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Influence of the Genotype on Growth of Norway Spruce (Picea abies L.) in in Vitro Meristem Culture

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(Received 30th June 1993)

Summary

The influence of the genotype for long term cultivation of meristematic tissues of *Picea abies* was investigated. For this aim, different *in vitro* clones were compared concerning their behaviour during (i) establishment, (ii, iii) their efficiencies to propagate and to elongate and (iv) their reaction to application of IAA.

Apparent phenotypical differences among individual clones could be observed. They remained stable during long term culture (4 years).

Induction of elongation of the shoot buds could be achieved with different success up to 100 %. This is the 1st report which describes an efficient induction of shoot elongation for *Picea abies* although the morphology of elongated shoots is still unsatisfactory.

Key words: Picea abies, genotype, *in vitro* clones, permanent multiplication, shoot buds.

Zusammenfassung

Es wurde der Einfluß des Genotyps auf die *In-vitro* Langzeitkultivierung von meristematischem Gewebe von *Picea abies* untersucht. Zu diesem Zweck wurden einige *In-vitro* Klone hinsichtlich ihres Verhaltens während ihrer Etablierung (i), (ii, iii) der Effizienz, mit der sie sich vermehren bzw. ihre angelegten Sproßknospen strekken und (iv) der Reaktion auf IAA-Applikation verglichen. Dabei zeigten die untersuchten Klone z. T. sehr auffällige Unterschiede, die während der Langzeitkultivierung (4 Jahre) stabil erhalten blieben.

In Abhängigkeit vom Genotyp konnten bis zu 100 % der angelegten Sproßknospen zur Streckung induziert werden. Es konnte damit zum ersten Mal eine effiziente Induktion der Sproßstreckung bei *Picea abies* erreicht werden.

Abbreviations

BA — 6-benzylaminopurine; KIN — N^6-furfuryladenine; ZEA — zeatin; 2ip, 6-(γ, γ-dimethylallyl)-aminopurine; IAA — indoleacetic acid; NAA — 1-naphthaleneacetic acid.

Introduction

The importance of the genotype for the regeneration potential of *in vitro* cultures of conifers has been described repeatedly. For example, explants like zygotic embryos or segments of seedlings of different genotype were stimulated by single application of growth regulators (Perez-Bermudez and Sommer, 1987; Mohammed and Vidaver, 1988; Aitken-Christie et al., 1988; Sen et al., 1989; von Arnold et al., 1988). The effect of the genotype on permanently growing shoot bud cultures of *Picea abies* has not yet been described.

Meristematic cultures can be established and permanently subcultured according to the method depeloped by Kunze et al. (1993). The principle of this method is the cultivation of segments of seedlings in 2 phases (without and with growth regulators) which alternate permanently. During the long term cultivation apparent phenotypical differences among individual clones were visible. The behaviour of genotypically different clones concerning their morphology, establishment, multiplication rates, reaction to IAA application and efficiency of induction of shoot elongation are described in the present paper.

Material and Methods

Material

Seeds of Norway spruce (*Picea abies* L) were kindly supplied by the Institute for Breeding of Forest Plants in

Waldsieversdorf/Eberswalde (FRG). They had been collected from the forest in Elstra/Kamenz (FRG) and were stored at 4 °C until use.

Sterilization

Seeds were surface sterilized in 0.2 % $HgCl_2$ for 6 min and washed carefully in sterilized double distilled water.

Culture media for continuous in vitro multiplication of shoot buds

The basic medium was developed by slight modification of the MCM (= medium for conifer morphogenesis) described by BORNMAN and JANSSON (1982). The concentrations of $(NH_4)_2SO_4$ and Fe-EDTA were reduced to 100 mg/l and 2,76 mg/l, respectively. The concentrations of the remaining MCM-components were reduced to one half. The medium contained 1 % sucrose and 0.7 % agar.

Media with different concentrations of 1:1 combinations of BA and KIN or ZEA and media prepared without cytokinins or with 0.05 μM KIN, BA, ZEA or 2 ip were alternative during cultivation. Media referred to as differentiation phase were prepared without or with 0.05 μM cytikinin were used for interphases.

Culture medium for the induction of elongation of shoot buds

This culture medium was called "BEM" and contained the following macronutrients: KH_2PO_4, 170 mg/1; KNO_3, 950 mg/l; HN_4NO_3, 200 mg/l; $MgSO_4X6H_2o$, 185 mg/l; $CaCl_2X2H_2O$, 77 mg/l.

Micronutrients of BEM were half concentrated of those of the medium "MCM" (BORNMAN and JANSSON, 1982) and vitamins used for BEM were half concentrated of those described by BOULAY (1979).

In vitro culture

Sterilized seeds were placed on agar-solidified growth regulator containing medium in Petri dishes. The growth regulator combinations used during germination and the following growth regulator phases were identical. The dishes were sealed with parafilm and germination was allowed to proceed at 25 °C in the dark for 4 weeks at which time the seedlings were dissected. In the case of seedlings larger than 2 cm, cotyledonary needles, hypocotol segments and/or whorl with 2 mm to 3 mm hypocotyl and about 1 mm of the cotyledons were prepared. In the case of smaller seedlings only the roots were removed. These explants were placed separately on cytokinin-free medium or on medium supplemented by 0.05 μM KIN, BA, ZEA or 2 ip and kept in red light (16 h photoperiod; 650 nm to 700 nm, irradiance 40 μmol m^{-2} s^{-1}) for 3 to 4 weeks. Subsequently a growth regulator phase of 1 or 2 weeks and a 3 to 4 week differentiation phase alternated permanently. The germination phase is considered as the 1st growth regulator phase. One growth regulator phase and 1 differentiation phase will be referred to as 1 cycle.

Clumps of buds or primordia larger than about 1 cm in diameter were dissected before transfer.

Statistical analysis

Estimation of the standard error was performed with the help of the computer program "Sigma Plot". Multiplication of single clones was statistical analysed by the

Table 1. — Average rates of multiplication of shoot buds of some established in vitro clones for 1 cycle [1 growth regulator phase and 1 interphase (for all cones 0.05 μM KIN)]. The formation of shoot buds of each clone was estimated during 4 cycles. The ages of A-, B- and C-clones at the beginning of the experiment were 7, 15, and 4 cycles, respectively.

clone	average factor of multiplication for one cycle	growth regulator phases				
		BA (μM)	KIN (μM)	ZEA (μM)	NAA (μM)	(weeks)
B-1	1.62 ± 0.05	0.5	0.5	0	0.05	2
B-6	1.55 ± 0.04					
B-8	2.28 ± 0.06					
B-9	1.14 ± 0.12					
B-10	1.64 ± 0.03					
A-1	1.18 ± 0.02	1.1	1.1	0	0.05	1
A-2	1.54 ± 0.02					
A-3	1.63 ± 0.05					
D-1	1.84 ± 0.05	0	0	10	0.05	2
D-2	1.78 ± 0.04					
D-3	1.31 ± 0.12					

Table 2. — Number of shoot buds and average rates of multiplication of established in vitro clones depending on different cytokinins during 2 interphases. As growth regulator phase 10μM ZEA for 2 weeks was used. Shoot buds were induced and propagated using alternately growth regulator phases with 0.5 μM of BA and KIN for 2 weeks and interphases with 0.05 μM KIN for 3 to 4 weeks (see methods). The age of the clones at the beginning of the experiment was about 1 year.

clone		0.05μM KIN	0.05μM ZEA	0.05μM BA	0.05μM 2ip	without
B-20	a(a)	40	60	55	65	63
	b(b)	55	70	64	80	86
	c(c)	78	85	99	110	122
	d(d)	1.43	1.19	1.26	1.30	1.39
B-21	a(a)	25	24			35
	b(b)	30	34			40
	c(c)	38	36			44
	d(d)	1.24	1.24	n.d.	n.d.	1.12
B-22	a(a)	48	70	95		55
	b(b)	58	90	115		60
	c(c)	81	126	150		73
	d(d)	1.30	1.34	1.26	n.d.	1.16
B-24	a(a)	44	26		50	57
	b(b)	68	32		69	84
	c(c)	98	45		131	123
	d(d)	1.50	1.32	n.d.	1.38	1.47

(a) — number of shoot buds at the beginning of the experiment; (b, c) number of shoot buds after the 1st and the 2nd cycle, respectively; (d) average rates of multiplication for 1 cycle.

„Mann Whitney U-test". An according computer program was developed by W. Jank.

Results

(i) Establishment of clones

It was possible to establish 5 % to 10 % of the tested seedlings as meristematic *in vitro* cultures by intermittent application of growth regulators (Kunze et al., 1993). These lines have now produced shoot buds over a period of more than 4 years.

Such genotypes, from which no established cultures could be obtained, either did not react at all to the permanently applied growth regulators or produced callus in addition to buds, which turned brown. Such cultures finally died.

Cultures grown over a period of at least 1 year were well established; in general, we did not observe deficiencies among them.

Meristematic cultures derived from 1 single seedling were named as 1 clone and considered as 1 genotype.

(ii) Multiplication rates of some clones

Individual clones differed in their efficiencies of propagation. Rates of multiplication within one cycle of cultivation (1 growth regulator phase and 1 interphase) ranged from 1.14 to 2.28 (*Tab. 1*).

All of the 3 variants of growth regulator phase tested (*Tab. 1*) resulted in an analogous spectrum of multiplication rates. The average multiplication rate was about 1.57 for 1 culture cycle. The efficiency of propagation of each clone was stable during the long term culture (4 years tested), that means clones growing very slowly retained this property during all of the conditions tested (increasing the level of applicated cytokinins, additional application of auxins during the growth regulator phases and/or the interphases (*Tab. 2 and 3*).

(iii) Effect of IAA on two in vitro clones

Two clones (B-8 and B-9) were randomly chosen to test the effect of IAA application. These clones differed widely from each other in their response. B-8 could be propagated

Table 3. — Number of shoot buds and average rates of multiplication of established in vitro clones for 1 cycle depending on different concentrations of ZEA and NAA during 3 growth regulator phases (each for 1 week). During 3 to 4 weeks interphases 0.05 μM KIN was applicated. Shoot buds were induced and propagated using alternately growth regulator phases with 10 μM ZEA for 2 weeks and interphases with 0.05 μK KIN for 3 to 4 weeks (see methods). The age of the clones at the beginning of the experiment was about 10 month.

clone		10µM ZEA		clone	20µM ZEA	
		0µM NAA	0.05µM NAA		0µM NAA	0.05µM NAA
D-52	a[(a)]	60	15	D-55	18	58
	b[(b)]	78	19		39	92
	c[(c)]	132	33		104	275
	d[(d)]	1.30	1.30		1.75	1.68
D-53	a[(a)]	25	18	D-56	29	31
	b[(b)]	42	35		34	35
	c[(c)]	77	65		77	43
	d[(d)]	1.45	1.55		1.15	1.12
D-54	a[(a)]	21	24	D-57	29	45
	b[(b)]	30	35		47	71
	c[(c)]	43	40		62	84
	d[(d)]	1.28	1.20		1.31	1.25

(a) number of shoot buds at the beginning of the experiment;
(b,c) number of shoot buds after the 1st and the 3rd cycle;
(d) average multiplication rate for 1 cycle.

with high efficiency (*Tab. 1*) but it is characterized of relatively small buds (*Fig. 1a*). In contrast to B-8 the propagation of the clone B-9 was very difficult (*Tab. 1*) but it consisted of robust bunds (*Fig. 1b*).

At the beginning of the experiment both clones were 1 year old. The plant growth regulators BA (0·5 μM) and KIN (0.5 μM) and NAA (0.05 μM) were applied during growth regulator phases and KIN (0.05 μM was used in interphases. About 100 buds of both clones were cultured for 3 weeks on medium with 0 μM IAA, 0.57 μmIAA, 28.5 μm IAA and 57 μM IAA, respectively. Up to 57 μM IAA we could not observe any alteration in the morphology of the B-9 buds (*Fig. 1c*). B-9 cultivated for a longer period than 3 weeks turned brown and died. Callus proliferation has never been observed. Buds of the clone B-8 cultured on 5.7 μM IAA showed altered morphology of the needles at 3 weeks (*Fig. 1b*). These alterations were the 1st phase for an extensive callus proliferation.

(iv) Induction of elongation of shoot buds of two in vitro clones

Of the buds which multiplied efficiently (factor of multiplication higher than 1.5 for 1 cycle) less than 1 % started to elongate during permanent multiplication of the buds. In contrast, inefficiently multiplying *in vitro* clones (factor of multiplications for 1 cycle lower than about 1.2) had a higher rate of bud elongation· Induction of elongation could be achieved by cultivation of the propagated buds on BEM. As can be seen from *table 4*, the elongation started after a 10 weeks culture on BEM and, depending on the genotype, up to 100% induction of elongation could be achieved. Spontaneous elongation of the control buds cultivated for propagation in 2 phases was lower than 1 %. Unfortunately, all these elongated shoots were weak and most of them showed vitrification. The problem of vitrification could partly be overcome by using Gelrite instead of agar.

Up to date it was not possible to root these shoots.

Discussion

A major objective in *Picea abies* tissue culture is the development of a meristematic tissue culture system allowing infinite subculture and continuous production of adventitious buds and finally plants. For *Picea abies* regeneration systems via organogenesis have been published by several authors (for example Jansson and Bornman, 1980; von Arnold, 1982), but a method for permanent propagation is not available. The 1st system for continuous multiplication of shoot buds induced on segments of seedlings was described by Kunze et al. (1993). By means of this method, sufficient meristematic tissue of 1 single genotype can be provided. Among individual clones ap-

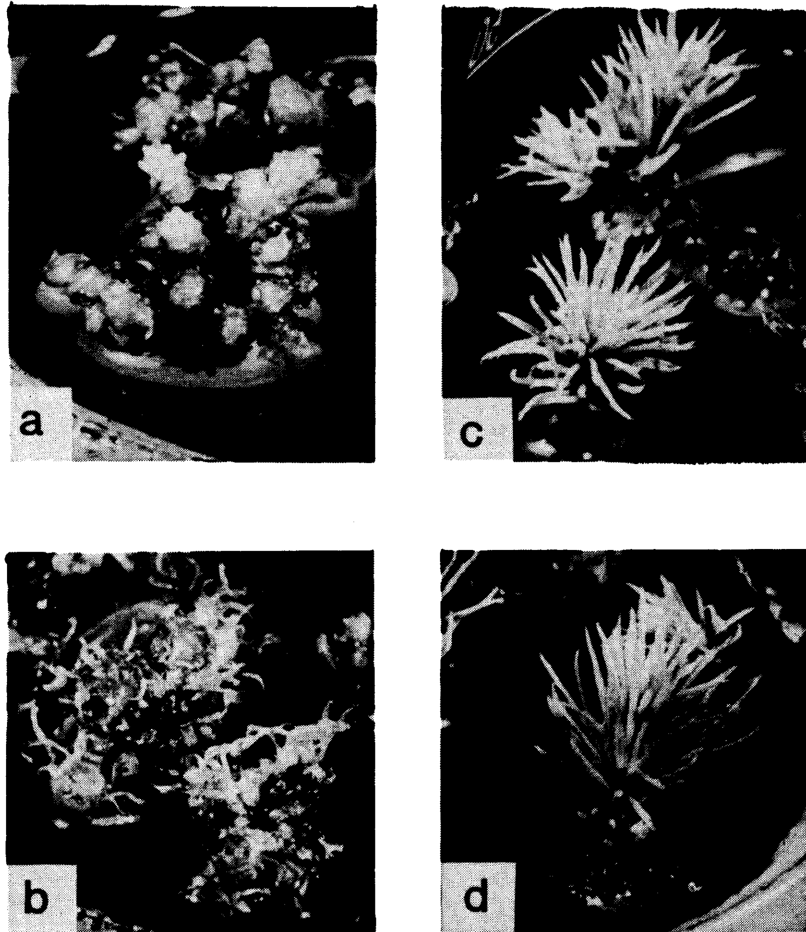

Figure 1. — Clone B-8 (a) and B-9 (c) during cultivation in 2 phases (with and without growth regulators as described in results) and B-8 (b) and B-9 (d) after a 3 weeks period on medium with 5.7 μM IAA and 57 μM IAA, respectively (X 1.7).

parent phenotypical differences could be observed. These characteristics have not changed during the whole time of cultivation. Therefore a high genetic stability of the cultures during long term cultivation can be suggested. There have also been some reports showing that tissue cultures of *Picea abies* and plantlets derived from it via organogenesis retain stable nuclear DNA content (VON ARNOLD, 1982; HAKMAN et al., 1984) in contrast to cultures of *Pinus coulteri* (PATEL and BERLYN, 1982).

On one hand genetic stability is a prerequisite for commercial use, because plantlets and at the end trees produced from meristematic tissue should be faithful copies of

Table 4. — Induction of buds elongation of 2 in vitro clones by cultivation on "BEM".

period	clone: B-1		clone: D-2	
(weeks)	number of elongated buds from total number	%	number of elongated buds from total number	%
0	1/51	2	0/78	0
10	18/35	52	33/130	25
25	25/50	50	176/176	100

the selected clone. On the other hand, certain genotypical properties could be an invincible barrier for the establishment and regeneration potential of some trees.

Another regeneration principle like somatic embryogenesis would lead to the same problem as organogenesis According to our experiences the genotype, in addition to the age of the explants, is the decisive criterion which determines whether explants (in our case zygotic embryos) are suitable for induction of embryogenetic cultures and for differentiation for somatic embryos or not.

Among other things the genotype may affect endogenous hormone activity (THORPE, 1978). Therefore, it can be assumed that the clone B-8, which responds very fast in the presence of IAA, contains or metabolizes more auxins like IAA or its derivatives than B-9. All of the conditions tested to improve the response of B-9 (for example to get a more efficient multiplication of the buds) failed. A higher multiplication rate and/or induction of callus could not be achieved. This means that the effect on the endogenous level of hormones could not be the only influence of the genotype which determines propagation and regeneration potential.

The extensive research on phenotypical properties (morphology, isoenzyme pattern and so on) wold allow an early selection of suitable genotypes for tissue culture but would not allow to overcome the inaccessibility of some genotypes for *in vitro* cultivation.

Acknowledgements

I am grateful to G. SAALBACH and K. MÜNTZ for their helpful discussion and critical reading of the manuscript. I also thank ILSE KNOSPE and CHRISTA WOLFF for technical assistance.

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Gains in Fusiform Rust Resistance and Height Growth in a Second Generation Slash Pine Seedling Orchard

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(Received 1st September 1993)

Summary

In 1979, seedlings obtained from wind-pollinated slash pines in a first-generation clonal seed orchard, from a second-generation seedling seed orchard established with polymix progenies from the clonal orchard, and from an unimproved check lot were planted in a progeny test. The study was installed on one site on the Upper Coastal Plain (UCP) and one site in the Flatwoods of Georgia. The UCP test site showed more differences among seedlings lots than did the other site, especially for fusiform rust. The clonal orchard produced seedlings more susceptible to rust than the check seedlings. Seedlings from the seedling seed orchard, however, were considerably more resistant to rust than were the check or the clonal orchard lots. Gains in height growth were obtained at all stages of selection for the first- and second-generation seed orchards. Gains in rust resistance were obtained where progeny testing for the trait was included in the selection process.

Key words: Pinus elliottii var. *elliottii, Cronartium quercum* f. sp. *fusiforme*, selection, breeding.

Introduction

When major tree improvement programs for the southern pines were begun in the 1950's, tree size and form were considered to be the most important traits that could readily be evaluated on candidate and comparison trees in natural or planted stands. Even those who were concerned about resistance to southern fusiform rust (caused by *Cronartium quercuum* (BERK.) MIYABE ex SHIRAI f. sp. *fusiforme*) had no practical way to evaluate resistance. Candidate trees with rust infections were avoided, but past