatable Formation of Elongating Adventitious Buds in Norway Spruce Tissue Cultures

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Abstract

Seedlings of Norway spruce (*Picea abies* L. Karst.) were induced with cytokinin combinations to form adventitious buds. A repeatable induction and formation of elongating buds was obtained via an induction cycle with cytokinins showing low bud-inducing capacity (zeatin, kinetin, 2iP), followed by subculture periods on media without plant growth regulators. Newly formed adventitious buds could be induced again without loosing their ability for elongation. One of the most effective phytohormone combinations for induction of elongating buds was 0.5 zeatin + 0.05 kinetin (mg l^{-1}) for 3 weeks. Zeatin in a concentration range (0.1 mg l^{-1} to 0.2 mg l^{-1}) stimulated the shoot elongation of preexisting terminal meristems in adventitious buds used for repeated cytokinin induction. 2iP promoted shoot formation and elongation in a concentration range from 0.1 mg l^{-1} to 0.5 mg l^{-1} depending on the explant type (adventitious bud/shoot) used.

Key words: Norway spruce, adventitious buds, repeatable formation, shoot elongation, gelling agents.

Zusammenfassung

Sämlinge von Fichte (*Picea abies* L. Karst.) wurden zur Adventivknospenbildung mit verschiedenen Cytokininkonzentrationen induziert. Eine wiederholbare Induktion und Bildung sich streckender Adventivknospen wurde durch den ständigen Wechsel eines Induktionszyklus mit Cytokininen, die eine geringe Induktionswirkung hinsichtlich der Anlage neuer Adventivknospen aufwiesen (Zeatin, Kinetin, 2iP), gefolgt von Subkultivierungsperioden auf phytohormonfreien Nährmedien erreicht. Neugebildete Adventivknospen konnten erneut induziert werden, ohne ihre Fähigkeit zur Sproßstreckung zu verlieren.

Eine der wirksamsten Cytokininkombinationen zur Induktion sich streckender Adventivknospen war 0,5 mg l^{-1} Zeatin + 0,05 mg l^{-1} Kinetin über einen Zeitraum von 3 Wochen. Zeatin im Konzentrationsbereich von 0,1 mg l^{-1} bis 0,2 mg l^{-1} stimulierte die Sproßstreckung bereits existierender Meristeme in den zur wiederholten Adventivinduktion eingesetzten Adventivknospen.

2iP förderte die Sproßbildung und Streckung in einem Konzentrationsbereich von 0,1 mg l^{-1} bis 0,5 mg l^{-1} in Abhängigkeit vom verwendeten Explanattyp (Adventivsproß/-knospe). Abbreviations: BA — 6-benzylaminopurine; PVP — polyvinylpyrrolidone, 2iP — 6-($\gamma\gamma$-dimethylallylamino)purine.

Introduction

The formation of adventitious buds in different spruce species has been described by several authors (Bornman, 1983; von Arnold, 1984, 1985; Rumary and Thorpe, 1984; Patel and Thorpe, 1986). A repeated induction of adventitious buds has also been described by Bornman (1987).

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Nevertheless, there is no system for spruce as effective as that of *Pinus radiata* (AITKEN-CHRISTI et al., 1988) for multiplying adventitious buds in tissue culture. Several attempts have been made to achieve a repeatable formation of elongating buds for continuous propagation (Mc COWN et al., 1988). The utilization of high cytokinin concentrations especially BA, alone or in combination with kinetin) has sucessfully been established for a single uniform induction process (VON ARNOLD and ERIKSSON 1985). The necessary 2. regeneration step, shoot elongation, for the development of a continuous propagation system has been inhibited by the repeated utilization of this induction process. Based on an observation during our adventitious bud induction experiments that zeatin/kinetin combinations led to an axial elongation in the shoot apex of seedlings in vitro, we attempted to determine the necessary plant growth regulator combinations and nutrient medium solidifiers for the development of a repeatable system of adventitious bud and shoot formation.

Materials and Methods

Seeds of *Picea abies* originated from Kamenz Lot. No 2216 and from controlled crosses of selected trees that survived in spruce populations in the Saxon Ore Mountains in south eastern Germany heavily damaged by air pollution.

The overcome difficulties caused by the different germination rates of embryos within seeds of one progeny we used only uniformly developed plant material. Seeds were sterilized for 5 minutes with a 0.25% solution of mercury chloride and rinsed 3 times with sterile water. Sterilized seeds were germinated on a mineral solution of half-strength basic medium according to BORNMAN (MCM, 1983, without the addition of urea). After 4 weeks fully developed seedlings were chosen for the experiments.

For adventitious bud induction the apical part of the seedling (cotyledons with a 5 mm hypocotyl segment) was used as the explant. These segments were placed individually for 24 h in a solution of a half-strength MCM-medium with 50 mg l^{-1} each of BA [2.21 x 10^{-4} M] and kinetin [2.32 x 10^{-4} M] at pH 5.5 (primary induction). The induction was followed by subculture on agar-agar solidified MCM medium with the addition of glutamine and ariginine (100 mg l^{-1}) and 0.5% (w/v) activated charcoal (EPN technicum, Jenapharm). After 4 weeks explants were subcultured to basic medium (BEMB) containing macroelements used by VON ARNOLD (LP — 1981) with a reduced concentration of ammonium nitrate (2.5 mM = 200 mg l^{-1}). The microelement composition used was published by BOULAY (1979). This medium, without plant growth regulators, was supplemented by addition of 100 mg l^{-1} PVP. After 3 passages on this medium, fully developed adventitious buds were separated and induced again secondary induction steps) on half-strength MCM-medium with 100 mg l^{-1} ariginine and different combinations of zeatin, kinetin and 2iP for 3 weeks. The control variant was placed on a medium without phytohormones and counted along with those on different cytokinin treatments. Clusters of adventitious buds developed on a medium without plant growth regulators (BEMB) within the following 2 to 3 subcultures (8 to 12 weeks). Afterwards, the bud clusters were divided into single buds and those were separated into 2 groups: elongated buds and fully developed spherical ones. The elongating buds were cultured on a medium without plant growth regulators (BEMB) but with an enhanced concentration of ammonia nitrate (7.5 mM = 600 mg l^{-1}) in order to support shoot formation. The spherical buds were selected for renewed induction steps with different cytokinins (zeatin, kinetin, 2iP). The scheme of experiments is demonstrated in *figure 1.*

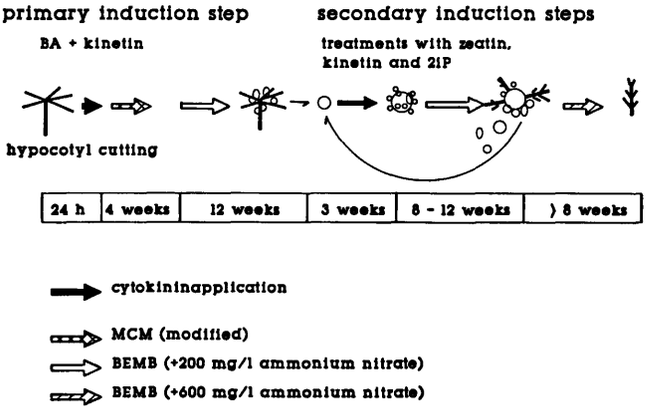


Figure 1. — Scheme of adventitious bud induction experiments with Norway spruce hypocotyl cuttings.

Two established clonal lines of adventitious bud clusters (*Picea abies* [KAMENZ] propagated in this manner and showing different elongation behaviour (Gat1 — no visible occurence of shoot elongation in vitro, Gat2 -visible occurence of shoot elongation in vitro) were additionally used to select optimal cytokinin combinations for adventitious bud propagation as well as shoot elongation and to demonstrate the clonal influence.

Chi-square test was used to compare the number of formed adventitious buds/treatment over different size ranges. To test the influence of zeatin concentrations versus kinetin concentrations and the influence of different 2iP concentrations ,one clonal line (7[11/88]) was used.

All explants were cultured under continuous red fluorescent light (35 μE m^{-2} s^{-1}, fluorescent lights LS 65 red 93; NARVA) and at a constant temperature of 22° C. The media were solidified with 0.55% Agar-agar (w/v, SERVA high-gel strength, Nr. 11936) or 0.45% gelrite (w/v, SERVA gelrite gellan-gum K9 A-40 Nr. 22168). Test tubes with 28 mm diameter and 9 cm height were used as cultivation vessels.

Results

Table 1 shows the results of an experiment with seeds of *Picea abies* (KAMENZ). A single primary induction step (control, 0/0 treatment) without additional subinduction cycles led to a low number of adventitious buds. An increase in cytokinin concentrations (zeatin/kinetin) used for the subinduction steps diminished the number of inactive buds and enhanced the number of elongating buds. The influence of higher cytokinin concentrations for subinduction also prevented the normal competitive reaction between nonmorphogenic tissues (visible as turning brown) and adventitious buds during the period of culture on a medium without cytokinins.

An optimal concentration for the induction of high numbers of elongating buds (number and distribution in size ranges) in our experiments was 0.5 mg l^{-1} zeatin [2.27 x 10^{-6} M] + 0.05 mg l^{-1} kinetin [2.32 x 10^{-7} M] (*Table 2*). By following the fate of formed adventitious buds on plant growth regulator-free media, we observed that low cytokinin concentrations used for subinduction promoted

Table 1. — Average number of elongating and inactive buds per explant 200 days after the primary induction and 2 subinduction cycles depending on the cytokinin concentration used for the subinduction. 30 explants of *Picea abies* were used per treatment.

Treatment combination zeatin/kinetin	Average number of adventitious buds/explant within size range						Percentage explants (adventitious bud forming callus) which started to turn brown (%)
($mg\ l^{-1}$)	elongating buds (mm)			inactive buds (mm)			
	0-2.	2-4	>4	0-2	2-4	>4	
0/0 (contr.)	2	0.1	0.3	0.1	1.8	0.6	0
0.1/0.01	2.9	0.3	0.6	0.4	5.8	0	28.6
0.2/0.02	5.6	0.1	0.2	0.4	2.9	0	63.4
0.5/0.05	8.4	0.4	0.4	0.6	0.7	0	8
1.0/0.1	5.9	0.1	0	0.1	1.5	0	0

Table 2. — Number and size of adventitious buds 410 days after primary induction and 3 subinduction cycles, followed by subculture periods without plant growth regulators. 30 explants of *Picea abies* were used per treatment

Treatment combination zeatin/kinetin ($mg\ l^{-1}$)	Number of adventitious buds per 30 explants	Percentage buds in each size range (mm)		
		0-2	2-4	>4
0/0 (control)	129	60	6.7	33.3
0.1/0.01	521	55	24.7	20.1
0.2/0.02	521	70.8	15.4	13.8
0.5/0.05	643	54.6	27.4	17.9
1.0/0.1	1594	83.2	10.5	6.2

their elongation into shoots. The highest concentration (1.0 mg l^{-1} zeatin + 0.1 mg l^{-1} kinetin) tested for subinduction led to a rapid formation of adventitious buds of smaller size. These buds had to be subinduced with lower cytokinin concentrations to ensure their elongation, otherwise they died. To prove that the results obtained with different genotypes (30 genotypes/treatment) showed the same trend with already established clonal lines (adventitious bud clusters) bud clusters of 2 lines, showing different elongation capacity, were induced (*Table 3*) for 1 subinduction cycle. The results in clone Gat2 confirmed the previous experimental data. The treatment with 0.5 mg l^{-1} zeatin + 0.05 mg l^{-1} kinetin led to higher numbers of elongating adventitious buds whereas 1.0 mg l^{-1} zeatin + 0.1 mg l^{-1} kinetin significantly diminished the amount of elongating, shoot forming buds (2 mm to 4 mm and >4 mm). Nevertheless the different cytokinin treatments could not overcome the reduced ability of shoot elongation of the clone Gat1. The pattern of growth and development of induced spherical buds (diameter and average number of formed adventitious buds) were relatively constant for one established clone (*Table 4*). The propagation of several clonal lines of *Picea abies* over a 3-year-period showed that it should be possible to reach constant propagation factors (depending on genotype) if this system (choice of the explant for subinduction, subinduction conditions) is optimized.

Preliminary experiments to estimate concentration ranges effective in repeated adventitious bud induction as well as for their later elongation included the 2 cytokinins zeatin and kinetin in a constant proportion. In a 2. series of experiments we varied the concentrations of both cytokinins to determine their influence on bud induction and later elongation into shoots. Based on the results of the first experiments we restricted the cytokinin concentrations to 0.3 mg l^{-1} zeatin and 0·03 mg l^{-1} kinetin. We avoided the use of concentrations higher than 0.5 mg l^{-1} zeatin and 0.05 mg l^{-1} kinetin because they enhanced the number of newly formed adventitious buds without stimulating their later shoot elongation.

After 130 days of subculture free of phytohormones we counted the number and size (*Fig. 2*) as well as the origin of buds and shoots formed.

Zeatin concentrations of 0.2 mg l^{-1} and 0.3 mg l^{-1} led to an increase in the number of shoots formed. Kinetin in the highest concentration (0.03 mg l^{-1}) in combination with zeatin supported the enhanced formation of smaller buds (*Fig. 2*).

Table 3. — Number and size of adventitious buds depending on the cytokinin combination used for 1 subinduction step 55 days after induction period. Explants (18 equal sized clusters with spherical buds per treatment) of 2 established adventitious bud-forming clones (Gat1 and Gat2) with different elongation capacity were examined. These clusters were cultured 6 weeks without plant growth regulators before induction.

Clone	Treatment combination zeatin/kinetin (mg l^{-1})	Number of counted buds in size ranges (mm)		
		0-2	2-4	>4
Gat 1	0.1/0.01	222	0	0
	0.2/0.02	290	1	0
	0.5/0.05	220	4	0
	1.0/0.1	178	0	0
Gat 2	0.1/0.01	181	40	19
	0.2/0.02	132	20	18
	0.5/0.05	241	54	37
	1.0/0.1 *	180	24	2

*) Distribution of buds over all size ranges is significantly different from other variants of the same clone, chi-square test (α=0.001)

Elongating shoots in a system of repeated adventitious bud formation have 2 possible origins of their formation.

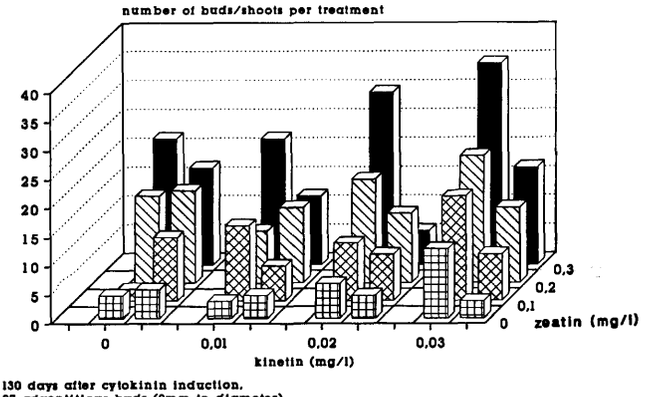
The first is an already existing terminal meristem in every adventitious bud used for induction and its cytokinin promoted elongation.

The second is the cytokinin-induced stimulation of existing lateral meristems or the formation of new meristems on the surface of the used adventitious buds.

To determine the influence of cytokinin concentration on the elongation of terminal meristems or on lateral shoot induction we categorized shoots as either terminal or lateral shoot origin on the surface of induced adventitious buds. The term "lateral" was used only to describe a place different from the existing terminal meristem.

Table 4. — Parameters of induced and adventitious bud-forming spherical buds 40 days after a 3-week subinduction period with 0.5 mg l^{-1} zeatin and 0.05 mg l^{-1} kinetin (comparison of 2 experiments). The spherical buds used were derived from 1 clone of *Picea abies*.

	experiment 1	experiment 2
Number of used spherical buds(explant)	48	45
Average diameter of the adventitious bud bearing explant (mm ± SD)	5.7 ± 2.2	4.7 ± 2.2
Average number of adventitious buds/explant (± SD)	6.8 ± 3.2	5.0 ± 3.2
Average number of formed adventitious buds of all explants bearing buds (± SD)	7.2 ± 2.8	5.6 ± 2.3



130 days after cytokinin induction.
27 adventitious buds (2mm in diameter) were used per treatment

left columns: buds of 2 mm to 4 mm length
right columns: shoots > 4 mm

Figure 2. — Influence of zeatkin/kinetin-combinations on repeated adventitious bud formation and elongation of *Picea abies*.

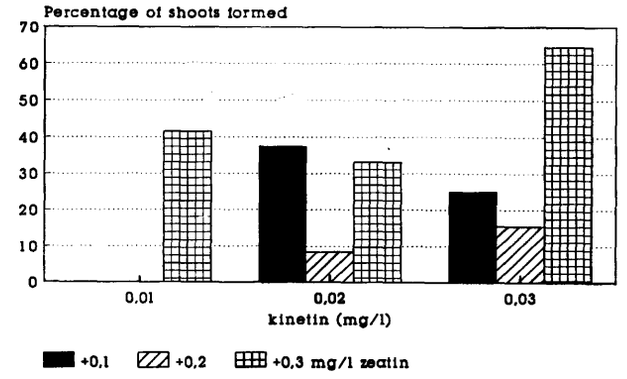
Figure 3 shows the percentage of all shoots formed with lateral origin depending on the cytokinin combinations used. Comparing the origin of shoots formed, zeatin concentrations higher than 0.2 mg l^{-1} in combination with kinetin led to the formation of a higher percentage of buds with lateral origin whereas zeatin alone or in combination with the lowest kinetin concentration mainly stimulated the elongation of the existing terminal meristem.

We also examined the effect of 2iP on the process of adventitious bud induction and development with one clonal line of adventitious bud clusters. 2iP (6-$\gamma\gamma$-dimethylallylaminopurin) is a cytokinin which has been used occasionally for adventitious bud induction (Patel and Thorpe, 1986) but has often showed a lower bud inducing capacity compared with BA.

Two different types of explants (spherical adventitious buds with 2 mm diameter and elongating adventitious shoots 3 mm to 5 mm long) were used to test the behaviour of this phytohormone on the adventitious bud induction. A broad concentration range (0.1 mg to 10.0 mg l^{-1}; 0,49 x 10^{-6}M — 49 x 10^{-6}M) was chosen. Both shoot formation and later shoot development were observed. This experiment was observed for 130 days and 250 days. Comparing the influence of 2iP on the 2 types of explants (*Fig. 4* and *5*) we observed that both explant types formed larger numbers of very small adventitious buds with an increasing 2iP-concentration after 130 days.

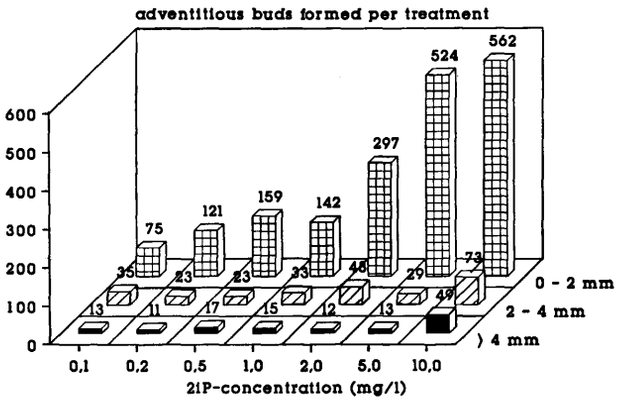
Adventitious buds (2mm in diameter) formed the highest number of longer shoots (>4 mm) after induction with 10.0 mg l^{-1} 2iP whereas the formation of elongating shoots derived from induced small adventitious shoots (3 mm to 5 mm length) were stimulated most by the lowest 2iP concentration (0.1 mg l^{-1}).

Following the fate of these buds and shoots formed, we used the same classification criteria after 250 days. It was found that without phytohormone application most of the preformed adventitious buds were unable to survive. Different cytokinin optima for the repeated induction of adventitious buds (2 mm in diameter) or adventitious shoots (3 mm to 5 mm length) were necessary for the production of growing shoots (longer than 6 mm). Adventitious buds treated with the different 2iP concentrations formed most shoots after induction with 0.5 mg l^{-1} to 1.0 mg l^{-1} 2iP (*Fig. 6*) whereas treated adventitious shoots formed most shoots at the lowest concentration to 0.5 mg l^{-1} 2iP (*Fig. 7*). These results showed the necessity of observing induction systems until the shoot formation has



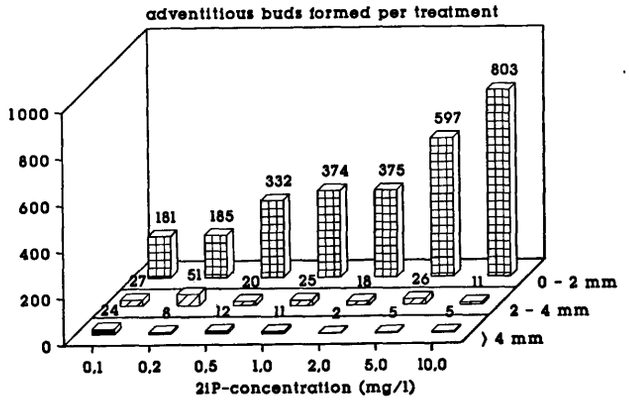
130 days after cytokinin induction,
27 adventitious buds (2mm diameter)
were used per treatment

Figure 3. — Percentage of shoots (>4 mm) with lateral origin formed per treatment depending on cytokinin combination.



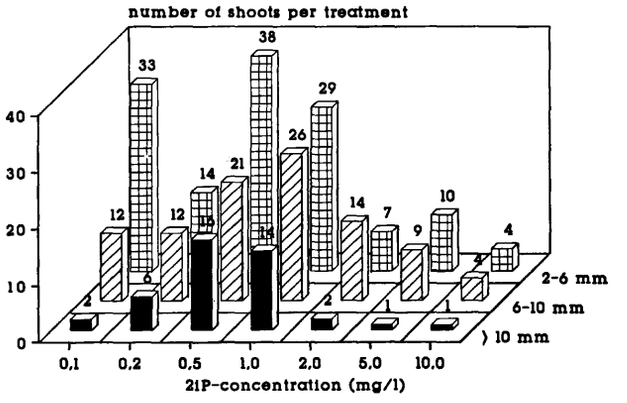
130 days after cytokinin induction,
(I.= 90 adventitious buds (2mm diameter)
were used per treatment)

Figure 4. — Influence of 2iP-concentration on the repeated formation of elongating adventitious buds (I.) of *Picea abies*.



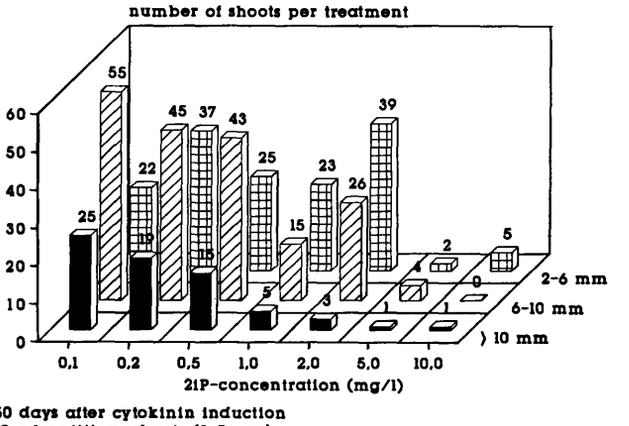
130 days after cytokinin induction,
(II.= 60 adventitious shoots (3-5mm)
were used per treatment)

Figure 5. — Influence of 2iP-concentration on the repeated formation of elongating adventitious buds (II.) of *Picea abies*.



250 days after cytokinin induction
(90 adventitious buds (2 mm in diameter)
were used per treatment)

Figure 6. — Influence of 2iP-concentration of the formation of shoots derived from induced adventitious buds of *Picea abies*.



250 days after cytokinin induction
(60 adventitious shoots (3-5 mm)
were used per treatment)

Figure 7. — Influence of 2iP-concentration on the formation of shoots derived from induced adventitious shoots of *Picea abies*.

Table 5. — Comparison of agar-agar and gelrite on the development of adventitious buds and shoots of different size ranges after 160 days in continuous red light (27 explants were used per variant).

	number of surviving shoots		shoot length on average (mm)	
shoot length (mm)	gelrite	agar-agar	gelrite	agar-agar
2 - 3	18	8	7,2	5,5
3 - 4	25	24	6,9	5,7
5 - 6	22	11	9,8	8,8
8 - 10	27	23	14,0	15,4

	number of vitrified shoots		number and size range of newly formed adventitious buds			
shoot length (mm)	gelrite	agar-agar	gelrite		agar-agar	
			0-2 (mm)	2-4 (mm)	0-2 (mm)	2-4 (mm)
2 - 3	18	7	15	2	-	-
3 - 4	25	23	89	8	42	19
5 - 6	18	9	5	1	14	1
8 - 10	25	23	15	9	26	7

crossed a critical size range for their further development (6 mm to 10 mm).

Using different gelling agents (gelrite, agar-agar) in elongation media for adventitious buds or shoots (*Table 5*), we found that gelrite stimulated the development of small adventitious buds, whereas there was no real influence visible on shoots of larger size.

Discussion

There exists a wide variety of induction procedures for the production of adventitious buds in different spruce species (Bornman, 1987). Their primary objective has been to increase the number of produced buds. Preliminary attempts showed that the induction procedure via a liquid medium containing cytokinins stimulated the formation of adventitious buds on nearly 100% of all explants if BA was used alone or in combination with other cytokinins (eg. kinetin). However an increasing number of these buds lost their ability to elongate. Attempts to induce a repeated adventitious bud cluster propagation using the most effective cytokinin combinations failed because of the formation of numerous small adventitious buds showing signs of dormancy (bud scale formation). These buds often lost the capacity to flush again. However, in our experiments for primary bud induction, cytokinins with a lower bud-inducing capacity (kinetin, zeatin) were able to support the elongation of induced buds. The ability of these cytokinins to stimulate shoot elongation agrees with the results of Pierik et. al (1991) for lilac and our findings that low concentrations of these cytokinins promote elongation of the shoot apex in spruce seedlings. Therefore, the use of low cytokinin concentrations stimulates the formation of buds that are able to elongate immediately. A culture on media lacking cytokinins and a change of basic medium after the induction period were necessary preconditions for stimulating elongation and decelopment of shoots.

The ability of the explant to react to the cytokinin and the rate of growth of formed buds depended highly upon the genotype used. Despite a high number of adventitious buds in some clones, the average number of adventitious buds per size range listed in *table 1* (30 clones/treatment) was low.

Establshing adventitious bud producing clones without knowledge of their elongation behaviour may lead to the selection of fast-multiplying clonal lines (adventitious bud formation) with low elongation frequency. A negative correlation between propagation rates and elongation behaviour in Norway spruce tissue cultures has already been described by von Arnold and Hakman (1988). For a rapid multiplication of established adventitious buds (bud clusters) without preventing the later shoot elongation 0·5 mg l^{-1} zeatin + 0.05 mg l^{-1} kinetin among the tested cytokinin combinations was useful for Norway spruce in our experiments. First experiments with other spruce species (*Picea jezoensis* [Sieb. et Zucc.] Carr. and *Picea pungens* var. glauca Bleiss) showed that they responded similarily.

The experiments using kinetin and zeatin showed that lower concentrations of these cytokinins stimulated the elongation of preexisting terminal meristems. Higher amounts of shoots after treatment with lower concentrations of zeatin, kinetin and 2iP, depending on the explant type used (adventitious buds or small adventitious shoots), may indicate that low concentrations of endogenous cytokinins are responsible for the stimulation of shoot elongation. An enhancement of the endogenous cytokinin concentration 3 weeks before bud break has been reported for Douglas fir (Britz, 1983). This indicates that cytokinins seem to be one factor which stimulates

shoot elongation and flushing of buds as well as the process of bud induction. Differences in the appearance and elongation behaviour of adventitious buds derived from induction steps was influenced by the type of cytokinin used. Additional research is needed to determine the general mechanisms of the dependence of cytokinin concentrations including their interaction with other metabolites influencing formation and development of shoots (e. g. nitrogen, SELBY and HARVEY, 1990). The positive effect of gelrite used as gelling agent on propagation has also been reported for other tree species (*Eucalyptus* — MACRAE and VAN STADEN, 1990). The stimulation of the development of small adventitious buds using gelrite could be valuable for juvenile and adult spruce explants.

The formation and propagation of adventitious bud clusters of juvenile spruce explants can serve as a means to multiply seed material from controlled pollination as well as selected somatic embryos (e. g. transgenic plants).

Stimulation of shoot elongation in adventitious buds of juvenile spruce explants via a cytokinin induction might also offer a way to overcome the difficulties in shoot elongation existing with adult plant material of Norway spruce in vitro.

Despite these first encouraging results much more research work work is needed to determine the general mechanisms of shoot elongation in spruce and put it to practical use.

Conclusions

1. The type and concentration of cytokinins used for a repeatable process of adventitious bud induction influences the later elongation behaviour of newly formed buds in Norway spruce.

2. Treatments of already formed spherical adventitious buds with 0.5 mg l^{-1} zeatin + 0.05 mg l^{-1} kinetin or 0.5 mg l^{-1} to 1.0 mg l^{-1} 2iP allow the propagation of adventitious bud clusters as well as the elongation of newly formed buds.

3. The elongation of preformed terminal meristems in adventitious buds is stimulated by a treatment with 0.1 mg l^{-1} to 0.3 mg l^{-1} zeatin [4.55 x 10^{-7} M— 1.36 x 10^{-6}] ± 0.01 mg l^{-1} kinetin [4.64 x 10^{-8} M].

4. Comparing the influence of gelrite and agar-agar on adventitious bud formation gelrite supported the formation of adventitious buds.

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Time Trends in Age-Age Covariances and Correlations – Examples from Norway Spruce Clones

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Summary

A Norway spruce clonal test, established with 5 clones on 4 extremely contrasting sites in 1967 and remeasured for height 10 times until 1981 has been used for an investigation of the following 3 topics:

1. Description of juvenile-mature correlations by LAMBETH's formula.
2. Investigation of time trends in age-age covariances, age-age correlations and standard deviations.
3. Proposal of a new formula for juvenile-mature correlations dependent on age.

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