Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro

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VOORWOORD

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STELLINGEN

1. De in de Nederlandse bosbouw vaak gebezigde term "natuurlijk" wekt ten onrechte de esthetisch hooggewaardeerde indruk dat er in de meeste bossen nog tamelijk oorspronkelijke ecologische processen plaatsvinden. (P.R. Schütz en G. van Tol (red.). Aanleg en beheer van bos en beplantingen. p.110-112 (1981))
2. Bij de selectie van klimatologisch aan de Nederlandse situatie goed aangepaste individuen van exotische boomsoorten op grond van het uitlooptijdstip van knoppen kan de topophysis en de daarmee samenhangende groeisnelheid en groeiperiode niet buiten beschouwing gelaten worden gezien de consequenties voor de boomvorm.
   Dit proefschrift.
3. De slechte resultaten met houtige gewassen in vitro zijn in ieder geval deels gelegen in het feit dat de lichtintensiteit in veel kweekcellen te laag is om een positieve netto fotosynthese van de explantaten te bewerkstelligen.
   Dit proefschrift.
4. Uit het feit dat knoppen van de douglas in vitro orthotroop groeien kan worden afgeleid dat het mechanisme van de plagiotropie niet alleen geïnitieerd wordt vanuit de scheutinitiaal.
   Dit proefschrift.
5. De scheutinitialen van coniferen, te beschouwen als gecomprimeerde takken, maken onder tot op heden best bereikte omstandigheden in vitro een dermate langzame ontwikkeling door dat geconcludeerd moet worden dat het onmogelijk is de in vivo situatie met in vitro technieken te imiteren.
   Dit proefschrift.
6. Er van uitgaande dat het instituut van onderzoeksassistenten bedoeld is om de wetenschap op een bepaald gebied te bevorderen betekent dat het stringent vasthouden aan een maximale duur van het tijdelijk dienstverband met deze assistenten van 3 tot 4 jaar een ontoelaatbare beperking is van het onderzoek aan houtige gewassen.
7. Gezien het feit, dat de resultaten in het onderzoek tot stand komen na inspanningen door velen, is het ongewenst bij het gereed komen van een proefschrift een op zich al te kostbare formele plechtigheid te houden waarbij alleen de promovendus geëerd wordt.
8. Het loslaten van de collectieve landbouw is een bedreiging voor het socialistische systeem in de volksrepubliek China.
9. Het gebruik van privé personenauto's om naar anti-kernenergiedemonstraties te gaan brengt de toepassing van kernenergie dichterbij en spreekt dus het doel van zo'n actie tegen.

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10. Het aantal actief betrokkenen bij maatschappelijke vernieuwingen in Nederland in de laatste decennia wordt bij terugblikken door de pers schromelijk overdreven.

Proefschrift P.W. Evers
Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro
Wageningen, 14 maart 1984

CONTENTS

CONTENTS Page

CONTENTS Page

III Photosynthesis in vitro

IV Influence of topping, forcing, the sucrose concentration and the light intensity.

CONTENTS Page

CONTENTS Page

VI Comparison of growth and development in vivo and in vitro

INTRODUCTION

All trees possess a growth programme in space and time in which every meristem plays a precise role. In general, the organization of growth in plants reflects the precisely controlled genetic programme that determines their development (Hallé et al., 1978). The growth programme in time, the architectural model, determines the successive architectural phases. Architecture is here defined as the visible, morphological expression of the genetic blueprint of a tree at any one time (Hallé et al., 1978).

During the development of a certain type of architecture, a complex of morphogenetic gradients is realized (Borchert, 1976). In the subsequent phases of the architectural model the degree of differentiation of the meristems increases: the distinctions between plagiotropic versus orthotropic growth and rhythmic versus continuous growth become more and more apparent (Figure 1). Furthermore, physiological gradients increasingly emphasize contrasts in the functioning of different parts of the tree; an example of this is the differentiation into "sun" shoots and "shade" shoots (Leverenz and Jarvis, 1980). However, only a minority of trees in a population show growth that is "ideal" in terms of their inherited basic model (Hallé and Oldeman, 1970) because the precise growth patterns are often disrupted by exogenous environmental factors during their long lives (Hallé at al., 1978).

In trees showing a basic growth programme, architecture can be used to pinpoint physiological gradients. The effect of these gradients is commonly referred to as topophysis. Both architecture and topophysis are thus closely related to basic principles such as physiological ageing (Romberger, 1976); it is therefore obvious that these properties of the tree will often determine the success of vegetative propagation. Thus the growth and differentiation of a tree species have to be studied before methods of vegetative propagation can be developed (Durzan, 1982).

Most physiological processess in tree growth are still not understood, particularly in the gymnosperms. There is therefore much to be gained from studying the growth and morphogenesis of a member of this group. Using the technique of micropropagation (a new development in the vegetative propagation of trees) offered the opportunity of studying morphogenesis of parts of the tree and the consequences of tree physiology in a controlled environment (Campbell and Durzan, 1976). In the in vitro system, the morphogenesis of tree meristem products can be studied and thus the consequences of endogenous physiological gradients of the tree can be ascertained (Borchert, 1976): furthermore, in the in vitro situation fluctuating gradients are stablized.

Before meristems or shoot initials can be successfully cultured, information on the condition of the tree, its basic growth programme and the inferred topophysis of the species has to be gathered. However, since most tissue culture scientists are in a hurry to establish methods of mass propagation by means of tissue culture, the starting tissues are usually isolated with no thought of tree architecture, physiology, or condition (Brown and Sommer, 1975). An example of how the condition of the tree can influence the physiology is the variation in photosynthesis that results from climatic factors (Larcher, 1969; Künstle, 1971). If tissues are isolated from trees in different states of photosynthesis, different types of morphogenesis will occur in vitro.

It is therefore difficult to draw conclusions about the in vivo situation in the tree from the in vitro experiments: not only is there genetic variation, but explants are taken during the "flow" of gradients in the transition from one architectural phase to the next, a transition which, moreover, is modified by environmental factors. In temperate zone trees, the growth programme for the next season is already prepared in the autumn; development is retarded in winter and accelerated in spring until the buds actually flush. If the architecture of a tree is damaged of modified as a result of climatic or traumatic changes, a change in priorities will occur in the preparations for growth of the various bud types. It is therefore apparent that the point of time during the development of the tree at which the tissues are isolated is crucial for the later behaviour of the tissues in vitro. Also, if the architectural phase is artificially changed, reaction patterns and physiological gradients of the tree can be studied.

Endogenous growth regulators and transported sugars play an important role during the preparations for flushing and for a change in priorities in the tree (Kozlowski, 1971). However, the action of growth regulators in trees in vivo seems to differ importantly from that in meristems or shoot initials in vitro (Zaerr and Mapes, 1982).

It is clear that a study of the influence of growth regulators on the in vitro morphogenesis of explants from trees can give reliable information about the regulation of growth in vivo. Moreover, the way sugars from the medium are used by the explants from trees in relation with the production of sugars in these tissues in vitro through photosynthesis has not yet been studied. It has often been stated that photosynthesis is not an important process during in vitro culture (Hughes, 1981); this thesis (III) shows that cultures can have an in vivo rate of CO_2 consumption.

Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) was chosen to study the morphogenesis of shoot initials in vitro for three main reasons: because it has a

well-known architectural model, Massart's model (Edelin, 1977); because it was thought that tissue culture could overcome problems in classic vegetative propagation; and because the in vitro system enables endogenous physiological gradients in a gymnosperm species to be studied simultaneously.

The first paper describes the optimalization of micropropagation; in the second paper the seasonal changes in optima for nitrate and sucrose in the medium, and in the optimum for the light intensity are described. From the second to the sixth papers the differences in morphogenesis between shoot initials from buds in 10 topophysical positions on 2-year-old trees are described. These differences in morphogenesis were influenced by the medium (II, III, IV, V), the light intensity (II, III, IV), forcing (IV) or by topping (IV, V) mother trees or treating them with growth regulators (V). In the final (VI) paper a first attempt is made to compare in vivo flushing with the morphogenesis of shoot initials in vitro.

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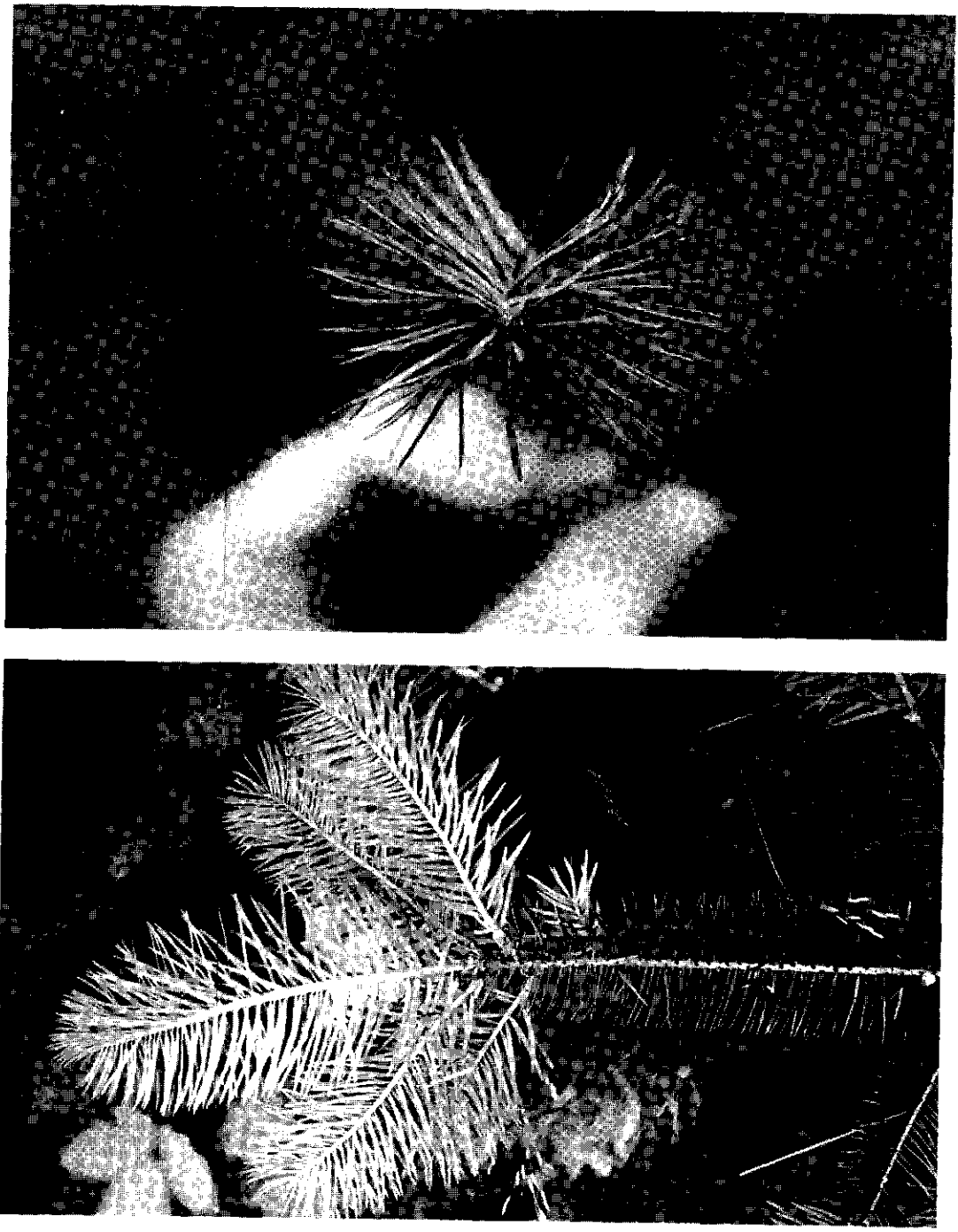

Figure 1. Plagiotropy in young twigs of Douglas fir *in vivo*. Note the spiral phyllotaxis (orthotropy) near the apex (A) and secondary dorsiventral architecture on the older parts of the growth unit (B). Naturally regenerated tree, approx. 6 years old, Sprielderbos 1983 (photograph: Oldeman).

SUMMARY

An optimalized method of micropropagation of Douglas fir is described. Seasonal changes were found in optima for nitrate and sucrose in the medium and in the optimum for the light intensity during the culture of shoot initials. Differences in morphogenesis were obtained from shoot initials that had been isolated from buds in 10 topophysical positions on 2-year-old trees. These differences between the shoots were influenced by the medium, the light intensity, and by forcing or topping the mother trees or treating them with growth regulators. Shoots from each of the topophysical positions had a characteristic photosynthesis. A first attempt is made to compare in vivo flushing with the morphogenesis of shoot initials in vitro.

SAMENVATTING

De architectuur van Pseudotsuga menziesii (Mirb.) Franco is herkenbaar als het model van Massart. Bij het doorlopen van de verschillende architecturele fasen spelen de knoppen en dus de meristemen een belangrijke rol.
De topofyse van de knoppen is gerelateerd aan de architectuur van de boom en die van de assen ervan. De ontwikkeling van scheutinitialen uit deze knoppen in vitro start aan de basis en schrijdt voort naar de apex: deze graduele progressie begint met de ontwikkeling van primordia, waarna de strekking van de primordiale as in gang wordt gezet. De groei in vitro is altijd langzamer dan in vivo; plagiotrope groei blijft achterwege en de naalden oriënteren zich niet in een vlak. De snelste ontwikkeling in vitro vindt plaats na isolatie van de initialen van de laagste takken of het laagste deel van de stam van jonge bomen in de periode direct voor het uitlopen in vivo. Ook voorbehandelingen van de moederboom (forceren, toppen, hormoonbehandelingen) kunnen de ontwikkeling van scheuten in vitro positief beïnvloeden. De groeisnelheid van scheuten van geforceerde bomen in vitro kan die van scheuten van bomen waarbij de rust op natuurlijke wijze verbroken wordt overtreffen. De optimale in vitro omstandigheden zijn: een constante temperatuur van 25° C, een vrij hoge lichtintensiteit (± 30-40 Wm^{-2}) geproduceerd door een lichtbron met een hoge opbrengst in het rode gebied (Son T; Gro Lux) gedurende 16 uur per dag en het gebruik van zoutarme media zoals dat van Heller. In tegenstelling tot de situatie bij het uitlopen van knoppen aan de boom vindt er bij de scheuten in vitro vaak een ontwikkeling van het apicale meristeem plaats, hetgeen, weer teruggeprojecteerd op de ontwikkeling in vivo, te beschouwen is als vrije of ritmische groei. De uitloop van het apicale meristeem in vitro is afhankelijk van diverse factoren, zoals topophysis, tijdstip in het seizoen, leeftijd van de boom, fysische groeiomstandigheden en de samenstelling van de media. Zowel bij de groeisnelheid als bij het optreden van vrije of ritmische groei in vitro blijkt de oorspronkelijke plaats van de knop in de boom een belangrijkere oorzaak van de daarin optredende variatie dan de genetische invloed.

In de zoutensamenstelling van het medium zijn vooral het nitraat en het ammonium essentieel voor een goede morfogenese van de scheuten van tweejarige bomen. De optimale nitraatconcentratie is afhankelijk van de topofysische positie en het tijdstip in het seizoen. Bij een lage lichtintensiteit (9 Wm^{-2}) in de kweekbuis neemt de optimale nitraatconcentratie af van 10,4 mM in november tot 4,5 mM in april terwijl bij een hogere lichtintensiteit (29 Wm^{-2}) deze concentratie toeneemt van 10,9 mM in januari tot 15,3 mM in april voor de gemiddeld beste groei en ontwikkeling. Ook de optimale saccharoseconcentratie voor de groei en ontwikkeling

van de scheuten neemt af in de loop van het seizoen, namelijk van 37,5 g/l in januari tot 33,0 g/l in april. Enkele posities wijken echter af van dit gemiddelde beeld. Een hoge saccharoseconcentratie remt de ontwikkeling van de scheuten. Dit komt mede tot uiting in een lagere maximum netto fotosynthese (P_{Nm}) en een lagere fotochemische efficiëntie (L_n). De scheutinitialen van de douglas reageren veelal niet of negatief op hormoontoevoegingen aan het medium (Cytokinines, auxines, GA_3). In 8,4% van de explantaten is het mogelijk met BAP (1,1-5,6 µM) knopvorming tussen de bladprimordia te induceren. In een aantal gevallen kan de groei van de scheuten in vitro met IAA gestimuleerd worden, vooral in de periode direct voor het uitlopen in vivo. Het IAA effect in vitro wordt bij scheuten van een aantal posities pas duidelijk na een voorbehandeling van de moederboom met BAP of GA_3 tijdens het forceren en vóór de isolatie van de knoppen; op deze wijze werd veel informatie over de topophysis verkregen. Deze gecombineerde in vivo - in vitro hormoonbehandelingen zijn effectief bij het accentueren en induceren van verschillen in morfogenese tussen scheuten van de verschillende topofysische posities. Tevens was het mogelijk met IAA wortelvorming in vitro te induceren, maar de efficiëntie was laag (maximaal 12%).

De optimale lichtintensiteit voor de groei en ontwikkeling van de scheuten in vitro fluctueert in de loop van het seizoen; op dit gemiddelde beeld zijn wederom topofysische variaties mogelijk. De optimale intensiteit in januari is 36,4 Wm^{-2}, neemt hierna toe, maar daalt in april weer tot 24,5 Wm^{-2}. De invloed van de lichtintensiteit op de fotosynthese van de scheuten in vitro hangt af van de saccharoseconcentratie van het medium; scheuten van elke topofysische positie hebben een eigen karakteristiek in de parameters van de fotosynthese. Verlaging van de lichtintensiteit van 22 tot 8 Wm^{-2} tijdens de kweek van de scheuten vertraagt de groei en ontwikkeling; deze remming kan niet gecompenseerd worden door een verhoging van de saccharoseconcentratie van het medium. Ondanks de kunstmatige in vitro omstandigheden blijken de scheuten een P_{Nm} te kunnen bereiken die goed vergelijkbaar is met die van uitlopende knoppen aan de boom. Scheuten uit knoppen in de meest "beschutte" posities vertonen de hoogste P_{Nm} en L_n en de laagste I_c (compensatiepunt).

In het algemeen groeien scheuten uit knoppen aan de laagste takken sneller dan die uit knoppen aan hogere takken; scheuten uit knoppen in terminale posities groeien sneller dan die uit axillaire knoppen. Op grond van deze generalisaties kunnen de scheuten in horizontaal en verticaal verdeelde groepen posities van knoppen ingedeeld worden. Ook bij de indeling in stadia van ontwikkeling van de scheuten (van initiaal tot vrije of ritmische groei) zijn de explantaten in positiegroepen in te delen, maar de verschillen in progressie tussen de groepen

zijn niet noodzakelijk analoog aan die bij de vergelijking van de groeisnelheid. De groepsindeling in posities van scheuten uit knoppen voor de groeisnelheidsverschillen van de explantaten in vitro blijkt ook goed op te gaan voor de rangorde in groei bij de knopuitloop aan de boom. De verschillen tussen de posities bij de ontwikkeling in vitro blijken maar voor een deel te verklaren uit de verschillen in volume van de scheuten ten tijde van de isolatie. Het aantal naaldprimordia op de scheutinitialen per eenheid van oppervlak blijkt bij de diverse posities een vrijwel constante factor.

Het forceren van de bomen voor de isolatie van scheutinitialen beïnvloedt vooral de scheuten uit knoppen in de hoogste posities in de boom. Het forceren van in plastic verpakte bomen in de kweekcel (25° C) werkt nivellerend op een aantal topofysische verschillen in ontwikkeling in vitro. Het forceren van moederbomen stimuleert vooral de kwantitatieve groei; de kwalitatieve ontwikkeling wordt eveneens positief beïnvloed, maar blijft veelal onder het niveau van scheuten geïsoleerd van bomen waarbij de rust op natuurlijke wijze werd verbroken. Het forceren van bomen remt de groei en ontwikkeling van scheutinitialen geïsoleerd uit knoppen op de stam tussen de takken; deze knoppen lijken meer voor reiteratie geprogrammeerd te zijn. De optimale duur van het forceren hangt dus af van de topofysische positie vanwaar de scheuten worden geïsoleerd alsmede van de saccharoseconcentratie in het medium.

Het toppen van de moederbomen stimuleert de groei van scheuten uit de resterende terminale posities; de gemiddelde groei van scheuten uit alle posities van getopte bomen is alleen sneller dan dat van scheuten van intacte bomen indien de kweek plaatsvindt bij een lage lichtintensiteit (8 Wm^{-2}). Het toppen remt echter het zwellen van de knoppen zodat de scheutinitiaal ten tijde van de isolatie een geringer volume heeft; scheutinitialen van getopte bomen hebben dus een groter aantal primordia per eenheid van oppervlak. Het is mogelijk de morfogenese van scheuten van enkele posities te modificeren door toediening van IAA of BAP aan het wondvlak van getopte bomen; toediening van groeiregulatoren aan beschadigde wortelstelsels van de bomen heeft echter meer consequenties voor de ontwikkeling van scheutinitialen in vitro.

Het gedrag van scheutinitialen in vitro uit knoppen in diverse topofysische posities aan douglasbomen heeft veel gegevens over de endogene fysiologische gradiënten aan het licht gebracht, maar ook vele nieuwe vragen opgeroepen. Door kweek van scheutinitialen van bomen waarbij modificaties aangebracht zijn in de architectuur is een beeld verkregen van de potentiële reactiepatronen van de knoppen onder bepaalde omstandigheden in de natuur.

Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro
I Plant, nutritional and physical factors

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CONTENTS

SUMMARY

The influence of plant, seasonal, hormonal, nutritional and physical factors on the growth and morphogenesis of shoot initials excised from vegetative buds of mainly 2 years old Douglas fir trees was studied in vitro. Growth and morphogenesis of shoot initials were best when grown on low strength salt media such as Heller's and when they had been excised from the parent tree just before flushing.

The $NaNO_3$ requirement of shoot initials from 2 years old trees decreased from November to April according to the topophysical position of the buds from which they had been isolated. The relative growth rate of shoot initials from buds on the stem appeared to be faster than that of initials from buds in other topophysical positions. The application of growth regulators did not accelerate the growth rates of shoot initials significantly.

The optimum temperature for growth and morphogenesis was 25°C; lowering the temperature during the 8 hour dark period reduced the growth rate of the shoot initials. The use of high pressure sodium lamps as light source improved morphogenesis of shoot initials considerably, compared to the development in light from other sources.

A restricted number of shoots derived from 2 years old trees were rooted on media containing a high concentration auxin; others were rooted in vivo after being dipped in a 0.2 mM IBA solution.

Key words:
Pseudotsuga - in vitro culture - morphogenesis - topophysical position - physical factors - nutrition - shoot initials.

ties of meristems at a certain age and in various topophysical positions (Romberger et al., 1970; Romberger, 1976).

The in vitro culture of shoot initials, embryo and callus tissues has been used with some success for the micropropagation of Douglas fir. (Winton, 1972; Cheng, 1975a, 1975b, 1977; Reilly and Brown, 1976; Boulay and Franclet, 1977a, 1977b;

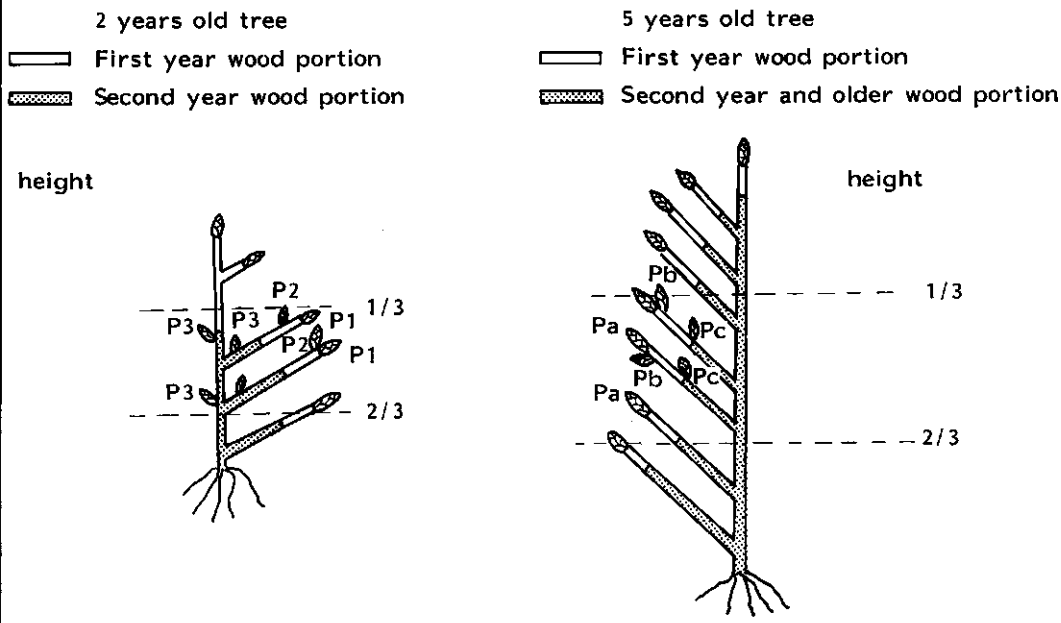
Brown and Sommer, 1977; Chalupa, 1977a, 1977b; Cheah and Cheng, 1977; Cheng and Voqui, 1977; Johnson and Carlson, 1977; Kadkade and O'Conner, 1977; Venketeswarau and Hubtinen, 1977, 1978; Winton and Verhagen, 1977a, 1977b; Kadkade and Jopson, 1978; Boulay, 1979). Since genetic abberrations are less likely to occur during the culture of shoot initials than during that of callus tissues and also because the offer the opportunity to study morphogenetic gradients in trees we decided to use these organs as starting material for the micropropagation of Douglas fir and to study their ontogenesis and physiology.

Using shoot initials Boulay and Franclet (1977a) developed a three-phase micropropagation system. First the initials undergo volume enlargement, secondly, elongation takes place and finally they form roots on specific media. Furthermore, they developed a method of multiplying the microcuttings in vitro by decapitation resulting in axillary branching. These microcuttings were also rooted in vivo (Boulay, 1979) but the percentages rooting were low. In our study the nutritional conditions required for the in vitro culture of shoot initials in the first phase of Boulay and Franclet's system (1977a), were optimalized. Particular attention was paid to the influence of the topophysical positions of the buds and the effect of the physical conditions in the culture room on growth and morphogenesis of shoot initials in vitro.

2 MATERIALS, METHODS AND DEFINITIONS

Plant material Vegetative buds were excised from 2 years old (provenance Arlington), 5 years old (provenance Courtenay), 12 years old (provenance Kootwijk) 40 years old (grafts of provenance Kootwijk) trees. The ages of the parent trees used in the various experiments are given in Table 1. In the 2 and 5 years old trees we distinguished three topophysical positions, i.e. P1, P2, P3 and Pa, Pb, Pc respectively (see Figure 1). Trees of all ages were either forced in the greenhouse at 18°C or packed in plastic bags and stored at 4°C. Intact 2 years old plants and of older trees only cut branches were stored (Table 1).

Figure 1. Distribution of topophysical positions of vegetative buds from which shoot initials were isolated for some of the experiments (specified in Table 1).



Position:
P1 : terminal bud
P2 : axillary buds near terminal bud
P3 : axillary buds on second year part of the tree

Position:
Pa : terminal bud
Pb : axillary bud near terminal bud
Pc : axillary buds near to the transition from first year to older wood

Sterilisation and preparation After the buds had been cut from the branches they were sterilized by submerging them in ethanol (70%) for about 10 seconds, followed by 20 minutes in bleaching liquor containing 2% NaOCl. However, the time in bleach was shortened to 13 minutes and the concentration lowered to 1% when the buds were isolated in March, April or June. After sterilisation the buds were washed 3 times in sterile demineralized water during totally 30 minutes.

The shoot initials, or embryonic shoots (Romberger, 1963) of Douglas fir buds are surrounded by soft white inner scales and hard brown outer scales (Allen and Owens, 1972, see Figure 2). Preliminary experiments showed that the scales were not sterile after immersion in bleaching liquor which apparently only sterilized the surface layers of the complete buds. Dissected scales could however be sterilized. When a homogenate of these sterilized scales was added to the medium it inhibited the development shoot initials in vitro. For these reasons, both inner and outer scales were removed in a laminar airflow cabinet according to the technique described by Romberger et al., (1970). The shoot initial, which is normally not infected (Kenner, 1951), is separated from the vascular tissues of the branch by a differentiated cell layer called the crown (Venn, 1965, Allen and Owens, 1972, see Figure 2) and excised in a plane just above this layer.

We always attempted to excise the shoot initials close to the crown, so that as large as possible part of the shoot was cultured (see Figure 2). However, the upper or lower half or even smaller parts of the shoot initials could also be cultured which indicates that mistakes made in preparing the shoot initials do not necessarily cause lethal effects, because the standard procedure probably concerned a quantity of tissue well above the "minimum critical mass" for survival.

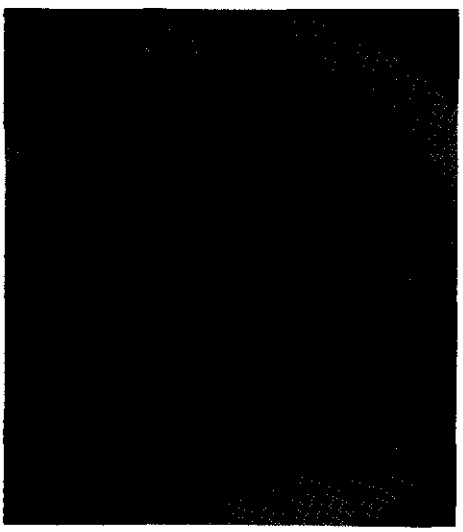


Figure 2.
Longitudinal section of a terminal vegetatieve bud (P1) of a 2 year old tree in vivo in February (12x). BS=brown scales; WS=white scales; AD=apical dome; NP=needle primordia; PA=primordial axis; CR=crown; PE(dotted line)= plane of excision.

Table 1. Summary of experiments; for further explanation see text

Experiment	Factor	Age parent tree (years)	Number of genotypes	Pre-treatment of the plant material	Time of isolation	Topophysical positions	Addition of 1mM $(NH_4)_2SO_4$	Treatments number	Treatments quality-quantity	Number of shoots per age
3.1.	Plant factors	2,5,12,40	4 x 16	various	Dec-May	-	-	1	Heller's medium, 5 isolations *	360
3.2.1.1.	Macro salt media	2,5	2 x 25	forced 3 weeks 18°C	January	-	-	10	5 media full and half strength	720
3.2.1.2.	Heller's medium	2,5	2 x 25	forced 3 weeks 18°C						
3.2.1.3.1.	NH_4^+-salts	2,5	2 x 25	forced 4 weeks 18°C	January	-	-	7	0,0.1,0.5,1,2.5,5,10x	336
3.2.1.3.2.	NH_4^+/NO_3^- ratio	2,5	2 x 25	forced 6 weeks 18°C	January	-	-	20	1,2,3,4mM of 5 salts	480
					February	-	-	9	combinations of 0.75, 1.5,3mM NH_4Cl and 3, 5,7,14mM $NaNO_3$	216
3.2.1.3.3.	$NaNO_3$	2	8	1-3 weeks plastic dark 4°C (trees)	Nov.Feb.Apr.	P1,P2,P3	+	4	0,3.5,7,14mM $NaNO_3$	1152
		5	8	4-7 weeks plastic dark 4°C (branches)	Nov.Feb.	Pa,Pb,Pc	+	4	0,3.5,7,14mM $NaNO_3$	768
3.2.1.4.	$MgSO_4.7H_2O$	2	4	trees from nursery						
3.2.1.5	$CaCl_2.2H_2O$	2	4	trees from nursery	April	-	+	3	0,1,3mM $MgSO_4.H_2O$	144
3.2.1.6.	$NaH_2PO_4.H_2O$	2	8	1-3 weeks plastic dark 4°C (trees)	April	-	+	4	0,0.5,1,3mM $CaCl_2.2H_2O$	192
		5	8	4-7 weeks plastic dark 4°C (branches)	February	P1,P2,P3	+	4	0,1,3,5mM $NaH_2PO_4.H_2O$	384
3.2.2.	Micro salt media	2,5	2 x 25	forced 5 weeks 18°C	February	Pa,Pb,Pc	+	4	0,1,3.5mM $NaH_2PO_4.H_2O$	384
	$CuSO_4.5H_2O$	2	25	forced 5 weeks 18°C	January	-	-	8	2 media at 0,1,10,100x	192
3.2.3.	Vitamins	2,5	2 x 25	forced 7 weeks 18°C	January	"	-	5	0,0.2,2,20,200mM $CuSO_4.5H_2O$	120
3.2.4.	Carbohydrates	2,5	2 x 25	forced 7 weeks 18°C	February	-	-	3	vitamin mixtures	144
					March	-	+	9	sucrose, fructose, glucose: 15,30,60 g/l	432
3.3.1.1.	IAA	2,5	2 x 25	forced 3 weeks 18°C	January	-	-	6	0.4,1.2,3.6,10.8,32.4,97.2 µM IAA	144
3.3.1.2.	IBA: parent trees	2,5,40	3 x 16	forced 3 weeks 18°C	January	-	-	6	0,2,10,25,50,100µM IBA	144
	positions	2	8	1-3 weeks plastic dark 4°C (trees)	Nov.Feb.Apr.	P1,P2,P3	+	4	0,2,8,16 µM IBA	1152
		5	8	4-7 weeks plastic dark 4°C (branches)	Nov.Feb.	Pa,Pb,Pc	+	4	0,2,8,16 µM IBA	768
3.3.2.	Cytokinin	2,5	2 x 25	trees from nursery	March	-	+	10	0,0.02,0.2,2,20 µM BAP + or - 0.3 µM GA	480
3.3.3.	Gibberellin	2,5,40	3 x 16	trees from nursery	Jan.Feb.Apr.	-	-	15	0,0.03,0.3,1.3,2.3 µM GA_3 3 lamp types	1020
3.4.1.	Temperature	2,5	2 x 25	trees from nursery	November	P1,P2,P3, Pa,Pb,Pc	+	4	light/dark: 17/17,21/21, 25/25,25/17°C	384
3.4.2.	Light source	2	8	1-4 weeks plastic dark 4°C	May	P1,P2,P3	+	4	light regimes	384

* includes general observations of all experiments

Figure 2. Longitudinal section of a terminal vegetative bud (P1) of a 2 years old tree in vivo in February (12x). BS=brown scales; WS=white scales; AD=apical dome; NP=needle primordia; PA=primordial axis; CR=crown; PE(dotted line)=plane of excision.

Tubes and basic medium The shoot initials were cultured in 22 x 150 mm pyrex test tubes containing 15 ml medium; the plane of contact between the shoot initial and nutrient solution was situated at a maximal depth of 0.5 mm below the surface of the medium. After isolation of the shoots the tubes were plugged with cotton wool. The plugs were then flamed and aluminium foil wrapped around the top of the tubes to reduce evaporation from the medium.
Unless otherwise stated, the basic culture medium contained the following components: macrosalts of Heller (1953) apart from $FeCl_3$, NaFe EDTA (68.1 µM), microsalts of Gautheret (1959), vitamins according to Quorin et al. (1974), sucrose (30 g/l), Difco Bacto agar (8 g/l) and pyrex distilled water. In part of the experiments 1 mM $(NH_4)_2SO_4$ was added (Table 1). The pH was adjusted to 5.8 before autoclaving.
The same basic medium was used for the initial culture of shoot initials as well as for subculturing for the elongation phase. The media, in some experiments also containing growth regulators, were sterilized by autoclaving for 20 minutes at 115°C and stored at 4°C upto 10 days wrapped in aluminium foil until inoculation.

Climate during the experiments The experiments were carried out in a culture room with a daylength of 16 hours supplied by fluorescent tubes (Philips TL 57/40W. When measured vertically the mean light intensity inside the test tubes (except in 3.3.3. and 3.4.2.) was 9 Wm^{-2} at the level of the explant. However, since the test tubes were placed in rows of 12 in a rack between and perpendicular to two fluorescent tubes, the light intensity in the tubes varied from 4 up to 14 Wm^{-2}. Although the room temperature was maintained at 25 ± 0.5°C, it was 1.3 ± 0.1°C higher in the test tubes nearest to the fluorescent tubes and 0.6 ± 0.1°C higher in the test tubes situated farthest from them. During the 8 hours dark period the temperature inside the test tubes was 25 ± 0.1°C.

The influence of temperature in artificial light and daylight (DL) on shoot growth and morphogenesis in vitro in experiments 3.4.1. and 3.4.2., respectively was studied in the phytotron of the Department of Horticulture, Agricultural University, Wageningen (Doorenbos, 1964). Apart from DL-light the mean light intensity inside the tubes was 27 Wm^{-2}.

Test tubes with excised shoot initials were always placed randomly in the racks both in the culture room and phytotron.

Variables tested The influence of several factors on growth and morphogenesis of shoot initials in vitro was tested; a extensive survey is given in Table 1. The shoots that survived the first phase (volume enlargement) were subcultured in the second phase to study elongation (Boulay and Fanclet, 1977a). Some of the elongated shoots were used in rooting experiments in vitro and in vivo.
Duration Experiments with shoot initials that had been isolated from 2 years old trees lasted 4 weeks in the first phase and 6 weeks in the second. In the second phase, we also studied the development of axillary shoort during 6 weeks according to the decapitation method of Boulay and Franclet (1977a). Experiments with shoot initials from older trees lasted 6 weeks in the first and 6 weeks in the second phase. The preliminary rooting experiments lasted 2 to 4 months.

Parameters measured Both the height and diameter of each shoot were measured at the end of the first and the second phase. In experiments 3.2.1.1. and 3.4.2. the initial length of the shoot initials was also measured so that a co-variance analysis of the extension growth could be carried out. Absolute and relative growth rates were calculated. In the statistical analysis the influence of the original topophysical position of the shoot initials of the mean growth rates in vitro was considered as dependent or independent of the collected genotypes or both. Linear and quadratic polynomes were tested in graphs of the growth measurements from which we were able to deduce maximal growth and optima.

Qualitative approach Qualitative standardized descriptions were made of the shoots in vitro according to a table of stages of development (Figure 3) also called an architectural diagram, as proposed by Edelin (1977).
The resulting architecture of the developing shoots in vitro is shown schematically in Figure 3, the stages drawn according to the criteria mentioned in the legend. The drawings represent a high degree of abstraction: minor details or even growth principles, not yet clear in this stage of research, may have been omitted. The significance of the influence of the different treatments on the occurrence of these qualifications was calculated using a chi-square test.

Figure 3. Stages of development of shoot initials in vitro described in terms of lenght of internodes, growth rhythm, foliar differentiation and quality of the cut surface (for further explanation see text).
The main phases of development are represented by letters A-F and variation within these basic stages by numbers 1-9; more than one variation may occur in a shoot in a certain phase.

A: little or no volume enlargement; poor or no development of needle primordia; no elongation of the pre-formed primordial axis; no extension of the apical meristem.

B: volume enlargement; slight to considerable elongation of the basal part of the primordial axis; poor to considerable development of needle primordia, being less towards the apex; no extension of the apical meristem or nearby primordia (Figures 9 and 11B).

C: poor elongation of the primordial axis; poor development of needle primordia in the apical region of the morphogenetic unit; beginning of needle development by the apical meristem (see Figures 4A, B, C and 8).

D: considerable basal and apical elongation of the primordial axis; good needle development in basal and apical part of the shoot; no extension of the apical meristem; the most apical part visible as a distinct unit.

E: good elongation of the axis, but with one or more stagnations (see Figure 5A, B and 7A, B, C); good needle development; the elongation of the pre-formed primordial axis can be poor (E1) or considerable (E2); stagnation zones marked by bud scales (total stagnation), short brown needles or just shorter needles (partial stagnations).

F: very good, simultaneous development of needles, axis and apical meristem; no stagnations, no contrasts between apical and basal part; non-predetermined apical development: free growth in vitro (Jablanczy, 1971; Cannell and Johnstone, 1978; Figures 6A, B).

1: basic shapes (A-F).

2: more elongation of the basal part of the primordial axis than in the basic shape, that in A, B and C coincides with a better needle development.

3: high needle density, sometimes caused by poor elongation of the axis.

4: basal or totally unorganized proliferation resulting in succelent-like, aqueous friable tissues.

5: partly or totally necrotic basal callus and/or cur surface, limited to the basal part of extending to the apex (indicated by ☐, see Figure 6B).

6: green callus at the base (indicated by ▦).

7: necrosis in the apical meristem and/or apical region.

8: short brown needles or bud scales around the apical region and/or along the axis (Figures 5A, B and 7B).

8a: bud scale formation resulting in a terminal bud (see Figures 7A and 8).

9: needle length resembling in vivo morphology, sometimes upward curling, thus hiding the apical region (stages A9 and B9).

Figure 3.

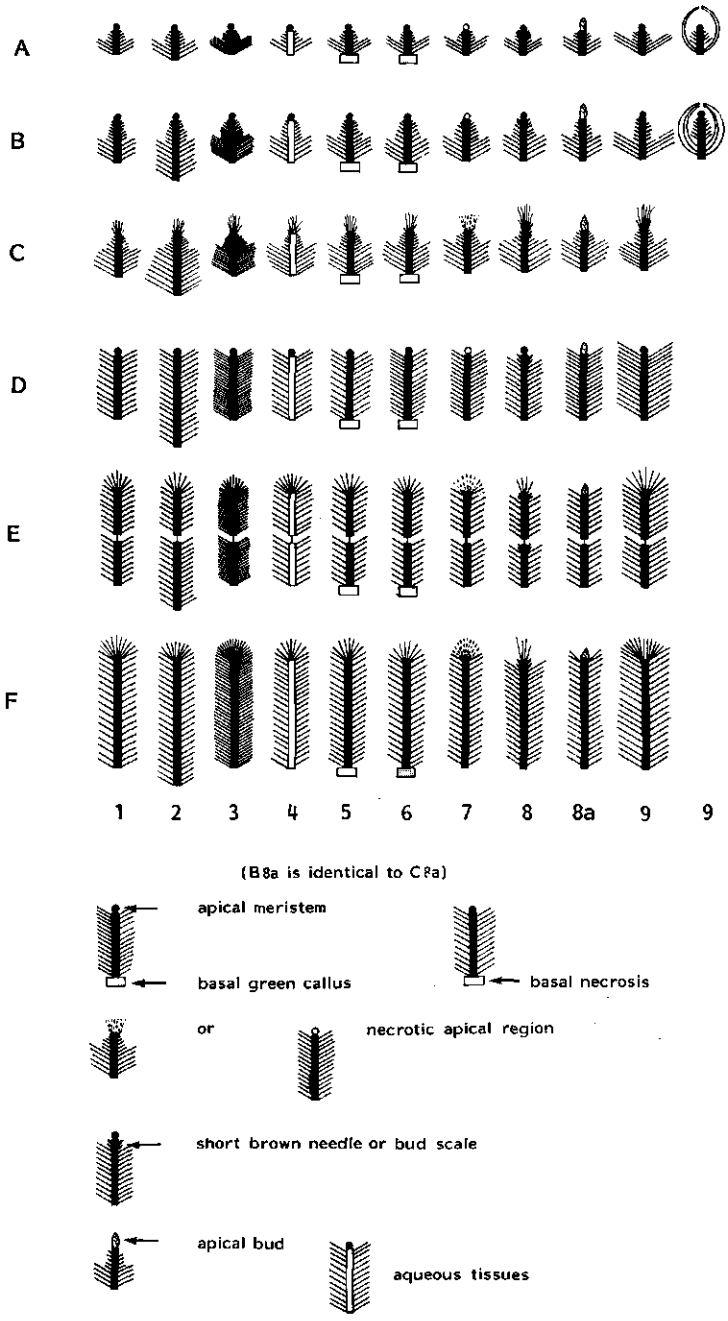

A
B
C
D
E
F
1 2 3 4 5 6 7 8 8a 9 9
(B8a is identical to C?a)
apical meristem
basal green callus
basal necrosis
or
necrotic apical region
short brown needle or bud scale
apical bud
aqueous tissues

Figure 4 Examples of cultures of type C (see Figure 3), grown from shoot initials from P1- (4A), P2- (4B) and P3-buds (4C) on a 2 years old tree, 8 weeks after isolation in February.

Figure 5 Examples of cultures of type E (see Figure 3), grown from shoot initials from a P1-bud on a 2 years old tree (5A) and a Pa-bud on a 5 years old tree (5B), 6 weeks after isolation in May.

Figure 6 Examples of cultures of type F (see Figure 3), grown from shoot initials from a P3-bud (6A) and a P2-bud (6B) on a 2 years old tree, 4 weeks after isolation in March. The P2-shoot shows basal necrosis (variation 5).

Figure 7 P1-shoot initials of type E, isolated in April from 2 years old trees, showing growth stagnations after 8 weeks in culture.
7A: partial and total stagnation (arrows); 7B: partial stagnation; 7C: old total stagnation marked by budscales (variation 8, arrow).

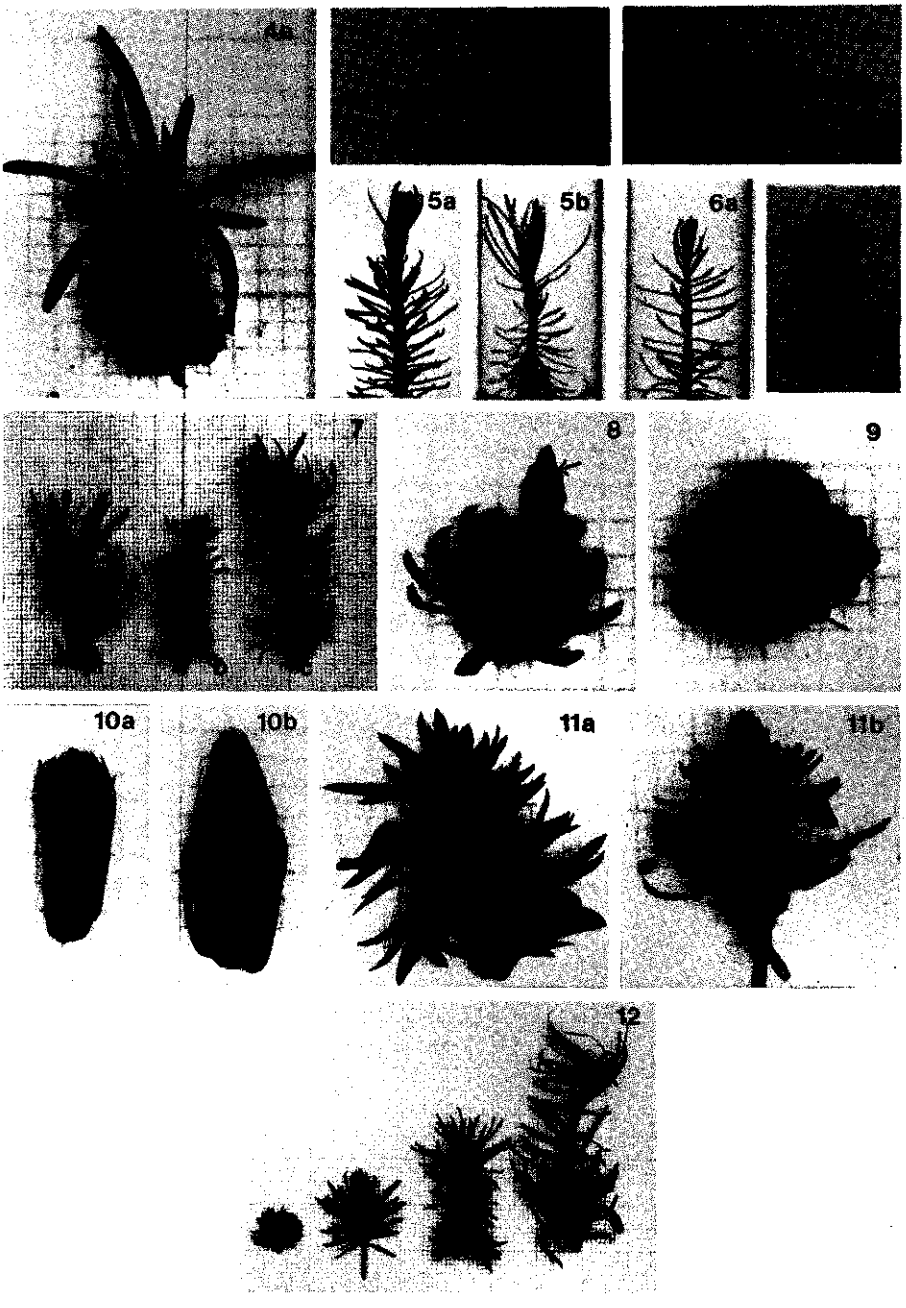
Figure 8 Example of a dormant culture of type C (see Figure 3), grown from a shoot initial from a P3-bud on a 2 years old tree, 8 weeks after isolation in March. Short brown needles (variation 8) and an apical bud (variation 8a) are visible (arrows).

Figure 9 Example of a culture of type B (see Figure 3), grown from a shoot initial from a 12 years old tree, 12 weeks after isolation in February.

Figure 10 Shoot initial isolated in May from a P1-bud on a 2 years old tree (10A) and a Pa-bud on a 5 years old tree (10B) just after excision. Note the difference in apical development.

Figure 11 Examples of cultures of type F (see Figure 3), grown from the shoot initial shown in Figure 10A, after 4 weeks in culture (11A) and of type B2 (see Figure 3) grown from the shoot initial shown in Figure 10B, after 6 weeks in culture (11B). Note the difference in the development of the apical meristems and needle primordia.

Figure 12 Example of the wide genetic variation in the cultures, in this case grown from shoot initials isolated from P2-buds on 2 years old trees in April, after 4 weeks in culture.


4a
5a
5b
6a
7
8
9
10a
10b
11a
11b
12

Definitions

Absolute growth. Growth of the shoot initials expressed as increase in height or diameter after the explants were isolated i.e. final minus initial dimensions.

Basic shape. Basic picture of the stages of development of a shoot initial, as shown in Figure 3 by stages A–F in variation 1.
These are the most observed stages during morphogenesis of shoot initials, but they do not necessarily form a sequence as they reflect different pathways of morphogenesis. Minor qualitative changes in the basic shapes are shown in the variations 2–9 in Figure 3.

Basifugal rhythm of morphogenesis. Morphogenesis of a shoot initial starting at the base and continuing towards the apex; once this basifugal development is either interrupted or completed an apical bud can be formed, often followed by the start of a new basifugal development (stages C and E in Figure 3).

Crown. Cell layers with thickened cell walls that separate the shoot initials from xylem and phloem of the branch during dormancy (Allen and Owens, 1972).

Dormancy. Period in vitro in which shoots with an apical bud (variation 8a in Figure 3) do not show any visible growth activity and cannot be reactivated by subculture. It is also referred to as total stagnation.

Extension unit. See Units of morphogenesis.

Free growth. This type of growth occurs in shoots with an extended apical meristem which leaves no visible traces on the axis (stage F in Figure 3). Shoots showing free growth have more needles than would be expected from the number of primordia, present on the morphogenetic unit (Jablanczy, 1971).

Growth regulators. IAA = indoleacetic acid; IBA = indolebutyric acid; NAA = naphthaleneacetic acid; BAP = benzylaminopurine; GA_3 = gibberellic acid

Internode. One unit of the basifugal type of growth rhythm of the shoots; an internode can be the result of the development of a pimordial axis and the needle primordia of the morphogenetic unit.

Morphogenesis. The total of quantitative and qualitative development of the shoot initials including extension growth, diameter growth and the various stages of development and modifications (variations in Figure 3).

Morphogenetic unit. See Units or morphogenesis.

Normal development. Morphogenesis of shoot initials which most resembles the rhythmic or free growth of initials that takes place after flushing in vivo; details are given in chapter 3.1.1.

Optimalization. An alteration in the culture medium or physical growth conditions that results in an improvement in growth and/or morphogenesis in terms of normal development.

Phases of morphogenesis. The 3 phases of morphogenesis of shoot initals, according to Boulay and Franclet (1977a), that result in rooted plantlets. For description see introduction.

Primordial axis. Axis of the shoot intial at the time of isolation, thus the axis of the morphogenetic unit (Romberger et al., 1970).

Relative growth. Growth of the shoot initials expressed as percentage of their initial height or diameter.

Relay axis. Axillary shoot, formed in the absence of the apical shoot meristem as a result of the lack of apical dominance (adapted from the definition of Hallé et al., 1978 for a comparable situation in vivo).

Rest. Period of arrested visible growth, for whatever or exogenous reason; often referred to as stagnation (see Stagnation). This period may be ended by subculture of may cease without any obvious reason. After re-activation, bud scales or short brown needles left on the axis show that respectively total or partial stagnation have taken place.

Stagnation. Period of rest during the sequence of the basifugal growth rhythm of the shoots in vitro, which may or may not be ended by subculturing these explants. It is not certain whether this arrested growth coincides with the termination of the development of the morphogenetic unit or whether it is an interruption in its morphogenesis. The areas of earlier stagnations are referred to as stagnation zones and it can be marked by deviate needles or bud scales.

Topophysical positions. Positions of vegetative buds in relation to the complex of growth relationships of the tree, here defined within the architectural diagram (Edelin, 1977) of the plant.

Units of morphogenesis in explants (adapted from Hallé and Martin, 1968).

a. Morphogenetic unit. Development of a shoot initial as a result of elongation of the primordial axis and development of needle primordia (stages A, B and D in Figure 3).

b. Extension unit. The part of the shoot in vitro which is the result of

extension of the apical meristem of the morphogenetic unit (upper parts of stages C, E and F in Figure 3).

Unorganized proliferation (variation 4 in Figure 3). Partial or total transformation of shoot initials into callus-like structures, resulting in destruction of part of or the whole initial. This type of morphogenesis results in succulent-like, aqueous tissues in the shoots and fast volume enlargement.

3 RESULTS

3.1. Plant factors

3.1.1. Introduction

When shoot initials of Douglas fir are isolated in vitro the development of leaf primordia into needles starts at the base of the primordial axis and subsequently proceeds towards the apex of the shoot (type A in Figure 3). Soon after the needles begin to develop, basal elongation of the primordial axis also starts proceeding in a basifugal direction (type B in Figure 3). The rates of these two processes show important differences in cultured shoots according to the age of the parent trees (see 3.1.2.). After the subculture of morphogenetic units, necessary for the completion of needle development and axis elongation up to the apical meristem (type D in Figure 3), the development of the shoots may either be interrupted by a growth stagnation (type E2 in Figure 3), or the apical meristem starts morphogenesis of a free growing extension unit (type F in Figure 3). In some cases however, the elongation of the axis is delayed until a growth stagnation occurs (type C in Figure 3). The stagnation zone is marked by short green or brown needles or bud scales on the axis (type E2 with modification 8 in Figure 3). When rhythmic growth did not yet begin after a stagnation an apical bud or short brown needles occur around the apical dome (types D8 and D8a in Figure 3, respectively). Rhythmic and/or free growth of fully developed internodes are thus considered 'normal' for shoot development in vitro.

3.1.2. Parent trees, time of isolation and topophysical position

When shoot initials were periodically isolated between October and June, there was a marked increase in the rates of growth and development and the numbers that survived in vitro. A comparable increase in morphogenetic activity was observed during the same period when shoot initials were examined in longitudinally cut vegetative buds under the stereo microscope. From June until September, none of the very small cultered shoot initials showed normal morphogenesis (see 3.1.1.) unless they had been taken from the expanding buds of 2 years old trees preparing for the flush in August.

The growth potential and survival in vitro of shoot initials, isolated from trees of different ages varied considerably; the growth potential and amount of free

growth decreased the older the parent tree. In contrast to shoot initials of 2 years old trees, the apical meristem of shoots from older trees did not extend in the first phase and, after subculture on fresh medium, irregulary in the second phase (Figure 9). Shoot initials, isolated from 12 and 40 years old trees often formed a terminal bud around the apical dome during morphogenesis in the second phase (variation 8a in Figure 3); this occurred on both fully and poorly developed morphogenetic units. We thus concluded that extension growth of shoot initials from 12 and 40 years old trees is limited in vitro by a poor elongation potential of the primordial axis. However, elongation of this axis improved the nearer flushing approached.

In shoot initials isolated from 5 years old trees, we observed a type of morphogenesis that seemed to be intermediate to that of shoots from 2 and 12 years old trees. Those initials which were isolated between October and February showed a similar development in the first phase to the shoots from 12 years old trees, growth depending on the morphogenesis of the primordial axis. However, in the second phase, extension of the apical meristem often occurred leaving short brown needles in the stagnation zone (variation 8 in Figure 3). In contrast, initials isolated in April or May, frequently resulted in shoots with both good basal and apical development in the first phase, comparable with cultures of the F-type (Figure 3) derived from shoots of 2 years old trees in the same period of isolation.

When we compared shoot initials from parent trees of different ages in the period from December to April, the best growth and morphogenesis was always observed in shoots from 2 years old trees. Free growth was often seen in these shoots; if there were bud scales or short brown needles on the axis, the preceding extension growth was often substantial, which suggests that the free growth of the morphogenetic unit was complete.

However, the difficulty remained that the shoot initials were not identical. The differences between them persist in the first culture phase (Figures 10 and 11): the progress in basifugal development of needle primordia and the primordial axes in vitro slowed down with the increasing age of the parent tree.

Apart from the genotype and age of the parent tree and time of year that the shoots were isolated, the topophysical position of the buds in vivo before excision of shoot initials also was very important for the course of morphogenesis in vitro. The shoot initials of terminal buds were always bigger at excision than those of axillary buds; the rank order of mean height of the shoot initials, starting with the largest was P1-P2-P3 and Pa-Pb-Pc (see Figure 1 and 3.4.2.). It

was impossible to eliminate these initial differences in height and diameter at the start of the experiments since not enough shoot initials of approximately the equal size could be found in buds of these positions. However, the quantitative and qualitative differences in morphogenesis in vitro between shoots of different topophysical origins could not be explained principally by just the progress of needle development or rate of volume enlargement at the time of isolation.

In general the shoot initials from terminal buds developed more slowly in the apical region and faster in the basal region than the initials from axillary buds. Other topophysical differences in morphogenesis in vitro will be described in chapters 3.2.1.3.3, 3.2.1.6, 3.3.1.2, 3.4.1 and 3.4.2.

3.2. Nutrition

3.2.1. Macrosalts

3.2.1.1. Macrosalt media

In January, shoot initials were isolated from 2 and 5 years old trees and cultured on the following macrosalt media at both full and half strength (Table 1): Heller (1953), Murashige and Skoog (1962), Harvey (1967), Sommer (1975b) and Boulay and Franclet (1977a). Elongation of the primordial axes and extension of apical meristems were best on Heller's and Harvey's medium both at full and half strength and the shoot initials had hardly any necrotic or chlorotic parts. On the other media the explants were mostly darker green and rather more compact in shape due to slow elongation of the primordial axis. This was particularly the case on Sommer's (1975b) medium where morphogenesis resulted in very dark green, compact shoots. On Murashige and Skoog's (1962), Sommer's (1975b) and Boulay and Franclet's (1977a) media the elongation of the primordial axes was better at half rather than at full strength. After subculture on Heller's medium at full strength the best elongation of the primordial axes was observed in shoots that had first been cultured on media with a relatively low salt concentration, such as Heller's or Harvey's medium at full or half strength. It can be concluded, that optimum morphogenesis of the shoot initials in the first phase is a prerequisite for a rapid elongation in the second phase. Because shoot elongation was more rapid on Heller's than on Harvey's medium, Heller's medium at full strength was used in all other experiments.

Differences in morphogenesis resulting from various media were less pronounced in shoot initials from 5 years old trees than those observed between the

more rapidly developing shoots from 2 years old trees.

3.2.1.2. Heller's medium

A comparison of development of shoot initials on media containing different concentrations of Heller's salts (Table 1) showed that morphogenesis only took place on half (0.5x) of full (1.0x) strength media. In preliminary experiments minor fluctuations in macrosalt content between 0.5x and 1.5x did not significantly change morphogenesis of the shoots.

The remaining concentrations (Table 1) resulted in necrosis and the cessation of growth and primordial development.

3.2.1.3. Nitrogen

In media used for the in vitro culture of conifers described in the introduction, NO_3^--salts were present in concentrations ranging from 2 mM up to 53 mM. In many cases NH_4^+-salts were also present in concentrations from 0.17 mM up to 30 mM. The broadness of these ranges and the important role of N-salts for the morphogenesis of shoot initials of Douglas fir observed in preliminary experiments, seemed to justify paying particular attention to the effect of NO_3^- and NH_4^+ in Heller's medium (Table 1).

3.2.1.3.1. NH_4^+-salts

Since NH_4^+-salts are not constituents of Heller's medium we investigated their effect on the morphogenesis by culturing shoot intitials on full strength Heller's medium to which 1.0, 2.0, 3.0 or 4.0 mM of $(NH_2)_2SO_4$, NH_4Cl, NH_4NO_3, $(NH_4)_2MoO_4$ or $(NH_4)_6Mo_7O_{24}$ had been added (Table 1). The best results, with neither necrosis nor chlorosis, were obtained on media containing 2mM NH_4Cl, 2mM NH_4NO_3 or 1mM $(NH_4)_2SO_4$. However, when shoots were subcultured several times on fresh media with only $NaNO_3$ as the source of nitrogen many turned yellow, seized growth and eventually died. These developments could be prevented, and in may cases reversed by adding low concentrations of NH_4^+-salts. Apical necrosis (variation 7 in Figure 3) was reduced from 32% tot 6% in shoot initials from 2 years old trees and from 60% to 12% in shoots from 5 years old trees when cultured on Heller's medium containing 2mM NH_4^+.

As a result of the promotive effects of NH_4^+-salts on morphogenesis of shoot

initials both in the initial and later culture phases it was decided to use 1mM $(NH_4)_2SO_4$ as an additional nitrogen source (Table 1).

3.2.1.3.2. NH_4^+/NO_3^- ratio

The effect of the $NH_4Cl/NaNO_3$ ratio was tested at the same time as that of the NH_4^+-salts (Table 1). Since the best development of shoot initials occurred on media with a $NH_4Cl/NaNO_3$ ratio of 1.5mM/7mM or 3mM/7mM, it was not necessary to change the standard N-nutrition (1mM $(NH_4)_2SO_4$ - 7mM $NaNO_3$) decided upon the previous section.

3.2.1.3.3. $NaNO_3$ and topophysical positions

Next the influence of the $NaNO_3$ concentration on morphogenesis of shoot initials was periodically investigated (Table 1). Since storage problems caused necrosis in branches and buds of 5 years old trees in April, this material could not be used.

Topophysical positions The differences in growth rates of shoot initials from different topophysical position (Figure 1) were generally significant. No significant differences were found between initials from positions P2 and P3 and those from Pa and Pb when isolated in November and February. The influence of the genotype was in most cases a random effect. The only significant correlation between genotype and treatment was found in shoot initials, isolated in February from 2 years old trees. Variance due to the topophysical positions was generally more important than that due to the genotypes; in such cases genotype and positions were considered to be independent of each other. However, interpretation of the results was complicated by the considerable variance in initials from the same topophysical position; that was probably caused by the fact that explants had been taken from trees with different architectures, i.e. with different development histories.

Two years old trees A quadratic correlation could be calculated between the $NaNO_3$ concentrations and the extension and diameter growth. The extrapolated optimum concentrations for $NaNO_3$ and accompanying growth are given in Table 2.

Table 2. Optimum $NaNO_3$ concentrations for extension and diameter growth of shoot initials averaged over the topophysical positions and genotypes.

Month of isolation		November			February			April		
Age of parent trees (years)	Type of growth	$NaNO_3$ conc. (mM)	SE	Optimum dimension (mm)	$NaNO_3$ conc. (mM)	SE	Optimum dimension (mm)	$NaNO_3$ conc. (mM)	SE	Optimum dimension (mm)
2	extension	10.4	0.45	6.5	9.1	0.47	10.1	4.5	0.55	20.9
5	extension	8.4	0.46	3.9	8.6	0.42	4.7	-	-	-
2	diameter	10.1	0.55	7.2	9.7	0.41	10.5	8.4	0.48	20.0
5	diameter	9.0	0.60	4.0	9.8	0.43	4.9	-	-	-

Extension growth of shoot initials from P1-, P2- and P3-buds increased from the isolation in November to April. The average maximum extension growth of shoots, isolated in April was extrapolated at a lower $NaNO_3$ concentration (4.5 mM) as compared to isolations in November (10.4 mM) or February (9.1 mM). The results of the diameter growth, however, did not show a significantly lower optimum for $NaNO_3$ for shoot initials, that where isolated in April (Table 2). No significant differences were found in extension and diameter growth of P1-, P2- and P3-shoots isolated in November and February between media containing 7 mM and 14 mM $NaNO_3$.

The extension growth figures for shoots that had been lated in April from the separate topophysical positions are given in Table 3.

Table 3. The effect of the $NaNO_3$ concentration on extension growth of shoot initials, isolated in April from P1-, P2- and P3-buds of 2 years old trees. See also Figure 13.

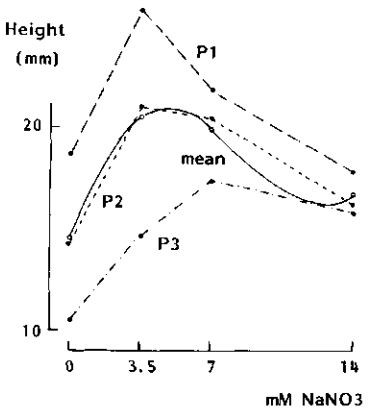


Figure 13. Graphs of the figures of Table 3.

$NaNO_3$ conc.

	Position			
(mM)	P1	P2	P3	Mean
0.0	18.69	14.22	10.53	14.48
3.5	25.87	21.00	14.75	20.54
7.0	21.78	20.38	17.41	19.85
14.0	17.78	16.13	15.81	16.57
Mean	21.03	17.03	14.63	17.86

SE treatments 0.92, same level 1.98
SE positions 1.07, same level 1.30
SE treatments/positions 1.78

Per position 128 and per concentration 96 shoots were cultured.

Most rapid extension growth of P1-shoot initials was observed on a medium containing 3.5 mM $NaNO_3$ (Table 3). The lower mean optimum concentration in April (4.5 mM), as calculated in Table 2, was mainly due to this relatively low value for P1-shoots. Comparison of the sodiumnitrate optima for the 3 topophysical positions in Table 3 shows that there is a nutritional gradient in the branch that is reflected by a shift of the optimum for extension growth in vitro from 7 mM to 3-5 mM, from P3-shoots towards P1-shoots. No such shift in optimum concentrations was found for diameter growth.

Qualitative variations The $NaNO_3$ concentrations of the medium affected the amount of basal and apical necrosis in shoot initials from 2 years old trees (Table 4). When the shoots had been isolated in November and February those that

Table 4. Influence of the $NaNO_3$ concentration on the frequency of occurrence of apical (variation 7 in Figure 3) or basal (variation 5 in Figure 3) necrosis in shoots, isolated from 2 years old trees. During November, February and April 384 shoot initials were cultured but infected shoots are omitted for each month from the results.

	$NaNO_3$ concentration (mM)	November	February	April
Number of shoots with basal necrosis	0.0	84	92	38
	3.5	14	51	14
	7.0	8	17	18
	14.0	8	23	29
Total		114	186	99
Shoots without basal necrosis		259	182	276
Number of shoots with apical necrosis	0.0	93	92	6
	3.5	18	13	15
	7.0	3	12	20
	14.0	8	14	28
Total		121	130	69
Shoots without apical necrosis		252	238	306

were least affected by necrosis (variations 5 and 7) were shoots cultured on media containing 7 mM or 14 mM $NaNO_3$; for shoots isolated in April the best media in this respect were those with 3.5 mM of 7 mM $NaNO_3$. There were significantly fewer shoots with unorganized proliferation, basal and apical necrosis (variation 4, 5 and 7), when they were grown on a medium with 7 mM $NaNO_3$ if isolated in November or February or with 3.5 mM $NaNO_3$ when isolated in April.

The occurrence of other qualitative variations (2, 3, 6, 8, 9) was not affected by the $NaNO_3$ concentration. The basal part of the P3-shoots that were isolated in November, February and April was healthier during morphogenesis than that of P1- and P2-shoots, showing less necrosis (variation 5) and better development of green callus (variation 6). When shoots had been isolated in November or February, there was more apical necrosis in cultures of P2-shoots than in those of P1- or P3-shoots.

Stages of development When shoots were isolated in November, they only developed as far as the stages A and B of Figure 3. In the succeeding periods of isolation important changes were observed in the frequency distribution of the shoots in the 6 stages of development, which was also strongly influenced by the $NaNO_3$ concentration (Tabel 5).

Table 5. The effect of $NaNO_3$ concentration and topophysical position on frequency distribution of the shoots in 6 stages of development of Figure 3 after isolation in February (1) and April (2 and 3) from 2 years old trees. Per concentration 96 and per position 128 shoots were cultured (+or- : $p<0.05$; ++or-- : $p<0.01$). Infected shoots were omitted from the results.

$NaNO_3$ conc. 1. Isolation in February

		Number of shoots								
	(mM)	stages A	B	C	D	E	F	A+B	C+D+E+F	C+E+F
	0	26+	56	1	8-	0	1	82+	10-	2-
	3.5	8	49	7	25	2	3	56	37	12
	7.0	1	45-	6	21	2	16+	46-	45+	24+
	14.0	1	60+	4	23	0	4	61	31	8
	total	35	210	18	77	4	24	245	123	46
Position	P1	6	81	2	28	0	6	87	36	18-
	P2	14	67	5	30	0	7	81	42	12
	P3	15	62	11	19	4	11	77	45	26+

$NaNO_3$ conc. 2. Isolation in April

		Number of shoots								
	(mM)	stages A	B	C	D	E	F	A+B	C+D+E+F	C+E+F
	0	2	35	0	48	0	3-	37+	51-	3-
	3.5	0	18-	1	51	2	17+	18-	71+	20+
	7.0	0	23	1	45	2	19+	23-	67+	22+
	14.0	0	38+	0	44	0	6-	38+	50-	6-
	total	2	114	2	188	4	45	116	239	51
Position	P1	1	46+	1	59	1	8-	47+	69	10-
	P2	1	38	0	69	1	10-	39	80	11-
	P3	0	30-	1	60	2	27+	30-	90	30+

$NaNO_3$ conc. 3. Isolation in April

		Number of shoots															
	stages	B				D				F				D+F			
	(mM)	P1	P2	P3	Total	P1	P2	P3	Total	P1	P2	P3	Total	P1	P2	P3	Total
	0	10	7	18+	35	16	19	13	48	2	0	1-	3-	18	19	14	51
	3.5	9	5-	4	18-	15	18	18	51	5	6	6	17+	20	20	24	68
	7.0	9	9	5	23	17	17	11	45	1	4	14+	19+	18	21	25	64
	14.0	18+	17+	3-	38+	11	15	18	44	0	0	6	6-	11	15	24	50
	total	46	38	30	114	59	69	60	188	8	10	27	45	67	79	87	233

For shoots isolated in February an optimum $NaNO_3$ concentration of 7 mM was found for apical development (C+D+E+F) and apical meristem extension (C+E+F) (Table 5.1). Most of the shoots with extension of the apical meristem developed as far as stage F thus showing free growth. However, since in February there was no difference in extension growth of the cultures on media containing either 7 or 14 mM $NaNO_3$, it was concluded that a faster elongation of shoots without apical meristem extension (stages B+D) on media containing 14 mM $NaNO_3$ compensated the lower number of free growing shoots (stage F). In February significantly more P3-shoots showed extension of the apical meristem than shoots from P2- and P1-buds; however, P3-shoots did not show a higher extension growth rate.

After isolation in April (Table 5.2) apical development of the shoots strongly improved as compared with February; it resulted in a reverse of the ratio of the numbers of shoots in the stages A+B/C+D+E+F. Morphogenesis on media containing 3.5 or 7 mM $NaNO_3$ was better than on 0 or 14 mM $NaNO_3$: a higher number of shoots with good spical development (stage C+D+E+F) and apical meristem development (stage C+E+F) was found.

It was concluded that the optimum $NaNO_3$ concentration for qualitative morphogenesis of the shoots in April was lower than after isolation in February; generally there existed a correlation between the optimum concentration for apical activity of the shoots and their extension growth rate.

When the topophysical positions were compared in April the highest number of shoots with extension of the apical meristem, almost exclusively in the F-stage, occurred after isolation from P3-buds. The number of P3-shoots which developed as far as the F-stage in April increased in comparison to February. In April the mean numbers of the stages of development of the 3 positions (Table 5.2) masked positional differences (Table 5.3). Table 5.3 demonstrates the differences between the positions, as affected by the $NaNO_3$ concentration, in the numbers of shoots reaching stages B, D and F. Although the number of long shoots reaching the F-stage is small, the optimum $NaNO_3$ concentration for free growth (Table 5.3) and for extension growth (Table 3) seem to coincide: for P1 3.5 mM, for P2 either 3.5 mM of 7 mM and for P3 7 mM. In spite of the high number of free growing P3-shoots on a medium containing 7 mM $NaNO_3$ the mean extension growth rate of these shoots was lower than the growth rates of P1- or P2-shoots on media containing either 3.5 mM or 7 mM $NaNO_3$ (Tables 3 and 5.3).

We concluded that a contrast existed between the positions in the effect of the $NaNO_3$ concentration on the progress of development and the dimensions of

the shoots.

5 Years old trees After isolation of shoot initials in November and February from 5 years old trees, extension growth of these cultures, as affected by the $NaNO_3$ concentration, resulted in a similar pattern of response as described for 2 years old trees (Table 3). However, the optimum $NaNO_3$ concentration for extension growth in November (8.4 mM) was lower than that of shoots from 2 years old trees (10.4 mM). The shoots did not differ significantly in extension growth on media containing 3.5 mM, 7 mM and 14 mM $NaNO_3$. Interpretation of the growth rates was impossible due to the high percentage of shoots with necrotic areas (70%). In November and February we found no extension of the apical meristem in the first culture phase. In February, extension and diameter growth on media containing 7 mM $NaNO_3$ was faster than on media containing either 3.5 mM or 14 mM $NaNO_3$; this reflected itself in the high number of shoots in the B stage at the expense of the A stage on a medium containing 7 mM $NaNO_3$ (Table 6). This elongation of the primordial axis on media containing 7 mM $NaNO_3$ coincided with an increase in the amount of apical and basal necrosis and unorganized proloferation (variations 4+5+7) in comparison to the development on media containing 0 mM, 3.5 mM or 14 mM $NaNO_3$.

Table 6. Numbers of shoot initials, isolated in February from 5 years old trees, developing as far as stages A and B. Per concentration 96 shoots were cultured; infected shoots were omitted from the results. (+,- : $p < 0.05$)

$NaNO_3$ conc. (mM)	Stage A	B
0	73+	23-
3.5	26	69
7.0	10-	85+
14.0	20	74
Total	129	251

3.2.1.4. $MgSO_4.7H_2O$

All of the shoot initials died on media lacking $MgSO_4.7H_2O$, whereas extension growth of the shoots did not change when the $MgSO_4.7H_2O$ concentration was increased from 1 mM to 3 mM (Table 1). After subculture of these shoots on the basic medium (1 mM $MgSO_4.7H_2O$) the extension growth of shoots previously cultured on 3 mM $MgSO_4.7H_2O$ was faster: 43 mm instead of 34 mm. However, this faster growth coincided with a high amount of unorganized proliferation (variation 4): 40 %.

3.2.1.5. $CaCl_2.2H_2O$

Since all of the shoot initials died on media lacking $CaCl_2.2H_2O$ and also because increasing the concentration of this salt during the first and/or the second phase (Table 1) had no significant effect on the extension growth of the shoots, its concentration in the basic medium was kept at 0.51 mM.

3.2.1.6. $NaH_2PO_4.H_2O$

Growth and qualitative morphogenesis of the shoots was poor on media lacking $NaH_2PO_4.H_2O$. Shoot initials isolated from both 2 and 5 years old trees did not differ in either extension growth or qualitative morphogenesis when cultured on media containing 1 mM, 3 mM or 5 mM $NaH_2PO_4.H_2O$ (Table 1), apart from the differences due to age and topophysical position. For that reason, the concentration of $NaH_2PO_4.H_2O$ was kept at 1 mM.

3.2.2. Micro salts

The micro salts of Gautheret (1959) and Murashige and Skoog (1962) (Table 1) showed no influence on growth and morphogenesis of shoot initials when added in 4 different concentrations to the basic medium. Since no effects of the micro salts could be established the basic medium was not altered.

In vivo experiments have often resulted in a special role being described to copper in the growth of Douglas fir trees (van Goor and Henkens, 1966). $CuSO_4.5H_2O$ did not seem to have any effect on the morphogenesis of shoot initials in vitro (Table 1).

3.2.3. Vitamins

A comparison of the vitamin mixtures (Table 1) of Quorin et al. (1974), Greshof and Doy (1972) and Jacquiot (Gautheret, 1959) showed that they only improved the qualitative morphogenesis of shoot initials in comparison to the control and that there was no difference in their respective effects.

3.2.4. Carbohydrates

In preliminary experiments, the influence of 3 concentrations of sucrose, fructose and glucose on the morphogenesis of shoot initials was examined (Table 1). The growth rates of the shoots on media containing sucrose were higher and the number of necortic areas in the explants lower than on media containing equal concentrations of fructose or glucose. Growth of shoot initials from 2 years old trees was slower on media containing 60 g/l sucrose than that containing 15 g/l or 30 g/l. The growth rate of shoot initials from 5 years old trees was not affected by raising the concentration sucrose from 15 g/l to 30 g/l or even to 60 g/l but it reduced the number of shoots showing necrosis.

3.3. Growth regulators

3.3.1. Auxins

3.3.1.1. IAA

The effect of the auxin IAA was tested in a broad range of concentration (Table 1). It did not improve the extension growth of shoot initials significantly in the first or second culture phase. However, increasing the IAA concentration resulted in more extensive formation of callus tissue at the base of the shoot.

3.3.1.2. IBA

Parent trees IBA, tested in the range of concentrations, given in Table 1, generally stimulated extension growth of shoot initials from 2, 5 and 40 years old trees isolated in January. However, when the shoots on media containing 25 µM, 50 µM or 100 µM IBA were compared with those on 0 µM up to 10 µM IBA, many more had developed undesirable properties (variations 4+5+7). At each concentration tested IBA stimulated the development of green callus at the base of shoots from 2 years old trees. The basal needles of these shoots curled downwards sometimes growing into the medium causing the whole shoot to be lifted from the surface of medium. Since these lifted shoots survived we presume the nutrient supply took place through the needles.

Topophysical positions The results of a more extensive experiment into the influence of IBA at 3 different periods of isolation (Table 1) were rather poor. No significant differences in growth as affected by the auxin concentration were observed either between shoots from P2- and P3-buds of 2 years old trees or between those from Pa- and Pb-buds of 5 years old trees.

IBA did not affect the diameter growth of shoot initials, isolated in December from both 2 and 5 years old trees. However, shoots isolated in February and April did show a small and equal stimulation of diameter growth on media with 2 M, 8 M or 16 M IBA. IBA also slightly stimulated the extension growth of shoot initials from trees of both ages in each of the isolation periods but in most cases the increase was insignificant.

Apical development of shoots isolated from trees of both ages, was also improved when 2 µM, 8 µM or 16 µM IBA was present in the medium (number of shoots reaching stages C+D+E+F) as compared to media lacking auxin.

3.3.2. Cytokinin

The addition of the cytokinin BAP (6-benzylaminopurine) tested in the range of concentrations given in Table 1 had a negative effect on extension growth of shoot initials. BAP often induced the formation of necrotic areas in the basal part of the shoot (variation 5). Its effect in the first culture phase of the shoots was not altered by adding 0.3 µM GA_3 to the medium.

3.3.3. Gibberellin

The influence of GA_3 in the range of concentrations given in Table 1 on morphogenesis of shoot initials was tested in 3 different periods of isolation using various light sources i.e. TL 57/60 W, ML/400 W and incandescent 60 W lamps with intensities of 12, 38 and 42 Wm^{-2} respectively.
Low concentrations GA_3 (0.03 µM and 0.3 µM) had no effect on growth of the shoots in the first and second phase, whereas higher concentrations (1.3 µM and 2.3 µM) inhibited extension growth. GA_3 had no effect on the occurrence of growth stagnations (stage E) nor did it reactivate dormant shoots. No interaction between the influence of the light source and GA_3 was observed.

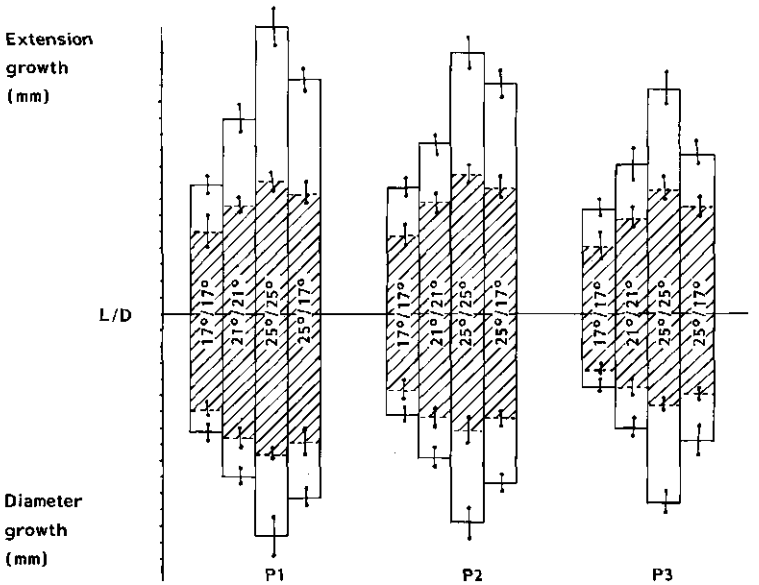
3.4. Physical growth factors

3.4.1. Temperature

The influence of four temperature regimes was investigated on morphogenesis of shoot initials from 3 topophysical positions also comparing the initials from 2 and 5 years old trees (Figures 14 and 15).

Two years old trees Extension and diameter growth were measured after the first and second culture phases (Figure 14).

Figure 14. The influence of 4 temperature regimes on extension and diameter growth of shoot initials isolated in November from P1-, P2- and P3-buds on 2 years old trees. The shoots were measured after 4 weeks in the first phase and 6 weeks in the second phase. L/D: temperature during the 16 hour light period/temperature during the 8 hour dark period. ▨ : first phase; □ : second phase.



As shown in Figure 14 a constant temperature of 25°C during the first culture phase in which the shoots increase in volume and begin to elongate resulted in higher rates of extension growth in P1-, P2- and P3-shoots than when the temperature was maintained at 21°C or 17°C for both light and dark periods during this phase.

However, when the temperature was lowered from 25°C to 17°C during the night there was no significant difference in the ectension growth rate from those of shoots kept at a constant temperature of 25°C or 21°C. When shoot initials were cultured at a constant temperature of 17°C their growth was always lower than that of those in the other regimes.

In the second phase, the differences in extension growth of the shoots in the 4 temperature regimes were more pronounced. A regime of 25°C / 17°C resulted in less extension growth than that in 25°C / 25°C, but apart from the P3-shoots was more than that in 21°C / 21°C.

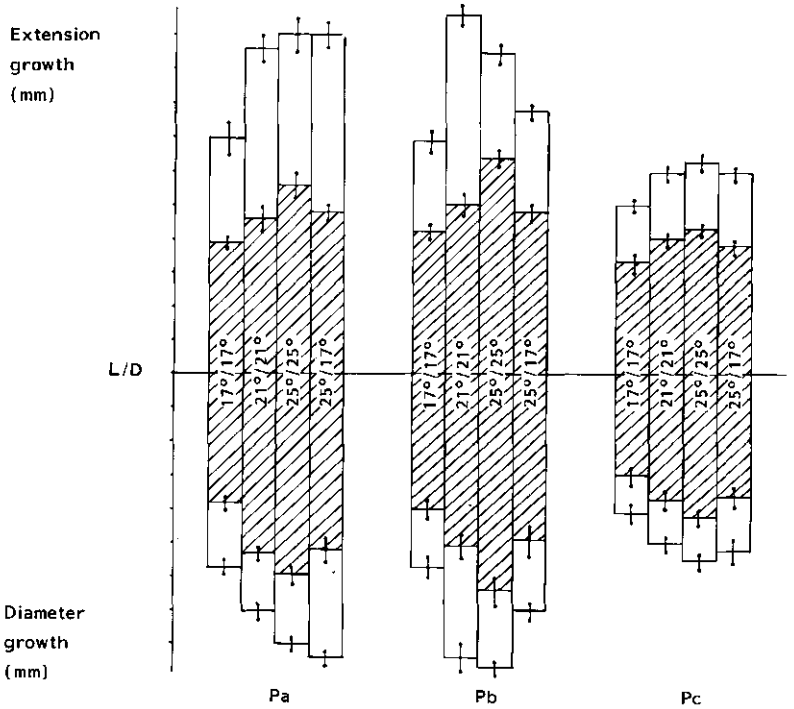
As shown in Figure 14 P1-, P2- and P3-shoots reacted similarly to the various temperature regimes. In the first phase the average extension growth of P1-, P2- and P3-shoots was about the same. However, in the second phase, P1-shoots showed a faster extension growth rate than the P2-shoots and the P2-shoots a faster rate than the P3-shoots.

Compared to the other temperature regimes a constant temperature of 25°C also proved to be best for diameter growth but the differences were not significant until the second phase.

The diameter growth rates were affected by the temperature regimes in the same way as the extension growth rates. Furthermore, necrotic areas were most seldom found on shoots that had been cultured at a constant temperature of 25°C.

Five years old trees The results of a comparable experiment with shoot initials from 5 years old trees are given in Figure 15. Height and diameter of the shoots were measured at the end of the first and the second culture phases. We again found that the optimum regime for growth during the first culture phase was a

Figure 15. The influence of 4 temperature regimes on extension and diameter growth of shoot initials isolated in November from Pa-, Pb- and Pc-buds on 5 years old trees. The shoots were measured after 6 weeks in the first phase and 6 weeks in the second phase. L/D: temperature during the 16 hour light period/temperature during the 8 hour dark period.
▨ : first phase; □ : second phase.



constant temperature of 25°C, although Pc-shoots showed the same amount of extension growth at a constant temperature of 21°C. A dark period at 17°C inhibited extension and diameter growth in all shoots to that shown by shoots cultured at a constant temperature of 21°C. Extension and diameter growth of shoots cultured at 17°C / 17°C was slower than in the other regimes. However, taken together, the mean growth rates of Pa- and Pb-shoots did not differ significantly.

Compared to the results in the first phase the mean extension and diameter growth of the shoots in the second phase present a rather divergent pattern of growth response to temperature. In Pa-shoots, the extension growth in the 21°C / 21°C, 25°C / 25°C and 25°C / 17°C regimes was equal, the fastest diameter growth taking place at 25°C / 17°C. Pb-shoots demonstrated their highest extension growth rate at 21°c / 21°C and maximum diameter growth rates at

21°C / 21°C and 25°C / 25°C. In Pc-shoots there were also no differences in the amount of extension growth at 21°C / 21°C, 25°C / 25°C and 25°C / 17°C and diameter growth was maximum both at 25°C / 25°C and 25°C / 17°C. This divergent pattern in responses of growth to temperature resulted in a treatment-position-genotype correlation in diameter growth.

We found that the mean growth rates in the second phase of shoots from all 3 positions cultured at 21°C / 21°C and 25°C /25°C no longer differed significantly. This result contrasts with that found for growth rates in the first culture phase and those from shoots from 2 years old trees. The high growth rate in regime 25°C / 17°C was mainly caused by the fast extension and diameter growth of Pa-shoots.

It was concluded that a constant temperature of 25°C was best for morphogenesis of shoot initials.

3.4.2. Light source

In preliminary experiments it was found that growth rates of shoot initials in a 8 hour light period were lower than those kept in light periods of 16 or 24 hours. Shoots cultured in a 16 hour light/8 hour dark period, had a better habit than those that had been subcultured frequently in constant light. We therefore chose 16 hours as the standard light period.

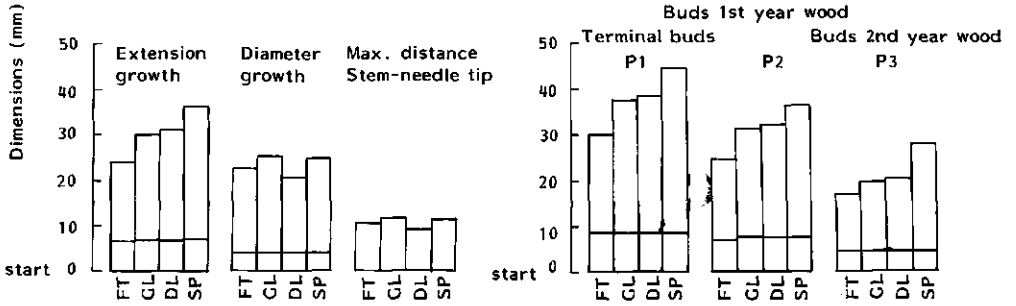
Subsequently the influence of four different light sources on growth and morphogenesis of shoots isolated in May from 2 years old trees was investigated (Table 1). The following light sources were used: daylight (DL) with a mean light intensity of 190 Wm^{-2} during the 15.5 hour light period inside the culture tubes, Philips fluorescent tubes 57/40 W (FT), Sylvania Gro-lux fluorescent tubes/40 W (GL) and Philips sodium high pressure lamps Son T/400 W (SP) with light intensities of 9, 8 and 40 Wm^{-2} respectively. Apart from the light intensities, all other conditions of the experiments were standardized; however, the DL-experiment took place in the controlled daylight room of the phytotron of the Department of Horticulture of the Agricultural University at 25°C (see chapter 2).

Figure 16. Influence of four light sources (FT, GL, DL and SP, see text) on growth of shoot initials, isolated in May from 2 years old trees, after 4 weeks in the first culture phase.

A: Influence on mean extension growth, diameter growth and maximum distance from the stem to the needle tips of P1- + P2- + P3-shoots. Start = initial dimension of the shoots.

B: Influence on extension of shoot initials, isolated from buds in the topophysical positions P1, P2 and P3.

C: Influence on relative extension and diameter growth of shoot initials from P1-, P2- and P3-buds as a percentage of the initial dimensions. SED = standard error of difference of means.



C position

		1	2	3	1+2+3
Relative extension growth (%)	FT	273.4	319.4	295.4	296.1
	GL	349.0	338.5	468.6	385.3
	DL	378.8	370.2	443.2	397.1
	SP	448.6	406.2	565.8	473.7
SED treatment/position 49.6					SED 31.9
	Mean	362.5	358.7	443.2	
SED 23.7					
Relative diameter growth (%)	FT	536.2	538.5	690.1	588.3
	GL	631.2	609.5	840.6	693.9
	DL	527.1	501.1	658.9	562.4
	SP	602.5	592.8	886.5	693.9
SED treatment/position 57.6					SED 32.3
	Mean	574.3	560.5	769.0	
SED 34.5					

The shoots showed the highest extension growth rate in SP light (Figure 16A). In GL- and DL-light the growth rates were lower but there was no significant difderence between them; however, the growth rate in FT-light, used in all other experiments except the temperature (3.4.1) experiments, was lowest. The effect of the light source was not correlated to that of the topophysical position on the morphogenesis of shoot initials in vitro, P1-, P2- and P3-shoots (Figure 16B) responding to the different light sources in similar way as that described above (Figure 16A). However, the mean relative extension growth of P3-shoots was significantly faster than that of P1- and P2-shoots (Figure 16C), which was mainly due to the high relative rate of extension growth shown by the P3-shoots in GL- and SP-light. When the relative growth rates were calculated for P1- and P2-shoots, differences in growth rate were no longer apparent.

The shoots had the maximum rate of diameter growth and distance between stem and needle tip in SP- and GL-light. The rate of the diameter growth was lower in FT-light and slightly even lower still in DL-light (Figure 16A). However, when the rates of diameter growth were expressed in relative terms this small difference between the growth of shoots in FT- and DL-light disappeared. The relative rate of diameter growth of P3-shoots was faster than that of P1- and P2-shoots in all light sources. In contrast to the extension growth there was a distinct difference betwen the diameter growth of P2- and P3-shoots when expressed as relative rates but this was not apparent in the absolute rates.

In general, no differences were found when the rates of growth in different light regimes were either expressed in mm height and diameter or as a percentage of the initial height and diameter. In a covariance analysis, which used the initial and final dimensions of the shoots, we were unable to demonstrate differences in mean growth of P1- and P2-shoot initials; larger initials gave rise to larger shoots in vitro. However, the mean starting height and diameter of P1-, P2- and P3-shoot initials were significantly different.

The light source had a marked effect on the development of the apical part of the shoot initials. In SP-light more shoots developed to the stages D, E and F (Figure 3) than in GL- and DL-light; far fewer of these shoots occurred in FT-light. P3-initials gave rise to a higher and P2-initials to far fewer shoots reaching the stages D+E+F or only F as compared to P1-shoots.

In spite of the fast rates of extension and diameter growth and the good apical development of shoots in SP-light, there was more basal and apical necrosis and unorganized proliferation (variations 4, 5 and 7) in the shoots than in GL-, DL- and FT-light.

We concluded that sodium high pressure lamps are the best light source for growth and optimum architecture of shoots in the first culture phase, although GL is the best light to use if necrosis and unorganized proliferation are to be avoided.

3.5. Morphogenesis after subculture

When elongation shoots derived from 2 years old trees in the second phase were decapitated frequently 1-3 axillary shoots or relay axes extended after the shoots had been transferred onto fresh basic medium. This procedure rapidly increased the number of shoots.

The shoots were rooted in vitro on media containing 100 µM IAA or IBA or in vivo after a 12-hour dip in a 0.2 mM IBA solution. However, the maximum percentages of shoots that rooted were still low: 12% in vitro and 19% in vivo, respectively.

4 DISCUSSION

Since the first publications on the morphogenesis of shoot initials of Douglas fir in vitro (Al-Talib and Torrey, 1959, 1961) appeared, considerable progress had been made in micropropagating this species using shoots from dormant juvenile trees (Boulay and Franclet, 1977a, 1977b, Chalupa, 1977a, Boulay, 1979). These authors used Murashige and Skoog's (1962) medium, but in our experiments (3.2.1.1) extension growth of the shoot initials was inhibited on this medium when compared to Heller's salts. Moreover, the NO_3^- and NH_4^+ concentrations of 43 mM and 20 mM respectively in Murashige and Skoog's medium proved to be supraoptimal for growth and qualitative morphogenesis of shoots from 2 and 5 years old trees (3.2.1.3). The optima for NO_3 depended on the month the initials were isolated and the topophysical position of the parent buds. However, Chalupa (1977a) and Boulay (1979) did not take these variables into account in their experiments and therefore the shift of the optimum concentration NO_3 for shoots from 2 years old trees from 10.4 mM in November to 4.5 mM in April went unnoticed.

Because of the immense genetical variability the statistical analysis of experiments concerning the micropropagation of conifer species (Brown and Sommer, 1975) present a severe problem. The use of randomly collected explants, thus abolishing topophysical properties, further obfuscates this variability. It was also found that the morphogenetic responses differed strongly between genotypes. The variance in growth and morphogenesis can expected to be less pronounced when there are more genotypes or when the plant material is genetically more homogeneous (Wochok and Abo El-Nil, 1978). Since we found that morphogenetic variance due to topophysis was even greater than that due to genotype, we feel that increasing the number of genotypes in each experiment would probably emphasize these positional differences. In some experiments shoots from topophysical positions in only one height level in the tree were used and thus these results can only give limited information about growth relationships in 2 and 5 years old trees. The differences and relations in the growth of the shoot initials in vitro can be better defined if topophysis is correlated to the periodicity in growth and architecture of the trees. Such an approach will enable a finer distinction to be made between the positions of the buds.

Two years old trees have between one and three vegetative growth periods each year and 5 years old trees just one. During each growth period elongation of the primordial axis, development of needles and the formation of terminal and

axillary buds take place. A comparable sequence of development can occur in shoots growing in vitro (stage E), some shoots forming terminal buds (variation 8a) but only rarely axillary ones. Bud scales, which indicate rest intervals amy be formed during or at the end of a growth period. Since conditions in vitro are still far from those required for a 'normal' growth period of shoot initials, the premature rest features are considered as a sign of stagnation due to a limiting environment (Hallé et al., 1978). In vitro shoots do not normally receive a cold treatment after a certain period of 'flushing' and 'free growth' (Jablanczy, 1971) that they would undergo in vivo. Death of some shoots after subculturing may therefore be due to a lack of rest periods.

The topophysical differences between shoot initials strongly depend on the treatments they receive in vitro; in some cases these differences may be completely cancelled out. The differences in morphogenetic response to the $NaNO_3$ concentration between the means of the various topophysical positions (3.2.1.3c) suggest an interaction between the salt and physiological gradients (Nozeran et al., 1971) that existed in vivo had persisted in vitro. Care should be taken in making such assumptions, however, since the most pronounced gradient in the $NaNO_3$ experiments (3.2.1.3c) was found in shoot initials that had been isolated from 2 years old trees just before flushing. As it is just in this period that fast and important changes in nitrogen metabolism are taking place (Kreuger, 1967, Durzan, 1976) it is difficult to adjust the medium to the physiological gradient at the time of isolation. The growth activity gradient or time of flushing gradient that at the time of their isolation may be present in buds in different topophysical positions in vivo, may also have consequences for the development of the initials in vitro.

In general, shoot initials isolated in April and May, have already completed in vivo a considerable part of development that takes place in the first phase of culture in vitro i.e. volume enlargement and development of needle primordia (Figures 10A and 10B). This seasonal morphogenetic gradient makes it difficult to compare this in vitro development with that of shoots isolated in other months of the year. Furthermore, there is a morphogenetic gradient that depends on the age of the tree and this affects the start of increased activity that takes place in vivo just before flushing. Therefore, the comparison made in our experiments of the effects of various treatments on the morphogenesis of shoot initials of trees of different ages is not possible directly.

In shoot initials from 5, 12 and 40 years old trees, the extension of the apical meristem depends on the vigour of the primordial axis and needle primordia; the development of the apical part of the shoots is poor, except when they are isolated

in April or May. In these shoots no new apical primordia are formed in vitro. Because of the poor development and lack of free growth we were unable to demonstrate the finer differences in morphogenesis due to topophysical position. The decline in the vigour of shoot initials in vitro with the increase in age of the parent tree also restricts further morphogenesis after subculture.

The important changes in morphogenesis of shoot initials in vitro brought about by altering the physical growth environment (3.4), imply the need for further investigations into the influence of related factors such as temperature and daylength and light intensity and quality. When physical factors are no longer limiting for morphogenesis, relatively small modifications of the medium may also be effective in inducing changes in the development of shoot initials. This is probably true for the use of growth regulators, even though we found them to be ineffective in most of the experiments. The optimum physical environment needs to be determined before a system in which the amounts of the different components in a particular medium can be varied to meet the needs of the shoot initials as they change according to the seasonal morphogenetic gradient.

In the light source experiment (3.4.2) both absolute and relative growth rates were determined and it was noted that the effect of the light sources on morphogenesis of shoot initials was not obscured by differences in the initial dimensions of the shoots. From this we may conclude that such differences do not mask the effects of various other treatments in earlier experiments.
In contrast to the mean absolute extension growth data of P1-, P2- and P3-shoots the relative growth figures bring to light deviate differences due to position, P3-shoots appearing to have a higher instead of a lower growth rate. This can possibly be explained by assuming that their tissues are at a different stage of differentiation than those of the P1- and P3-shoots. If differences will be found in rooting abilities of different types of shoots in future experiments this will be a likely explanation (Borchert, 1976, Romberger, 1976).

The stimulation of the growth rates of the shoots by increases in light intensity indicates that photosynthesis plays an important role, even under the artificial circumstances of organ culture. This is confirmed by preliminary experiments on photosynthesis of shoot initials in vitro where the uptake of CO_2 by the explants increases with increasing light intensity (unpublished results). A special role may be indicated for sucrose from Bonga's (1979) observation of the importance of CO_2 for the morphogenesis of the shoots.

5. ACKNOWLEDGEMENT

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Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro
II Growth factors, topophysis and seasonal changes

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CONTENTS

SUMMARY

The influence of the $NaNO_3$ concentration, the light intensity and the sucrose concentration on the growth and morphogenesis of shoot initials excised from vegetative buds of 2-year-old Douglas fir trees was studied in vitro.

The mean optimum $NaNO_3$ concentration for the extension growth in vitro of shoot initials increased from 10.9 mM in January to 15.3 mM in April. The optimum light intensity for quantitative morphogenesis of the shoots increased from 36.4 Wm^{-2} in January to a value above 43 Wm^{-2} in March followed by a decrease to 24.5 Wm^{-2} in April. The optimum sucrose concentration for maximal absolute increase in length of the shoots decreased from 37.5 g/l in January to 33 g/l in April.

The shoots were isolated from buds in 10 topophysical positions. Shoot initials from axillary buds are smaller than those isolated from terminal buds. In some positions the optima for growth and development did not coincide with the mean optima for all positions. In general, the morphogenesis of shoots from buds in different vertically or horizontally distributed groups of positions in the tree contrasted quantitatively and qualitatively. In January the best apical development was observed during morphogenesis of shoots isolated from buds on the stem or the lover branches; in April the shoots from terminal positions also showed active apical morphogenesis.

For each of the bud positions there was a relation between a fast extension growth rate of the shoots and the absence of callusing tissues and basal necrosis. Fast extension growth of the whole shoot i.e. morphogenetic unit and extension unit also often correlated with high apical morphogenetic activity of the shoot initials.

Key words:
Pseudotsuga - in vitro culture - morphogenesis - topophysical position - $NaNO_3$ concentration - light intensity - sucrose concentration.

1 INTRODUCTION

The architecture of a tree is the result of a hereditary differentiation programme and environmental influences starting at germination. The complexity increases as the number of meristems increases and as they show higher degrees of differentiation. The meristems initiate biomass and thus the architecture of the tree; moreover, the influence other meristems and thus generate morphogenetical gradients. Tree architecture is the visible consequence of meristem activity; types of architecture can therefore be summarized in models such as Massart's e.g. for Douglas fir (Edelin, 1977). These models include growth and differentiation properties such as rhythmic growth, plagiotropic growth, branching, flowering, etc. Each resting meristem has a special morphogenetic potential which changes with age. Since these meristems influence each other during and after initiation many types of morphogenetical gradients can be described (Wareing, 1959, 1970; Borchert, 1976; Romberger, 1976). The visible and invisible activities of meristems are strongly interrelated; architecture can therefore be used to pinpoint gradients which is commonly referred to as topophysis (Hallé et al., 1978). Romberger (1976) has stated that culturing shoot apical meristems and a study of their manifestation in vitro is a suitable technique for direct comparison of meristems properties, their degree of differentiation and thus the physiological age. In an earlier study (Evers, 1981) it was found that there are some persistent meristem properties of Douglas fir remaining in vitro which means that at least part of the differentiation was attained at the time of excision. One of those properties was the ability to grow rhythmically in vitro. Care should be taken, however, since differentiation effects concerning orthotropy and plagiotropy may be linked to rhythmic functioning or not. Rhythmic growth in Douglas fir is an endogenous rhythm in vivo and probably also in vitro which after some time becomes synchronized with climate.

Shoot initials isolated from vegetative buds somewhere in the architectural construction have often been used as a starting point for the production of micro-cuttings in vitro (Brown and Sommer, 1975). Many authors did not describe the origin of the explants nor estimate the physiological state of the initial tissues throughout the season. However, Borchert (1976), Romberger (1976) and others did not point out that morphogenesis of branches and their ability to form adventitious roots can be correlated with the architectural construction i.e. topophysis in vivo. Doorenbos (1965), Kozlowski (1971), Nozeran et al. (1971), Borchert (1976), Fortanier and Jonkers (1976) and many others have

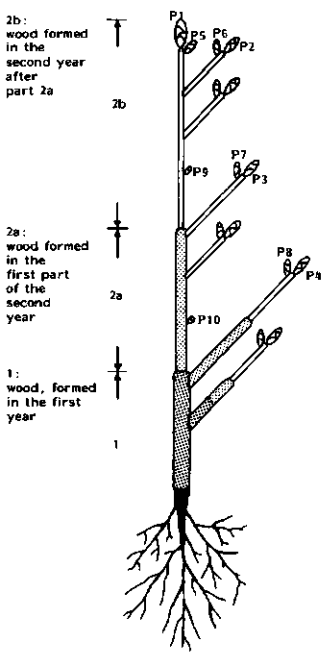
discussed the relations between physiological ageing, the concepts of juvenility and their success in inducing adventitious roots on cuttings and micro-cuttings. In Douglas fir trees it has also been found that the physiological state throughout the season (Kleinschmidt et al., 1976) and the position in the tree from which the cuttings were taken (Black, 1972; Brix, 1974; Bhella and Roberts, 1975) influenced rooting ability.

Apart from the influence of topophysis on morphogenesis and rooting of explants in vitro also dedifferentiation can increase the efficiency in producing micro-cuttings. Dedifferentiation occurs easier in very young, very small quantities of meristematic tissues. Total dedifferentiation of meristems in a high degree of differentiation can only be reached by frequent subculturing and by reducing the quantity of tissue for each subculture (Nozeran et al., 1971).

In an earlier study (Evers, 1981) the morphogenesis of shoot initials of Douglas fir in vitro was described whereas the possibility of micropropagating this species (Boulay, 1979) was confirmed. It was also demonstrated that there were differences in morphogenesis between shoots that had been isolated from vegetative buds in 3 positions in 2- and 5-year-old trees. These observations confirm the existence of morphogenetic gradients, accompanied by physiological gradients, which had been found in other plants from other evidence (Kozlowski, 1971). In this paper, the influence of 10 topophysical positions on the morphogenesis of shoot initials from 2-year-old trees in 3 periods of isolation are described in relation to the influence of the light intensity and the concentrations of $NaNO_3$ and sucrose in the medium.

2 MATERIALS AND METHODS

Plant material and pretreatments In November 1978, March 1979 and April 1979 500 trees were selected from groups of 4000 2-year old Pseudotsuga menziesii (Mirb.) Franco provenance Arlington. The root system of the trees was undercut in the field after the first year (1a1), The architectural image of the selected trees i.e. Massart's model (Edelin, 1977) and a further explanation of wood formation and bud positions is given in Figure 1. Trees with Lammas growth were not selected. An essential prerequisite tree selection was the presence of buds in 10 topophysical positions as shown in Figure 1. This selection was essential to make clear the sequence of differentiation in trees used in the experiments. All trees which did not display Massart's model, which is hereditary in Douglas fir, were considered as having a mutilated form as a result of stressed environments. The use of non-stressed trees enables normal endogenous processes to be studied.



A. Terminal positions:
Position 1(P1): Terminal buds of the tree
" 2(P2): " buds of the highest branch on stem part 2b
" 3(P3): " buds of the highest branch on stem part 2a
" 4(P4): " buds of the highest branch on stem part 1

B. Axillary positions close to terminal buds:
Position 5(P5): Axillary buds close to the P1 buds
" 6(P6): " " " " " P2 buds
" 7(P7): " " " " " P3 buds
" 8(P8): " " " " " P4 buds

C. Positions on the stem:
Positons 9(P9) : Axillary buds on the stem part 2b, closest to part 2a (Figure 2)
" 10(P10) : Axillary buds on the stem part 2a, closest to part 1

Figure 1. Positions of buds in selected 2-year-old Douglas fir trees (1a1), displaying Massart's model of architecture.

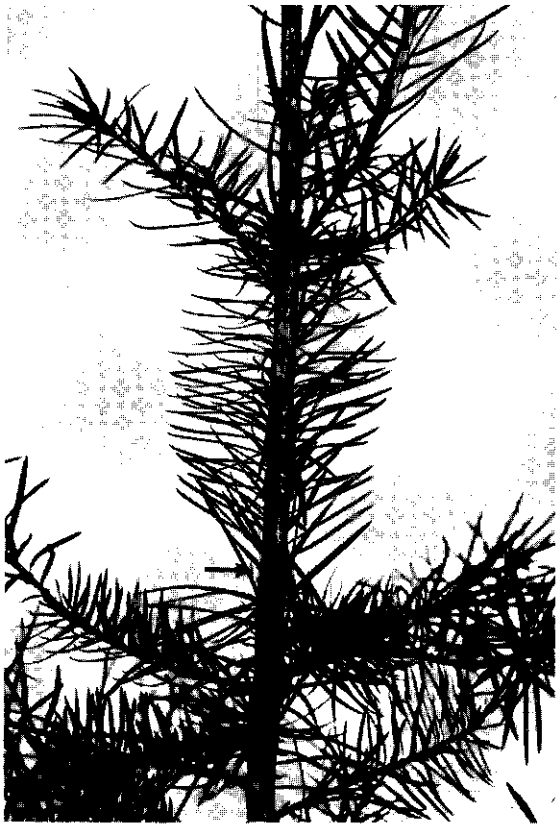


Figure 2.
2-Year-old Douglas fir tree showing the transitions of stem part 1 to part 2a, and part 2a to part 2b, marked by bud scales (arrows).

The selected trees were placed in the greenhouse at a temperature of approximately 10°C for 4 (chapter 3.2), 5 (chapter 3.3) and 6 weeks (chapter 3.4) starting the last week of November 1978, followed by a period of 2 weeks at 20°C. In the first week of March 1979, after a severe frost period of 8 weeks, a second group of trees was kept at 10°C for 1 (chapter 3.2) and 2 weeks (chapter 3.3) followed by a period of 2 weeks at 20°C. The last group of selected trees was stored in plastic bags in subgroups of 25 plants from the third week of April 1979 at 4°C for an average of 1 (chapter 3.2), 2 (chapter 3.3) and 3 weeks (chapter 3.4). Each subgroup was sufficient for 1 day's inoculation of shoot initials (see: Variables tested), thus enabling the day effect for various parameters of the pretreatments to be assessed.

Tubes and medium The preparation of shoot initials and the sterilization of media and buds were described earlier (Evers, 1981). Shoot initials were cultured in new soft glass test tubes (24 x 150 mm) that had been rinsed in distilled water.

Transparant caps (Afiplastex®, Roubaix, inner diameter 25 mm) were used to close the tubes in order to enhance the light intensity at the explant level. The gap of 1 mm between tubes and caps was covered with transparent PVC foil (Vitafilm®, Goodyear, maximum pore size 0.2 microns) permeable to oxygen and carbon dioxide. Unless otherwise stated the basic medium contained:

a. macrosalts described by Heller (1953). The $NaNO_3$ concentration was increased to 10 mM; 1 mM $(NH_4)_2SO_4$ was added; 68 µM NaFeEDTA replaced $FeCl_3$. To ensure comparability, in April the basic medium also contained 10 mM $NaNO_3$, although in an earlier paper (Evers, 1981) a lower optimum concentration was described for this period.
b. microsalts described by Gautheret (1959) for Heller's medium (1953).
c. organic compounds described by Quorin et al. (1974).
d. sucrose (30 g/l).
e. Difco Bacto® agar (8 g/l).
f. activated charcoal (Merck 2186®; as advised by Boulay, 1979). In January and March 20 g/l activated charcoal was added to the medium; in April only 5 g/l was added since it was found in preliminary experiments in this period that only low charcoal concentrations promoted growth of shoot initials.
g. pyrex distilled water.

The pH was adjusted to 5.8 before autoclaving; each tube contained 17 ml medium.

Temperature and light intensity The experiments were carried out in a culture room with 16 hours sodium high pressure light (Philips Son T®, 400 W) daily. The mean light intensity inside the culture tubes at the explant level was 29 Wm^{-2}. In the light period the temperature in the tubes was 25°C (± 0.5); in the dark period it fell to 23°C (± 0.5).

Variables tested In January, March and April, shoot initials were isolated from buds in 10 positions in the trees (Figure 1). In March, however, the experiment on the influence of sucrose was omitted because the trees had been damaged by frost. Three seperate experiments were done in which only the $NaNO_3$ concentration, the sucrose concentration and the light intensity, respectively, were varied. Four treatments of each of these factors were applied: 2.5 mM, 5 mM, 10 mM (control) and 20 mM $NaNO_3$, 7.5 g/l, 15 g/l, 30 g/l (control) and 60 g/l sucrose and 8 Wm^{-2}, 18 Wm^{-2}, 29 Wm^{-2} (control) and 43 Wm^{-2} light. The light intensity was changed by varying the distance between the culture tubes and the lamps above. In each of the 3 periods 25 shoot initials per treatment and

position were cultured: 3 x 25 x 4 x 10 = 3000 shoots per experiment. In each treatment and position of each of the experiments these groups of 25 shoots were cultured in 5 days, thus 5 per day resulting in 5 repetitions. A few shoots became infected: it was assumed that their growth would have been average and they were eliminated from the tables.

Parameters measured Before inoculation of the shoots their length was measured using millimetre paper. After 6 weeks in culture, the height and diameter of the explants were measured. The maxima in absolute and relative increase in length as well as optimum concentrations and intensities were extrapolated using linear and quadratic polynomes of the optimum graphs. It was possible to recognize the contrasts between the influences of the topophysical positions and between the periods of isolation of the shoot initials. The quantitative measurements as well as the qualitative estimations were made according to the systems of stages of development and their modifications described in a previous paper (Evers, 1981; Appendix 1). The significance of the occurrence of shoots in these stages and their modifications were tested using the chi-square method. The influence of the 10 topophysical positions was tested separately, both in horizontally (P1+P5, P2+P6, P3+P7, P4+P8) and in vertically (P1+P2+P3+P4, P5+P6+P7+P8, P9+P10; see Figure 1) distributed groups. It was possible to recognize interactions between the influence of the positions and the average effects of the treatments.

3 RESULTS

3.1. The initial length

The initial lengths of the shoot initials from buds in each of the 10 positions were randomly distributed in all treatments (section 3.2-3.4) which was later confirmed in the statistical analysis. Figure 3 clearly demonstrates the topophysical contrasts in length between the shoot initials at the successive periods of isolation. Between January and March the length of P4 shoots increased more than the mean whereas the length of P9 shoots increased less than the mean. Between March and April, the length of P1, P3, P4 and P5 shoots increased more than the mean, whereas P6, P7, P8 and P10 shoots, originally from axillary positions increased less than the mean. The in vivo growth of P2 and P9 shoots in this period did not deviate from the mean increase. The small P10 shoots from part 2a of the stem demonstrated a larger increase in length between March and April than shoots from buds in the other axillary positions; yet this increase was still less than the average extension growth in vivo in this period.

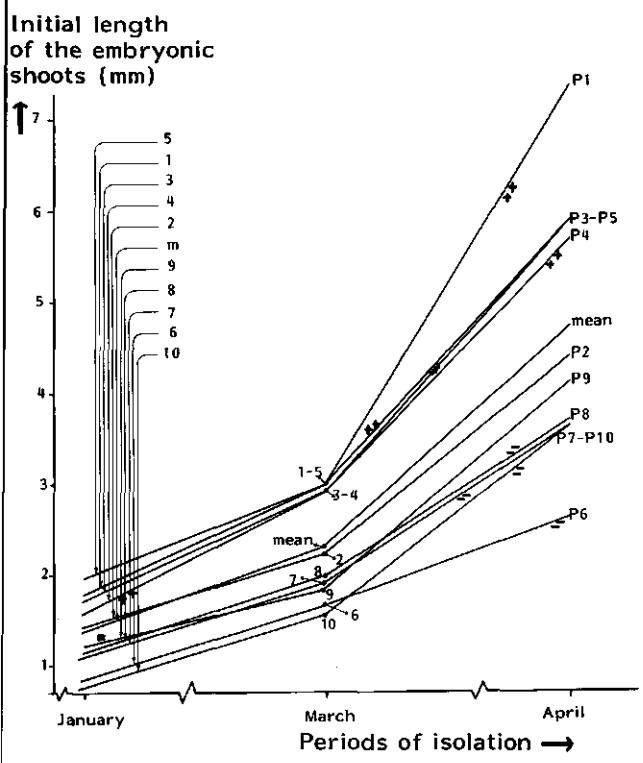


Figure 3.
Initial length of shoot initials from the positions P1-P10. Comparison of length in 3 periods of isolation averaged over all experiments (see Figure 1).

++: increase more than mean ($p < 0.01$)
--: increase less than mean ($p < 0.01$)

3.2. $NaNO_3$

3.2.1. Growth measurements

Extension growth The influence of the $NaNO_3$ concentration on the absolute increase in length of the shoot initials in 3 periods of isolation is shown in Figure 4.

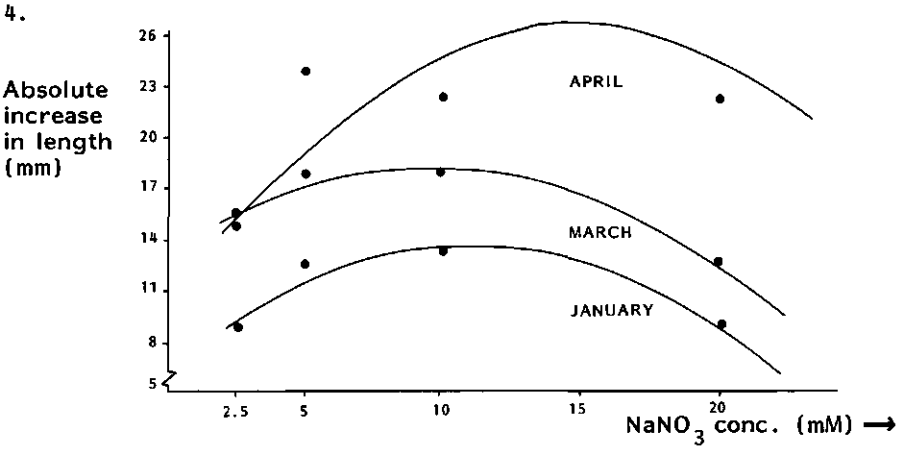


Figure 4. Influence of the $NaNO_3$ concentration on the mean extension growth of shoot initials represented in quadratic fits of the measurements in 3 periods of isolation. The calculated means of the actually used concentrations are represented by points.

The extension growth of shoot initials on all media increased from January to April, with one exception (from March to April at 2.5 mM $NaNO_3$). In none of the periods of isolation could any significant difference be observed between the mean extension growth of shoots on media containing 5 or 10 mM $NaNO_3$. In April, no significant differences in the mean extension growth rates of the shoots on media containing 5, 10 and 20 mM $NaNO_3$ were observed because of a large increase in growth between March and April on media containing 20 mM $NaNO_3$. The fast growth rate on 20 mM $NaNO_3$ resulted in the extrapolated optimum $NaNO_3$ concentration for extension growth being higher (15.3 mM) compared with the optimum concentration in March (Table 1).

Table 1. Extrapolated optimum $NaNO_3$ concentrations for the mean absolute increase in length of shoot initials in 3 periods of isolation. SE=standard error

Period of isolation	Extrapolated optimum $NaNO_3$ concentration (mM)	SE optimum $NaNO_3$ concentration (mM)	Limit of optimum in quadratic fit (mM)		Extrapolated length on optimum $NaNO_3$ concentration (mm)	SE length on optimum $NaNO_3$ concentration (mm)
			Lower	Upper		
January	10.9	0.3	10.3	11.4	13.6	0.1
March	9.6	0.7	8.1	11.0	18.0	0.1
April	15.3	2.2	11.8	18.8	27.5	1.4

Interactions between the influence of the topophysical positions and the average effect of $NaNO_3$ on the extension growth only occurred in March and April. It appeared, however, that the quadratic fits of the extension growth of shoots in these cases demonstrated contrasts in shape but no significant shifts of the *optimum* $NaNO_3$ *concentration*.

Table 2. Mean increase in length in mm of shoot initials from buds in 10 positions averaged over all $NaNO_3$ concentrations in 3 periods of isolation.

Topophysical position	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	SED
Period of isolation											
January	10.9	10.9	10.5	11.1	10.5	9.5	10.8	11.3	11.5	9.7	0.5
March	22.9	15.4	16.7	19.4	15.1	11.6	12.1	13.3	14.5	15.8	1.2
April	31.1	21.2	27.7	25.2	22.0	11.4	15.9	13.5	18.4	17.7	2.4

January When the mean extension growth figures were considered, only small differences between the positions were observed after isolation in January (Table 2). Only the initially smallest P6 and P10 shoots showed a lower increase in length than average.

March In March the graphs of the extension growth of P1 and P10 shoots demonstrated a steeper slope (Figure 5) than the graph of the mean increase in length (Figure 4) in the applied concentration range. In this range, no maximum could be established for the growth of P8 shoots in vitro (Figure 5). Nevertheless, as

is indicated by Figure 5 and Table 2, the mean increase in the length of the shoots differs considerably, according to the topophysical positions.

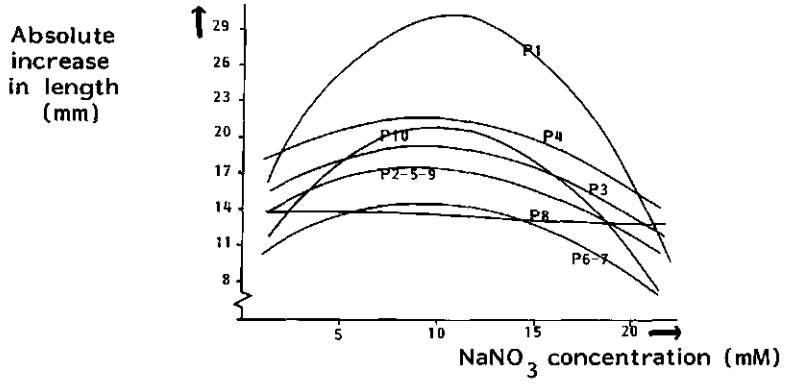


Figure 5. Influence of the $NaNO_3$ concentration on the increase in length of shoot initials, isolated in March from buds in 10 positions.

The mean increase in length of P6, P7 and P8 shoots, isolated from axillary buds on branches, was significantly less as compared with the extension growth of shoots from buds in terminal positions and from P9 and P10 buds. In spite of their small initial length (Figure 3) shoots isolated from buds on the stem (P9 and particulary P10), demonstrated a relatively fast extension growth rate in vitro. In March, P1 and P4 shoots demonstrated the fastest growth rates. Shoots isolated in March from P10 buds on the stem showed a striking increase in length compared with their relatively slow growth rate in January (Table 2).

April The quadratic fits of the extension growth of P2, P3 and P5 shoots showed a deviant slope (Figure 6) compared with the graph of the influence of the $NaNO_3$ concentration on the mean extension growth of shoots from all positions (Figure 4). The graph of the P2 shoots was flatter, whereas the graphs of the extension growth of P3 and P5 shoots, had a steeper slope than the graph of the average effect of $NaNO_3$ on the increase in length. For growth of P3 and P5 shoots an indication was observed ($p<0.1$) for the existence of a lower and for P2 shoots of a higher optimum concentration than the mean calculated 15.3 mM $NaNO_3$.

As was observed in March, shoots isolated from the axillary P6, P7 and P8 buds increased in length less in vitro than those from the other positions; shoots from buds in terminal positions again demonstrated the fastest extension growth rates. Shoots from buds in all positions demonstrated a striking improvement in growth

compared with the length increment measured in March on media containing 20 mM $NaNO_3$.

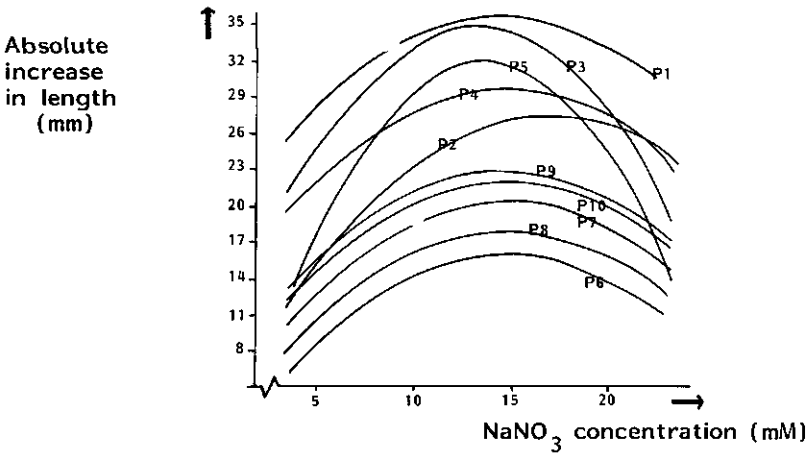


Figure 6. Influence of the $NaNO_3$ concentration on the increase in length of shoot initials isolated in April from buds in the positions P1-P10.

The contrasts in growth between shoots from buds in terminal and axillary positions became increasingly clear in April, except for P5 shoots (Table 2, Figure 6). As isolation time proceeded this divergence in the growth rates of shoot initials in vitro according to their place of origin, was generally observed.

When the increases in length of shoots, that had been isolated in March and April from buds on branches at 3 levels in the tree (low: P4+P8; middle: P3+P7 and high: P2+P6, see Figure 1) were compared, the rates of extension growth were found to decrease the higher the level from which the buds had originated. In April the contrasts in growth in vitro between shoots from buds from the 2 lower levels (P4+P8, P3+P7) disappeared.

Diameter growth The data on the diameter growth of the shoot initials (Table 3) also show a strong tendency for the optimum $NaNO_3$ concentration to rise in April compared with January. The positional contrasts in mean diameter growth of the shoots were similar to the pattern observed in the results of the extension growth.

Table 3. Extrapolated optimum $NaNO_3$ concentration for the mean increase in diameter of the shoot initials in 3 periods of isolation, averaged over all topophysical positions.

Period of isolation	Extrapolated optimum $NaNO_3$ concentration (mM)	SED optimum $NaNO_3$ concentration (mM)	Limit of optimum in quadratic fit (mM)		Extrapolated diameter in optimum (mm)	SED diameter on optimum NaNO concentration (mm)
			Lower	Upper		
January	11.9	0.3	11.2	12.6	11.6	0.1
March	11.5	0.3	10.8	12.1	17.0	0.1
April	13.3	0.8	11.6	15.0	19.9	0.3

3.2.2. Qualitative morphogenesis

Optimum concentration The $NaNO_3$ concentration strongly influenced the numbers of shoots in the standardized stages of development (Table 4).

After isolation in January most shoots with apical development (D+E+F) occurred on media containing 5 mM and 10 mM $NaNO_3$, coinciding with fewer shoots reaching stage B (Table 4). In general a better qualitative development coincided with a faster extension growth rate (Table 4, Figure 4). After isolation in March a strong increase in the number of shoots with an extended apical meristem (E+F) was observed in comparison with morphogenesis in January, in particular on media containing 5 mM and 10 mM $NaNO_3$. Morphogenesis on the 5 mM $NaNO_3$ medium was better, because there were more shoots in stage D. However, in April, a medium containing 10 mM $NaNO_3$ proved to be best for the shoots to develop to the D+E+F stages. As in the case of the growth measurements in April qualitative morphogenesis on media containing 20 mM $NaNO_3$ also improved enormously compared with the development of the shoots on this medium in March.

Topophysical positions There were differences in the qualitative morphogenesis of shoot initials isolated from buds in horizontally distributed groups of positions (P1+P5, P2+P6, P3+P7, P4+P8) and those isolated from vertically distributed groups of positions (P1+P2+P3+P4, P5+P6+P7+P8, P9+P10; Table 4).

After isolation in January (Table 4) a poor apical development was observed in shoots in vitro that had been isolated from buds in terminal positions (P1+P2+P3+P4) and from buds in the highest horizontal level in the tree (P1+P5). The maximum number of shoots in vitro with an extended apical meristem (E+F stages) occurred after isolation from buds on the stem (P9+P10), again coinciding with a

Table 4. Influence of the $NaNO_3$ concentration on the numbers of shoot initials in the various stages of development (I) with some modifications (II) in January, March and April and the distribution of these numbers in horizontally and vertically distributed groups of topophysical positions. For further explanation see appendix 1. B: no apical development; D: apical development; E+F: extending apical meristem. Decrease, increase: decrease or increase of the number after the preceding period on the same medium. -,+: $p<0.05$; --,++: $p<0.01$.

I

Period	$NaNO_3$ conc. (mM)	Numbers of shoots: in stage B	decrease B shoots	in stage D	increase D shoots	in stages E+F	increase E+F shoots	in stages B+D+E+F	in stages A+C
January	2.5	186++		36--		1--		223	27
	5	147--		69++		18+		234	16
	10	145--		69++		21++		235	15
	20	177+		42-		3--		222	28
	Total	655		216		43		914	86
March	2.5	100	--	107	++	23--	++	230	20
	5	88--	--	83	++	62++	++	233	17
	10	110		65--		61++	++	236	14
	20	146++	--	76	++	14++	++	236	14
	Total	444	--	331	++	160	++	935	65
April	2.5	82++		112++		30--		224	26
	5	54	--	95		83		232	18
	10	41--	--	92	++	97++	++	230	20
	20	53	--	83		88+	++	224	26
	Total	230	--	382	+	298	++	910	90
January	Positions								
	P1+P2+P3+P4	310++		57--		5--		372	28
	P5+P6+P7+P8	261		92		16		369	31
	P9+P10	84--		67++		22++		173	27
	P1+P5	173++		14--		0--		187	13
	P2+P6	139		37		6		182	18
	P3+P7	131-		46+		8		185	15
	P4+P8	128--		52++		7		187	13
	Total P1-8	571		149		21		741	59
March	P1+P2+P3+P4	176	--	123	++	74	++	373	27
	P5+P6+P7+P8	193+	--	141	++	40--	++	374	26
	P9+P10	75-		67		46++	++	188	12
	P1+P5	102	--	55	++	30	++	187	13
	P2+P6	97	--	65	++	18-	++	180	20
	P3+P7	86	--	79+	++	26	++	191	9
	P4+P8	84	--	65	++	40+	++	189	11
	Total P1-8	369	--	264	++	114	++	747	53
April	P1+P2+P3+P4	76--	--	147		146++	++	369	31
	P5+P6+P7+P8	120++	--	153		80--	++	353	47
	P9+P10	34-	--	82		72	++	188	12
	P1+P5	43	--	93++	++	51	++	187	13
	P2+P6	83++		50--		41-	++	174	26
	P3+P7	36-	--	75		64	++	175	25
	P4+P8	34--	--	82		70+	++	186	14
	Total P1-8	196	--	300	+	226	++	722	78

II

Modifications	$NaNO_3$ conc./positions	Numbers of shoots: Period of isolation: January	March	April	Total
	$NaNO_3$ conc. (mM)				
Callusing of the shoots (4)	2.5	171++	169++	147++	487++
	5	119--	114--	112	345
	10	105--	112--	93--	310--
	20	123	130	104	357
	Total	518	525	456	1499
	(4) absent	461	422	457	1340
Basal necrosis (5)	2.5	146++	117	144++	407++
	5	108	126	149++	383
	10	105-	123	113-	341--
	20	135	150++	106--	391
	Total	494	516	512	1522
	(5) absent	485	431	401	1317
Basal green callus (6)	Position				
	P1+P2+P3+P4	219++	208	192-	619
	P5+P6+P7+P8	194	220	223++	637
	P9+P10	81-	88-	97	266
	$NaNO_3$ conc. (mM)				
	2.5	98--	159++	106-	363
	5	146++	143	112	401
	10	138+	149	138+	425+
	20	101--	107--	135+	343-
	Total	483	558	491	1532
	(6) absent	496	389	422	1307
Apical necrosis (7)	2.5	24++	127+	105++	256++
	5	10	97-	61	168
	10	2--	80--	50--	132--
	20	10	147++	38--	195
	Total	46	451	254	751
	(7) absent	933	496	641	2070
Short and/or brown needles sometimes scale-like on part(s) of the axis (8)	2.5	45++	17-	15--	77-
	5	16	44++	62	122+
	10	9--	40++	68+	117
	20	17	8--	69++	94
	Total	87	109	214	410
	(8) absent	892	838	699	2429

low number of the B stage. In the horizontally distributed groups of positions, only the numbers of shoots in the D stage demonstrated significant contrasts.

There were more shoots in stage D in material that had been isolated from the lower branches in the tree (P3+P7, P4+P8). As was observed in March the maximum number of shoots in the E+F stages occurred in material isolated from P9+P10 buds; however, these shoots did not exhibit the highest growth rate. In March and April it was found that the number of E+F shoots had increased when a comparison was made of morphogenesis after isolation from buds on the highest (P2+P6) branches vis à vis buds on the lowest (P4+P8) branches (Table 4).

In March and April, P2+P6 and P5+P6+P7+P8 shoots demonstrated a strikingly low tendency for apical meristem extension in comparison with other groups of positions.

The morphogenesis in April differed from that in January and March: in April most shoots in stages E+F were observed after isolation from P1+P2+P3+P4 buds. In this period of isolation the $NaNO_3$ concentration did not influence apical meristem extension (E+F stages) in the same way in the vertically distributed groups of positions (Table 5). P1+P2+P3+P4 shoots demonstrated no optimum whereas P5+P6+P7+P8 and P9+P10 shoots showed a preference for 10 mM and 20 mM $NaNO_3$, respectively.

Table 5. Influence of the $NaNO_3$ concentration on the numbers of shoots in the E+F stages in vertically distributed groups of positions in April.

Positions		P1+P2+P3+P4	P5+P6+P7+P8	P9+P10
$NaNO_3$ conc. (mM)	2.5	18--	4--	8--
	5	43	24	16
	10	43	43++	22
	20	42	20	26++
Total	EF	146	80	72
	BD	223	273	116

3.2.3. Modifications of stages of development

Callusing of the shoots The relative frequency of uncoordinated proliferation of the shoot tissues (modification 4, Table 4) was modified by the $NaNO_3$ concentration. On media containing 10mM $NaNO_3$ more "normal" shoots were observed com-

pared with material on media containing 2.5 mM, 5 mM and 20 mM $NaNO_3$, respectively in the 3 periods of isolation, "normal" shoots occurred less often shoots from buds in terminal positions and more in P6 shoots.

Necrotic base In January there was a correlation between the $NaNO_3$ concentrations in the media (5 mM or 10 mM $NaNO_3$) that promoted the fastest growth rates and the absence of necrosis at the base of the explants (modification 5, Table 4). In April the fewest shoots with basal necrosis occurred on media containing 10 mM or 20 mM $NaNO_3$. When the vertically distributed groups of positions were considered, the fewest shoots with a necrotic base appeared to coincide with the most shoots with extension of the apical meristem (modification 5 and E+F stages; Table 4).

Green basal callus The formation of green callus on the cut surface of the explants (modification 6, Table 4) did not exclude basal necrosis in all shoots. In January the highest numbers of shoots with green basal callus coincided with the highest rates extension growth which occurred on media with 5 mM or 10 mM $NaNO_3$. In March and April P10 shoots demonstrated green callus at the base more often than expected statistically whereas P5+P6+P7+P8 shoots showed this phenomenon less frequently.

Apical necrosis Table 4 demonstrates that the $NaNO_3$ concentration that promoted extension growth the strongest (Figure 4) more often prevented the apical meristem from dying: these concentrations were 10 mM $NaNO_3$ in January and March and 10 mM or 20 mM $NaNO_3$ in April. This suggests that extension growth of the morphogenetic unit and the extension unit influenced apical necrosis. After isolation in April the fewest shoots with apical necrosis were observed in the position groups P1+P2+P3+P4 and P1+P5.

Brown and/or short needles Table 4 clearly demonstrates the increase from January to April in the number of shoots that had some needles with an unusual morphology. These needles indicates the occurrence of a rhythmic type of growth. In this period an increase was observed in the optimum $NaNO_3$ concentration for the maximum number of shoots with this phenomenon. In March and April P5+P6+P7+P8 shoots had unusual needles less often than shoots from the other vertically distributed groups of positions, whereas in January most unusual needles occurred in P1+P2+P3+P4 shoots.

3.2.4. Conclusions

Between January and April there was a rapid increase in extension growth rates of shoots initials in vitro; the optimum $NaNO_3$ concentration for the increase in height of the explants was calculated as having risen from 10.91 mM (±0.28) to 15.26 mM (±2.15, see Table 1). One of the causes for this rise was the improved morphogenesis of shoot initials on media containing 20 mM $NaNO_3$ in April compared with the development on this medium in January and March: this improved morphogenesis was accompanied by faster extension growth (Figure 4). A substantial increase in the number of shoots in the E+F stages (Table 4) and less basal and apical necrosis (Table 6). The graphs of the absolute increase in length of shoots from buds in each of the 10 positions did not significantly demonstrate optima diverging from the mean optimum concentrations (Figure 5 and 6). However, the curves of extension growth in vitro of P1 and P10 shoots in March and P2, P3 and P5 shoots in April differed from the graph of the average effect of $NaNO_3$ (Figure 4). In April there were indications of a lower optimum for P3 and P5 shoots and for a higher optimum for P2 shoots. The steeper slopes of the curves and thus narrower optima did not result in unexpected numbers of E+F shoots on the four media compared with the remaining positions of the vertically and horizontally distributed groups, as evidenced by the chi-square test.

In March and April the growth rates of P5+P6+P7+P8 shoots significantly lagged behind the rates of the remaining positions. Concomitantly there were fewer apical meristem extensions (E+F stages). Qualitative morphogenesis in vitro improved markedly depending on the progress of activation of the buds in March and April; the topophysical distinction between the shoot initials became increasingly clear. The optimum concentration of $NaNO_3$ for qualitative morphogenesis was 10 mM in all periods of isolation. The ranking in topophysical positions for optimum qualitative morphogenesis in January was: shoots isolated from buds on the stem - axillary buds on branches - terminal buds; in April this rank order had changed to: terminal buds - buds on the stem - axillary buds on branches.

Generally the shoots that had been induced to grow fastest by the $NaNO_3$ concentration less frequently showed callusing of their tissues, basal necrosis and apical necrosis and more often had basal green callus and unusual needles on their axes. In each of the positions the fastest extension growth rates often coincided with the occurrence of the largest number of D and E+F shoots.

In March and April most shoots in the E+F stages were observed in material

isolated from the lowest branches (P4+P8). This number decreased when shoots were isolated from P3+P7 buds (i.e. from higher branches) and diminished further in P2+P6 shoots from the highest branches (Table 5). This decrease resulted in falling extension growth rates. When vertically distributed groups of positions were compared, most shoots with an extended apical meristem were observed after isolation in January and March from P9+P10 buds, i.e. on the stem; in April, isolation from P1+P2+P3+P4 buds, i.e. in terminal positions resulted in the best apical morphogenesis. The apical development of P1+P2+P3+P4 shoots was initiated later than in the P9+P10 shoots; nevertheless the growth rates of the former shoots were always faster. In general the differences in the numbers of shoots with apical meristem extension i.e. C+E+F shoots could not account for the major part of the positional differences in the mean extension growth of all types of shoots.

3.3. Light intensity

3.3.1. Growth measurements

Extension growth In contrast with the experiments described in 3.2, Figure 4, there was no increase in the mean extension growth rate of the shoot initials in April as compared with March (Figure 7). At 43 Wm^{-2} even the reverse was true: in April the extension growth was significantly slower than in March.

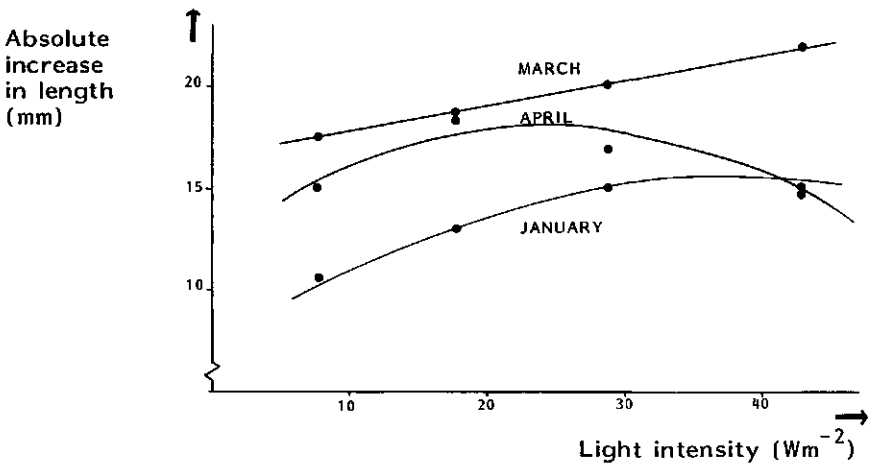


Figure 7. Influence of the light intensity inside the culture tubes on the mean absolute extension growth of shoot initials in 3 periods of isolation. The calculated means of the actually used intensities are represented by points.

After raising the extrapolated optimum light intensity for extension growth of shoot initials from 36.4 Wm^{-2} in January to an unknown (for explanation see below) value above 43 Wm^{-2} in March, measurements in April resulted in a significant decrease of the optimum to 24.5 Wm^{-2} (Table 6, Figure 7). The optimum in March could not be established in the applied intensity range: the extension growth figures resulted in a linear graph. In our system it was technically impossible to apply intensities above 43 Wm^{-2}.

Table 6 . Extrapolated optimum light intensities for the mean absolute increase in length of shoot initials in January and April.

Period of isolation	Extrapolated optimum light intensity (Wm^{-2})	SE optimum light intensity (Wm^{-2})	Limit of optimum in quadratic fit (Wm^{-2})		Extrapolated length in optimum (mm)	SE length on optimum light intensity (mm)
			Lower	Upper		
January	36.4	2.1	32.2	40.5	15.3	0.1
April	24.5	1.9	20.7	28.2	18.1	0.2

January In January interactions were found between the influence of the light intensity on the extension growth of shoots and their original topophysical position. This occurred in shoots that had been isolated from buds on the highest branches (P2 and P6), in shoots from the lowest axillary buds on a branch (P8) and in shoots from the lowest axillary buds on the stem (P10). When the growth of shoots from these positions had been measured it was found that the optimum light intensities differed from the average (36.4 Wm^{-2}). There was a contrast in the response pattern between P2 and P6 shoots from buds on the highest branches. The optimum light intensity in January for extension growth of P2 shoots decreased to 26.5 Wm^{-2} (SE 3.5) whereas the optimum for P6 and P10 shoots increased above the highest applied intensity: 43 Wm^{-2}. P8 shoots also demonstrated a decrease of the optimum light intensity for extension growth: 30.8 Wm^{-2} (SE 2.1).

These observations imply that in January there was a vertical gradient in light intensity optima for shoot initials from axillary buds on branches: from 30.8 Wm^{-2} (P8) via 36.4 Wm^{-2} (P7) to above 43 Wm^{-2} (P6).

March The influence of the light intensity on the extension growth of shoots from buds in 10 topophysical positions in March is represented in the linear graphs of Figure 8.

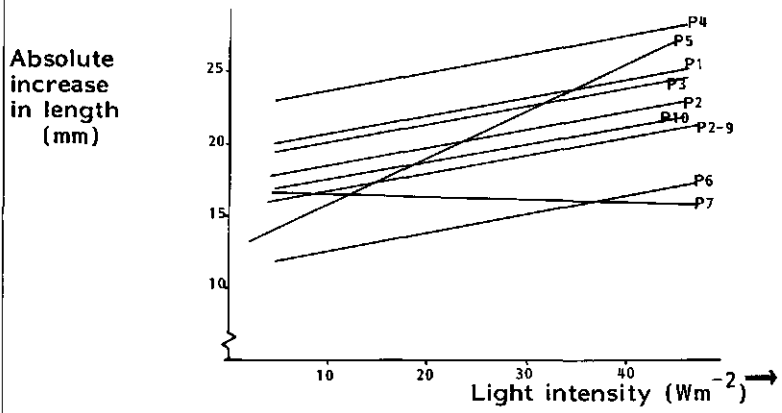


Figure 8. Influence of the light intensity on the extension growth of shoots initials isolated in March from buds in 10 topophysical positions (P1-P10).

As was already observed in the $NaNO_3$ experiments in March (see Table 2), the P1+P2+P3+P4 shoots had a faster extension growth rate than the shoots from the other positions. In particular, the P4 shoots showed a fast increase in length in vitro. It should be remembered, however, that the initial length of P4 shoots in vivo had already increased more than average between January and March (Figure 3). However, P1 shoots had a slower extension growth than in the $NaNO_3$ experiments in March. The mean increase in height of P2+P6 shoots was less than in the P4+P8 shoots.

As shown in Figure 8, only P5 and P7 shoots deviated in their growth response to the light intensity. In P7 shoots no significant influence of the light intensity was observed between 8 and 43 Wm^{-2} whereas the extension growth of P5 shoots increased faster than average in the intensity range applied (Figures 7 and 8).

April In April, the graphs of the extension growth of P1, P2 and P5 shoots (Figure 9) as affected by the light intensity deviated from the average graph (Figure 7). As was observed in January, extension growth of P2 shoots resulted in a lower extrapolated optimum light intensity than the mean optimum: 16.9 Wm^{-2} (SE 5.2) instead of P5 shoots had not yet been reached at 43 Wm^{-2} (Figure 9).

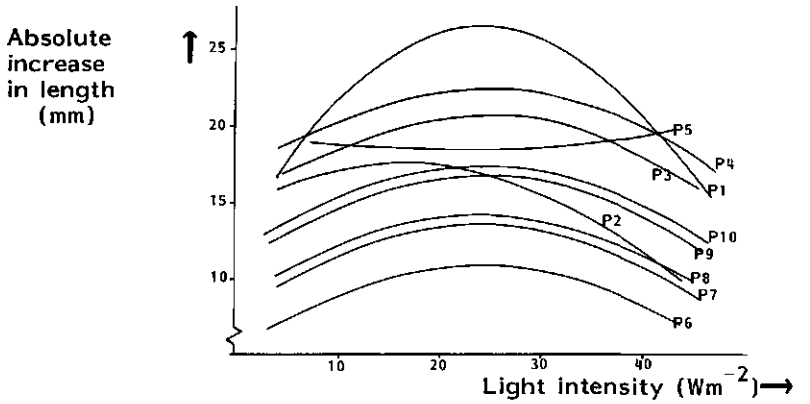


Figure 9. Influence of the light intensity on the extension growth of shoot initials isolated in April from buds in 10 topophysical positions (P1-P10).

The graph of the increase in length of P1 shoots had a steeper slope in April compared with the increase in length of shoots from other positions (Figure 9). Although the extrapolated optima of extension growth (i.e. the light intensity that gave the maximum increase in length of P1, P3 and P4 shoots) did not diverge from the mean maximum (24.5 Wm^{-2}), the actually measured extension growth at a light intensity of 18 Wm^{-2} was significantly faster than during morphogenesis at the other light intensities. The decrease of the mean extension growth rate in April at high light intensities (29 Wm^{-2} and 43 Wm^{-2}) compared with low intensities (8 Wm^{-2} and 18 Wm^{-2}) was especially clear in P1+P2+P3+P4 shoots.

As shown by the figures of the mean absolute increase in length of each of the positions (Table 7) the growth of P5 shoots resembled the growth of shoots from terminal buds (P2 shoots excluded) more than the growth of shoots from axillary positions of the buds.

Table 7. Mean increase in absolute length (mm) of shoot initials isolated from buds in 10 positions in 3 periods averaged over all light intensities.

Position		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	SED
Period of isolation	January	13.9	11.8	12.7	14.2	13.5	12.1	13.9	14.8	14.6	12.5	0.9
	March	22.3	20.1	21.6	25.2	20.4	14.3	15.9	18.3	18.3	19.1	2.9
	April	23.6	16.1	19.0	19.5	19.3	11.0	13.3	13.7	15.0	15.9	0.7

Diameter growth The influence of the light intensity on the diameter growth of the shoot initials in January and April resembled the pattern of extension growth

(Table 8). Again there was a decrease in the optimum light intensity required for growth in April, compared with the increase in diameter in January. The light intensity did not influence the diameter growth in March.

Table 8. Influence of the light intensity on the mean diameter growth (mm) in vitro in 3 periods of isolation averaged over all topophysical positions.

Light intensity (Wm^{-2})		8	18	29	43	SED
Period of isolation	January	10.9	12.5	12.8	12.2	0.3
	March	16.8	16.8	17.6	17.7	0.7
	April	16.8	18.2	16.6	15.0	0.4

3.3.2. Qualitative morphogenesis

Optimum light intensity The light intensity strongly influenced the architecture of the shoot initials in vitro in all periods of isolation (Table 9). After isolation in January and March, morphogenesis at 43 Wm^{-2} resulted in the highest number of shoots with an extended apical meristem (E+F stages) at the expense of the number of shoots that lacked apical development (B stage).

The numbers of shoots in the E+F stages observed in January and March decreased the lower the light intensity. In contrast with the observations of E+F shoots in the $NaNO_3$ experiments on media containing 10 mM of this macrosalt, the numbers of these shoots did not increase from March to April at 29 Wm^{-2} (Table 9). In this period the number of E+F shoots decreased at a light intensity of 8 or 43 Wm^{-2}.

Table 9. Influence of the light intensity on the numbers of shoot initials in the various stages of development (I) with some modifications (II) in January, March and April and the distribution of these numbers in horizontally and vertically distributed groups of topophysical positions. For further explanation see appendix 1. B: no apical development; D: apical development; E+F: extending apical meristem. Decrease, increase: decrease or increase of the number after the preceding period on the same medium. -,+: $p < 0.05$; --,++: $p < 0.01$.

I

Period	Light intensity (Wm^{-2})	in stage B	increase/ decrease B shoots	in stage D	increase/ decrease D shoots	in stages E+F	increase/ decrease E+F shoots	in stages B+D+E+F	in stages A+C
January	8	126++		51		1--		178	72
	18	120		45		20		185-	65
	29	95--		62		31+		188	62
	43	98--		55		55++		188	62
	Total	439		213		87		739	261
March	8	118++	--	78		39--	++	235	15
	18	102+	--	62		70	++	234	16
	29	78	--	67		82	++	227	23
	43	52--	--	81		103++	++	236	14
	Total	350	--	288		294	++	932	68
April	8	131++		87		15--	--	233	17
	18	74--	--	92		68+		234	16
	29	88		72-		72++		232	18
	43	76-	++	93		61	--	230	20
	Total	369		344	+	216	-	929	71
January	Positions								
	P1+P2+P3+P4	243++		51--		8--		302	98
	P5+P6+P7+P8	156--		98+		42		296	104
	P9+P10	40--		64++		37++		141	59
	P1+P5	141++		11--		0-		152	48
	P2+P6	91		42		14		147	53
	P3+P7	89		45		12		146	54
	P4+P8	78--		51++		24++		153	47
	Total P1-P8	399		149		50		598	202
March	P1+P2+P3+P4	113--	--	121	++	139++	++	373	27
	P5+P6+P7+P8	175++		93--	-	96--	++	364	36
	P9+P10	62		74+		59		195	5
	P1+P5	74	--	57	++	50	++	181	19
	P2+P6	84	--	58		46-	++	188	12
	P3+P7	78	--	54		50	++	182	18
	P4+P8	52--	--	44		89++	++	185	15
	Total P1-P8	288	--	214	++	235	++	737	63
April	P1+P2+P3+P4	127--		162++	++	86	--	375	25
	P5+P6+P7+P8	179++		123	++	64--	--	366	34
	P9+P10	63		59		66++		188	12
	P1+P5	67		92++	++	27-	--	186	14
	P2+P6	110++	++	52--		20--	--	182	18
	P3+P7	74		75		38		187	13
	P4+P8	55--		66	+	65++		186	14
	Total P1-P8	306		285	++	150	--	741	59

II

Modifications	Light intensity/ positions	Number of shoots: Period of isolation January	March	April	Total
	Light intensity (Wm^{-2})				
Callusing of the shoots (4)	8	112++	131	161++	404++
	18	81	126	96--	303
	29	57--	103	114	274--
	43	47--	122	132	301
	Total	297	482	503	1282
	(4) absent	480	459	428	1367
Basal necrosis (5)	8	78--	74--	46--	198--
	18	94--	106	90	290
	29	102	125+	98	325
	43	151++	147++	111++	409++
	Total	425	452	345	1222
	(5) absent	352	489	586	1427
	Positions				
	P1+P2+P3+P4	189+	170	130	489
	P5+P6+P7+P8	158	201++	170++	529+
	P9+P10	78	81-	45--	204-
Basal green callus (6)	Light intensity (Wm^{-2})				
	8	12++	180++	102++	494++
	18	104	154	158	416
	29	98	128-	142-	368
	43	57--	117--	132--	306--
	Total	381	579	624	1584
	(6) absent	396	362	307	1065
Short and/or brown needles sometimes scale-like on part(s) of the axis (8)	8	5	19--	3--	27-
	18	14	36	21	71
	29	16	43	32++	91+
	43	10	65++	28	103+
	Total	45	163	84	292
	(8) absent	732	778	847	2357

In all periods of isolation there was a correlation between large numbers of shoots in the E+F stages and rapid mean extension growth rates induced by the light intensity. In April the most E+F shoots occurred at a light intensity of 18 or 29 Wm^{-2} whereas in January and March they occurred a light intensity of 43 Wm^{-2}. This coincided with a decrease in the optimum light intensity for extension growth.

Topophysical positions As shown in Table 9 the general conclusions on the influence of the positions of the buds, as described in the experiments 3.2.2., were confirmed. In contrast with the results of the $NaNO_3$ experiments, in March there were more P1+P2+P3+P4 shoots than P9+P10 shoots in the E+F stages.

In March the number of E+F shoots in material from terminal (P1+P2+P3+P4) and axillary (P5+P6+P7+P8) positions increased compared with the apical development in January (Table 10); there were fewer E+F shoots in April. At the same time, the numbers of D shoots increased; the sensitivity of the shoots to high light intensity inhibited apical meristem development. As a result of this sensitivity the most E+F shoots in April occurred in the P9+P10 group. Between March and April the numbers of the E+F shoots decreased in the other groups of positions (Table 9). The topophysical shifts of the highest number of apical meristem extensions in the vertically distributed groups of positions did not occur in the horizontally distributed groups: in all periods of isolation most E+F shoots occurred in the P4+P8 group. Morphogenesis of P1+P5 shoots in January and of P2+P6 shoots in March and April, i.e. from the upper part of the tree, resulted in the fewest explants with an extended apical meristem. In April, E+F shoots were replaced by explants in the D stage (P1+P5) or the B stage (P2+P6). Most of the shoots reached the E+F stages at a light intensity of 18 Wm^{-2} in material isolated from P1+P2+P3+P4 buds and at 29 Wm^{-2} in material isolated from P5+P6+P7+P8 buds; P9+P10 shoots did not demonstrate a preference for 18 Wm^{-2}, 29 Wm^{-2} or 43 Wm^{-2} for apical meristem extension.

3.3.3. Modifications of stages of development

Callusing of shoot parts The influence of the ligth intensity on the numbers of shoots that showed this phenomenon is given in Table 9, modification 4. In January and April the occurrence of fewer shoots with callusing parts again coincided with

the occurrence of the fastest extension growth rates (see 3.2.3.). In these periods P2+P6 shoots more often demonstrated callusing parts than the shoots from other groups of positions. In all periods of isolation, shoots from buds in terminal positions showed the least tendency to the uncoordinated proliferation of their tissues.

Necrotic bases The number of shoots with a necrotic base increased as light intensity increased. In March and April, necrosis occurred less in P9+P10 shoots than in shoots from other groups of positions (Table 9, modification 5).

Basal green callus The number of shoots with green callus at the base decreased with increasing light intensity, and thus had no correlation with the growth rates. In P9+P10 shoots, green callus was observed more often than in shoots from the other vertically distributed groups of positions. P10 shoots isolated in March and April, frequently had basal green callus. P5+P6+P7+P8 shoots demonstrated the fewest green calluses (Table 9, modification 6).

Brown and/or short needles After isolation in March and April there were fewer shoots with unusual needles at 8 Wm^{-2} than at higher light intensities. The high total number of shoots with unusual needles at 43 Wm^{-2} in March was mainly caused by the high number of these shoots in the P1+P2+P3+P4 group. When horizontally distributed groups of positions were compared in March and April unusual needles occurred more often on P4+P8 shoots. In April this phenomenon was less frequent on P1+P5 and P2+P6 shoots.

3.3.4. Conclusions

The optimum light intensity for the absolute increase in length of shoot initials rose from 36.4 Wm^{-2} (SE 2.1) in January to a value above 43 Wm^{-2} in March followed by a decrease to 24.5 Wm^{-2} (SE 1.9) in April. The results of the diameter growth measurements demonstrated a comparable trend (Figure 7, Tables 6 and 8). The extension growth in April at a light intensity of 29 Wm^{-2} was slower than under conditions in the $NaNO_3$ experiments (on media containing 10 mM).

In all periods of isolation, shoots from buds in a few positions demonstrated extension growth responses that differed from the average response to the light intensities (Figures 8 and 9). In January the optimum light intensities for extension growth of P2 and P8 shoots were found to be lower than average: 26.5 Wm^{-2}

(SE 3.5) and 30.8 Wm^{-2} (SE 2.1), respectively; for growth of P6 and P10 shoots this intensity rose to an unknown value above 43 Wm^{-2}. In January there was a gradual increase in light intensity for extension growth, the further down in the tree the shoots from buds in axillary positions on branches had been isolated (P6, P7 and P8, respectively). In March, P5 shoots elongated faster than the average whereas P7 shoots elongated more slowly than the average as light intensity increased. In April as in January, P2 shoots had a lower optimum light intensity for extension growth than average: 16.9 Wm^{-2} (SE 5.2). In contrast with the average increased light sensitivity of the shoots in April, extension growth of P5 shoots resulted in the optimum intensity being inferred as more than 43 Wm^{-2}. There were more shoots with an extended apical meristem (E+F stages) in January than in the $NaNO_3$ experiments; this was mainly because many of these shoots occurred at a light intensity of 43 Wm^{-2} (Tables 4 and 9). The fact that there were more shoots in the E+F stages at a light intensity of 29 Wm^{-2} in January was one of the reasons for the faster growth rate compared with elongation on media containing 10 mM $NaNO_3$ in the experiments described in section 3.2 in this period. In all periods of isolation the fastest extension growth rates of the explants induced by the light intensity were correlated with the occurrence of the shoots in the E+F stages. In contrast with the observations in the $NaNO_3$ experiments, shoots that had been isolated in March from buds in terminal positions already demonstrated extension of the apical meristem more frequently than shoots from buds on the stem. This increase in March resulted from the large number of P1+P2+P3+P4 shoots in the E+F stages occurring at a light intensity of 43 Wm^{-2}. As was observed in January, the morphogenesis in April of shoots isolated from buds on the stem, resulted in the highest number of explants in the E+F stages.

In contrast with the observations in the $NaNO_3$ experiments, no correlation was established between the fastest extension growth rates and the lowest number of shoots with apical or basal necrosis; however, the absence of callusing of parts of the shoots coincided with faster growth rates. The condition of the basal part of the shoots worsened with increasing light intensity: more necrotic parts and less green callus occurred (Table 9).

3.4. Sucrose

3.4.1. Growth measurements

Extension growth The influence of the sucrose concentration on the absolute

increase in length during morphogenesis of shoot initials is graphed in Figure 10. In March no shoots were isolated because the trees were damaged by the frost.

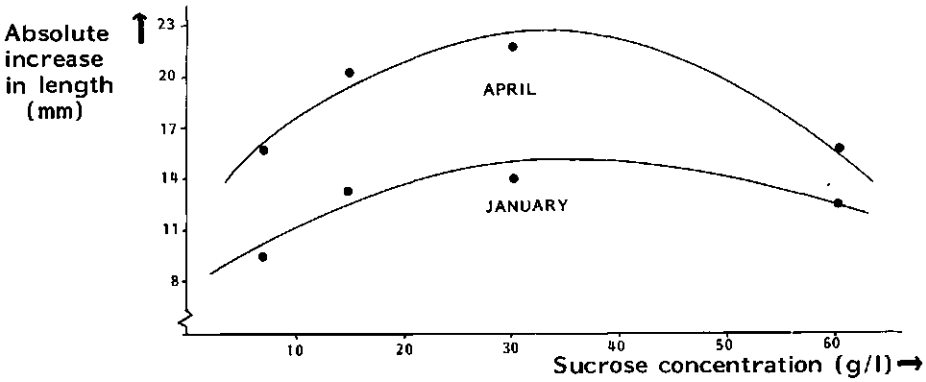


Figure 10. Influence of the sucrose concentration on mean extension growth of shoot initials in January and April. The means of the actually used concentrations are represented by points.

In January there were no interactions between the influence of the sucrose concentration on extension growth and the influence of the topophysical positions. The differences in mean growth rates of the shoots from buds in 10 positions were small (Table 11). The mean extension growth in January and April on the shoots on media containing 15 g/l and 30 g/l sucrose was significantly faster than on media containing 7.5 g/l and 60 g/L sucrose respectively. From January to April the optimum concentration for the absolute increase in length of the shoots decreased from 37.5 g/l to 33 g/l (Table 10).

Table 10 . Extrapolated optimum sucrose concentrations for the mean extension growth of shoot initials in January and April.

Period of isolation	Extrapolated optimum sucrose concentration (g/l)	SE optimum sucrose concentration (g/l)	Limit of optimum in quadratic fit (g/l)		Length on optimum sucrose concentration (mm)	SE length on optimum sucrose concentration (mm)
			Lower	Upper		
January	37.5	1.1	35.3	39.5	14.8	0.1
April	33.0	1.4	30.2	35.8	22.2	0.1

In April the shape of the graph of the absolute increase in length of shoots from buds in 6 of the positions (Figure 11) did not correspond with the graph of the

average effect of sucrose (Figure 10). For morphogenesis of P6 and P7 shoots no maxima of the quadratic fit of the extension growth could be established. The graphs of P3 and P5 shoots sloped more steeply than average; no shift from the mean sucrose concentration (33 g/l) could be established. When the increase in length of P2 shoots was considered, a tendency towards maximum growth at a lower optimum sucrose concentration was observed whereas P1 shoots demonstrated maximum growth at a significantly higher sucrose concentration (45.0 g/l, SE 3.6).

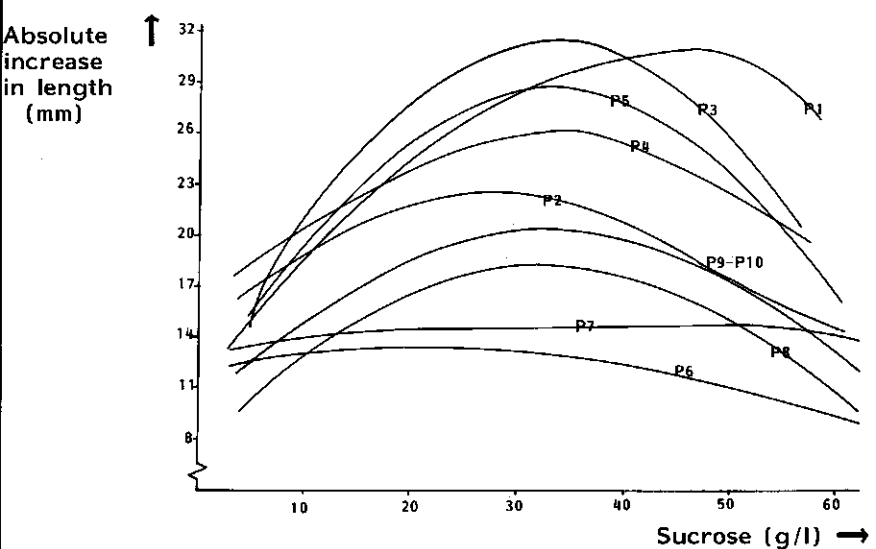


Figure 11. Influence of the sucrose concentration on extension growth of shoot initials isolated in April from buds in the positions P1-P10.

Tabel 11. Mean increase in absolute length (mm) of shoot initials isolated in January and April from buds in 10 positions averaged over all concentrations.

Period of isolation	Position P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	SED
January	12.6	12.0	12.5	13.4	12.5	11.3	12.6	12.6	13.3	11.0	0.9
April	23.9	18.4	22.9	21.7	21.6	12.0	14.1	13.6	16.4	15.5	1.6

Diameter growth The optimum sucrose concentration for diameter growth of shoot initials was 15 g/l in January and 15 g/l or 30 g/l in April (Table 12).

Table 12. Influence of the sucrose concentration on the mean increase of the diameter (mm) of shoot initials in January and April averaged over all topophysical positions.

Period of isolation	Sucrose concentration (g/l)				
	7.5	15	30	60	SED
January	10.8	13.8	12.3	10.7	0.4
April	17.4	18.8	18.9	15.3	0.5

3.4.2. Qualitative morphogenesis

Optimum concentration The best qualitative morphogenesis of shoot initials in January was observed on media containing 30 g/l sucrose, since on these media most of the shoots reached stages D and E+F and only a few reached stage B (Table 15). The qualitative morphogenesis on media containing 15 g/l and 60 g/l sucrose was very similar although extension growth of the shoots on the former medium was much faster (see 3.4.1). The influence of the sucrose concentration on the increase in length of shoot initials did not seem to be directly correlated with its influence on the apical development of these shoots.

The number of E+F shoots in April increased on all media as compared with January (Table 13). Most of these shoots were observed on media containing 15 g/l and 30 g/l sucrose; in January a corresponding number of shoots in the stage D also occurred on these media. The most D shoots in April were observed on media containing 7.5 g/l sucrose; extension of the apical meristem lagged behind, compared with shoots on media containing higher sucrose concentrations.

Table 13. Influence of the sucrose concentration on the numbers of shoot initials in the various stages of development (I)with some modifications (II) in January and April and the distribution of these numbers in horizontally and vertically distributed groups of topophysical positions. For further explanations see appendix 1. B: no apical development; D: apical development; E+F: extending apical meristem. Decrease, increase: decrease or increase of the number after the preceding period on the same medium. -,+: $p < 0.05$; --,++: $p < 0.01$.

I

Period	Sucrose conc. (g/l)	Number of shoots: in stage B	decrease B shoots	in stage D	increase D shoots	in stages E+F	increase/ decrease E+F shoots	in stages B+D+E+F	in stages A+C
January	7.5	189++		38--		4--		231	19
	15	155		60		21		236	14
	30	125--		78++		34++		237	13
	60	164		45		22		231	19
	Total	633		221		81		935	65
April	7.5	57	--	116++	++	55--	++	228	22
	15	39--	--	92	++	104+	++	235	15
	30	41--	--	83		119++	++	243	7
	60	87++	--	75-	++	78	++	240	10
	Total	224	--	366	++	356	++	946	54
January	Positions								
	P1+P2+P3+P4	324++		52--		14--		390	10
	P5+P6+P7+P8	231-		102+		34		367	33
	P9+P10	78--		67++		33++		178	22
	P1+P5	178++		12--		1--		191	9
	P2+P6	130		41		14		185	15
	P3+P7	124--		48		18+		190	10
	P4+P8	123--		53++		15		191	9
	Total P1-P8	555		154		48		757	43
April	P1+P2+P3+P4	62--	--	158	++	158+	++	378	22
	P5+P6+P7+P8	119++	--	141	++	117--	++	377	23
	P9+P10	43	--	67		81	++	191	9
	P1+P5	40	--	87	++	65	++	192	8
	P2+P6	65++	--	81	++	46--	++	192	8
	P3+P7	44	--	62-		83+	++	189	11
	P4+P8	32-	--	69	+	81++	++	182	18
	Total P1-P8	181	--	299	++	275	++	755	45

II

Modification	Sucrose conc./ positions / Sucrose conc. (g/l)	Number of shoots: Period of isolation: January	April	Total
Callusing of the shoots (4)	7.5	158++	119	277++
	15	103--	84--	187--
	30	130	96--	226
	60	141	142++	283++
	Total	532	441	973
	(4) absent	439	510	949
	Position			
	P1+P2+P3+P4	213	114--	327--
	P5+P6+P7+P8	236++	219++	455++
	P9+P10	83--	108++	191
	P1+P5	124	69-	193
	P2+P6	122	103++	225+
	P3+P7	104	91	195
	P4+P8	99-	70-	169-
	Total P1-P8	449	333	782
Basal necrosis (5)	Sucrose conc. (g/l)			
	7.5	97--	78	175--
	15	137	74--	211
	30	150	101	251
	60	188++	113++	301++
	Total	572	366	938
	(5) absent	399	585	984
Green basal callus (6)	7.5	156++	163	319++
	15	129	176+	305
	30	115	161	278
	60	65--	147-	212--
	Positions			
	P1+P2+P3+P4	206+	271	477+
	P5+P6+P7+P8	175	235--	410-
	P9+P10	84	143+	227
	Total	465	649	1114
	(6) absent	506	302	808
Short and/or brown needles sometimes scale-like on part(s) of the axis (8)	Sucrose conc. (g/l)			
	7.5	1--	8--	9--
	15	4--	29--	33--
	30	24	79++	103++
	60	58++	64++	122++
	Total	82	180	262
	(8) absent	889	771	1660

Topophysical positions In January the most shoots in the D and E+F stages occurred in material isolated from P9+P10 buds (Table 13). After isolation from buds in the highest positions (P1+P5) fewer shoots in the D and E+F stages were observed than in the lower horizontally distributed groups of positions.

In April the numbers of P1+P2+P3+P4 shoots in the D and E+F stages increased substantially compared with those in January. In this period the number of extended apical meristems in P5+P6+P7+P8 shoots lagged behind those in shoots isolated from buds in terminal positions and buds in positions on the stem. More E+F shoots occurred in material isolated from buds on the lower branches (P3+P7, P4+P8), while shoots from buds on the highest branches (P2+P6) lagged behind in this development (Table 1).

In April interactions were observed between the influence of the sucrose concentration on the extension of the apical meristem (E+F stages) and the influence of the groups of positions. P1+P2+P3+P4, P2+P6 and P4+P8 shoots demonstrated a preference for 15 g/l sucrose in the medium for the extension of the apical meristem, whereas more P5+P6+P7+P8, P9+P10, P1+P5 and P3+P7 shoots occurred in the E+F stages on media containing 30 g/l sucrose.

3.4.3. Modifications of the stages of development

Callusing shoot parts Media containing 15 g/l sucrose proved to be optimum in preventing the formation of callusing parts on the shoots (Table 13). In January P9+P10 and P4+P8 shoots less often gave rise to uncoordinated growth that shoots from the other groups of positions; in April uncoordinated growth occurred less in shoots from P1+P2+P3+P4, P1+P5 and P4+P8 buds.

Necrotic base The occurrence of necrotic parts at the base of the explants during morphogenesis in vitro increased with a rising concentration of sucrose (Table 13).

Green basal callus Most shoots with basal green callus occurred in January on media containing 7.5 g/l and in April on media containing 15 g/l sucrose (Table 13). In January, shoots from buds in terminal positions more often demonstrated green callus at the base than shoots from axillary buds; in April this phenomenon was often observed in P9+P10 and P1+P5 shoots.

Apical necrosis In both periods of isolation fewer shoots with apical necrosis were observed on media containing 15 g/l sucrose than on the other media.

Brown and/or short needles or bud scales In both periods of isolation the number of shoots with unusual needles or bud scales increased with a rising sucrose concentration in the medium (Table 13). When shoots from buds in horizontally distributed groups of positions were compared in April most unusual needles were found in P3+P7 shoots.

3.4.4. Conclusions

The optimum sucrose concentration in the medium for the absolute increase in length of shoot initials decreased from 37.5 g/l (SE 1.1) in January to 33 g/l (SE 1.4) in April (Table 10). In April, changes in the sucrose concentration exerted little influence on P6 and P7 shoots. The graphs of the extension growth of P3 and P5 shoots showed a steeper slope than average, whereas P1 shoots demonstrated a substantial increase in the optimum sucrose concentration for extension growth (45 g/l (SE 3.6)).

In January, the extension growth of shoot initials on media containing 15 g/l or 30 g/l sucrose was the same; however, diameter growth was significantly faster on media containing 15 g/l sucrose (Table 12).

In both periods most shoots in the E+F stages were observed on media containing 30 g/l sucrose (Table 13). In April P1+P2+P3+P4, P2+P6 and P4+P8 shoots preferred 15 g/l sucrose in the medium for a good apical development. As in the $NaNO_3$ experiments, the best apical development (D and E+F stages) occurred in P9+P10 shoots in January and in P1+P2+P3+P4 shoots in April. In both periods of isolation a better apical development of the shoots was observed after isolation from buds on the lower branches (P3+P7, P4+P8) compared with shoots from horizontally distributed groups of buds higher in the tree.

There was a correlation between the slower extension growth of the shoots on media containing 60 g/l sucrose, the poor conditions of the base of the explants and the occurrence of stagnations in growth as indicated by unusual needles (Table 13).

5 DISCUSSION

The results clearly indicated, that there were considerable contrasts in size and architecture in vitro between shoot initials isolated from buds in different topophysical positions in the tree. By correlating some of the characteristics of this architecture (stages of development) with growth of shoots from buds in horizontally and vertically distributed groups of positions in the tree, gradual shifts in morphogenesis became evident.

Between January, when reiteration (Hallé, et al., 1978) is best prepared, and April, when architectural model growth is best prepared, the greatest activity of the apical part of the shoot initials shifted from shoots from positions on the stem to shoots in terminal positions. Meanwhile, the slower apical activity of shoots from the upper branches, compared with shoots from the lower branches, was maintained. Shoot initials in terminal buds seemed to start their morphogenesis activity in vivo and thus in vitro at a later stage than the other shoots.

It may be that shoots demonstrating a high apical activity under most of the experimental conditions and broad optima for extension growth in vitro, are in a physiological younger state (Romberger, 1976; Borchert, 1976). As the above summarized results suggest, this may hold true for P4+P8 and P9+P10 shoots.

The influence of the various treatments on extension growth and the influence of the topophysical positions produced interactions that indicated that the shoots have topophysically specific requirements for their cultural conditions in each of the periods. However, a problem was that optimum treatments for the increase of the dimensions did not necessarily coincide with the optima for apical development and apical meristem extension. These multivariable problems are still being studied.

The contrasts in morphogenesis of the shoots between the three periods of isolation as a result of changing endogenous physiological gradients in vivo were possibly related to an asynchronous onset of bud activity. The apical part of shoots from terminal buds, for instance, was activated at a later stage than in shoots from buds on the stem. Within the group of terminal positions, P1 and P4 shoots often started apical development in March whereas P2 and P3 shoots began in April (unpublished results). The way this contrast manifested itself was influenced by the pretreatments of the plant material, as indicated by slower growth rate in P1 shoots in March in the light experiments compared with the $NaNO_3$ experiments.

In January the topophysical contrasts in morphogenesis were less pronounced

because of the absence of sufficient extension units: contrasts in the development of the basal part of morphogenetic units were relatively small. In April good apical development, including neoformation, occurred which accelerated extension growth.

The occurrence of free growth (Jablanczy, 1971) at the apex of the shoots was difficult to observe since this de novo characteristic of morphogenesis resembled fast development of the apical region. To solve this problem a comparison should be made between the number of needle primordia specifically arranged on the shoot initial (Williams, 1975) and the number of needles that subsequently develop. Thus in shoots in the E stage the development of the preformed or more recent apical meristem temporarily stagnated.

It was often observed that the initially small P2 and P6 shoots from proleptic branches developed worse than shoots from the other positions. Because these buds were formed later in the season they were one endogenous growth unit younger than P3 and P7 shoots; however, they were not a result of Lammas growth; possibly a smaller amount of reserve substances in the shoots caused the slower in vitro development. This architectural history may have been the reason that P2 shoots had different optima for the light intensity and sucrose concentration than the other shoots.

The various treatments had different influences on the modifications of the stages of development. The absence of necrosis in the apical part of the shoots only correlated with a fast extension growth rate in the $NaNO_3$ experiments.

Photosynthesis and respiration do play important roles in morphogenesis of the shoots, as was indicated by the influence of light and sucrose and by the photosynthesis in vitro (Evers, in preparation). The decrease of the mean optimum sucrose concentration for extension growth of the shoots in April when compared with morphogenesis in January (Table 12) suggested that the photosynthetic apparatus was operational. The increased optimum sucrose concentration for P1 shoots in April suggests that these shoots initially use sucrose from the medium rather than photosynthetic carbohydrates, although there was a normal growth response to the light intensities (Figure 9).

The contrasts in the mean growth rates of the shoots in March and April in comparable circumstances in the $NaNO_3$, light and sucrose experiments were at least partly due to the pretreatments of the plant material and the preceding period of severe frost.

In contrast with earlier experiments (Evers, 1981) the optimum $NaNO_3$ concentration for extension growth in April was higher than in winter, except perhaps

or P3 and P5 shoots. It is difficult, however, to compare those two groups of experiments since the experimental system was totally different. First of all in the previous experiments the shoots were cultured at a much lower light intensity supplied by a different light source from aside instead of from above. Even comparison with shoots in low light in section 3.3 of the present experiments is difficult since the use of different caps on the tubes and the use of activated charcoal increased the number of shoots in the E+F stages in all periods of isolation. Only in April the extension growth rates are comparable under these circumstances. Care should be taken, however, because in previous experiments only 3 instead of 10 topophysical positions were compared. Those 3 positions probably were mixtures of 2 or even 3 of the positions, used in the present study. Furthermore, it is not known whether the in vitro manifestation of shoots from the different positions was influenced to the same extent by the improved cultural conditions. Another important difference was the condition of the trees from which shoots were isolated. In the previous larger experiments trees were not forced in winter; in the present experiments a frost period retarded flushing in April which means that the material was isolated farther from the time of flushing. It can therefore be concluded that as the time of flushing approaches the optimum $NaNO_3$ concentration for morphogenesis of shoot initials increases as compared with the optimum in winter (this study) which is followed by a decrease of the optimum even below the concentration in winter (Evers, 1981). Various stresses may have been applied on meristems from an intact architecture by sub-optimum and supra-optimum treatments as well as by the worse conditions in the previous experiments. If these results are translated back in vivo one may obtain provable stress scenarios explaining the Massart versions of plants which were not selected. The major part of the conclusions made after the previous experiments, however, were confirmed; a further step was made towards replacements of vascular supply to the various types of shoot initials throughout the year by a sequence of specific nutrient media. The complexity of the tree architecture (Edelin, 1977) and the biochemical changes during gymnosperm development (Durzan, 1976) need further research before we can think of studying the relationship between architecture, morphogenesis in vitro and rooting (Heaman and Owens, 1972; Bonga, 1974; Borchert, 1976).

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Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro
III Photosynthesis in vitro

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CONTENTS

SUMMARY

The influence of the sucrose concentration and the light intensity on photosynthesis of shoots, originally isolated from 2-year-old Douglas fir trees, was studied in vitro. The shoots were derived from initials which were situated in vivo in 10 topophysical positions.

A high sucrose concentration in the medium generally inhibited the maximum net photosynthesis (P_{Nm}) and the photochemical efficiency (α_n) of shoots cultured in vitro; it also resulted in a higher compensation irradiance (I_c) except when the initials of these shoots had originally been isolated from buds on the stem. Shoots grown at a high light intensity (43 Wm^{-2}) demonstrated a lower dark respiration (R_d) and P_{Nm} compared with shoots grown at a low light intensity (22 Wm^{-2}).

Shoots from all topophysical positions had their own characteristic parameters of photosynthesis; this was especially clear when the shoots were cultured at a low light intensity. Indications were found that in general shoots originating from buds in the least exposed places in the trees had a higher P_{Nm} and α_n and a lower I_c, which could be correlated with their high relative growth rates. Morphogenesis of shoots in Sylcania Lifeline Gro-Lux fluorescent light resulted in parameters for photosynthesis that more closely approached those of shoots grown in natural daylight than did the parameters of shoots cultured in Philips TL 57 fluorescent light.

The highest P_{Nm} values of shoots in vitro correspond to the P_{Nm} of 2-year-old trees in vivo.

Key words:
Pseudotsuga - in vitro culture - photosynthesis - topophysical position - light intensity - sucrose concentration - shoot initials.

1 INTRODUCTION

After bud break new shoots undergo a period of intensive growth. The energy for this process is supplied through respiration of carbohydrates stored mainly in the older parts of the tree (Neuwirth, 1959, Hallé et al., 1978) and through photosynthesis that occurs both in the current year's shoots and in the older shoots. When shoot initials are cultured in vitro, in most cases the supply of carbohydrates from the branches is replaced by sucrose in the medium (particularly shown for conifers by Brown and Summer, 1975). The new shoots are therefore in vitro subjected to a relatively constant supply of sucrose which is probably not the case in vivo.

The role of photosynthesis during the morphogenesis of conifer shoots in vitro is not known. The question is whether the parameters of photosynthesis that occur during the development of shoot initials in vitro are comparable with those in vivo. The climates in vivo and in vitro are totally different: maximum net photosynthesis in conifers usually occurs at an irradiance above 300 Wm^{-2} (Dijkstra, 1974) while in most culture rooms the mean light intensity is below 20 Wm^{-2}. Furthermore, the humidity in the culture tubes during the morphogenesis of shoots is very high and this will affect the stomata and transpiration.

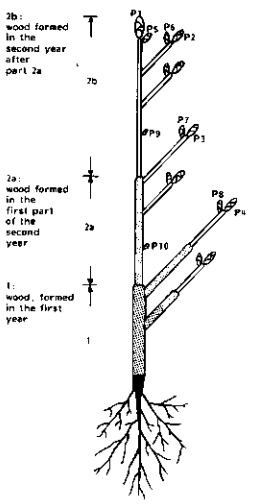
In earlier studies (Evers, 1981a, 1981b) it was found that the morphogenesis of shoot initials of Douglas fir in vitro was influenced by topophysis. Furthermore, it was found that the optimum sucrose concentration for the growth of the shoots varied according to the topophysical origin of the shoots. It seems very probable that there is some kind of relation between photosynthesis of shoots of Douglas fir in vitro and the sucrose concentration in the medium and that this relation will depend on the topophysical origin of the shoots. Among others, Leverenz and Jarvis (1980) found differences in photosynthetic efficiency between "shade shoots" and "sun shoots" of Sitka spruce; this also indicates that there are differences in the CO_2 exchange between different parts of trees. The consequences of topophysis for the photosynthesis of isolated parts in vitro still have to be studied. The detachment of parts itself does not necessarily have great impact on photosynthesis as among others Barden et al. (1980) demonstrated. Apart from topophysis the condition of the tree and the time of the year strongly influence the photosynthetic efficiency of Douglas fir (Helms, 1965, Larcher, 1969, Künstle, 1971) and will have consequences for the photosynthesis in vitro of detached parts.

To ascertain how shoot initials of Douglas fir photosynthetize in vitro, the CO_2 exchange was measured in a closed system. The influence of topophysis, sucrose

concentration, light source and light intensity were tested. The rates of photosynthesis were expressed on a dry weight basis which has the disadvantage that the results cannot be correlated with morphological parameters of the shoots.

2 MATERIALS AND METHODS

Plant material In April of 1978, 1979 and 1980 2-year-old Pseudotsuga menziesii (Mirb.) Franco trees were collected and stored in plastic bags at 4 °C for 5 weeks after which the buds were excised. In each of these periods an experiment was done: experiment A in 1978, experiment B in 1979 and experiment C in 1980. In experiment A unselected trees were used; buds were excised from the middle third part of the tree (Evers, 1981a). In experiments B and C trees were selected for a defined architecture from large groups of plants; shoot initials were isolated from buds in 10 topophysical positions (Figure 1); the reasons for this selection were given earlier (Evers 1981b).



A. Terminal positions:
Position 1(P1): Terminal buds of the tree
" 2(P2): " buds of the highest branch on stem part 2b
" 3(P3): " buds of the highest branch on stem part 2a
" 4(P4): " buds of the highest branch on stem part 1

B. Axillary positions close to terminal buds:
Position 5(P5): Axillary buds close to the P1 buds
" 6(P6): " " " " " P2 buds
" 7(P7): " " " " " P3 buds
" 8(P8): " " " " " P4 buds

C. Positions on the stem:
Positons 9(P9) : Axillary buds on the stem part 2b, closest to part 2a (Figure 2)
" 10(P10) : Axillary buds on the stem part 2a, closest to part 1

Figure 1. Positions of buds in selected 2-year-old Douglas fir trees (1a1), displaying Massart's model of architecture.

In vitro culture The in vitro culture procedures have been described earlier (Evers 1981a; 1981b). Shoots were cultured on media without activated charcoal. To assure comparability the nitrate concentration was always kept at 10 mM. In experiments A and B the same basal medium was used during the first and the second phase and the CO_2 measurements; in experiment C, however, three sucrose levels were used in both phases: 15 g/l, 30 g/l and 45 g/l. After 6 weeks in culture, the shoots were subcultured for the second phase. After 4 weeks in this phase the CO_2 measurements were done on shoots with an elongated primordial axis.

Temperature and light intensity Experiment A. The culture tubes were placed

in 3 light regimes: the first and the second group in a culture room with a day-length of 16 hours supplied by either of 2 types of fluorescent tubes, and the third group in the daylight compartment of the phytotron (Evers, 1981a). The light intensity at the explant level of the first group was 9 Wm^{-2} supplied by Philips 57/40 W tubes (FT); that of the second group was 8 Wm^{-2} supplied by Sylvania Lifeline Gro-lux/40 W tubes (GL) and that of the third group 160 Wm^{-2} supplied by daylight averaged over the light periods in June and July 1978 (DL). The temperature in all light regimes was kept at 25° ± 0.5° C.

Experiment B. The culture tubes were placed in a culture room with 16 hours sodium high pressure light (Philips Son T 400W) daily (Evers, 1981b) which resulted in an intensity of 43 Wm^{-2}. The temperature was kept at 25° ± 0.5° C; in the dark period it fell to 23° ± 0.5° C.

Experiment C. The culture tubes were placed in a culture room with 16 hours GL light daily. The mean light intensity was 22 Wm^{-2}. The culture tubes were placed between 2 horizontal rows of 10 fluorescent tubes, the rows being 30 cm apart. The fluroescent tubes were 4.5 cm apart in the rows. The culture tubes were placed in open racks at a 4.5 cm x 4.5 cm spacing; the surface of the medium was 22 cm below the upper row of tubes. The temperature was kept at 25° ± 0.3° C, however, during the dark period it fell to 23.8° ± 0.2° C.

CO_2 measurements These were done with the photosynthesis equipment of the Department of Horticulture (Agricultural University, Wageningen) which was described by van Holsteijn (1979) and Nilwik and Ten Böhmer (1981, see Figure 2).

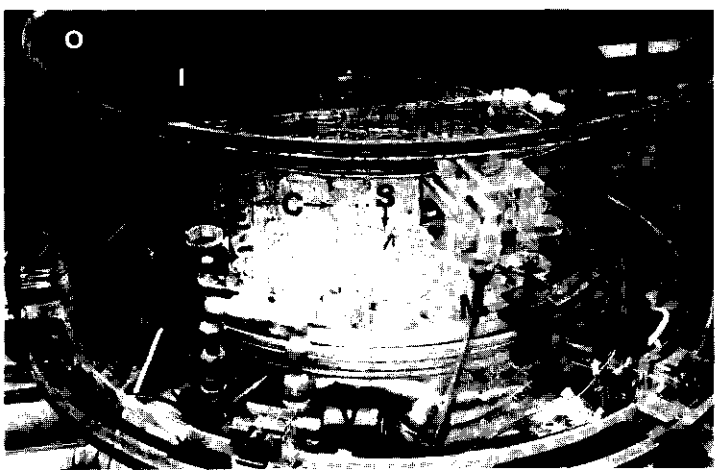


Figure 2. Apparatus for photosynthesis measurements. For further explanation see text. O=outer chamber; I=inner chamber; V=inlet valve;M=measurement tube to Uras; S=selenium photocell; C=culture tubes.

The volume of the measuring inner perspex chamber was adjusted to 30.3 l so that it would record the relatively small changes in the CO_2 content caused by the photosynthesis of Douglas fir shoots in vitro. All equipment was installed in and connected with this inner chamber. The outer chamber was only used to keep the temperature at 25° ± 0.4° C. The humidity, which was kept above 80%, was measured with a Vaisala humidity sensor (Suatola and Antson, 1973) placed outside the chambers in the measuring tube that led to the Uras. All the experiments were done in the concentration range between 280 ppm and 320 ppm CO_2. When the CO_2 content rose above 320 ppm, the inlet valve and the outlet valve were opened; subsequently the chamber was rinsed with an O_2/N_2 (20%/80%) mixture which was led through a 75 cm water column to prevent a major drop in humidity. When the CO_2 content fell below 280 ppm, CO_2 was injected into the gas tube which led to the chamber through a rubber seal.

For each measurement, 40 culture tubes containing Douglas fir shoots were put in the inner chamber; within 30 seconds before closing the chamber all caps were removed from the tubes. All groups of shoots were kept in darkness during 8 hours before they were placed in the chamber, except for the afternoon (=M2) measurement of experiment B. For each group of shoots the net CO_2 exchange was determined at 9 levels of irradiance (irradiance series) supplied by Son T lamps, from 0 Wm^{-2} stepwise, rising to approximately 160 Wm^{-2} at the explant level and ending with 0 Wm^{-2} again, to check irregularities. In experiment B, however, the maximum intensity was approximately 60 Wm^{-2} because in most cases higher intensities caused irreversible damage in these shoots. For each light intensity an adaptation period of 15 minutes was given followed by a 15-minute period in which the CO_2 exchange of the shoots was recorded. The adaptation time was extended if the CO_2 exchange did not attain a constant rate after 15 minutes. After each irradiance series the shoots were collected and their dry weight was determined at 70° C. The mean shoot dry weight on media with 15 g/l sucrose was 10.9 mg; on media with 30 g/l 12.9 mg and on media with 45 g/l 12.2 mg.

Variables tested Experiment A. Shoots grown in FT light, GL light and DL light, respectively were subjected to an irradiance series.

Experiment B. Shoots from buds in each of the topophysical positions P1 to P10 were subjected to an irradiance series. Each series was done after the dark period (M1) and repeated with the same material after a dark period of 2 hours (M2)

Experiment C. Shoots from each of the 10 topophysical positions were subjected to three irradiance series (Figure 1). All groups of shoots were cultured on

media with 15 g/l, 30 g/l or 45 g/l sucrose, respectively.

Parameters and calculations The responses of net CO_2 exchange to the irradiance (I) were described by means of an exponential function as outlined by Nilwik (1980). The values for P_{Nm} (maximum net photosynthesis at saturating irradiance), I_c (compensation irradiance, i.e. when net photosynthesis = 0), α_n (photochemical efficiency, i.e. the slope of the curve in I_c) and R_d (dark respiration, i.e. CO_2 exchange in I = 0) were calculated as described by Nilwik (1980), except that photosynthesis was expressed on a dry weight basis instead of a leaf area basis since the leaf area ratio of the densely leafed tiny shoots was difficult to determine.

3 RESULTS

3.1. Influence of the light source

The influence of the light source in the culture room on the photosynthesis of the shoots in the second phase is represented in Figure 3. The in vitro growth of shoots in GL light resulted in a maximum photosynthesis (P_{Nm}) that differed less from that of shoots grown in the daylight (DL) than the P_{Nm} of shoots grown in FT light. The P_{Nm} of DL, GL and FT shoots differed significantly. The differences in dark respiration (R_d), photochemical efficiency (α_n) and compensation irradiance (I_c) were not significant.

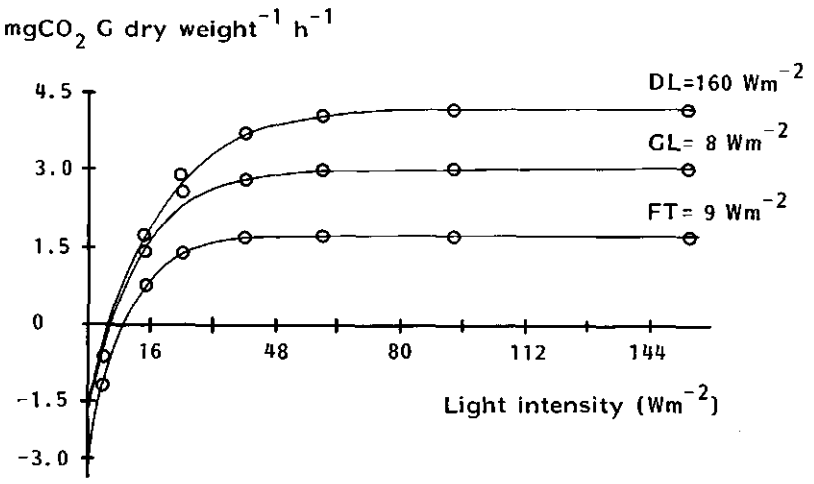
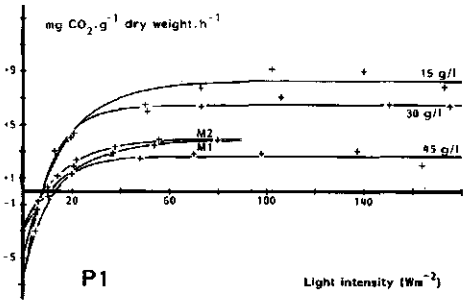
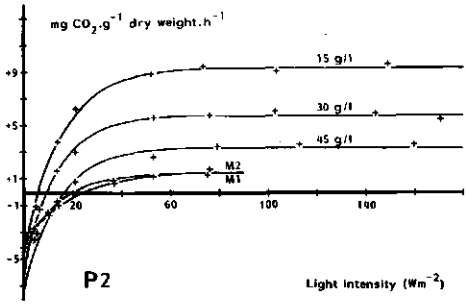


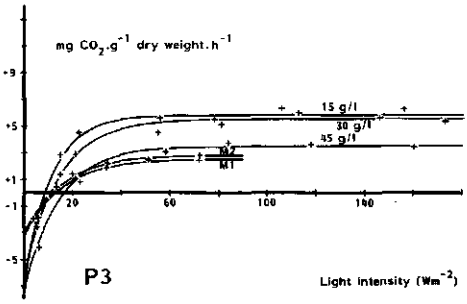
Figure 3. Influence of 3 light sources (FT, GL, DL) during pre-culture on photosynthesis of groups of elongated Douglas fir shoots in vitro, measured in Son T light.

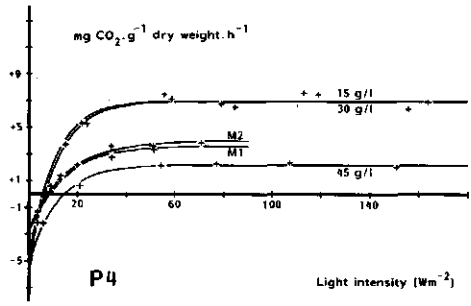
3.2. Influence of the light intensity during the culture of shoots

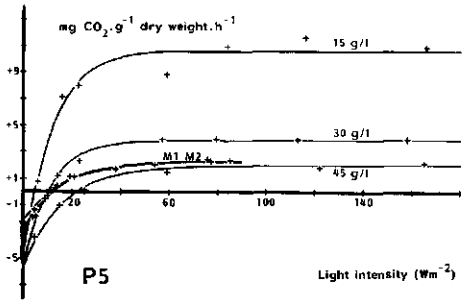
Shoots, cultured at a high light intensity (43 Wm^{-2}, Exp. B) can be compared with material grown at a low light intensity (22 Wm^{-2}, Exp. C) on media with 30 g/l sucrose (Figure 4 and Appendix 1). When grown at a high light intensity shoots from all the topophysical positions resulted in a significantly lower P_{Nm}, R_d (except for P2 shoots) and α_n (except for P5 and P6 shoots) compared with

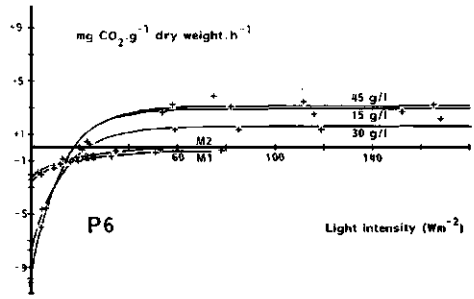

mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
30 g/l
45 g/l
M2
M1
P1
Light intensity (Wm^{-2})

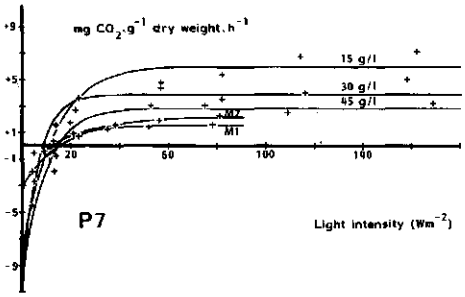

mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
30 g/l
45 g/l
M2
M1
P2
Light intensity (Wm^{-2})

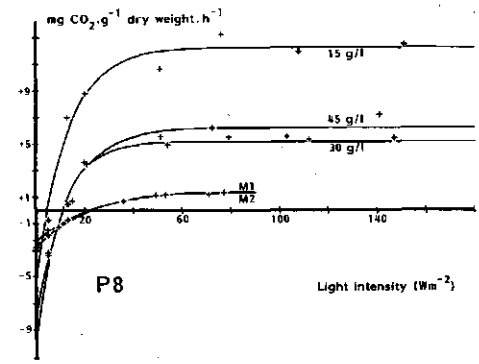

mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
30 g/l
45 g/l
M2
M1
P3
Light intensity (Wm^{-2})

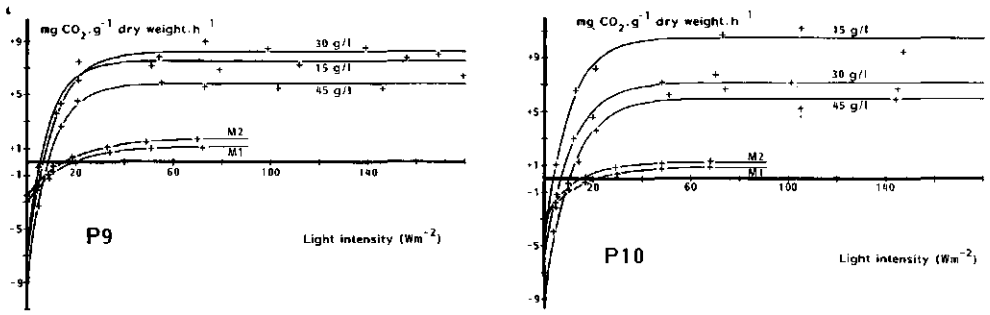

mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
30 g/l
45 g/l
M2
M1
P4
Light intensity (Wm^{-2})


mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
30 g/l
45 g/l
M1 M2
P5
Light intensity (Wm^{-2})


mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
45 g/l
15 g/l
30 g/l
M2
M1
P6
Light intensity (Wm^{-2})


mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
30 g/l
45 g/l
M2
M1
P7
Light intensity (Wm^{-2})


mg $CO_2 \cdot g^{-1}$ dry weight.h^{-1}
15 g/l
45 g/l
30 g/l
M1
M2
P8
Light intensity (Wm^{-2})



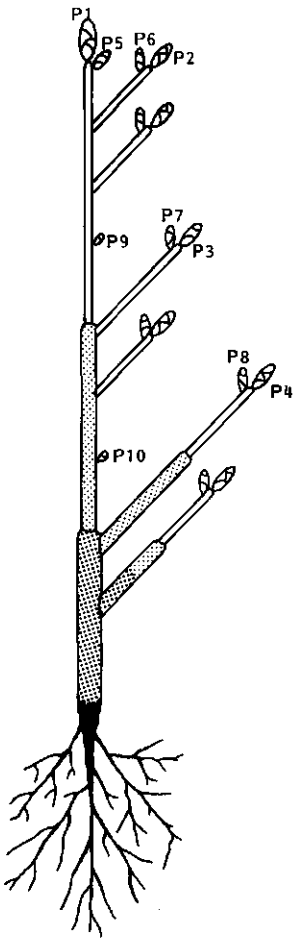


Figure 4. Graphs of the photosynthesis of shoots of Douglas fir in vitro as influenced by the sucrose concentration, the topophysical position, the light intensity during the measurements of photosynthesis and the light intensity during the pre-culture of these shoots. The irradiance series of shoots grown at a high light intensity was repeated: M1 morning measurement, M2 afternoon measurement. Vertical axes: CO_2 exchange in mg CO_2.g^{-1} dry weight.h^{-1}; horizontal axes: light intensity in Wm^{-2}. P1 to P10: topophysical positions 1 - 10. Shoots grown at a low light intensity (22 Wm^{-2}): sucrose concentration 15 g/l, 30 g/l and 45 g/l; shoots grown at a high light intensity (43 Wm^{-2}): M1 and M2 measurements.

the parameters of shoots, cultured at a low light intensity. Shoots from buds on the highest branches (P2 and P6 shoots) and from buds in the least exposed positions in the tree (P8, P9 and P10 shoots) also demonstrated a significantly higher irradiance compensation (I_c) when grown at a high light intensity. After the high light intensity pre-treatment, P1 and P4 shoots gave the highest P_{Nm} values. For the shoots originating from terminal positions (P2, P3 and P4) P_{Nm} and α_n increased and I_c decreased form P2 to P4 i.e. from the higher to the lower original position in the tree; this trend did not occur in the shoots isolated from axillary buds close to these terminal positions (P6, P7 and P8 shoots, respectively). There was no difference in the P_{Nm}, R_d, I_c and α_n between shoots from P9 and P10 buds grown at a low or a high light intensity. The I_c of P8 shoots was higher and the α_n lower than those of P7 shoots when grown at 43 Wm^{-2}; at 22 Wm^{-2} these differences were no longer apparent. The P_{Nm}, I_c (if any) and α_n values of P6 shoots are hypothetical when shoots were grown at 43 Wm^{-2}, because under these circumstances the P_{Nm} was reached at an intensity where in reality net photosynthesis became negative, even in the R_d value. This decrease in P_{net} above 60 Wm^{-2} also held true for shoots from the other positions, but to a far lesser extent: P_{net} decreased no more than 20% of the P_{Nm} value. Since shoots grown in daylight (Exp. A, DL light) showed no decrease of P_{net} at high light intensities, it may be concluded that the Son T lamps used in Exp. B increased the sensitivity of the shoots to light in the second culture phase.

The measurements done on shoots cultured at a high light intensity resulted in almost the same parameters in the morning (M1) as in the afternoon (M2). The only difference was a significantly lower I_c of P2 and P10 shoots in the M2 measurement compared with the M1 measurement.

3.3. Influence of the sucrose concentration

Again shoots from buds in all positions demonstrated a positive net photosynthesis in the irradiance series. In general the P_{Nm} decreased with increasing sucrose concentration. However, sucrose did not influence the parameters of photosynthesis of P6 shoots, except that the I_c of these shoots on media with 30 g/l sucrose was higher than on media with 15 g/l or 45 g/l sucrose (Figure 4, Appendix 1). Furthermore, the P_{Nm} of P3 shoots, P4 shoots and P9 shoots was the same on media containing 15 g/l or 30 g/l sucrose, and that of P7 shoots, P8 shoots and P10 shoots was the same on media with 30 g/l or 45 g/l sucrose. The P_{Nm} of shoots from buds in terminal positions (P1 - P4) showed no differences whether grown on media with 30 g/l or 45 g/l sucrose. On a medium with 15 g/l sucrose, however, shoots from buds on the highest branches (P2) demonstrated a higher P_{Nm} than terminal shoots from lower branches (P3 and P4). When shoots from buds in axillary positions were compared, the lowest P_{Nm} was always found in P6 shoots. On media with 45 g/l sucrose, axillary shoots from the lowest branches (P8) and from the stem (P9 and P10), i.e. the least exposed positions, showed higher maxima than the shoots from other axillary positions and from all terminal positions (P1-P7). On media with 30 g/l sucrose the P_{Nm} of P8 shoots was no longer higher than that of P5 and P7 shoots; it was lower than the P_{Nm} of P9 and P10 shoots. The maximum of P9 and P10 shoots on this medium was not higher than that of shoots from all terminal positions. The P_{Nm} increased from axillary shoots from the highest branches to axillary shoots from the lowest branches (P6, P7, P8, respectively) when grown on a low sucrose level (15 g/l). On this medium, the axillary P5 shoots, P8 shoots and P10 shoots demonstrated the highest maxima, excluding the difference with the terminal P2 shoots. It can be concluded that the highest P_{Nm} values occur in some of the axillary shoots, mostly from the lower parts and/or least exposed parts of the tree: P5, P8 and P10 shoots on media with 15 g/l sucrose, P9 shoots on media with 30 g/l sucrose and P8, P9 and P10 shoots on media with 45 g/l sucrose.

In preliminary experiments it was established that the P_{Nm} remained the same when the irradiance series were done in reverse (starting with the highest intensity) or when the groups of shoots were taken from the culture room during the light period.

The sucrose concentration exerted little influence on the dark respiration (R_d) of the shoots: a stimulation of R_d with increasing sucrose concentration was only found in P8 and P10 shoots.

In general, a high P_{Nm} was correlated with a low compensation irradiance (I_c). However, only the I_c of P5, P6 and P8 shoots on media with 15 g/l sucrose was significantly lower than that of the same shoots on media with 30 g/l sucrose. The I_c of shoots on media with 45 g/l sucrose increased compared with that of shoots on media with 30 g/l sucrose, except for the P6, P8, P9 and P10 shoots. The I_c of P10 shoots on media with 45 g/l sucrose was higher than the I_c of these shoots on media with 15 g/l sucrose. Thus only the I_c of P9 shoots was totally unaffected by the sucrose concentration. On media with 15 g/l or 30 g/l sucrose, the highest I_c occured in P6 shoots. This caused the mean I_c of the axillary P5+P6+P7+P8 shoots to be higher than that of the terminal P1+P2+P3+P4 shoots and the axillary shoots from the stem (P9+P10). Only after growth on media with 45 g/l was the mean in vitro I_c of the shoots that were least exposed in vivo (P8+P9+P10) lower than that of other groups of axillary or terminal shoots. On media with 15 g/l sucrose the I_c decreased from axillary shoots originally from the highest branches to that of shoots from the lowest branches: P6 shoots, P7 shoots and P8 shoots, respectively.

A positive correlation was found between the photochemical efficiency (α_n) and the P_{Nm}. The α_n of P5, P8 and P10 shoots was lower on media with 30 g/l compared with that on media with 15 g/l; the α_n of P1 and P4 shoots was lower on media with 45 g/l than that on media with 30 g/l sucrose. Shoots grown on media with 15 g/l sucrose or 45 g/l sucrose are among those with the highest α_n if they originate from the least exposed positions (P8, P9 and P10, respectively).

4 DISCUSSION

It was impossible to establish a correlation coefficient between shoot dry weight of material isolated from buds in different topophysical positions and P_{Nm} or $_{n}$. It has to be remembered, however, that the chlorophyll content of the shoots may differ according to the positions and that this content was influenced by the sucrose concentration and the light intensity during the culture of the shoots. It is necessary that the chlorophyll content will be determined on material that has been used in the photosynthesis measurements, since there are enormous genetic and seasonal differences (Neuwirth, 1959; Brix, 1972; Sorensen and Ferrell, 1973). Brix (1971) reported, that the condition of nutrition influences the chlorophyll content of Douglas fir trees. The consequences of these differences and conditions for the photosynthesis in vitro of shoots from these trees is not known. Furthermore, it has to be determined whether the chloroplasts are functioning normally, especially after cold storage of the trees (Öquist and Hellgren, 1976). In the present experiments photosynthesis was not expressed per unit leaf area because it is extremely difficult to measure the leaf area of the small in vitro shoots that have hundreds of needles often with a diameter of less than one millimetre. Moreover, it is doubtful whether leaf area is a relevant parameter for Douglas fir shoots in vitro, because the needles are oval and the light in the culture tubes did not predominantly come from one side as it does in nature. Therefore, shoot architecture particularly to its plagiotropic or orthotropic nature (Hallé et al., 1978) probably is more important than leaf surface. In Sitka spruce, this has been confirmed for the plagiotropic branches by Leverenz and Jarvis (1980) who reported that the direction of the light influenced photosynthesis of shoots more than photosynthesis of needles; this difference in CO_2 exchange was mainly caused by differences in the needle arrangement on the shoots, which indicates the important role of the architecture of the plant. It has to be remembered, that growth habit is physiognomically defined, contrarily to architecture which rests on precise morphological criteria. According to Sweet and Wareing (1968), growth habit shows a genetic variability, accompanied by photosynthetic variations, so that the CO_2 exchange should be measured repeatedly over longer periods in different plants perhaps with the $^{14}CO_2$ method (Gordon and Larson, 1968; Nielson, 1977). According to the theory of van Holsteijn (1981), who studied the influence of the growth habit of lettuce plants on photosynthesis, are differences in α_n mainly determined by physiognomic factors of the plant. In the present experiments the α_n was directly correlated with the P_{Nm}: the graph resulted in a straight line. This suggests that the observed topophysical differences in photo-

synthesis in vitro resulted from differences in shoot morphology. As a consequence of the theory of van Holsteijn (1981) photosynthesis of the shoots would then be fully determined by the shoot initial as it was initiated by the tree and the conditions during its outgrowth in vitro. Photosynthesis would in that case not be modified by physiological differences occurring in the tree during the initiation and during the development in vitro of shoots from various positions. In the present experiments, however, photosynthesis of isolated shoots in vitro as influenced by the sucrose concentration varied according to the original topophysical positions of the shoot. This suggests that the physiology of the different types of shoots is dissimilar, assuming that the in vivo and in vitro situations are comparable.

Another important issue in photosynthesis measurements is the water status of the plant. The caps of the culture tubes were removed just before the measurements, which may have caused water stress in the shoots; Douglas fir trees are extremely sensitive to this (Brix, 1979). Furthermore, it is not known whether the stomata of shoots in vitro are functioning normally. The in vivo and in vitro situations are comparable but not equal: in the present experiments the P_{Nm} was attained at a much lower I ($\pm$ 100 Wm^{-2}) than reported in in vivo experiments ($>$300 Wm^{-2}: Kreuger and Ferrell, 1965; Dijkstra, 1974) and the shoots in vitro experienced this I at P_{Nm} for the first time. However, the P_{Nm} values for Douglas fir trees in vivo are very comparable with the P_{Nm} of shoots on a medium with 15 g/l sucrose measured during a relatively short period. Zavitkovski and Ferrell (1970) report the same P_{Nm} values for 2-year-old Douglas fir seedlings as the mean P_{Nm} of the shoots from 10 positions in vitro in the present experiments. Künstle (1971) reports comparable P_{Nm} values for current year's shoots of 20-year-old Douglas fir trees in vivo; he measures a maximum P_{Nm} of 9 mg $CO_2.g^{-1}$ dry weight.h^{-1} in September. In the present experiments this value was attained by 6 of the 10 positions in vitro (Figure 4) when pre-cultured at a lower light intensity. The P_{Nm} of shoots grown at an I of 43 Wm^{-2} corresponds with Künstle's (1971) observations in June. In the present experiments the R_d of shoots in vitro was always higher than values reported for R_d in vivo. The isolation in vitro of shoots did not instantly decrease photosynthesis as is the case in cut branches of Douglas fir (Helms, 1965). The results strongly support the hypothesis that the physiological differences between shoots from various topophysical positions really exist and continue to exist in vitro: the shoots 'remember' where they came from.

The lower P_{Nm} and R_d values of the shoots cultured at a light intensity of 43 Wm^{-2} (Son T light) compared with those at 22 Wm^{-2} (Gro-lux light) suggest that a lower production of photosynthetic carbohydrates is not compensated by a

higher respiration of sucrose from the medium. It has to be taken in account, however, that the Son T light may have damaged the photosynthetic apparatus of the shoots (see section 3.2.). There are indications that the shoots become more sensitive for the Son T light after a few subcultures: the axillary micropropagation system (Evers, 1981a) is only possible at light intensities supplied by Son T lamps below 20 Wm^{-2}, but generally above the I_c (unpublished results). The damage effect does not occur in daylight at an high intensity (section 3.1.).

Provided there is no osmotic effect of high levels of sucrose in the medium, directly or through closure of the stomata, nor an influence of the sucrose concentration on the chlorophyll content, it can be concluded that a lower sucrose concentration is correlated with a potentially higher P_{Nm}. Perhaps the shoots use less sucrose from the medium when the rate of photosynthesis is high; a sort of compensation effect may occur. It is not possible, however, to translate P_{Nm} values directly into growth; the same goes for extrapolated P_{net} values at 22 Wm^{-2}. Furthermore, the optimum sucrose concentrations for the growth of the shoots were established at an earlier stage, at the end of the first culture phase (Evers, 1981b). In this study, high relative growth rates but not higher absolute rates were described for the originally very small shoots from buds in the positions P8, P9 and P10. These high relative growth rates may be correlated with the high P_{Nm} values of shoots after culture at 22 Wm^{-2}, observed in a number of cases. When grown on media with 45 g/l sucrose, the high P_{Nm} of P9 and P10 shoots coincided with a low I_c which may be the result of their originally shaded position on the stem. Shaded needles from the lower plagiotropic branches receive diffuse light (Zelawski, et al., 1973), which is more comparable with the in vitro situation than the direct illumination of the terminal parts of the branches; maybe the stomata of shoots from buds in originally more shaded positions in the tree are better adapted to the conditions in the culture tube. Leverenz and Jarvis (1980) compared photosynthesis of 'shaded' and 'sun' parts of Sitka spruce trees. They found a high P_{Nm} and α_n, and a low I_c for the least exposed parts of the tree. Generally this also holds true for shoot initials of Douglas fir in vitro from the least exposed buds (P8, P9 and P10) especially when the mean P_{Nm}, α_n an I_c of these shoots grown at 22 Wm^{-2} are compared with those of shoots from buds in terminal positions (P1, P2, P3 and P4) whose absolute growth rate is faster. The P_{Nm} of shoots from axillary buds on branches grown on a medium with 15 g/l sucrose, increased from the upper (P6) to the middle (P7) and hence to the lowest branches (P8). The same vertical increase was reported for the extension growth of these shoots (Evers, 1981b). In this study it was found that the absolute growth rates of terminal shoots from branches were faster the lower they had been

isolated from these exposed places in the tree (P2, P3 and P4 shoots, respectively). When grown at 43 Wm^{-2}, this faster growth coincided with a higher P_{Nm} and α_n and a lower I_c. When grown at 22 Wm^{-2}, however, no differences were found between the parameters of photosynthesis of these shoots from terminal buds. It can be concluded that contrasts between shoots from the least exposed positions became clearer when they were grown at a low light intensity and the contrasts between shoots from exposed positions became more marked when grown at a high light intensity.

In preliminary experiments that lasted 8 hours, no rhythms were observed in the CO_2 exchange; This contrasts with in vivo studies (Helms, 1965; Salo, 1974) where a strong diurnal rhythm was observed during the whole day.
There does not seem to be a long lag in the CO_2 uptake in vitro after the light is turned on as was observed in vivo (Larcher, 1969) since it made no difference whether the measurements were done on light-adapted or dark-adapted shoots. Photosynthesis in vitro thus seems to become a more constant process when explants are studied outside the influence of endogenous tree rhythms and environmental factors in vivo.

Since it takes much time to do rooting experiments with Douglas fir shoots produced in vitro, it would seem better to use more 'self-supporting' explants i.e. shoots that are less dependent on sucrose from the medium. The shoots with a high α_n may have been less dependent on the sucrose in the medium. It is not known whether the more self-supporting shoots were of a different physiological age.

To ascertain that normal photosynthesis occurs during the development of Douglas fir shoots in shoots in vitro free gas exchange should be made possible with the interior of the culture tubes.

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APPENDIX 1

The influence of the sucrose concentration and the light intensity on the parameters of the photosynthesis of Douglas fir shoots during their culture: R_d (dark respiration, mg $CO_2.g^{-1}$ dry weight.h^{-1}), P_{Nm} (maximum net photosynthesis at saturating irradiance, mg $CO_2.g^{-1}$ dry weight.h^{-1}), I_c (compensation irradiance, Wm^{-2}) and α_n (photochemical efficiency, µg $CO_2.J^{-1}$). THSD=Tukey's honest significant difference ($p<0.05$). M1=Morning measurement, M2= Afternoon measurement

Light intensity during the culture of shoots	Low (22 Wm^{-2}) - Gro-lux light												High (43 Wm^{-2}) - Son T light							
Sucrose conc. (g/l)	15				30				45				30 (M1)				30 (M2)			
Position	R_d	P_{Nm}	I_c	α_n	R_d	P_{Nm}	I_c	α_n	R_d	P_{Nm}	I_c	α_n	R_d	P_{Nm}	I_c	α_n	R_d	P_{Nm}	I_c	α_n
1	4.82	8.32	7.67	0.50	6.88	6.49	7.61	0.61	6.57	2.61	13.01	0.25	3.00	3.88	10.87	0.20	2.78	4.00	8.69	0.24
2	4.43	9.40	5.90	0.61	5.94	5.84	9.41	0.44	7.14	3.38	15.79	0.24	3.69	1.49	23.71	0.08	4.17	1.51	19.74	0.10
3	6.52	5.84	8.31	0.53	5.92	5.55	10.75	0.38	7.29	3.50	16.14	0.24	3.03	2.54	12.47	0.16	2.96	2.83	11.17	0.18
4	5.23	7.02	5.82	0.67	5.90	6.98	6.68	0.63	5.52	2.19	14.52	0.19	2.87	3.62	8.10	0.26	2.62	4.02	7.90	0.26
5	5.20	10.67	4.75	0.89	5.86	3.90	9.88	0.36	5.72	1.98	21.92	0.12	2.95	2.24	11.70	0.16	2.33	2.32	11.43	0.14
6	9.51	2.94	17.15	0.25	7.80	1.59	23.19	0.12	9.92	3.09	17.69	0.25	2.45	-0.26	-	-0.02	2.14	0.28	83.22	0.001
7	8.00	5.98	10.87	0.47	9.84	4.04	8.19	0.61	10.35	3.00	13.93	0.32	2.78	1.72	10.77	0.15	2.51	2.40	13.28	0.13
8	5.34	12.25	4.52	0.98	10.63	5.19	10.84	0.53	8.18	6.24	10.87	0.48	2.77	1.42	21.87	0.07	2.30	1.44	20.97	0.06
9	7.00	7.54	5.35	0.93	7.25	8.25	6.44	0.81	8.58	5.82	8.82	0.64	3.00	1.15	19.11	0.08	2.62	1.75	15.59	0.10
10	5.30	10.71	3.98	1.11	6.34	7.19	6.81	0.67	9.22	6.17	9.98	0.57	2.90	0.99	21.61	0.06	2.96	1.30	14.76	0.10

THSD (22 Wm^{-2}) R_d: 3.42; P_{Nm}: 1.64; I_c: 5.01; α_n: 0.310

(43 Wm^{-2}) R_d: 0.88; P_{Nm}: 1.01; I_c: 3.82; α_n: 0.049 (P6 shoots excluded)

(Total) R_d: 2.74; P_{Nm}: 1.53; I_c: 4.86; α_n: 0.250

Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro

IV Influence of topping, forcing, the sucrose concentration and the light intensity.

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CONTENTS

SUMMARY

The influence of the light intensity, the sucrose concentration, topping and the period of forcing on the growth and morphogenesis of shoot initials excised from vegetative buds of 2-year-old Douglas fir trees was studied in vitro. Before isolation in vitro shoot initials from terminal buds had the largest surface area and those from the lower stem buds had the smallest surface area. Topping the trees reduced the increase of the surface area of the remaining initials during forcing in the greenhouse but not the number of needle primordia. The number of needle primordia per mm^2 surface area of the shoot initials was almost constant for shoots from the 10 topophysical positions; this number was larger in initials from topped trees.

The optimum light intensity and sucrose concentration for growth and morphogenesis in vitro of shoot initials were 22 Wm^{-2} and 15 g/l, respectively. A lower light intensity and a higher sucrose concentration reduced extension growth, diameter growth and needle development. Lowering the light intensity could not be compensated by a higher sucrose concentration. However, shoots from the lowest buds on the stem grew faster on a medium containing 45 g/l sucrose than on a medium with 15 g/l sucrose. Topping the trees only stimulated growth of the shoots at a low light intensity (8 Wm^{-2}) when compared with shoots from untopped trees; the development of the needles was not influenced. In shoots from buds on the lower stem part of the second flush of the second year, all the primordia developed into needles but in those from other buds only some of the primordia developed into needles; the lowest number of needles were produced on shoots that originated from the highest bud positions. After the trees were topped the apical meristem extension of shoots from terminal buds was stimulated.

Forcing the trees resulted in the top part of the shoot initials growing faster than after natural breaking of dormancy; this was particularly noticeable in shoot initials isolated from the highest buds in the tree. However, these shoots developed fewer needles and showed less frequently extentension of the apical meristem. In January the optimum period of forcing was 6 weeks for trees that had previously been stored at 4° C. The optimum sucrose concentration for the growth of shoots from forced trees and that of shoots taken from trees in April was not always the same for shoots from all positions.

Key words:
Pseudotsuga - in vitro culture - morphogenesis - topophysical positions - topping - forcing - sucrose concentration - light intensity.

1 INTRODUCTION

Recent developments in in vitro techniques as a tool in forest tree breeding have enabled many authors to predict the success of the application of this technology (Bonga, 1980; Erikson and von Arnold, 1980; Girouard, 1980; Guinaudeau, 1980; Karnosky, 1981; Sommer and Brown, 1979; Thorpe, 1977; Winton, 1978). However, the results obtained from species that hardly root, such as Douglas fir, point to the neccessity for a more fundamental approach (Cheah and Cheng, 1978; Cheng, 1979; Kirby, 1980; Kirby and Frank, 1980; Yasuda and Cheng, 1978; Yasuda et al., 1980; Wochok et al., 1980).

In earlier studies on the morphogenesis of shoot initials of Douglas fir (Evers, 1981a, 1981b) it was found that the condition and the dimensions of the original explants played a major role in their growth dynamics in vitro. Some of the original differences between explants were a direct result of their topophysical position, which, together with genetic differences explained most of the variance in the experiments. It was difficult to distinguish between factors that could be modified by experimental treatments and those that were genetically fixed. It was suspected that unfixed properties had a relation of some kind with juvenility and thus the ability to root. Experiments were designed to modify endogenous gradients to be able to determine which morphogenetic phenomena were genetically fixed. However, the results strongly depended on the time in the season. Firstly, this factor influenced the growth activity of the shoot initials; secondly, the optima of the nitrate and sucrose concentrations as well as those of the light intensity for morphogenesis in vitro changed during the season. The question arose whether growth and apical extension could be stimulated by a temperature treatment before excision, since the latter is the main cause of breaking dormancy in young Douglas fir trees (Allen and Owens, 1972). During the breaking of dormancy the mobilization of sucrose plays an important role (Kozlowski, 1971). In the present study, morphogenesis of shoot initials in vitro was therefore investigated in relation to temperature treatments of trees in vivo and the sucrose concentration in vitro.

The sucrose concentration also influenced free growth (possibly exogenously programmed) and rhythmic growth (probably endogenously programmed) of shoot initials (Borchert, 1973; Hallé and Martin, 1968) in vitro. For most tree species it is not yet clear how environmental factors and endogenous rhythms are working together in determining the occurrence of these types of growth (Borchert, 1973; Hallé et al., 1978). For that reason the role of sucrose, light and other factors needs to be determined. Beyond the growth type, sucrose

also influences the growth rate, at least partly, by modifying photosynthesis (Evers, 1982) and thus the influence of the light intensity. The influence of the sucrose concentration on the morphogenesis of shoot initials was therefore studied in relation to the light intensity. Furthermore, an attempt was made to modify endogenous gradients of the tree by topping some trees.

2 MATERIALS AND METHODS

Introduction The first experiment, which investigated the influence of topping the light intensity and the sucrose concentration on morphogenesis of shoot initials in vitro, will hereafter be referred to as experiment I. A second experiment, experiment II, assessed the influence of the sucrose concentration and the period that the trees were forced on the morphogenesis of shoot initials. The experimental details are summarized in Table I.

Plant material and pre-treatments In October 1979 (Experiment I) and in January 1980 and the first week of April 1980(Experiment II), 1300, 500 and 100 trees respectively were selected from groups of 4000 2-year-old Pseudotsuga menziesii (Mirb.) Franco provenance Arlington (1a1) according to the criteria of tree architecture described in an earlier paper (Fig. 1, Evers, 1981b). The selected trees were packed in plastic bags and stored at 4° C. All trees were forced in the greenhouse at a constant temperature of 20° C. In experiment I half of the trees were topped (Type 2) before being forced. All other trees (Exp. I and II) remained untopped (Type 1). In the topping procedure the 2B-parts (Figure I; Evers, 1981b) i.e. those parts formed during the second flush of the second year were cut off: the P1, P2, P5, P6 and P9 shoot initials were not cultured. The wounds were treated with lanolin on spot A (Fig.I, type 2).

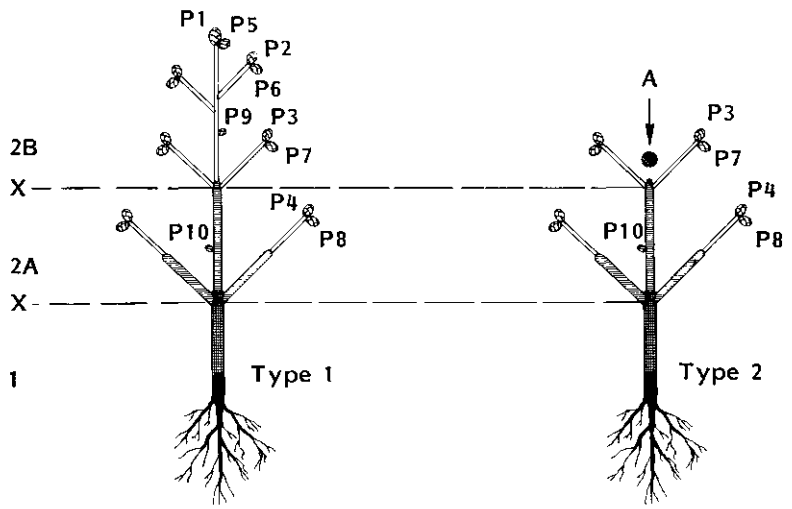

Figure 1. Distribution of topophysical positions of buds on Type 1 (untopped) trees and Type 2 (topped) trees of 2-year-old Douglas fir. A= the cut surface treated with lanolin paste.

Type 2 trees only contained the terminal positions P3 and P4 and the axillary positions P7, P8 and P10. In experiment I the period of forcing was 4 weeks for all trees (type 1 and type 2); since there were 15 days of inoculation of buds there were also 15 days of transfer of trees from 4° C to 20° C. In experiment II (only type 1) trees were forced during 3, 5, 6 and 7 weeks. This material was compared with trees stored at 4° C in early April; the trees were considered to have been "unforced" or "naturally forced" and are further referred to as the '0 weeks in the greenhouse at 20°C' treatment. These trees were not placed in the greenhouse; the buds were collected after 1–7 days of cool storage.

Media The preparation of shoot initials, sterilization procedures, the culture tubes and basal medium have been described in earlier papers (Evers 1981a; 1981b). In the present experiments, however, the sucrose solution was filter-sterilized and added to the autoclaved medium when its temperature had dropped to 50° C, since preliminary experiments had demonstrated that this procedure improved shoot morphogenesis. The concentration of activated charcoal (Merck 2186) in the basal medium was 20 g/l. In earlier experiments, seasonal changes in nitrate optima were found (Evers, 1981b); therefore in experiment I the basal medium contained optimalized dosages of 10.9 mM $NaNO_3$ and in experiment II it contained 9.6 mM $NaNO_3$. In both experiments, the sucrose concentration varied. In experiment I, half of the shoots from both tree types was cultured on media containing 15 g/l sucrose and the other half on 45 g/l sucrose; in experiment II three sucrose concentrations (15 g/l, 30 g/l and 45 g/l) were used after each of the 5 forcing periods.

Temperature and light intensity The experiments were done in a culture room with 16 hours Gro-lux fluorescent tube light (Sylvania Lifeline Gro-lux, 40W) daily. The mean light intensity with all tubes switched on was 22 ± 2 Wm^{-2} at the explant level. The culture tubes were positioned between an upper and a lower row of 10 fluorescent tubes. The light sources were 4.5 cm apart in the rows; the rows were 30 cm apart. The distance between the surface of the medium and the upper row of fluorescent tubes was 22 cm and between the medium and the lower row it was 8 cm. The culture tubes which were closed with transparant plastic caps were placed in open racks at a 4.5 x 4.5 cm spacing.

In experiment I, the shoot initials were cultured at a light intensity of 8 Wm^{-2} and 22 Wm^{-2}. The low intensity was achieved by switching off the bottom row of tubes and half the tubes in the upper row. All shoots in experi-

ment II were cultured at a light intensity of 22 Wm^{-2}.

In the light period the temperature in the culture tubes was 25° (±0.3) C; in the dark period it fell to 23.8° (±0.2) C.

Variables tested In experiment I two types of trees were used, Type 1, which had 10 bud positions and Type 2, which had 5 bud positions (Figure 1). To determine the original dimension of the shoot initials after the forcing period, 25 initials from each of the 15 bud positions were fixed in 96% ethanol. This procedure was repeated 4 times in 4 successive fortnightly periods to determine the influence of the increasing period of cool storage. All the shoot initials were photographed from above and from the side.

At the same time shoot initials from the same positions were isolated in vitro from a second group of Type 1 trees and Type 2 trees: 30 shoots per treatment. The 4 light intensity and sucrose concentration treatments were: 8 Wm^{-2} and 15 g/l (treatment A), 8 Wm^{-2} and 45 g/l (treatment B), 22 Wm^{-2} and 15 g/l (treatment C) and 22 Wm^{-2} and 45 g/l (treatment D). The initials were cultured in 15 days of inoculation.

Experiment II. Shoot initials were isolated from buds in 10 positions on Type I trees. For each of the 15 treatments (media with 15 g/l, 30 g/l or 45 g/l sucrose after 0 weeks, 3 weeks, 5 weeks, 5 weeks and 7 weeks at 20° C) 25 buds were cultured.

Parameters measured Experiment I. From the photographs (see variables tested) the original length, basal diameter, distal diameter and apical dome diameter were measured and the means were calculated. The numbers of short spirals of needle primordia (Williams, 1975) were counted, as well as the number of needle initials (minimum diameter 0.02 mm) in one short spiral per shoot initial (Figure 2). The number of primordia per mm^2 surface area of the shoot initials was determined according to the formula: $\frac{NP}{\pi(BD+DD).\frac{1}{2}(\frac{\sqrt{(BD-DD)^2}}{2}+L^2)}$

in which NP=number of primordia, BD=basal diameter, DD=distal diameter and L=length.

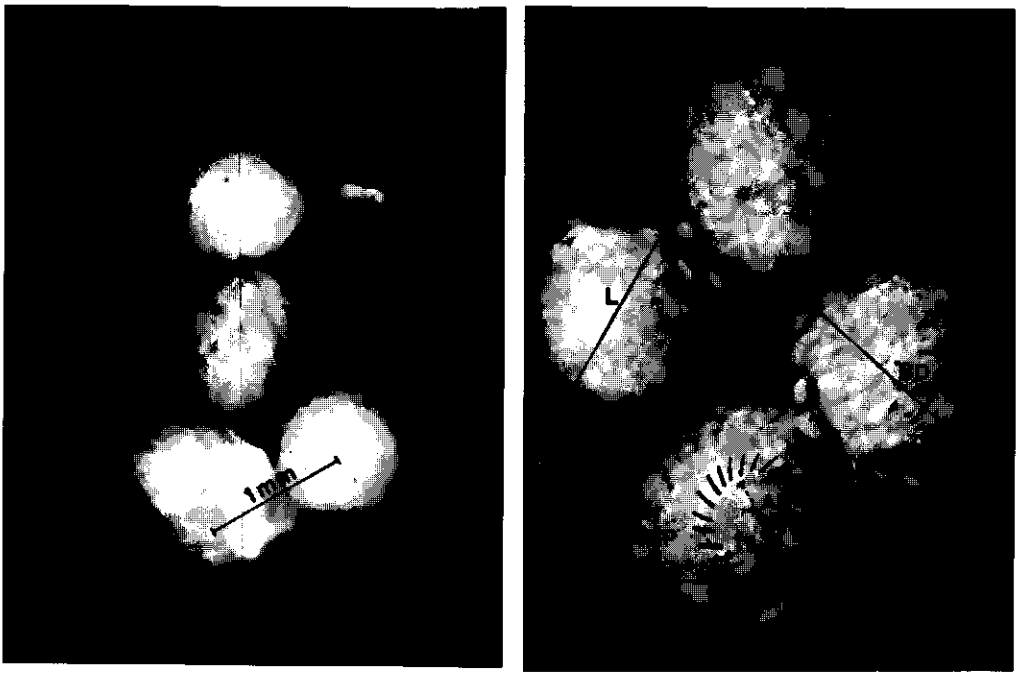

Figure 2. Top and side views of P1 shoot initials (left) and of P10 shoot initials (right) from Type I trees after a pre-treatment of 4 weeks at 20° C. L=length, BD=basal diameter, DD=distal diameter, P=primordium, sp=short spiral, AD=apical dome diameter

Experiments I and II. The cultured shoots were measured after 6 and 12 weeks; after the first measurements the explants were subcultured on a fresh medium of the same composition, because the necrotic base of the shoots had to be recut. The length, basal diameter and distal diameter of the shoots were measured using millimetre paper; the number of needles with a minimum length of 2 mm in a short spiral were counted. The variances of the differences of the means were analysed. Qualitative estimations of the shoots were also made according to the system of stages of development and their modifications described in an earlier paper (Evers, 1981a). The significance of the occurrence of shoots in these stages and their modifications were tested using the chi-square test. The influence of the 10 bud positions in Type 1 and the 5 bud positions in Type 2 trees were tested separately and in horizontally and vertically distributed groups.

Table 1. Summary of the experiments; for further explanations see text.

	pre-treatments						in vitro conditions							numbers		
Experiment	Months into dark 4° C	Weeks stored at 4° C	Number of trees stored	Tree type	Months into green-house 20° C	Weeks forced at 20° C daylight	Positions	Periods of inocu-lation	Weeks in culture	Heller's medium with $NaNO_3$ conc. (mM)	Activated charcoal conc. (g/l)	Sucrose conc. (g/l)	Light intensity (Wm^{-2})	Number of buds x treatments x positions	Total number of buds	Grand total numbers
I:Topping, light int. and sucrose conc.	Oct. '79	3-9	500	1	Nov.'79 Dec.'79	4	P1-P10	Dec.'79 Jan.'79	6	10.9	20	15,45**	8,22**	30x4x10	1200	1800
			500	2	Nov.'79 Dec.'79	4	P3,P4,P7, P8,P10	Dec.'79 Jan.'80	6	10.9	20	15,45**	8,22**	30x4x5	600	
	Oct. '79	3,5,7, 9	150	1	Nov.'79 Dec.'79	4	P1-P10	Fixed + photogr.	-	-	-	-	-	25x4x10	1000	1500
			150	2	Nov.'79 Dec.'79	4	P3,P4,P7, P8,P10	Fixed + photogr.	-	-	-	-	-	25x4x5	500	
II:Forcing and sucrose conc.	Jan. '80	4	500	1	Feb.'80	3,5, 6,7***	P1-P10	Feb.'80 March'80	6	9.6	20	15.30,14***	22	25x12x10	3000	3750
	April '80	0-1	100	1	-	"0"	P1-P10	April'80	6	9.6*	20*	15,30,45	22	25x3x10	750	
														Grand total		7050

* not adjusted to the values for April found in an earlier study (Evers, 1981b).
** combinations of light intensities and sucrose concentrations (treatments A, B, C and D).
*** combinations of weeks of forcing and sucrose concentrations.

3 RESULTS

3.1 Distribution of primordia and the surface area of the original explants

3.1.1 Dimensions of the explants

The mean surface areas of shoot initials from buds in the different positions are given in Figure 3. P1 initials had the largest area and P6, P7 and P10 initials had the smallest area. The mean areas of initials on the stem decreased progressively in a proximal direction: the rank order was P1 - P5 - P9 - P10. The mean areas of initials from the lowest branches (P4, P8) were larger than those of terminal and axillary initials from other branches. Topping the trees (Type 2) reduced the increase in mean areas of initials from the remaining positions during forcing, except for the P10 shoot initials. This reduction was mainly caused by the fact that initials on Type 2 trees were shorter, except for P10 initials. In P10 shoot initials of Type 2 trees only the diameter of the apical dome was smaller than that of Type 1 trees. Shoot initials from the other positions (P3, P4, P7 and P8) in Type 2 trees were shorter and had a smaller basal diameter than those of Type 1 trees. The mean length, basal diameter, distal diameter and diameter of the apical dome for the shoots from the 5 positions from Type 2 trees were smaller than those from Type 1 trees.

3.1.2 Number of needle primordia

The differences in the total numbers of needle primordia on shoot initials from the different positions resulted in a pattern similar to that described for the surface areas; however, the lower the terminal position of the original bud in the tree, the larger was the surface area of the shoot initial (P2<P3<P4), but shoots from these positions had similar numbers of spirals and primordia. The total number of needle primordia differed because there were contrasting numbers of short spirals on the shoot initials from the different positions as well as contrasting numbers of primordia per spiral (Figure 3).

As was expected topping the trees (Type 2) did not influence the number of spirals, the number of primordia per spiral and the total number of needle primorida per explant in any of the positions.

3.1.3 Density of needle primordia

The number of primorida calculated per mm^2 surface area of the shoot initials was almost constant for all positions; only the densities of P4 and P8 shoots were somewhat lower than those of P1 and P2 shoots (Figure 3). Topping the trees (Type 2) increased the density of primordia of the shoot initials except in ini-

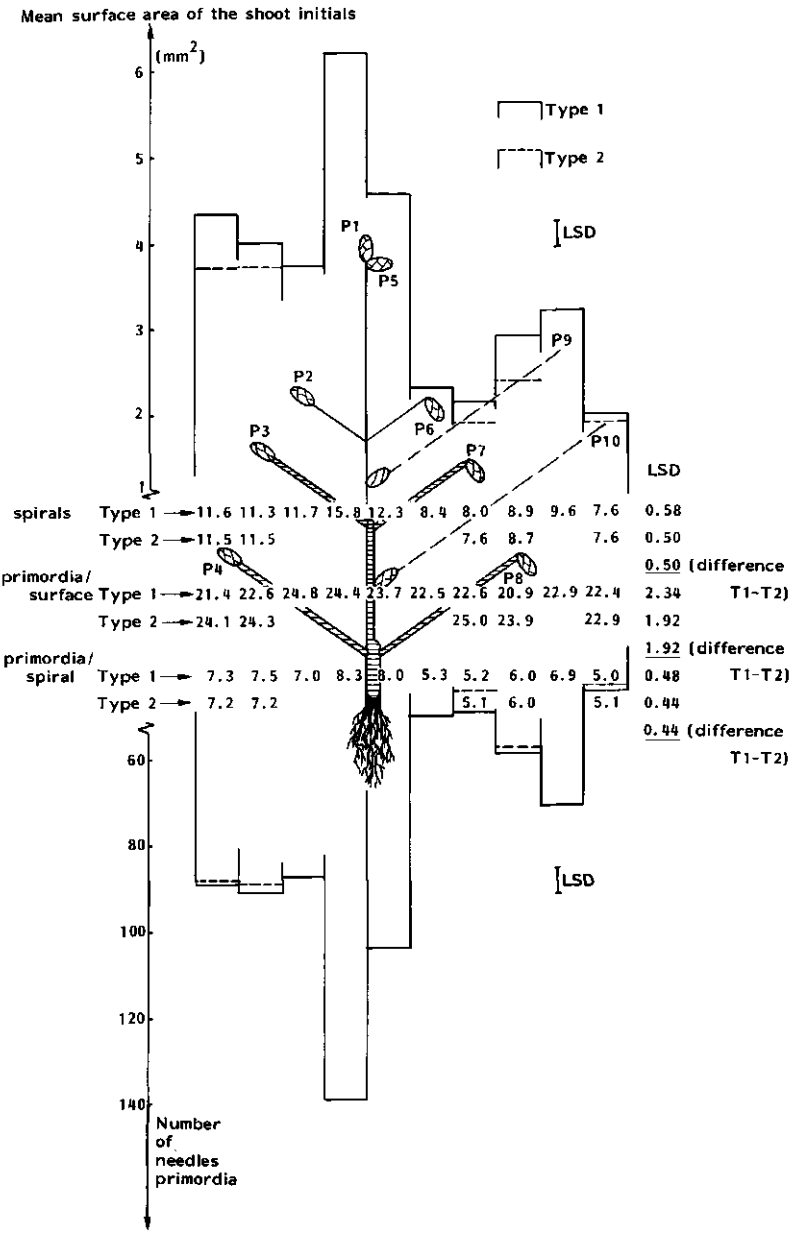

Figure 3. Diagrammatic representation of surface area and numbers of needle primordia of shoot initials isolated from 10 bud positions in type 1 trees and from 5 positions in type 2 Douglas fir trees. Data on the number of short primordia spirals per initial, the number of primordia per mm² surface area and the number of primordia per short spiral are also given. LSD: least significant difference. Type 1 and type 2 trees: see Figure 1.

therefore this increase in the density was solely caused by a reduction in the increase in mean surface area during forcing in initials of Type 2 trees.

3.2 The influence of the sucrose concentration and the light intensity

3.2.1 Extension growth

The mean extension growth of shoots from the 10 positions of Type 1 trees at 22 Wm^{-2} was faster than at 8 Wm^{-2} (Figure 4). The influence of the sucrose concentration on the mean extension growth of the shoot initials was only significant at 22 Wm^{-2}: the shoots on media with 15 g/l sucrose grew faster than those on media with 45 g/l sucrose. This overall conclusion did not hold true for all positions, as Figure 5 shows. Treatment C proved to be most promotive for extension growth of shoots from all positions; however, for P2 shoots also treatment A and for P3 shoots also treatment D proved to be most promotive.

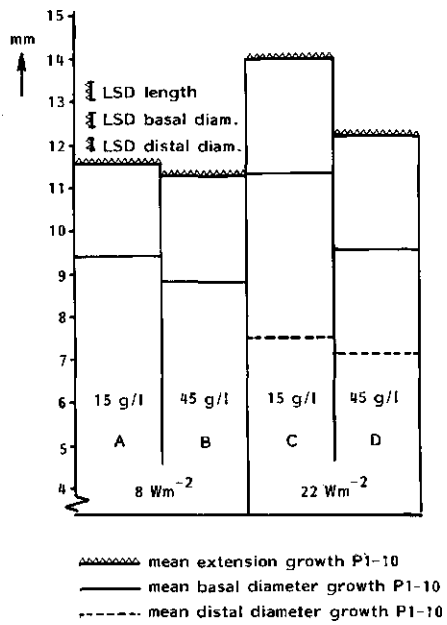

Figure 4. Mean extension growth and diameter growth of shoot initials from buds in 10 positions on Type 1 trees as influenced by 4 light intensity/sucrose concentration treatments.
LSD=Least significant difference

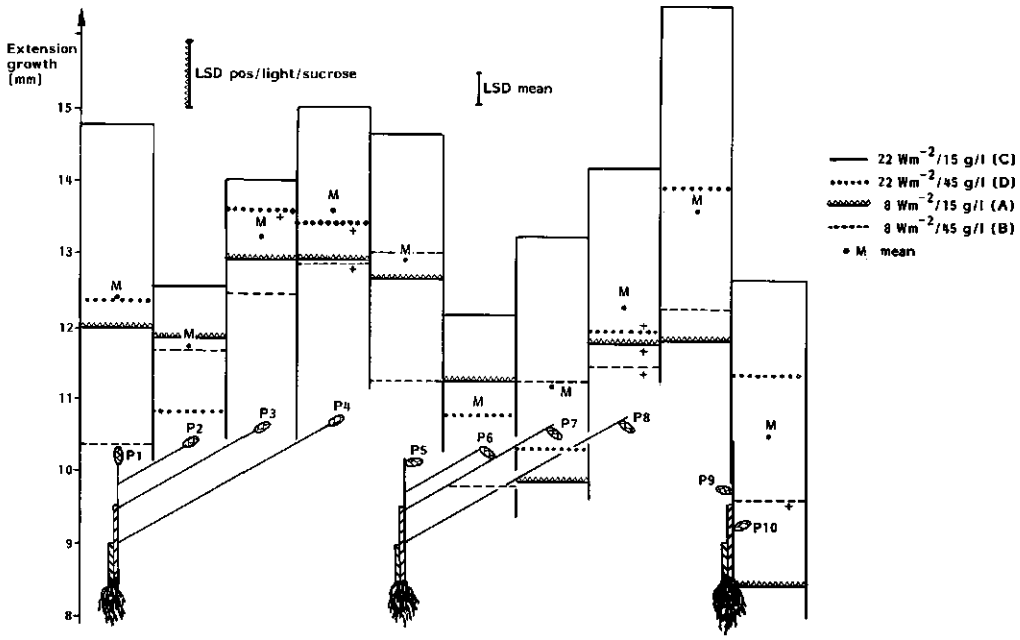

Figure 5. Extension growth of shoot initials from buds in 10 positions on Type 1 trees as influenced by 4 light intensity/sucrose concentration treatments LSD=Least significant difference.
+: significantly higher growth rate in shoots from the same position in Type 2 trees ($p<0.05$).

For shoots from 3 of the highest positions (P1, P5 and P6) treatment B was the least promotive for extension growth. Shoots from positions on the lowest branches (P4 and P8) performed equally on the least promotive treatments A, B and D; P10 showed the slowest extention growth in treatment A. Extension growth at 22 Wm^{-2} was faster than at 8 Wm^{-2} except for the P2 shoots; however, the difference in extension growth in shoots from the 10 positions between the treatments A and D was only significant in P9 and P10 shoots.
At 22 Wm^{-2} the extension growth of all shoots except P3 shoots was faster on media with 15 g/l sucrose than with 45 g/l sucrose.
At 8 Wm^{-2} this sucrose effect was only observed in P1, P5 and P6 shoots, for P7 and P10 shoots even the reverse was true.

Topping the trees stimulated extension growth of the shoots in vitro when

cultured at 8 Wm^{-2}; however, the growth rate remained lower than at 22 Wm^{-2} (Figure 6). Topping did not correlate with the influence of the sucrose concentration on extension growth (Figure 6): at both concentrations topping stimulated extension growth.

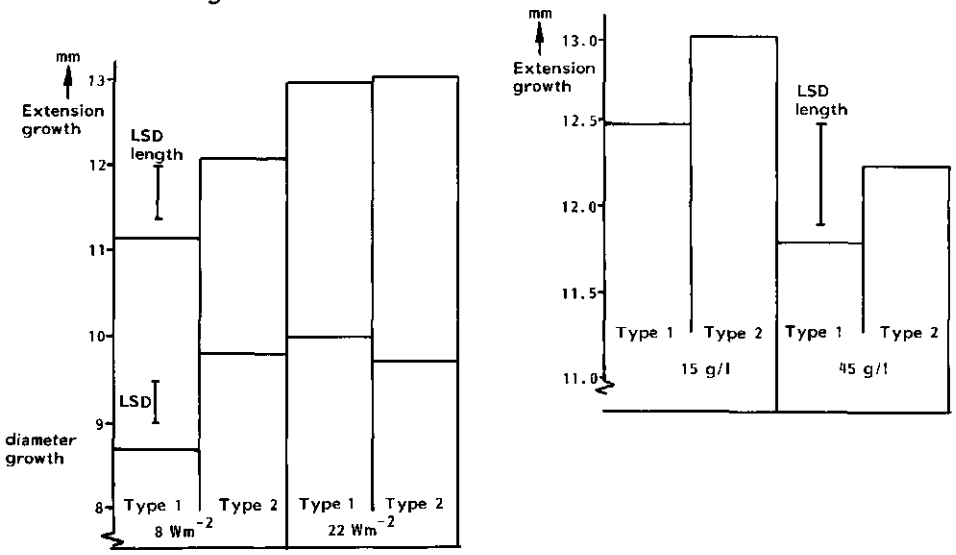

Figure 6. Mean extension growth and diameter growth of shoots initials from buds in the positions P3, P4, P7, P8 and P10 on Type 1 trees and (left, averaged over sucrose treatments) and Type 2 trees as influenced by the light intensity and the sucrose concentration (right, averaged over light treatments). LSD=Least significant difference.

It slightly changed the influence of the light/sucrose combinations on the extension growth of P3, P4, P7, P8 and P10 shoots (Figure 6). Topping the trees (Type 2) did not stimulate extension growth in treatment A except for that of the P8 shoots, vis à vis shoots from untopped trees (Type 1). The most substantial stimulation of extension growth resulting from topping was observed in treatment B (low light/high sucrose): P4, P8 and P10 shoots demonstrated a faster growth rate compared with the same shoots from Type 1 trees. In contrast to the growth of shoots from Type 1 trees the differences in growth rates between P3 and P4 shoots and between P7 and P8 shoots in treatment B were significant. The increase in the growth of shoots from Type 2 trees compared with those in Type 1 trees in treatment C was not significant. In treatment D, P3, P4 and P8 shoots demonstrated a small but significant increase in extension growth when isolated from Type 2 trees. This resulted in a significant difference between the growth of P3 and P4 shoots at 8 Wm^{-2} (Treatments A and B) and at 22 Wm^{-2} (Treatments C and D). The faster extension growth on media

with 45 g/l sucrose compared with 15 g/l sucrose at an intensity of 8 Wm^{-2} of P7 and P10 shoots also occurred after isolation from Type 2 trees.

Table 2. Influence of the light intensity and sucrose concentration on the diameter growth of shoot initials from buds in 10 positions on Type 1 trees and on the number of needles with a minimum length of 2 mm on a short spiral. LSD=Least significant difference.
*: highest value.

	basal diameter growth (mm)				distal diameter growth (mm)				number of needles on short spiral (>2 mm)			
Sucrose conc.(g/l)	15		45		15		45		15		45	
Light intensity (Wm^{-2})	8	22	8	22	8	22	8	22	8	22	8	22
Treatment Position	A	C	B	D	A	C	B	D	A	C	B	D
1	11.9	14.3*	9.9	11.8	8.1	9.3*	8.4	8.3	4.6	5.0	3.2	5.7*
2	11.1*	10.8*	8.6	9.7	6.9	7.8*	6.1	7.8*	4.6	4.4	4.3	4.1
3	10.5	13.5*	10.3	10.1	6.9	9.7*	6.8	7.0	5.2	5.5	4.2	5.2
4	12.4	13.8*	10.2	10.4	7.7	8.3*	6.8	7.5	5.1	5.1	4.8	5.6*
5	10.5	10.7	9.0	10.6	7.6	7.3	7.5	8.3*	5.1	5.8	4.0	5.4
6	7.6	8.7*	7.1	8.4*	6.2	6.8*	5.3	7.0*	4.6	4.2	3.7	4.0
7	7.0	9.3*	8.0	7.0	5.1	6.4*	5.4	5.2	3.2	4.2	4.2	3.9
8	7.8	10.3*	8.1	8.9	5.4	6.6	5.9	7.2*	4.3	5.3*	3.8	4.5
9	9.5	12.7*	9.6	10.3	6.3	7.6*	6.9	6.8	5.2	6.9*	3.8	5.2
10	5.7	9.1*	7.0	7.8	5.4	6.0	5.4	5.7	3.1	3.8*	3.2	3.8*
LSD position/ light/sucrose	0.68				0.47				0.48			

3.2.2 Diameter growth

The light intensity/sucrose concentration treatments had the same influence on the mean distal diameter growth and mean basal diameter growth as described for extension growth (3.2.1). One exception was found: there was a significant sucrose effect on the basal diameter growth of the shoots cultured at 8 Wm^{-2} (Figure 6). This effect did not occur in extension growth. For shoots from most positions, treatment C was the most promotive for basal diameter growth. For P5 shoots no clear optimum could be established; for P2 shoots no difference was found between the influences of treatments C and A (Table 2). A stimulating effect of a high light intensity (22 Wm^{-2}) compared with a low intensity (8 Wm^{-2}) was also found in shoots from most positions except for P2 and

P5 shoots when cultured on media with 15 g/l sucrose and for P3 and P4 shoots when cultured on media with 45 g/l sucrose. The basal diameter growth rate of the shoots cultured on media with 15 g/l sucrose was much higher than that of shoots cultured on media with 45 g/l sucrose except for P3, P6, P8 and P9 shoots at 8 Wm^{-2} and for P5 and P6 shoots at 22 Wm^{-2}. However, on media with 45 g/l sucrose, diameter growth of P7 shoots was curtailed at a high light intensity, and at 8 Wm^{-2} increasing the sucrose level had a positive effect on diameter growth. The P10 shoots whose extension growth unlike that of the other shoots responded positively to these treatments did not exhibit increased basal diameter growth like the P7 shoots.

Topping the trees (Type 2) stimulated mean diameter growth of the shoots from the 5 remaining positions only when grown at 8 Wm^{-2} (Figure 6). The same difference between the same shoots from the 2 types of trees occurred when they were cultured on media with 15 g/l sucrose. However, P4 shoots were the only shoots whose diameter growth was not stimulated at 8 Wm^{-2} after topping; as a result, P3 shoots from Type 1 trees grew more slowly than P4 shoots and those of Type 2 trees grew faster than the P4 shoots of the same trees (Table 2). It was striking to observe that topping inhibited the diameter growth of P10 shoots and possible also of P3 shoots at 22 Wm^{-2}, which strongly contrasted with the stimulation observed at 8 Wm^{-2}. At 22 Wm^{-2} the diameter growth of P3 shoots from Type 2 trees was slower than that of P4 shoots from the same trees. Growth in shoots from all positions on Type 1 trees was stimulated at a higher light intensity. However, this stimulation was only observed in P4 and P8 shoots when isolated from Type 2 trees; the light intensity had no influence on P7 and P10 shoots. The diameter growth of P3 shoots from Type 2 trees at 22 Wm^{-2} was clearly slower than at 8 Wm^{-2}.

Table 3. Influence of the light intensity, the sucrose concentration and tree type on mean growth and numbers of needles of shoot initials from the positions P3, P4, P7, P8 and P10. LSD=Least significant difference; * : highest value.

Light intensity (Wm^{-2})		8		22		
Tree type		1	2	1	2	LSD
Basal diameter growth (mm)						
position	P3	10.4	12.6*	11.8	11.3	
	P4	11.3	11.3	12.1*	11.9*	
	P7	7.5	8.5	8.1*	8.3*	
	P8	7.9	8.7	9.6*	9.5*	
	P10	6.4	8.0	8.5*	7.7	
LSD position/light/ sucrose						0.51
Distal diameter growth (mm)		6.1	6.9	7.0	6.7	0.34
Number of needles on a short spiral		4.1	4.3	4.7*	4.7*	0.32
Sucrose conc. (g/l)		15		45		
Tree type		1	2	1	2	
Basal diameter growth (mm)		9.5	10.3*	8.8	9.2	0.52
Distal diameter growth (mm)		6.8	7.2*	6.3	6.4	0.34
Number of needles on a short spiral		4.5	4.7	4.3	4.2	0.32

The fastest distal diameter growth was observed in treatment C; for P5 and P8 shoots treatment D resulted in the fastest growth whereas for P10 shoots no optimum was established. Distal diameter growth was faster at 8 Wm^{-2} than at 22 Wm^{-2} and even faster in shoots isolated from Type 2 trees (Tables 2 and 3).

3.2.3 Number of needles on the short spiral.

Treatment C also proved to be most promotive for the outgrowth of needles in vitro (Figure 7); at 8 Wm^{-2} or at a high sucrose concentration (45 g/l) leaf expansion was inhibited. However, important differences were found between the positions. Treatment C was the optimum for P8 and P9 shoots, whereas for P5 and P10 shoots there was no significant difference in the number of needles between treatments C and D. P1 and P4 shoots showed a clear preference for treatment D; P2, P3, P6 and P7 shoots had no optimum.

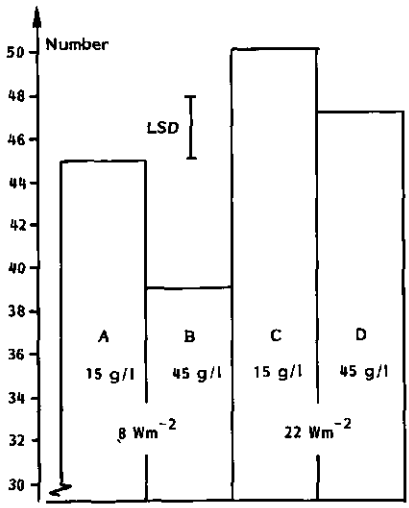

Figure 7. Mean numbers of needles with a minimum length of 2 mm on short spiral of shoot initials from buds in 10 positions on Type 1 trees as influenced by 4 light intensity/sucrose concentration treatments. LSD=Least significant difference.

There was no influence of the light intensity on the needle development of P2, P4, P6 and P7 shoots (mean of sucrose treatments) and no influence of the sucrose concentration on P2, P4, P7 and P10 shoots (mean of light treatments). Topping the trees did not affect the number of needles that developed on the shoots (Tables 2 and 3).

The number of needles that developed on the shoots (Table 2) was never higher than the number of primordia (Figure 3) and only the same on P9 shoots. This last observation indicated that neoformation of primordia had taken place: the results are the average of shoots that developed more needles than the number of primordia on the initial and those that developed less needles than the original number of primordia. Shoots from the other positions developed only some of their primordia into needles. P1 and P5 shoots had the highest number of primordia but developed the same number of needles as P3, P4 and P8 shoots in the optimum treatments. Shoots from axillary buds on branches (P6, P7 and P8) had fewer primordia than shoots from terminal buds of the same branches (P2, P3 and P4) but developed more of them into needles: 86% and 72%, respectively.

3.2.4 Stages of development

The mean number of shoots reaching the stages D and C+E+F was higher at

22 Wm^{-2} than at 8 Wm^{-2} after isolation from Type 1 and Type 2 trees. Most neoformation (stages C+E+F) occurred in P7, P9 and P10 shoots from untopped trees at 22 Wm^{-2} and in P3 and P4 shoots at 22 Wm^{-2} when isolated from Type 2 trees (Table 4). Topping only stimulated apical meristem extension (stages C+E+F) in P3 and P4 shoots cultured at 22 Wm^{-2}.

Table 4. Influence of the light intensity on the numbers of shoots in stages A+B, D and C+E+F (for explanation see Evers, 1981b) in material originally isolated from buds in 10 positions on Type 1 trees and in 5 positions on Type 2 trees.
-, +: $p < 0.05$ (Influence of the light intensity)
θ, θ: $p < 0.05$ (Influence of the tree type)

position	light intensity (Wm^{-2})	Tree Type 1 stages A+B	stage D	stages C+E+F	position	light intensity (Wm^{-2})	Tree Type 2 stages A+B	stage D	stages C+E+F
1	8	55+	2-	0					
	22	38-	20+	0					
2	8	44	12-	1					
	22	34	23+	3					
3	8	32+	23	4	3	8	35	20	4-
	22	18-	34	70		22	20	24	14+θ
4	8	34	21	1	4	8	35	20	3-
	22	25	27	60		22	31	17	10+θ
5	8	46	12-	0					
	22	34	25+	0					
6	8	32	18	7					
	22	25	29	4					
7	8	31	24	3-	7	8	29	25	4
	22	22	27	9+		22	18	31	8
8	8	30	23	4	8	8	29	24	6
	22	20	32	5		22	22	28	8
9	8	37+	18-	5-					
	22	14-	31+	12+					
10	8	34	20	4-	10	8	29	23	5
	22	23	23	9+		22	20	28	7

3.3 The influence of the length of the period of forcing and the sucrose concentration

3.3.1 Extension growth

The mean extension growth of shoots from forced trees (periods of forcing ranged from 3-7 weeks) was always faster than that of shoots from trees whose dormancy was broken naturally (Figure 8). The fastest extension growth occurred on media with 30 g/l sucrose, except after 7 weeks forcing where 15 g/l sucrose was the best treatment. An increase of the sucrose level from 15 to 30 g/l had no significant influence on the length of the shoots after 3 and 5 weeks forcing. When the shoots were cultured on media with 15 g/l or 30 g/l sucrose the growth rate after 5 weeks of forcing was slower than after 3 weeks forcing; however, on media with 30 g/l sucrose the growth rate after 6 weeks forcing resumed the level after 3 weeks forcing and on media with 15 g/l sucrose it was restored gradually after 7 weeks forcing.

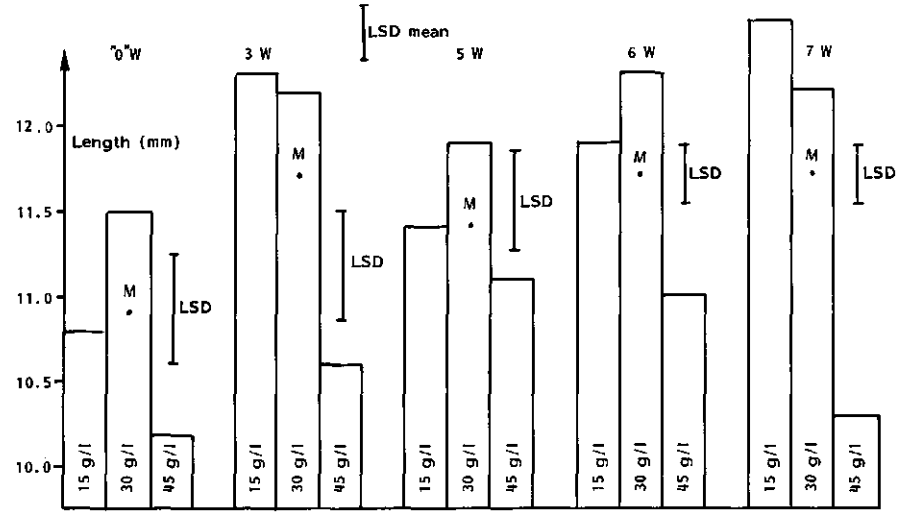

Figure 8. The influence of the sucrose concentration on the mean extension growth of shoot initials from buds in 10 positions on unforced trees and trees that were forced during 3, 5, 6 or 7 weeks. M=mean of the 3 concentrations. LSD=least significant difference.

Extension growth of shoots on media with 45 g/l sucrose reached a maximum after 5 and 6 weeks forcing but fell back to the "0" weeks level after 7 weeks forcing.

When shoots from the separate bud positions are considered it appears that forcing the trees only stimulated extension growth of P1, P2, P5, P6 and P7

shoots when compared with the same shoots from unforced trees (Figure 9). P3 and P4 shoots showed no preference at all, whereas P9 and P10 shoots (and to a lesser extend P8 shoots) demonstrated the fastest extension growth rate when they were isolated from unforced trees.

P8 shoots after 0, 3 and 7 weeks forcing grew the same amount but faster than those after 5 and 6 weeks forcing. It can be concluded that there was a gradient in the response to forcing in different parts of the tree: the in vitro growth of shoots from the highest positions (P1, P2, P5, P6) was stimulated whereas that of shoots from the least exposed positions (P9, P10) was inhibited by forcing. The most promotive duration of forcing was 3 or 6 weeks for P1 shoots, 6 weeks for P2 shoots, 3 weeks for P5 shoots, all periods of forcing for P6 shoots and 6 or 7 weeks for P7 shoots.

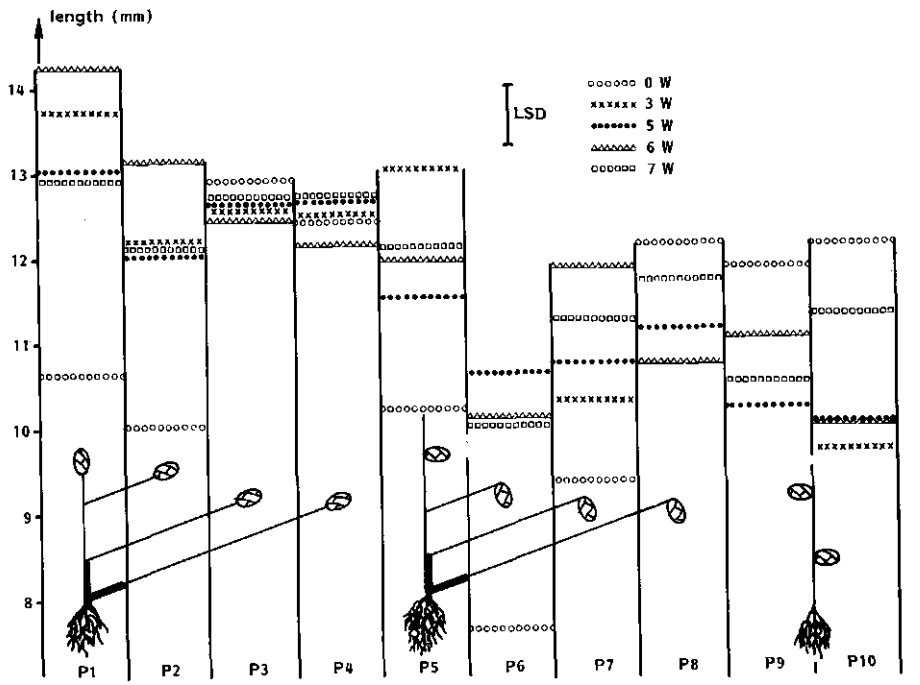

Figure 9. Mean extension growth of shoot initials isolated from buds in 10 positions on unforced trees in April ("0 weeks") and forced trees (forcing periods of 3 weeks, 5 weeks, 6 weeks or 7 weeks from January onwards) averaged over the sucrose treatments.
LSD=Least significant difference.

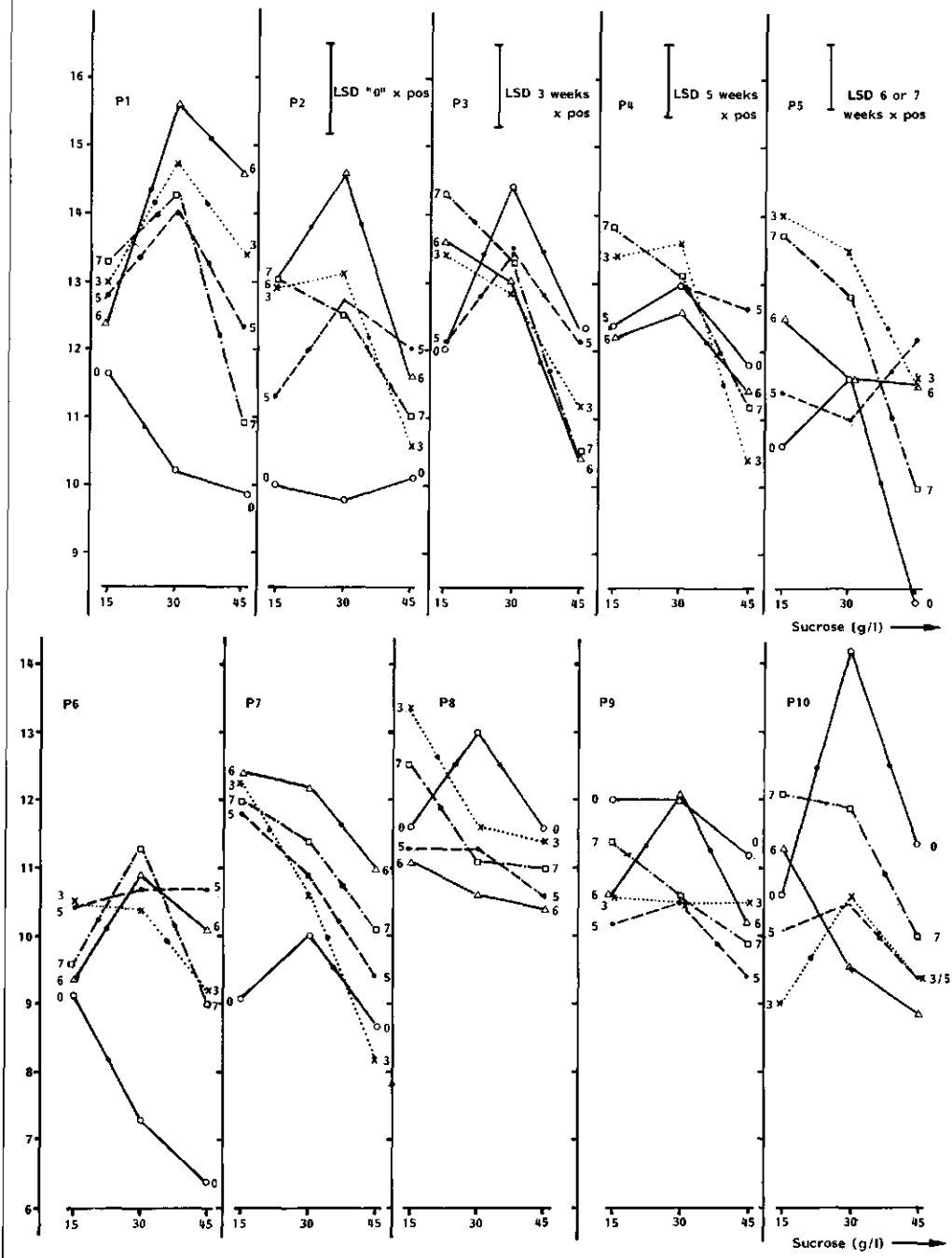

Figure 10. The influence of the duration of forcing and the sucrose concentration on the extension growth of shoot initials isolated from buds in the positions P1-P10, compared with those from trees after the natural breaking of dormancy. LSD=Least significant difference.
.: significant decrease or increase.

The influence of the duration of forcing on the extension growth of shoots from the 10 positions depended on the sucrose concentration (Figures 10 and 11) Growth on media with 45 g/l sucrose was never faster than on media with 15 g/l or 30 g/l sucrose, except for the growth of P5 shoots after 5 weeks forcing. After the trees had been forced, P1, P2 and P6 shoots showed the fastest extension growth on media with 30 g/l sucrose, while growth of P3, P4, P5, P7 and P10 shoots on media with 15 g/l was the same as that on media with 30 g/l sucrose. The effect of the sucrose concentration on the extension growth of P8 and P9 shoots of forced trees was not clear, if indeed there was any. Extension growth of shoots from unforced trees was fastest on media with 15 g/l sucrose for the P1 and P6 shoots or on media with 30 g/l sucrose for the P3, P8 and P10 shoots; no optimum was established for the shoots from other positions on unforced trees. It can be concluded that the average stimulating effect of forcing was especially clear in P1 and P2 shoots on media with 30 g/l sucrose, in P7 shoots on media with 15 g/l sucrose, in P6 shoots on media with 30 g/l or 45 g/l sucrose and in P5 shoots on media with 45 g/l sucrose. Forcing the trees clearly had an inhibiting effect on extension growth of P8 and P10 shoots on media with 30 g/l sucrose.

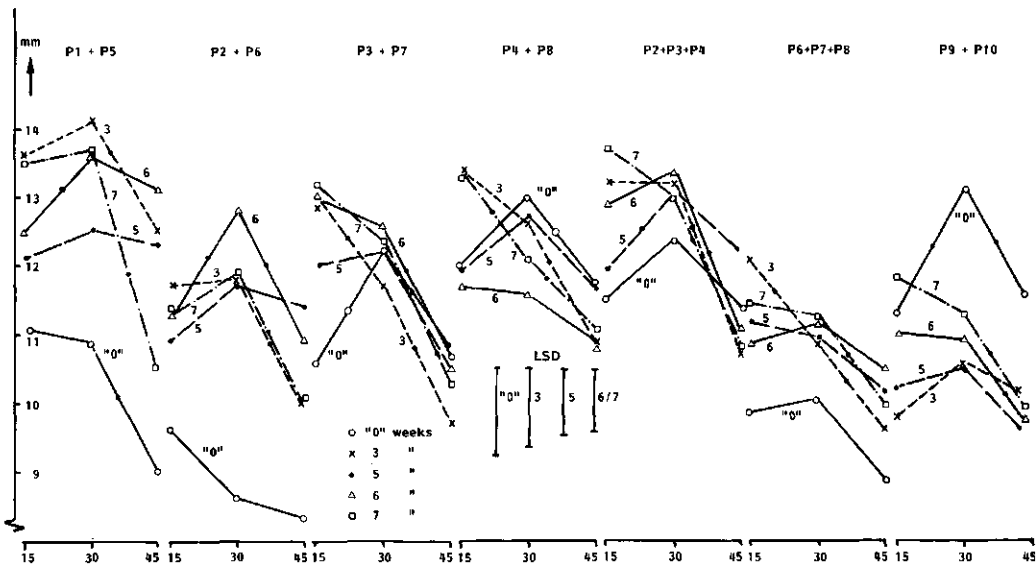

Figure 11. Influence of the duration of forcing and the sucrose concentration on the mean extension growth of shoot initials isolated from buds in horizontally and vertically distributed groups of positions. LSD=Least significant difference. .: significant increase or decrease.

Surprisingly the increases in the sucrose concentration from 15 g/l to 30 g/l stimulated the extension growth of P1 and P6 shoots from forced trees (at least after 6 weeks or 7 weeks forcing) whereas it inhibited the growth of the same shoots from unforced trees.

3.3.2 Diameter growth

The influence of forcing and of the sucrose concentration on diameter growth at the base of the shoots led to the same accents as described for the influence on extension growth (see Appendix 1). Forcing the trees stimulated the diameter growth of P1, P2, P6 and P7 shoots and inhibited that of P8, P9 and P10 shoots. However, the mean diameter growth of shoots from forced trees was not faster than that of shoots from unforced trees. Again, P1 shoots from unforced trees grew fastest on media with 15 g/l sucrose whereas the P1 shoots from forced trees grew fastest on media with 30 g/l sucrose.

Distal diameter growth (see Appendix 1) was stimulated by forcing the trees except in the case of the P1 and P10 shoots. It may be inferred that forcing the trees had the strongest influence on the volume increase of the apical part of the shoots because it stimulated extension growth and diameter growth at the top, but did not stimulate diameter growth at the base. Shoots from the various positions showed modifications of this general pattern. The influence of the sucrose concentration on distal diameter growth was far less clear than its influence on extension growth and basal diameter growth, especially in shoots isolated from unforced trees.

3.3.3 Number of needles on the short spiral

Averaged over shoots from the 10 positions on unforced trees developed more needles longer than 2 mm on the various sucrose treatments than shoots from forced trees (Figure 12). However, on media with 30 g/l sucrose there was no difference between shoots from unforced trees and shoots from trees that had been forced for 6 weeks. On media with 15 g/l sucrose there was no difference between shoots from unforced trees and shoots from trees that has been forced for 7 weeks. Shoots on media with 45 g/l sucrose always had the fewest needles longer than 2 mm. Media with 30 g/l sucrose were the most promotive for needle development of shoots from unforced trees. After 3 weeks or 5 weeks forcing, the most needles occurred on shoots on media with 15 g/l or 30 g/l sucrose; after 6 weeks forcing the shoots demonstrated a preference for 30 g/l sucrose, whereas after 7 weeks forcing 15 g/l sucrose proved to be the most promotive for needle development. Since the optimum sucrose concentrations for needle

development (Figure 12) corresponded with those for extension growth (Figure 8) little difference was expected in the lengths of the internodes of shoots on media with different sucrose concentrations. In fact there were substantial differences in lengths of the internodes between shoots from forced and unforced trees: forced trees produced longer shoots with a lower number of needles. Shoots from all the bud positions followed this pattern; in a considerable number

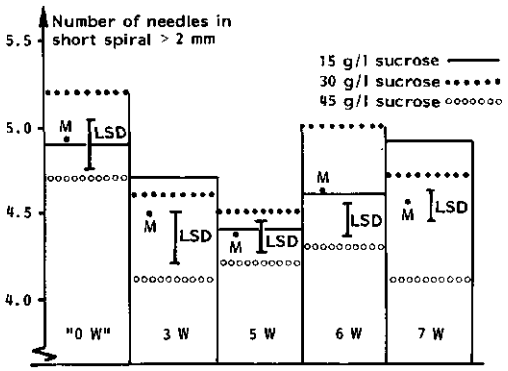

Figure 12. Influence of the sucrose concentration and the duration of forcing on the numbers of needles with a minimum length of 2 mm in short spirals on shoot initials averaged over the buds in 10 positions. LSD=Least significant difference. M=mean of the 3 sucrose concentrations.

of cases the sucrose concentration did not clearly influence the development of the needles (Appendix 1).

3.3.4 Stages of development

In shoots from unforced trees, terminal meristem extension was greatest after isolation from the positions P6+P7+P8 and P9+P10 (Table 5), while shoots from the terminal part of these trees (P1+P5) lagged behind in this development (stages C+E+F). The numbers of shoots reaching the stages C+E+F could not be modified by changing the sucrose concentration. The mean frequency of shoots reaching the stages C+E+F, averaged over the positions and the sucrose concentrations, was lower in shoots isolated from forced trees. The frequency of shoots without apical meristem extension (stages A+B and stage D) was higher after isolation from forced trees than after isolation from unforced trees. The shoots reaching stage D and the stages C+E+F accounted for the major part of the mean extension growth.

Table 5. Influence of the period of forcing and the sucrose concentration on the relative frequency of the stages of development reached by shoot initials isolated from buds in 10 positions. +,-: increase or decrease in the frequency, according to the chi-square test. ←: C+E+F frequency comparable to that in April. A+B: basal needle development; D: Axis development; C+E+F: terminal meristem extension (see Figure 1 in Evers, 1981b).

Time of tree collection		January						January		April	
Time of isolation in vitro		February						March		April	
Weeks of forcing		3		5		6		7		'0'	
Positions	Stage of development	Relative frequency	Tested for stage per week / '0'	Relative frequency	Tested for stage per week / '0'	Relative frequency	Tested for stage per week / '0'	Relative frequency	Tested for stage per week / '0'	Relative frequency	Tested for stage per week / '0'
P1+P5	A+B	0.48	+/	0.60	+/+	0.35	+/	0.40	+/	0.33	+
	D	0.47	/	0.37	-/	0.52	/	0.51	/	0.50	
	C+E+F	0.05	-/-	0.03	-/-	0.13←	-/	0.09	-/ns	0.17	-
P1+P3+P4	A+B	0.39	+/+	0.28	/+	0.20	/	0.23	/	0.12	
	D	0.51	/	0.53	/	0.53	/	0.59	/	0.45	
	C+E+F	0.10	/-	0.19	/-	0.27	/-	0.18	/-	0.43	
P6+P7+P8	A+B	0.13	-/+	0.14	-/+	0.03	-/	0.09	-/	0.04	-
	D	0.68	/+	0.58	/+	0.56	/	0.52	/	0.38	
	C+E+F	0.19	+/-	0.28	/-	0.41←	+/	0.38	+/-	0.58	+
P9+P10	A+B	0.16	-/+	0.08	-/	0.11	/	0.07	-/	0.04	-
	D	0.67	/+	0.54	/	0.51	/	0.57	/	0.38	
	C+E+F	0.17	/-	0.38←	+/	0.38←	+/	0.36	+/-	0.58	+
Sucrose conc. (g/l)		for stage per conc. / '0'		for stage per conc. / '0'		for stage per conc. / '0'		for stage per conc. / '0'		for stage per conc. / '0'	
15	A+B	0.24	/+	0.29	+/+	0.15	/	0.14	/	0.11	-
	D	0.63	/+	0.52	/	0.57	/	0.57	/	0.44	-
	C+E+F	0.13	-/-	0.19	-/-	0.28	/-	0.29	/-	0.45	+
30	A+B	0.33	+/+	0.24	/+	0.13	-/	0.16	/	0.11	-
	D	0.52	/	0.51	/	0.49	/	0.51	/	0.42	
	C+E+F	0.15	-/-	0.25	/-	0.38←	/	0.33←	/	0.47	+
45	A+B	0.29	/+	0.26	/+	0.20	/	0.27	/+	0.15	-
	D	0.60	+	0.52	/+	0.54	/	0.57	/+	0.41	-
	C+E+F	0.11	-/-	0.22	/-	0.26	/-	0.16	-/-	0.44	+
Total		for '0'		for '0'		for '0'		for '0'		for '0'	
	A+B	0.29	+	0.26	+	0.16		0.19	+	0.12	
	D	0.58	+	0.52		0.53	+	0.55	+	0.43	
	C+E+F	0.13	-	0.22	-	0.31	-	0.26	-	0.45	

It can therefore be concluded that the fact that shoots from forced trees were longer after culture in vitro than those from unforced trees was more a result of a stimulation of the extension growth than a result of a stimulation of extension of the apical meristem i.e. rhythmic and free growth. When the shoots had been isolated from trees that had been forced for 6 weeks or 7 weeks and cultured on media with 30 g/l sucrose, the relative frequency of shoots in C+E+F stages was lower than in shoots from unforced trees, but this difference was not statistically significant. A forcing period of 6 weeks proved to be the optimum for the shoots to develop into the C+E+F stages. However, the optimum forcing periods for P6+P7+P8 shoots were 6 or 7 weeks and for P9+P10 shoots were 5, 6 or 7 weeks. The relative frequencies of P1+P5, P6+P7+P8 and P9+P10 shoots in the C+E+F stages after 6 weeks forcing could not be distinguished from those of the corresponding shoots of unforced trees. For P9+P10 shoots after 5 weeks forcing, this relative frequency was also comparable with that of P9+P10 shoots from trees after a naturally broken dormancy. It can be concluded that it is very difficult to activate the terminal meristem of the shoots by forcing the trees, especially in terminal shoots from the branches (P2+P3+P4) and that tree forcing does not change the ratios of apical meristem extension between the positions.

4 DISCUSSION

At the start of the in vitro experiments the dimensions and primordium distribution of the shoot initials were examined in greater detail than in an earlier study (Evers, 1981b). This resulted in a better characterization of the differences and the identification of some similarities between shoot initials from buds in the 10 topophysical positions. Some original differences between the positions were modified during development in vitro, indicating that the hereditary programme of development for each bud also exerts its influence when isolated from the complex of physiological gradients in the trees in vivo. An example of this general observation was found in P1 shoots, which originally had the largest surface area (Figure 4) but did not have the highest growth rate later in vitro. At first the rank order in the surface area of shoot initials decreased the lower down the stem their original position had been: i.e. in the order P1-P5-P9-P10. This rank order was not reflected in the differences in growth rates of shoots from these positions. However, the larger surface area of shoot initials from the lower P4 and P8 buds compared with initials from the higher P2 and P6 initials was reflected in the difference in growth rate between shoots from these groups of positions. Not only the original dimensions and growth rates but also the original number of primordia and the number of developed needles were difficult to compare in relation to the positional differences. The numbers of primordia on shoots from terminal buds on branches (downwards P2-P3-P4) were the same, but P2 shoots developed fewer needles than P3 and P4 shoots: hence the percentages of outgrowth is a separate criterium. Topping the trees reduced the density of the needles by reducing the surface area, except in P10 shoots, perhaps because these shoots are most prepared for reiteration (Hallé et al., 1978).

In the light intensity/sucrose concentration experiments it was found that growth of the shoots at 22 Wm^{-2} and 15 g/l sucrose was always faster than at 8 Wm^{-2} and 45 g/l sucrose (Figure 5). This led to the conclusion that a reduction in the light intensity and thus in the production of photosynthetic carbohydrates cannot be compensated for a higher sucrose concentration. Care must be taken, however, since 45 g/l sucrose might have been a superoptimum concentration; furthermore, Barker and Osmond (1980) found that Eucalyptus shoots in vitro assimilated only about 10% of their (labelled) carbon by autotrophic CO_2 fixation. In an earlier study (Evers, 1982) it was demonstrated that Douglas fir shoots in vitro have the same photosynthetic potential as in vivo. The shoots use carbohydrates from the medium but apparantly also de-

pend on the autotrophic system. Only in P7 and P10 shoots was it found that at a lower light intensity (8 Wm^{-2}) more sucrose was used: growth on media with 45 g/l sucrose was faster than on media with 15 g/l sucrose. However, the overall growth at 8 Wm^{-2} was slower than at 22 Wm^{-2}. Shoots from 'shaded' positions (P4, P8) had no preference for 15 g/l or 45 g/l sucrose at 8 Wm^{-2} while 45 g/l sucrose even inhibited growth of shoots from 'sunny' positions (P1, P5, P6) in this light regime.

Topping the trees only stimulated the growth of the shoots at a low light intensity: trees shoots had a lower autotrophic production but had received more carbohydrates from reserves of the remaining part of the tree, which had been distributed over a smaller number of buds than in intact trees. A higher sucrose concentration did not compensate for this stimulation in shoots from untopped trees. The diameter growth of P3 shoots from Type 2 trees was inhibited and that of P4 shoots was stimulated when the light intensity increased from 8 Wm^{-2} to 22 Wm^{-2} (Table 2) while no effect of the light intensity was found in P7 shoots. Possibly by recovery of the tree from topping at a high light intensity was best prepared in buds on the lower branches (P4, P8) whereas buds on the middle branches (P3, P7) were best prepared for recovery at a lower light intensity. Topping influenced volume growth, but not the development of needles: the arrangement of needles on shoots from both types of trees must have been different. Furthermore, it changed the pattern of development into the CEF stages of shoots from the various positions: most CEF shoots at 22 Wm^{-2} occurred in the P7, P9 and P10 positions of Type 1 trees, whereas they were most numerous in the P3 and P4 positions of Type 2 trees (Table 4). Apical meristem extension was only stimulated in P3 and P4 shoots: recovery from the injury was best prepared in the terminal buds of the branches. However, the absolute number of shoots reaching the CEF stages was low (Table 4). These observations reveal the strategy of a damaged young tree conforming to Massart's model. It fits in the growth programme in space and time in which, as was proven before, every meristem plays a special role. Topping the trees indicated that the growth programme is functional under certain ecological circumstances, e.g. in controlling the surface that is functional in photosynthesis.

The best proof of neoformation of needles would have been for more needles to develop than the initial number of needle primordia present. However, shoots from all positions except P9 did not develop all their primordia into needles. The exceptional reaction of P9 shoots again indicated how well they are prepared for reiteration. Shoots from terminal buds, especially P1 shoots,

developed a low percentage of their primordia into needles but had the most substantial volume growth: apparently the two processes were not directly interrelated. Stimulation of the quantitative rate (volume growth) of the programmed development was easier than stimulation of the programme (needle development, stage of development) itself.

Forcing the trees resulted in the shoots growing faster than the shoots from unforced trees (Figure 8). It is not clear whether this difference would still have existed if the shoots had been taken from trees even closer to the time of flushing. The decrease in the optimum sucrose concentration, from 30 g/l after 6 weeks forcing to 15 g/l after 7 weeks forcing, mirrored the seasonal pattern of decrease found in an earlier study (Evers, 1981b). This decrease in the need for sucrose may be related to the restoration of the photosynthetic system after the cold period (Öquist and Hellgren, 1976). However, this decrease did not occur in shoots from unforced trees (Figure 8); possibly the trees that had been forced for 7 weeks were closer to the time of flushing than unforced trees. Forcing had most influence on shoots from buds high in the tree, which are the positions where flushing occurs last (Allen and Owens, 1972). The pre-treatment levelled the differences in growth rate between shoots from terminal buds and between shoots from axillary buds (Figure 9): forcing influenced the natural differences in the condition of swelling between the positions, as well as the quantitative variations of the programmed development. After forcing, the priorities in the tree were changed: the growth rate of shoots from buds on the stem and of P8 shoots was inhibited. Preparation for the flushing of terminal buds became more important than preparation for reiteration. Forcing influenced programmed growth (stages of development) less than the quantitative variations on this programme. The optimum sucrose concentration for shoots from forced trees was 15 g/l or 30 g/l (Figure 10). When the sucrose concentration was raised from 15 g/l to 30 g/l, the growth rate of P1 and P6 shoots was stimulated if they had been isolated from forced trees, but was inhibited if they had been isolated from unforced trees (Figures 9 and 10). Apparently the photosynthetic system of P1 and P6 shoots recovered better in trees where dormancy was broken naturally - even better than in an earlier study (Evers, 1981b) where the optimum sucrose concentration for P1 shoots was 45 g/l. However, in this earlier study flushing was retarded by a period of frost. Shoots from the top of forced trees must have had longer internodes than shoots from the same positions on unforced trees, since the pre-treatment stimulated the growth but not the development of needles (Figure 12). This divergence was not observed in the influence of the sucrose concentration:

the optimum for needle development coincided with the optimum for extension growth. Since shoots from forced trees reached the CEF stages less often (table 5), shoots from these trees in the D and CEF stages must have had longer internodes.

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Influence of the duration of forcing and of the sucrose concentration on the diameter growth and number of needles with a minimum length of 2 mm in shoot initials from buds in 10 positions.

LSD=Least significant difference. *: highest values.

Basal diameter growth (mm) Pre-treatment in weeks		'0'			3			5			6			7		
Sucrose conc. g/l		15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Position	P1	12.0*	10.0	9.7	12.4*	12.1*	11.4	12.8	14.2*	10.7	11.6	13.7*	11.0	12.6	13.9*	10.2
	P2	9.7*	7.9	9.4*	11.5*	9.6	8.4	10.4*	10.4*	9.2	10.6*	11.0*	7.8	11.7*	10.6*	8.3
	P3	11.7*	11.5*	9.9	11.5*	9.5	9.5	10.6*	11.1*	9.2	11.1*	9.4	8.4	11.8*	9.4	9.1
	P4	10.9	11.5	10.5	11.4*	11.1*	8.2	12.0*	11.3*	9.6	10.1*	9.9*	7.8	10.8*	10.3*	8.2
	P5	10.6*	10.8*	9.4	11.5*	10.3	8.8	9.3	9.0	10.6*	10.1*	8.3	8.8	10.7*	10.4*	8.6
	P6	5.7	6.2	6.2	7.4	7.3	7.1	8.2	7.4	7.5	6.4	7.2	7.0	7.8*	8.0*	7.2
	P7	7.3	7.2	6.8	9.2*	7.8	5.8	8.6*	7.5	7.4	9.8*	8.8	8.0	8.9*	8.2*	6.8
	P8	9.8	10.2	9.8	9.9*	8.9	9.7*	7.8	8.0	7.9	7.6	7.6	7.0	8.4	8.3	8.7
	P9	9.1	9.0	9.0	7.9	8.1	7.9	8.1*	8.4*	7.1	9.0*	8.9*	6.8	7.9	7.3	7.2
	P10	8.0	9.4*	8.8*	7.1	7.3	7.3	7.6	7.2	7.3	8.1*	6.9	6.9	8.6*	8.0*	7.2
	mean	9.5*	9.3*	9.0	10.0*	9.2	8.4	9.5*	9.4*	8.7	9.4*	9.2*	7.9	9.9*	9.4	8.2
LSD mean			0.21			0.40			0.23			0.21			0.18	
LSD pos/sucrose			0.70			0.85			0.76			0.68			0.57	
Distal diameter growth (mm)																
Position	P1	6.4	10.0*	6.1	9.1*	8.4	7.8	6.8	9.0*	7.2	7.0	7.2	6.6	7.4*	7.4*	6.1
	P2	5.5	4.7	4.7	9.1*	6.7	6.4	6.5	6.7	6.3	6.5*	6.4*	5.8	7.2*	6.4	6.1
	P3	5.5	5.2	5.2	7.8*	6.8	7.5*	6.4	7.3	7.0	7.6*	5.8	5.7	7.4*	6.9	6.8
	P4	5.5	5.6	5.1	7.6*	7.8*	5.7	7.3	6.5	6.8	6.2	6.7*	4.8	6.2	7.0*	5.0
	P5	5.9	6.1	5.8	8.3*	6.4	7.4	6.9	6.6	6.3	6.1	5.5	5.8	7.8*	7.4*	6.4
	P6	3.8	3.8	3.9	5.2	6.0	5.4	5.3	5.0	5.5	4.1	4.7	5.3*	6.0*	6.0*	5.4
	P7	4.3	3.6	3.5	6.0*	6.2*	4.8	5.6	5.3	5.2	5.2	5.1	4.8	4.8	5.2	4.5
	P8	5.0	4.7	4.4	7.4	6.3	6.7	5.6	5.4	5.8	4.6	5.0	4.8	6.4	5.9	6.6
	P9	4.3	5.2	4.6	5.8	5.8	6.2	5.0	6.0*	5.1	5.4	5.4	5.6	4.9	4.7	4.8
	P10	5.0	4.4	4.4	4.8	5.5*	5.8*	5.8*	4.6	4.4	5.5*	4.6	4.5	5.6	5.2	4.8
	mean	5.1	5.3	4.8	7.1*	6.6	6.3	6.1	6.2	6.0	5.8*	5.6	5.4	6.4	6.2	5.7
LSD mean			0.29			0.20			0.18			0.11			0.19	
LSD pos/sucrose			0.99			0.64			0.59			0.47			0.48	
Number of needles on short spiral min. 2 mm																
Position	P1	5.8	6.4*	5.3	4.3	6.0*	6.2*	4.1	5.2*	4.0	4.9	5.8*	5.7*	4.9	5.8*	4.3
	P2	4.7	4.7	4.9	4.4*	4.6*	3.7	4.0	4.3	4.0	4.7	5.7*	4.1	4.9*	4.8*	3.8
	P3	5.5	6.3*	5.1	5.6*	4.5	4.2	4.5	5.3*	5.0*	4.5*	4.6*	3.7	5.0*	4.3	3.8
	P4	5.2	5.9*	5.3	5.1*	5.0*	3.6	4.8	4.8	5.1	4.7*	4.8*	4.2	5.1*	5.0*	4.2
	P5	4.7	5.8*	4.0	5.4*	4.8	4.4	4.4	4.1	4.3	4.9*	4.2	4.4	5.4*	4.9	4.2
	P6	3.9	4.0	3.8	4.2*	3.6	3.6	4.3*	4.2*	3.8	4.0	4.2	3.6	3.6	4.4*	3.4
	P7	4.4	4.2	4.3	4.8*	4.0	2.9	4.2	4.0	3.8	4.8	4.5	4.6	4.8*	4.4	3.9
	P8	5.0	5.0	4.8	5.3*	4.4	4.3	4.6*	4.4*	3.8	4.4	4.4	4.4	5.1*	4.1	4.6
	P9	5.4	4.9	4.7	4.0	3.8	4.2	4.4*	4.6*	4.0	4.3	4.7	4.3	5.2*	4.2	4.2
	P10	5.0	4.9	4.8	3.7	3.8	3.6	4.3	4.3	4.0	4.3	5.0*	4.0	4.6*	4.7*	4.2
LSD pos/sucrose			0.43			0.47			0.33			0.37			0.31	

Growth and morphogenesis of shoot initials of Douglas fir, Pseudotsuga menziesii (Mirb.) Franco, in vitro
V The influence of growth regulators and their interaction with topophysis

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CONTENTS

SUMMARY

The influence of GA_3, IAA and BAP applied to the tree and of IAA and BAP applied in vitro on the growth and morphogenesis of shoot initials from buds in 10 positions on 2-year-old Pseudotsuga menziesii (Mirb.) Franco was investigated in vitro. Damaging the trees to enable the uptake of growth regulators occasionally influenced the morphogenesis of the shoots. BAP in the medium strongly inhibited the growth of shoots from untreated trees. It stimulated organogenesis on 8.4% of these explants. Applying GA_3 to the tree followed by IAA in vitro stimulated the growth of shoots from the terminal buds of the tree and of the highest branches, but inhibited the growth of shoots from axillary buds near these positions. Applying IAA to the tree but not to the medium stimulated the growth of shoots from buds on the middle branches. When GA_3 and IAA had been applied to the tree and IAA to the medium the growth of shoots from the terminal buds on the lowest branches was stimulated but the growth of shoots from axillary buds near this position was inhibited. The number of shoots that extended their apical meristem was changed by the combined in vivo - in vitro treatments with growth regulators. The mean extension growth of the shoots was stimulated after BAP had been applied to the roots and IAA to the medium; when BAP was applied to the top of a decapitated tree this stimulation only occurred on media without IAA. The BAP treatment of the roots boosted the extension growth of most shoots when cultured on media containing IAA. When BAP was applied both to the roots and the apex, only the growth of shoots from the remaining axillary positions was stimulated. The BAP treatments of the trees influenced the free growth of the shoots especially if the shoots had been isolated from terminal buds of the branches.

Key words:
Pseudotsuga menziesii - in vitro culture - morphogenesis - topophysical position - mother plant conditioning - growth regulators

Abbreviations:
IAA: 3-indoleacetic acid; GA_3: gibberellic acid; BAP: 6-benzylaminopurine

1 INTRODUCTION

The regulation of morphogenesis in bud cultures is determined by the characteristics of the explant at the moment of isolation and by the conditions during in vitro culturing. The physiological characteristics of tree buds are related to their topophysical position (Nozeran et al., 1971; Evers, 1981b, 1982a) and the environmental history during the development of the tree after the initiation of the buds. Environmental instructions influence the physiology of the shoot initials with endogenous hormones as intermediates. This adaptation of the various bud types, i.e. programming future morphogenesis, enables the tree to react on different environments with different strategies. Modification of the physiology of the buds by exogenous application of growth regulators to the tree followed by culturing them is an approach to simulate environmental changes. It can be expected that for normal morphogenesis of the shoot initials according to a growth program of the tree certain levels of growth regulators in the medium are necessary. The hormonal control of morphogenesis of shoot initials must be clear to be able to regulate the sequential steps (Brown and Sommer, 1975). A difficulty is that the hormones that are present in trees often seem to exert no influence on or negatively influence the natural morphogenetic processes (Zaerr and Mapes, 1982).

In earlier studies (Evers, 1981a) on the morphogenesis of shoot initials of Douglas fir in vitro no stimulating effects of IAA and GA_3 were reported which confirmed earlier results by Al-Talib and Torrey (1959), Boulay (1979) and Chalupa and Durzan (1973). However, it was demonstrated that IAA and GA_3 are present in tissues of Douglas fir (DeYoe and Zaerr, 1976; Pharis and Kuo, 1977). Morphogenetic effects of growth regulators in excised tissues and organs of Douglas fir in vitro have been described for callus induction and maintainance (2,4-D: Harvey and Grasham, 1969; Kinetin: Winton, 1972; NAA: Kirby and Cheng, 1979), shoot induction in embryonic tissues (2,4-D: Cheng, 1977; NAA: Cheah and Cheng), 1978; BAP: Cheng, 1975; 1979; Chalupa, 1977; Sommer, 1975) and root induction on microcuttings (IBA: Winton and Verhagen, 1977; Chalupa, 1977; IAA: Boulay, 1979; NAA: Sommer, 1975; Chalupa, 1977; Boulay, 1979; Cheng and Voqui, 1977). The more differentiated tissues of the shoot initials may have responded less clearly to the growth regulators in earlier studies because the physiological state of the explants (topophysis, pre-conditioning) was not defined (Evers, 1982b; David, 1982). The fastest development of shoot initials of Douglas fir in vitro occurred when they had been isolated just before flushing (Evers, 1981a). Therefore, the conditioning procedures in subsequent experiments (Evers, 1982b) intended to bring the trees in a physiological state similar to that just before flushing: as a

result the topophysical contrasts between the shoots in vitro became more pronounced. In preliminary experiments it was observed that IAA influenced the morphogenesis of shoot initials in vitro after the trees had been treated with growth regulators. Apparently the shoot initials will respond to growth regulators when the mother tree is in a certain physiological state. Therefore, in the present experiments shoot initials were isolated from trees that had been pre-treated in various manners; subsequently these initials were cultured on media with different concentrations of growth regulators.

2 MATERIALS AND METHODS

Plant material and pre-treatments Two-year-old Pseudotsuga menziesii (Mirb.) Franco provenance Arlington trees were selected according to the system described in an earlier paper (Evers, 1981b) and were used in the following experiments (Table 1):

- Experiment A: the influence of the concentration of BAP on the morphogenesis of shoot initials in vitro.
- Experiment B: the influence of the concentration IAA in the medium on the morphogenesis of shoot initials isolated from trees that had been pre-treated with IAA and/or GA_3.
- Experiment C: the influence of the concentration of IAA in the medium on the morphogenesis of shoot initials isolated from trees that had been pre-treated with BAP.

Before the pre-treatments, all selected trees were individually packed in plastic and stored in the dark at 4° C; the plastic was perforated at the top of the trees. After cool storage, the trees were subjected to pre-treatments to allow the growth regulators to be applied; this resulted in 4 types of trees (Figure 1):

- Type 1 (T1): intact trees. Used in all experiments. Roots in 150 ml water.
- Type 2 (T2): topped trees, i.e. the "2b-part" was cut off. The wound was dressed with 150 mg lanolin, in which the growth regulators were dispersed. Used in experiments B and C. Roots in 150 ml water.
- Type 3 (T3): half the root system severed, the remaining roots immersed in 150 ml water in plastic, in which the growth regulators were dissolved. Used in experiments B and C.
- Type 4 (T4): both the T2 and T3 pre-treatments were applied. Used in experiments B and C.

On T1 and T3 trees no buds were removed; on T2 and T4 trees only P3, P4, P7, P8 and P10 were present. All trees were repacked in plastic.

Table 1. Summary of the experiments; for further explanation see text.

PRE-TREATMENTS

Experiment	Trees uprooted	Days in plastic in dark at 4° C (intact)	Number of trees	Type of tree	Top part cut off; lanolin on wound	Half the roots severed; roots in water
A: BAP in vitro	April '80	10-17 (2 weeks)	160	T1	-	-
		24-31 (4 weeks)	160	T1	-	-
		38-45 (6 weeks)	160	T1	-	-
B: GA_3,IAA in vivo;	Nov.'80	55-90	134	T1	-	-
IAA in		55-90	167	T2	*	-
vitro		55-90	167	T3	-	*
		55-90	167	T4	*	*
C: BAP in vivo;	April '81	1-35	134	T1	-	-
		1-35	167	T2	*	-
IAA in		1-35	167	T3	-	*
vitro		1-35	167	T4	*	*

	PRE-TREATMENTS			IN VITRO CONDITIONS[+]		
Exp.	Month into culture room at 25° C	Days of positions forcing		Period of inoculation	Weeks in culture	Light intensity (Wm^{-2})
A:	-	-	P1-P10	April/May	6	24.5
	-	-	P1-P10	'80		
	-	-	P1-P10			
B:	Dec./Jan.'80/'81	21	P1-P10	Jan./Feb.	6	29[+++]
	Dec./Jan.'80/'81	21	P3,4,7,8,10	'81		
	Dec./Jan.'80/'81	21	P1-P10			
	Dec./Jan.'80/'81	21	P3,4,7,8 10			
C:	April/May '81	7	P1-P10	April/May	6	24.5
	April/May '81	7	P3,4,7,8,10	'81		
	April/May '81	7	P1-P10			
	April/May '81	7	P3,4,7,8,10			

	IN VITRO CONDITIONS				NUMBERS		
Exp.	in vivo treatments	Heller's medium[++] with $NaNO_3$ (mM)	Sucrose (g/l)	IAA (µM)	BAP (µM)	shoots per treatment	total	reserve[++++]
A:	T1	15.3	33.0	-	0/0.22/ 1.11/5.55	30	3600	1200
	T1	10.9	37.5	0/0.29/ 2.9/29	-	25		
B:	GA_3, IAA treated							
	T2,T3,T4	10.9	37.5	0/0.29 2.9/29	-	25	3500	1167
	untreated							
	T2,T3,T4	10.9	37.5	-	-	25		
	T1	15.3	33.0	0/0.29/ 2.9/29	-	25		
	BAP							
C:	treated							
	T2,T3,T4	15.3	33.0	0/0.29/ 2.9/29	-	25	3500	1167
	untreated							
	T2,T3,T4	15.3	33.0	-	-	25		

+: 25° C constant, 16 hours light, 8 hours dark.
+++: 29 Wm^{-2} was the highest intensity in the culture room
++: Heller's medium (Evers, 1981b) without activated charcaol.
++++: reserve shoots only replaced infected shoots
* : procedure carried out

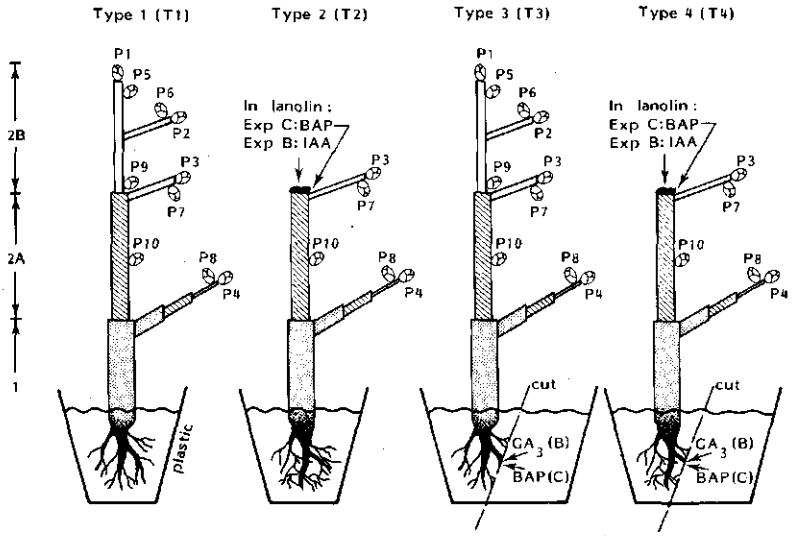

Figure 1. Pre-treatments carried out on trees in experiments B and C. T2 and T4 trees were topped; T3 and T4 trees only retained half their root system. P1-P10: Bud positions P1 up to P10 (Evers, 1981b). Part 1 was formed in the first year, Part 2a in the spring of the second year and Part 2b later in the second year. For further explanation see text.

In preliminary experiments trees were pre-treated at the topped apex (in lanolin) or at the severed roots (in water) with IAA or IBA (0.07-0.15-0.3 mg), GA_3 (0.25-0.5-1.0 mg) and BAP or kinetin (0.10-0.33-1.0 mg) and subsequently a sample of shoot initials from these trees were cultured on media without growth regulators. Only IAA (0.15 mg) and BAP (0.33 mg) applied to the apex or GA_3 (0.5 mg) and BAP (0.33 mg) applied to the roots stimulated the growth of the shoots in vitro. Therefore, in experiment B, 0.15 mg IAA was dispersed in the lanolin of each of the T2 and T4 trees in the treated groups; the T3 and T4 trees in the treated groups received 0.5 mg GA_3 each. In experiment C, T2 trees were given 0.33 mg BAP at the top (lanolin) and T3 trees at the base of the tree (water), respectively. T4 trees were treated at the top as well as at the base. Both the treated and untreated trees were forced in the culture room at 25° C and at a light intensity of 22 Wm^{-2} (= in vitro conditions) for 21 days (Exp. B: December) or 7 days (Exp. C: April), since in April longer forcing than 7 days resulted in flushing of the buds.

In vitro procedures The preparation of the shoot initials and the sterilization of media and buds were described earlier (Evers, 1981a).

Parameters measured After 6 weeks in culture, the height and diameter of the shoots were measured. The influence of the pre-treatments, of the topophysical positions and of the growth regulator concentration in the medium were determined. Shoots from T2, T3 and T4 trees were compared with shoots from T1 trees cultured on media with various IAA concentrations and the differences were statistically tested:

a. Shoots (P1-P10) from T1 trees cultured on media containing no IAA, were compared with shoots from treated or untreated T2 (P3, 4, 7, 8, 10), T3 (P1-P10) and T4 (P3, 4, 7, 8, 10) trees that had been cultured on media containing no IAA.
b. Shoots (P1-P10) from T1 trees cultured on media containing no IAA were compared with shoots from the same positions on growth regulator pre-treated T2, T3 and T4 trees cultured on media with 3 IAA concentrations.
c. Shoots from T1 trees cultured on media with 3 IAA concentrations were compared with shoots from pre-treated trees.

A classification of development was made according to the system described earlier (Evers, 1981a). The significance of the occurrence of shoots in certain classes of development was tested using the chi-square method. The same comparisons as described above (a, b, c) were made.

3 RESULTS

3.1. The influence of BAP in the medium on the morphogenesis of shoot initials

BAP inhibited the mean extension growth and the mean diameter growth of shoots from all positions after the 3 periods of cool storage. No relation was found between the influence of the concentration of BAP and the influence of the topophysical positions on the extension growth. Longer storage periods (4 and 6 weeks) resulted in slightly slower extension growth rates, especially in shoots from terminal positions (P1, P2, P3, P4), than after 2 weeks storage at 4° C. When BAP was present in the medium this difference almost disappeared. BAP inhibited apical meristem extension regardless of the topophysical origin; however, this extension was stimulated by cool storage on media containing 0.22 or 1.11 µM BAP. On media containing 0.22 µM BAP, after a pre-treatment of the tree of 4 weeks at 4° C, apical meristem extension was inhibited compared with that of shoots cultured on a medium containing no BAP: this inhibition disappeared when the cold pre-treatment was extended to 6 weeks.
Many shoots showed necrosis and vitrification. This condition improved the longer the in vivo cold storage period had lasted, except when the shoots were cultured on media containing 5.55 µM BAP. The higher the BAP concentration, the more shoots with apical and basal necrosis and with vitrification were observed. In 8.4% of the shoots cultured on media containing 1.11 or 5.55 µM BAP, organogénesis occurred which were slightly more common in shoots from P4 and P10 buds. Needles were transformed into scale-like structures; many (possibly adventitious) buds were formed on the developing shoots, often surrounded by short brown needles or bud scales. Since the high BAP concentrations stimulated necrosis and vitrification (Figure 2B, 2C) it was difficult to develop shoots (Figure 2A) from these new buds. Neoformation of these structures only occurred on the shoot initial in vitro and not on the shoot part formed as a result of free or rhythmic growth.

3.2. The influence of IAA in the medium on the morphogenesis of shoot initials from trees treated with GA_3 and/or IAA

3.2.1. Extension growth

Forcing As was already observed in preliminary experiments, the 25° C culture room pre-treatment reduced the differences in extension growth between the shoots that were caused by topophysis (Figure 3, Type 1, IAA oµM), especially in shoots from terminal positions (P1, P2, P3, P4). The extension growth

the same for shoots from T1 and hormone pre-treated T2 and T3 trees but was slower for shoots from hormone pre-treated T4 trees. This inhibition in the growth of shoots from T4 trees was only significant on media containing 2.9 or 29 µM IAA and only for shoots from the axillary group P7+P8.

Topping and root severing: topophysis (Figure 3) Only 2 cases of a significant influence of topping and/or root severing on extension growth were observed (shoots from T2, T3, T4 trees, not pre-treated with hormone, without IAA in the medium): P7 and P8 shoots from T4 trees grew faster than corresponding shoots from T1 trees. Topping the tree and/or cutting part of the root system itself thus had little effect on the potential growth of the various shoots in vitro.

IAA in vitro (not shown in graphs) The IAA concentration did not significantly influence the mean extension growth of shoots from intact (T1) trees. Only the extension growth of shoots from the P6+P7+P8 and P9+P10 groups was stimulated: this was ascertained by comparing mean growth on media containing 0 or 0.29 µM with that on media containing 2.9 or 29 µM IAA.

IAA in vitro - IAA/GA_3 in vivo interactions (figure 3) In several cases an interaction between the influence of GA_3 and/or IAA in vivo and the influence of IAA in vitro on extension growth was observed. The results will be described for all shoots.
- P1. The extension growth of P1 shoots from T3 trees pre-treated with GA_3 was faster than P1 shoots from T1 trees whether the P1 shoots were grown on media containing 29 µM IAA or on media not containing IAA; on media containing 0.29 or 29 µM IAA the extension growth of the P1 shoots from T3 trees was the same as that of P1 shoots from T1 trees.
- P2. The P2 shoots from T3 trees treated with GA_3 was slower than that of the controls, but only on media containing 29 µM IAA. This GA_3-IAA effect did not occur in shoots from other positions.
- P3. The P3 shoots did not react to the pre-treatment of the T3 trees with GA_3. The extension growth of P3 shoots on media containing 0 or 0.29 µM IAA was stimulated by the IAA pre-treatment of T2 trees; no stimulation of extension growth occured when the concentration of IAA in vitro was higher (2.9 or 29 µM). The mean extension growth of P3 shoots from T4 trees pre-treated with GA_3/IAA and cultured on media containing 4 levels of IAA was faster than that of the P3 shoots from T1 trees cultured on the same media; the effect of the seperate IAA concentrations was only significant at 0.29 µM.
- P4. The P4 shoots did not react to the pre-treatment of the T3 trees with GA_3. The mean extension growth of P4 shoots from T1, T2 and T3 trees was the same

on all media; it was only stimulated if the shoots had been isolated from T4 trees pre-treated with GA_3 and IAA and cultured on media containing 29 µM IAA.

- P5 and P6. The extension growth of P5 and P6 was inhibited if the shoots had been isolated from GA_3 pre-treated T3 trees and cultured on media containing 29 µM IAA.
- P7. The extension growth of P7 shoots was stimulated by the IAA pre-treatment of T2 trees especially in the shoots cultured on media without IAA or containing 0.29 µM IAA. In contrast, P7 shoots grew more slowly on media containing 2.9 or 29 µM IAA if they had been isolated from T4 trees pre-treated with GA_3 and IAA. Thus, extension growth was the same for P7 shoots from both T1 and T4 (+GA_3/IAA) trees on media without IAA and on media containing 2.9 µM IAA, and for P7 shoots from T1 and T2 (+IAA) trees on media containing 2.9 or 29 µM IAA. The P7 shoots from T2 trees always grew faster than the corresponding shoots from T4 (+GA_3/IAA) trees; the application of IAA in vitro had no effect on this difference.
- P8. The extension growth of P8 shoots from T1, T2 and T3 trees was the same. Pre-treating the T4 trees with GA_3 and IAA inhibited the growth of P8 shoots. The application of IAA in vitro had no effect on this inhibition of growth.
- P9 and P10. Neither the pre-treatments nor the IAA concentration in vitro had any observable effect on the extension growth of P9 and P10 shoots.

Summary All effects of the pre-treatments and hormones in vivo and in vitro on extension growth of the shoots in vitro are summarized in Table 2. In the column for the influence of IAA in the medium any effect (averaged or just one concentration) of this growth regulator is given; for further explanation see text.

Table 2. Influence of the tree type, hormone pre-treatment and IAA in vitro on the extension growth of shoots from 10 positions. All comparisons with shoots from T1 (intact) trees at IAA 0 µM.
n: bud not present. *: comparison of the mean extension growth of shoots from T1 and T4 trees on all media. o, +, −: similar, faster, slower extension growth than in T1 at IAA 0 µM.

Position	T1: intact trees IAA in vitro	T2			T3			T4		
		Topped	IAA + in vivo	IAA in vitro	Roots severed	Ga_3 + in vivo	IAA in vitro	Topped + roots severed	IAA + GA_3 in vivo	IAA in vitro
P1	o	n	n	n	o	o	+	n	n	n
P2	o	n	n	n	o	o	+	n	n	n
P3	o	o	+	o	o	o	o	o	o	+
P4	o	o	o	o	o	o	o	o	o	+
P5	o	n	n	n	o	o	−	n	n	n
P6	o	n	n	n	o	o	−	n	n	n
P7	o	o	+	o	o	o	o	+	o	−*
P8	o	o	o	o	o	o	o	+	−	−
P9	o	n	n	n	o	o	o	n	n	n
P10	o	o	o	o	o	o	o	o	o	o

3.2.2. Diameter growth

The pre-treatments and the IAA concentration in vitro had hardly any effect on the diameter growth of the various shoot initials. The mean diameter growth of all shoots was slightly stimulated by the GA_3 pre-treatment of T3 trees; the diameter growth of shoots from T2 trees was always faster than that of shoots from T4 trees.

3.2.3. Classes of development

Pre-treatments and IAA in vitro: average effects In shoots from T1 and T3 trees, extension of the apical meristem (classes C+E+F) was not stimulated by IAA in the medium. In shoots from the 5 positions in T2 trees the total number of shoots with apical meristem extension was promoted by IAA when applied both in vivo and in vitro; however, the same stimulation had already been achieved by topping only when compared with shoots from IAA pre-treated trees on media without IAA (Figure 4). It can therefore be concluded that IAA in vitro only restored the inhibiting effect of IAA in vivo on shoots from T2 trees. After application of IAA both in vivo and in vitro or only after topping, more shoots reached the C+E+F classes than those from T1 trees. It must be remembered, however, that the 5 bud positions in T2 trees usually produce relatively more shoots with an extended apical meristem than those from the 10 positions of T1 trees. Adding IAA to the medium inhibited the apical meristem extension of shoots from T4 trees that had been treated with GA_3 and IAA; *simultaneously topping and root severing had no influence.*

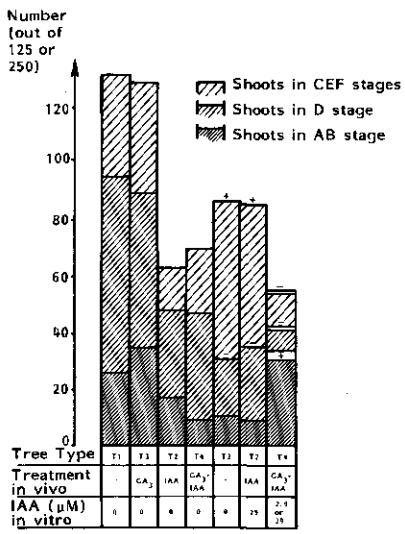

Figure 4. Numbers of shoots reaching the classes AB (no apical development), D (apical development and elongation) and CEF (apical meristem extension in rhythmic or free growth) as influenced by the type of the tree, the hormone pre-treatment and the IAA concentration in the medium. The numbers reflect the sum over all bud positions: 5 in T2 and T4 (total 125 per IAA conc.), 10 in T1 and T3 (total 250 per IAA conc.).

Hormone pre-treatments and topophysis: total numbers per position As Figure 5 shows, forcing resulted in the highest number of shoots with apical meristem extension (classes CEF) occurring in P2 and P4 shoots among the terminal positions (P1 up to P4) and in P6 shoots among the axillary positions (P5 up to P8). The hormone pre-treatments modified the ratio between shoots in the D class and shoots in the CEF classes, usually without changing the total number of shoots in the classes D+CEF; this only held true for the total numbers of shoots per position i.e. the sum of all shoots on media with 4 levels of IAA (Figure 5). The total numbers of P1, P3, P5, P6 and P9 shoots in the CEF classes were not altered by the hormone pre-treatments. GA_3 stimulated apical meristem extension in P2 shoots but inhibited this development in P7 shoots. Pre-treating T2 trees with IAA stimulated apical development in P4 and P8 shoots. The combined in vivo application of GA_3 and IAA promoted the apical meristem extension of P4 shoots from T4 trees but inhibited it in P7, P8 and P10 shoots from the same trees.

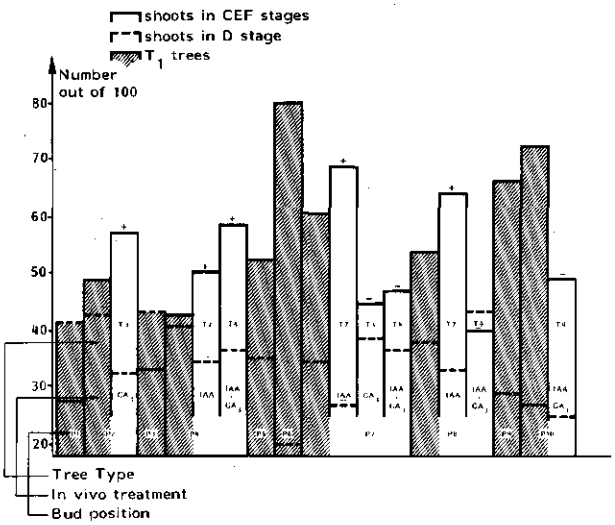

Figure 5. Influence of the tree type and hormone pre-treatment on the total numbers of shoots in the D and CEF classes in 10 topophysical positions cultured on media with 4 IAA concentrations. +, -: significant increase or decrease compared with corresponding shoots on T1 trees.

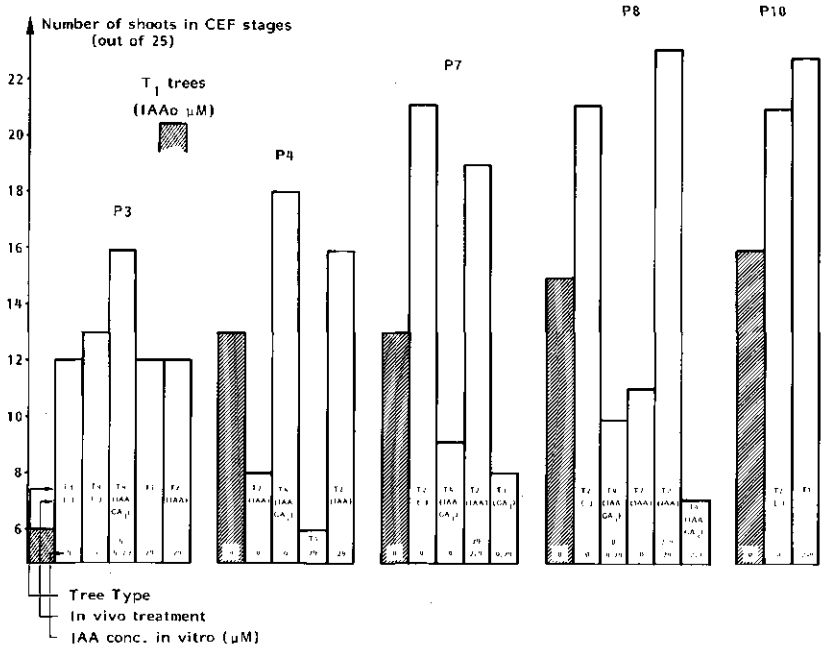

Figure 6. Influence of the tree type, hormone pre-treatment and IAA concentration of the medium on the numbers of P3, P4, P7, P8 and P10 shoots reaching the CEF classes. (-): not pre-treated with growth regulators. Only significantly higher or lower numbers are given. More concentrations in a bar: the same number of CEF shoots.

IAA in vitro - IAA/GA_3 in vivo interactions (Figure 5) When compared with the corresponding shoots from T1 trees on media without IAA the numbers of P1, P2, P5 and P6 shoots reaching the CEF classes were not significantly influenced by any of the pre-treatments nor by the IAA concentration in vitro (Table 3). The results of the other positions were:

- P3. The apical meristem extension of P3 shoots from T1 trees was stimulated by IAA in vitro. The same effect was observed in P3 shoots from T2 trees but could partly be attributable to topping. Topping and root severing the T3 and T4 trees also stimulated apical extension of P3 shoots. The GA_3 treatment of T3 trees counteracted some of this stimulation in P3 shoots; this counteraction did not occur after the GA_3/IAA treatment of T4 trees if the IAA concentration of the medium did not exceed 0.29 µM.
- P4. IAA in the medium reduced the number of P4 shoots from T1 trees with apical meristem extension to that of P3 shoots cultured on media without IAA. This extensio in P4 shoots was also inhibited if the T2 trees had been pre-treated with IAA. However, P4 shoots from T2 trees reacted strongly to the IAA concentration of the medium: when 0.29 µM IAA was present apical meristem extension was comparable with that of P4 shoots from T1 trees and with 29 µM IAA the number of P4 shoots in the CEF classes was even higher than in the shoots from T1 trees; this was not attrib-

utable to the topping of T2 trees. It can therefore be concluded that the IAA treatment of T2 trees counteracted the IAA effect on apical meristem extension in vitro. Pre-treating the T4 trees with IAA and GA_3 stimulated the apical meristem extension of P4 shoots. Once again, the presence of IAA in the medium counteracted this stimulation.

- P7. The topping of T2 trees stimulated apical meristem extension of P7 shoots; adding 2.9 or 29 µM IAA to the medium induced the same amount of extensions in corresponding shoots from IAA treated T2 trees. A concentration of 0.29 µM IAA in the medium inhibited the apical meristem extension of P7 shoots from T3 trees treated with GA_3; this development was also inhibited if they had been isolated from T4 trees treated with IAA and GA_3 but was cancelled out by IAA in vitro.
- P8. The topping of the T2 trees stimulated the apical meristem extension of P8 shoots but the IAA pre-treatment counteracted this effect; moreover, it was again restored by adding 2.9 or 29 µM IAA to the medium. Apical meristem extension of P8 shoots was inhibited if they had been isolated from T4 trees treated with IAA and GA_3; this effect was not cancelled out by IAA in vitro.
- P9. P9 shoots from T1 trees on media with 0.29 or 2.9 µM IAA reached the CEF classes more often than P9 shoots from T3 trees that had been treated with GA_3 on the same media.
- P10. IAA in vitro stimulated apical meristem extension of P10 shoots from T1 trees; about the same stimulation was obtained after topping the T2 trees.

Table 3. Influence of the tree type, hormone pre-treatment and IAA in vitro on apical meristem extension (CEF classes) of shoots from 10 positions. All comparisons with shoots from T1 trees at IAA 0 µM. IAA concentrations in vitro are specified in the text. n: bud not present. (+): stimulation not significant at $p<0.05$. *: comparison at IAA 0.29 and 2.9 µM. **: comparison of the total numbers from T1 and T3 or T4 (all media).

Position	T1	T2			T3			T4		
	IAA in vitro; intact trees	Topped	+ IAA in vivo	+ IAA in vitro	Roots severed	+ GA_3 in vivo	+ IAA in vitro	Topped + roots severed	+ IAA + GA_3 in vivo	+ IAA in vitro
P1	o	n	n	n	o	o	o	n	n	n
P2	o	n	n	n	o	o	+**	n	n	n
P3	+	(+)	(+)	+	+	o	o	+	+	o
P4	–	o	–	+	o	o	o	o	+	o
P5	o	n	n	n	o	o	o	n	n	n
P6	o	n	n	n	o	o	o	n	n	n
P7	o	+	o	+	o	o	–	o	–	–**
P8	o	+	–	+	o	o	o*	o	–	–
P9	(+)	n	n	n	o	o	–*	n	n	n
P10	+	+	o	o	o	o	o	o	o	–**

3.2.4. Condition

All hormone pre-treatments (T2, T3, T4) increased the incidence of shoots with basal necrosis, apical necrosis and vitrification, especially if the shoots had been

isolated from terminal positions. The GA_3 treatment of T3 trees resulted in more shoots with short brown needles or bud scales; the same was observed in shoots from T4 trees (untreated) when they were compared with those from T2 trees (untreated).

3.3. The influence of IAA in the medium on morphogenesis of shoot initials from trees treated with BAP

3.3.1. Extension growth

Forcing In this experiment the 25° C culture room pre-treatment reduced but not levelled the differences in extension growth between P2, P3 and P4 shoots observed in earlier experiments; in contrast with the results of experiment B the growth of shoots from the upper part of the tree (P1, P2, P5, P6) was not stimulated more than that of shoots from other positions when compared with non-forced trees in preliminary experiments in April.

Mean extension growth: IAA in vitro In contrast with the results in experiment B a stimulation of the mean extension growth (averaged over the positions) by IAA in vitro was observed. The mean extension growth of shoots from T1 and T3 trees was stimulated by IAA (Figure 7), while the mean growth of shoots from T2 and T4 trees (containing only 5 positions) remained almost unaffected. The same conclusions could be drawn when only the mean growth of the 5 positions present on all tree types was compared (not given in the graphs).

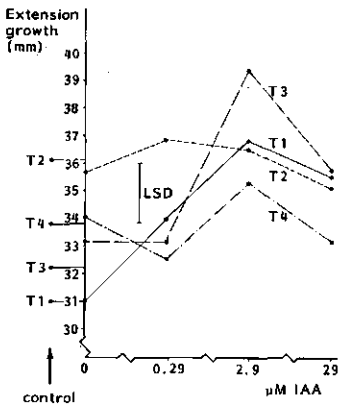

Figure 7. Influence of the IAA concentration on the mean extension growth (averaged over the positions) of shoots from T1 and BAP treated T3 trees (10 positions) and from BAP treated T2 and T4 trees (5 positions). LSD: least significant difference. Control: mean growth of shoots from trees not pre-treated with BAP on media without IAA.

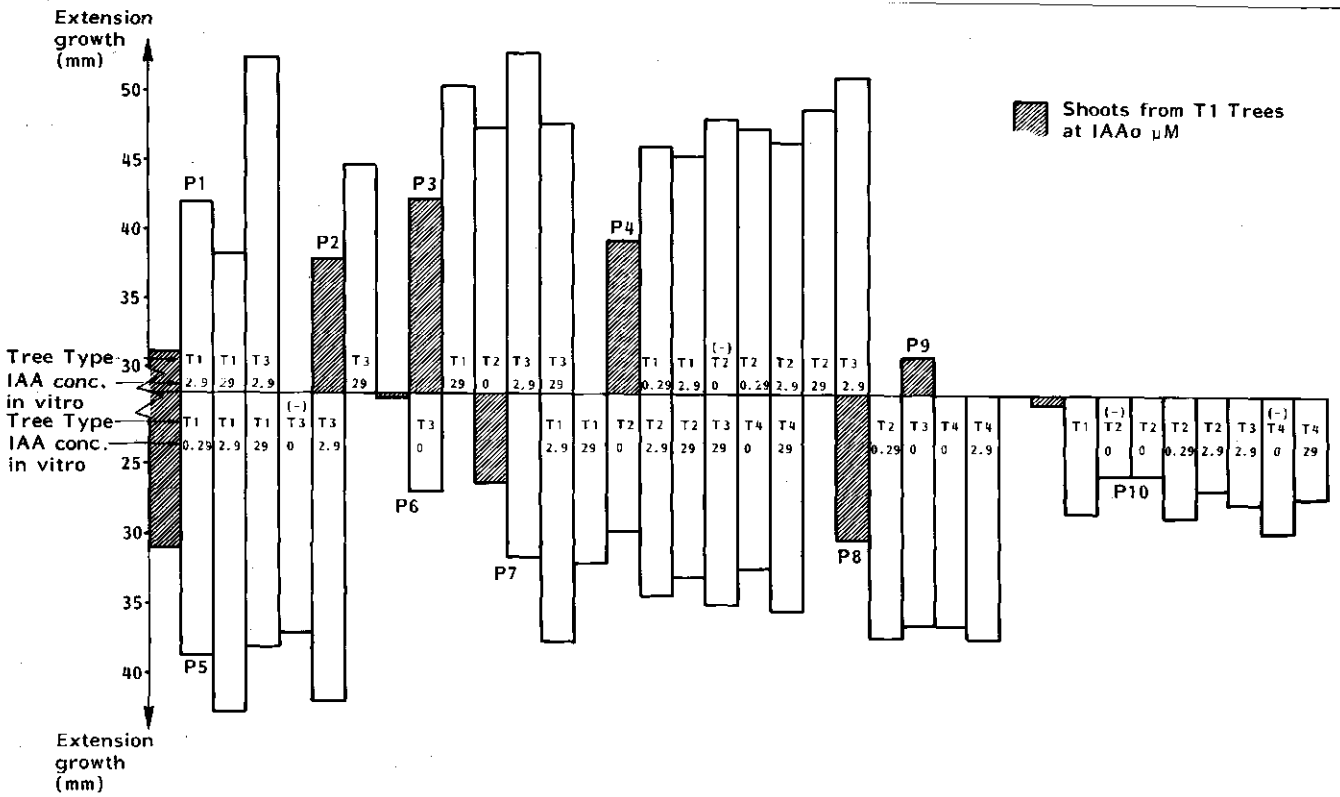

Figure 8. The extension growth of shoots from buds in 10 positions on intact trees (T1) on media not containing IAA, compared with significantly different growth of 1) the same shoots on media with 3 levels of IAA, 2) that of shoots from buds in 5 positions on T2 and T4 trees pre-treated or not pre-treated with BAP on all media and 3) that of shoots from buds in 10 positions pre-treated or not pre-treated with BAP on all media. (-): no hormone pre-treatment, IAA: IAA concentration in vitro (µM). 1), 2) and 3) were tested separately. Not significantly different growth from T1 at IAA o µM is not given.

Mean extension growth: topping and root severing Topping and root severing of T2 and T4 trees (control level of Figure 7) stimulated extension growth compared with that of shoots from T1 trees at 0 µM IAA which was not modified by IAA in vitro nor by BAP in vivo. The root severing and the BAP pre-treatment of T3 trees did not significantly stimulate extension growth of the shoots; however, with 2.9 µM IAA the growth of shoots from treated trees reached a higher maximum than that of shoots from all other tree types. When the growth of the shoots from the seperate positions was considered (Figure 8, Table 4), the mechanical pre-treatments only significantly influenced P4 shoots (T2), P5 shoots (T3) and P10 shoots (T2, T4).

BAP in vivo - IAA in vitro interactions: topophysis (Figure 8)

- T1. The extension growth of P1, P3, P4, P5, P7 and P10 shoots was stimulated by IAA .
- T2. Topping the trees stimulated the growth of P4, P8 and P10 shoots. The BAP treatment of the trees eliminated this effect in P4 and P8 shoots, stimulated the growth of P3 and P7 shoots but did not affect the stimulated growth of P10 shoots. Adding IAA stimulated the extension growth of P4 and P8 shoots from T2 trees treated with BAP, inhibited that of P3 shoots and did not affect the growth of P7 and P10 shoots when compared with the corresponding shoots on media without IAA.
- T3. Root severing of T3 trees only stimulated the extension growth of P5 shoots; this effect was eliminated by BAP and restored by IAA in vitro. The BAP pre-treatment stimulated the growth of P6 and P8 shoots which was no longer clear when IAA was added. The combined BAP - IAA treatment stimulated the growth of P1, P2, P3, P4, P7 and P10 shoots. The peak in the mean extension growth of shoots from T3 trees at 2.9 µM IAA (Figure 7) was mainly caused by shoots from terminal positions (P1-P4).
- T4. Topping and root severing the T4 trees stimulated the growth of P10 shoots; this growth was reduced by the BAP pre-treatment and restored by IAA in vitro. Both P7 and P8 shoots grew faster after the BAP pre-treatment. This stimulation disappeared on media containing 0.29 µM IAA but recurred on media containing 2.9 µM IAA (P8) or 29 µM IAA (P7).

Table 4. Influence of the tree type, BAP treatment in vivo and IAA treatment in vitro on the extension growth of shoots from 10 positions. All comparisons are made with shoots from T1 trees without IAA. n: bud not present. (s): strongest stimulation of extension growth. +: stimulation. -: inhibition. IAA concentrations in vitro are specified in the text.

Position	T1: IAA in vitro	T2: Topped	T2: BAP in vivo +	T2: IAA in vitro +	T3: Roots severed	T3: BAP in vivo +	T3: IAA in vitro +	T4: Topped + roots severed	T4: BAP in vivo +	T4: IAA in vitro +
P1	+	n	n	n	o	o	+(s)	n	n	n
P2	o	n	n	n	o	o	+(s)	n	n	n
P3	+(s)	o	+	o	o	o	+(s)	o	o	o
P4	+	+	o	+	o	o	+(s)	o	o	o
P5	+(s)	n	n	n	+	o	+(s)	n	n	n
P6	o	n	n	n	o	+(s)	o	n	n	n
P7	+(s)	o	+	+(s)	o	o	+(s)	o	+	+(s)
P8	o	o	o	+(s)	o	+(s)	o	o	+(s)	+(s)
P9	o	n	n	n	o	o	o	n	n	n
P10	+(s)	+(s)	+(s)	+(s)	o	o	+(s)	+(s)	o	+(s)

3.3.2. Diameter growth

The mean diameter growth of the shoots was stimulated by the BAP treatment of T2 and T3 trees. BAP stimulated the diameter growth of the P3, P8 and P10 shoots from T2 trees, P3, P4, P6, P7, P8 and P9 shoots from T3 trees and P3, P7 and P8 shoots from T4 trees.

3.3.3. Classes of development

Total numbers: tree type (Figure 9) The total numbers of shoots from all positions reaching the classes of development ABD, CE and F were not influenced by topping and/or root severing except in the case of shoots from T4 trees where free growth (F class) was reduced but resumed after the BAP treatment. The total number of shoots that reached the F class from all positions was much higher in T2 trees that had been pre-treated with BAP than in the T1 trees. In shoots from T2 trees and T1 trees it was possible to increase the number of rhythmically growing shoots (CE classes) by adding IAA to the medium. The BAP pre-treatment of T3 trees did not affect free growth; a combined BAP-IAA treatment stimulated free growth in shoots from these trees but did not stimulate rhythmic growth.

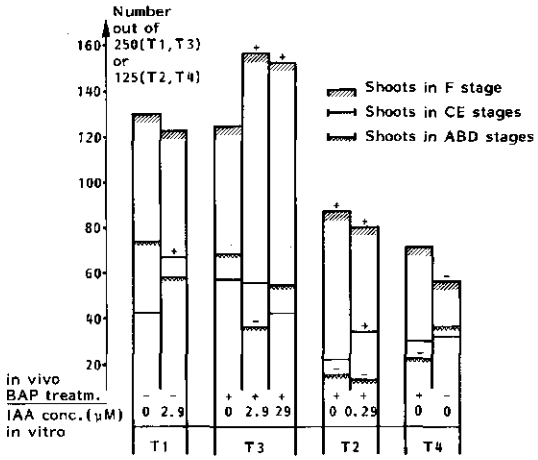

Figure 9. Influence of the tree type, BAP treatment and the IAA concentration on the total numbers of shoots from all positions (10 in T1 and T3; 5 in T2 and T4) reaching the classes ABD, CE and F, when compared with those of T1 trees on media without IAA.

Topophysis: BAP pre-treatment (Figure 10) The total numbers of shoots in the CE and F class on media with 4 concentrations of IAA, originally isolated from 10 bud positions are given in Figure 10. Neither the free nor the rhythmic growth of P1, P6 and P7 shoots were significantly influenced by the mechanical or the BAP pre-treatment; however, the toal number of CE+F shoots in the P7 group increased as a result of the BAP treatments.

- T2. The BAP treatment merely changed the CE:F ratio in P3 and P4 shoots without significantly changing the total number of rhythmically or freely growing shoots. Moreover, it did not affect the total number of shoots in the CE+F stages: more free growth occurred at the expense of rhythmic growth when compared with shoots from untreated trees. The BAP treatment significantly reduced rhythmic growth and stimulated free growth of P10 shoots.
- T3. In the P1 and the P6 groups the ratio between free and rhythmic growth altered as a result of the BAP treatment, but the total number of CE+F shoots remained unaffected: P1 shoots grew relatively more rhythmically and the P6 shoots grew more freely. The BAP treatment significantly stimulated the free growth of P2, P3, P4 and P9 shoots which was in P2 and P3 shoots accompanied by a reduction in rhythmic growth thus not affecting the total number of shoots with an extended apical meristem. This reduction did not occur in P4 and P9 shoots: the total number of CE+F shoots increased after the BAP treatment. In P5 shoots, the BAP treatment reduced rhythmic growth.

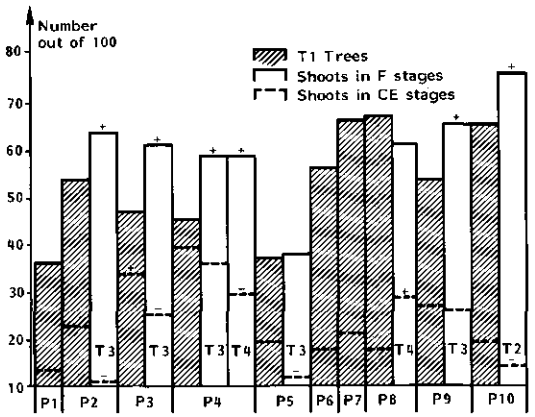

Figure 10. Influence of the tree type and the BAP treatment on the significant increase (+) or decrease (-) of the total numbers of P1–P10 shoots reaching the classes CE (rhythmic growth and F (free growth) on media with 4 concentrations of IAA (4x25 shoots). Changes in the CE:F ratio and the total number of CE+F are given in the text.

- T4. The rhythmic growth in P4 shoots was reduced by the BAP treatment; the total number of CE+F shoots remained the same as in the P4 group of T1 trees. The BAP treatment merely changed the CE:F ratio in P3 shoots without affecting the total number of shoots in the CE+F classes: more free growth occurred at the expense of rhythmic growth when compared with shoots from untreated trees. The BAP treatment significantly stimulated rhythmic growth of P8 shoots. It can be concluded that the BAP pre-treatment influenced free growth, rhythmic growth or the ratio between the growth types of shoots from all positions except P7 shoots.

BAP in vivo - IAA in vitro interactions: free growth (Figure 11, Table 5)

- Topping and root severing. The mechanical pre-treatments influenced the free growth of shoots from some of the positions. Topping (T2) reduced the free growth in P3 shoots and stimulated it in P4 and P7 shoots: severing the roots (T3) reduced free growth in P2 shoots and stimulated it in P9 shoots and both procedures (T4) reduced free growth in P3 and P7 shoots.

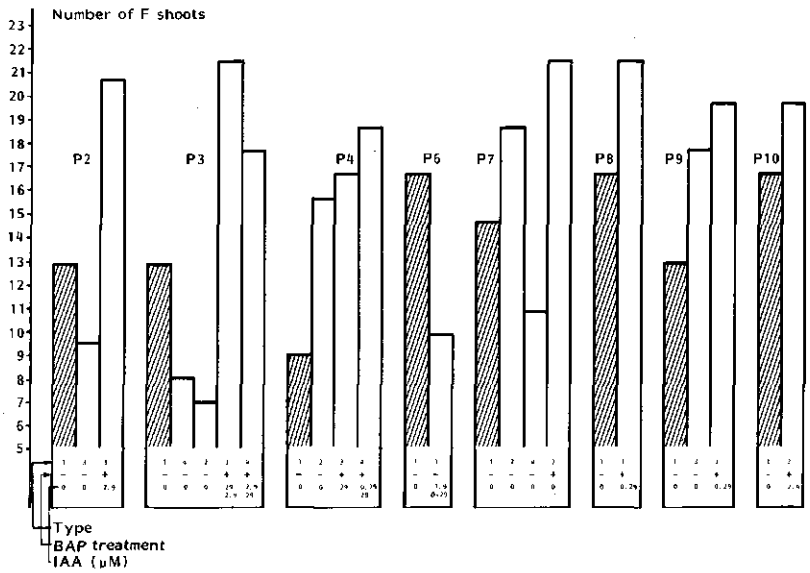

Figure 11. Influence of the tree type (T1-T4), the BAP treatment (+) and the IAA concentration on the numbers of shoots reaching class F (free growth) originally isolated from buds in the positions P1-P10. White bars: more or less free growth than expected when compared with T1 according to the chi-square test.

- T1. The concentration of IAA did not influence the free growth except in the case of P6 shoots on media with 0.29 or 2.9 µM IAA: less shoots reached the F class.

- T2. In P3 shoots the BAP pre-treatment eliminated the reduction of free growth caused by topping. As a result of topping the stimulation of the free growth of P4 shoots was also eliminated by BAP regardless of the IAA concentration. More P10 shoots reached the F class after the BAP pre-treatment if they had been cultured on media with 2.9 µM IAA.

- T3. In P2 shoots the BAP pre-treatment eliminated the decline in free growth caused by root severing; IAA at 2.9 µM even stimulated this type of growth. The IAA effect observed in P6 shoots from T1 trees did not occur in corresponding shoots from T3 trees. IAA at concentrations of 2.9 or 29 µM stimulated free growth of P3 shoots from BAP treated trees; at 29 µM it occurred in P4 shoots and at 0.29 µM IAA in P8 shoots. Free growth of P9 shoots was stimulated by root severing, which was counteracted by BAP but reappeared when 0.29 µM IAA was added to the medium.

- T4. In P3 shoots the BAP pre-treatment eliminated the reduction in free growth caused by topping and root severing. IAA at concentrations of 2.9 or 29 µM stimulated free growth in P3 shoots from treated trees; at 0.29 or 29 µM the P4 shoots were also stimulated.

Table 5. Summary of influences of the tree type, BAP treatment and IAA treatment on free growth (F class) of shoots in 10 positions. All comparisons are made with shoots from T1 trees at 0 µM IAA. n: bud not present; (m): most free growth; o: no effect; +: stimulation; -: inhibition. IAA concentrations are specified in the text.

Position	T1: IAA in vitro	T2 Topped	T2 + BAP in vivo + IAA in vitro	T3 Roots severed	T3 + BAP in vivo + IAA in vitro	T4 Topped and roots severed	T4 + BAP in vivo + IAA in vitro
P1	o	n	n n	o	o o	n	n n
P2	o	n	n n	-	o +(m)	n	n n
P3	o	-	o o	o	o +(m)	-	o +
P4	o	+	o o	o	o +(m)	o	o +(m)
P5	o	n	n n	o	o o	n	n n
P6	-	n	n n	o	o o	n	n n
P7	o	+	+(m) o	o	o o	-	o o
P8	o	o	o o	o	o +(m)	o	o o
P9	o	n	n n	+	o +(m)	n	n n
P10	o	o	o +(m)	o	o o	o	o o

BAP in vivo - IAA in vitro interactions: rhythmic growth The rhythmic growth of the P6+7+8+9+10 shoots isolated from T1 trees was stimulated on media with 29 µM IAA. P7 shoots from T2 and T3 trees treated with BAP reached the CE classes more often on media with 0.29 µM IAA. The rhythmic growth of P8 shoots from T3 trees treated with BAP was reduced on media containing 0.29 µM IAA. None of the other effects of IAA on rhythmic growth were significant.

3.3.4. Condition of the shoots

The BAP treatments in vivo improved the condition of P3 and P7 shoots. Treating T3 and T4 trees with BAP reduced apical necrosis and vitrification in P3 and P7 shoots in vitro. In general, vitrification, basal necrosis and apical necrosis in the shoots in vitro increased only slightly after the BAP treatments; the combined BAP - IAA treatment worsened the condition of the shoots, especially that of P1, P8 and P10 shoots. The stimulation of extension growth of the shoots in vitro by the pre-treatments was often accompanied by an increase in the incidence of these undesirable attributes.

4 DISCUSSION

Root severing and topping These pre-treatments caused considerable damage and thus resulted in a modification of Massart's model (Edelin, 1977); the ratios of shoot growth in the various positions could be expected to show a recovery strategy. In the trees that had been uprooted in November, only the extension growth of P7 and P8 shoots was stimulated by the T4 procedure; in the trees uprooted in April the extension growth of P4(T2), P5(T3) and P10(T2, T4) shoots was stimulated by root severing and topping. These modifications do not point to a drastic change in growth strategy but suggest that there was an intensified use of the space already occupied by the tree; the stimulation of P4 and P5 shoots may be of another nature. More P7, P8 and P10 shoots from trees uprooted in November reached the CEF classes after topping and more P3 shoots from the same trees extended their apical meristem after the T3 or T4 procedures. Topping the trees in April stimulated free growth in P4(T2) and P7(T2, T4) shoots and inhibited this in P3 shoots (T2, T4) Severing the lower half of the roots in April stimulated free growth in P9 shoots but inhibited it in P2 shoots. Again, "damaging" the trees stimulated the shoots from axillary buds more than those from terminal buds. The growth strategy after damaging was not reorganized to create a new leader in the tree; this may have been because of the plagiotropic nature of the terminal buds (Hallé et al., 1978) which is difficult to study in vitro since almost all shoots grow orthotropically (unpublished results). It can be concluded that applying the growth regulators in vivo via the roots was the best research strategy, because this caused the least damaging effects. The question remains how the growth regulators actually influenced the subsequent morphogenesis in vitro. It is certain, however, that applying growth regulators in vivo modified the topophysical relations in the tree and enabled the IAA to act in vitro.

Experimental gaps The experimental system described in Chapter 2 (Table 1) has some shortcomings. For the in vivo treatments with growth regulators (GA_3, IAA, BAP) only one dose was chosen (determined in preliminary research). The fact that for experiment B the trees were uprooted in November means that its results are not valid for the trees uprooted in April (experiment C): the physiological state of the trees in November and thus that of Massart's model was clearly different from that of plants in April notwithstanding the extensive conditioning of the trees in experiment B. Furthermore, IAA was not tested in vitro on shoots from T2, T3 and T4 trees that had not been hormone-treated; there were no control T4 trees with an in vivo growth regulator treatment to only the roots or only the apex.

Forcing The culture room pre-treatment in experiment B during 3 weeks did not stimulate the extension growth of the shoots in vitro more than the greenhouse forcing of trees for 4 weeks in October/November used earlier (Evers, 1982b). However, in the latter experiments different combinations of sucrose concentrations and light intensities were used. In experiment B the culture room forcing procedure stimulated extension growth, especially in shoots from the highest positions in the tree: P2, P6 and P5; there was no difference between the mean extension growth of shoots from the middle and from the lower branches (P3/P7-P4/P8) when compared with earlier experiments on shoots in November (Evers, 1981b, 1982b). These conclusions did not hold true for trees pre-treated for 1 week in the culture room in April (experiment C). Apparently the combined cold storage - culture room forcing procedure that started in November affected the "programming" and dormancy of the buds in the upper part of the tree. Since buds in the upper part of the tree are among those that flush the latest (unpublished results) it can be inferred that the growth strategy for the next season and the onset of dormancy were not yet fully prepared in November. The consequence of this would be that the determination of branch morphology in the various bud types (Allen and Owens, 1972) varies over time as well as in space. However, the pre-treatments that started in November could not stimulate apical meristem extension (classes CEF) of shoots from the upper part of the tree: the qualitative programming in that part of the tree may have been completed but fairly large quantitative variations on this strategy were still possible.

Influence of BAP: in vitro effects As summarized in the introduction, BAP induced organogenesis in mainly embryonic tissues of Douglas fir (Zaerr and Mapes, 1982). As was reported in an earlier study (Evers, 1981a) BAP inhibited the growth of shoot initials (exp. A); it did not affect the topophysical sequences. The formation of buds on the explants probably resulted from the outgrowth of meristematic regions: BAP stimulated bud development (bud initials for the next year) between the needles.

BAP in vivo - IAA in vitro BAP affected the influence of topophysis and the action of IAA in vitro in various ways (Tables 4 and 5, Figures 8 and 11), but an inhibition of growth of shoots in vitro was never observed.
- No influence of BAP. In shoots from P3, P5, P7 and P10 buds IAA stimulated extension growth in shoots isolated from T1 trees as well as T3 trees treated with BAP: the BAP pre-treatment had no influence. It may be concluded that if the BAP was absorbed by the roots and then distributed in the plant then this IAA

effect would also have occurred when the levels of endogenous cytokinin were higher.

- Intensified IAA effect with BAP. In P1 and P4 shoots BAP applied in vivo intensified the stimulation of extension growth by IAA that occurred when corresponding shoots were isolated from trees that had not been treated with BAP.
- BAP as a prerequisite for IAA effects. The stimulation of extension growth of P2, P6 and P8 shoots depended on whether BAP had been applied in vivo: in P2 shoots extension growth was stimulated only in combination with IAA in vitro; the stimulation of P6 and P8 shoots was a direct effect of BAP but was reduced by IAA. Other direct stimulations by BAP were found in P3 and P7 shoots from T2 trees and in P7 and P8 shoots from T4 trees.
- Relation growth - class of development. BAP also stimulated free growth (Jablanczy, 1971; Cannell and Johnstone, 1978), often at the expense of rhythmic growth (Hallé and Martin, 1968) of the shoots but only in the presence of IAA (except in the case of P7 shoots from T2 trees, see Figure 10). This stimulation occurred in P2, P3, P4, P8 and P9 shoots from T3 trees and P10 shoots from T2 trees. It can be concluded that the combined BAP-IAA treatment stimulated both extension growth and free growth in only P2 and P4 shoots; in all other cases quantitative and qualitative growth were stimulated in different ways.

Consequences for tree form Applying BAP in vivo resulted in a faster extensio growth of shoots in vitro from the terminal buds and from buds on the highest and lowest branches. The extension growth of shoots from buds on the middle branches could only be stimulated by BAP if the trees had been topped. Since in vitro growt and morphogenesis of the shoots may be a reflection of the potential strategy in development one can speculate that raising the level of endogenous cytokinin will drastically change the expression of the tree model.

GA_3 in vivo - IAA in vitro When GA_3 was supplied to the roots (exp. B) the extension growth of shoots from the highest terminal buds in the tree (P1, P2) was stimulated but that of shoots from the accompanying P5 and P6 buds was inhibited on media containing IAA; all other shoots were unaffected. When the total number of CEF shoots from T1 and T3 trees on all media and growth of the shoots were compared, it was found that the stimulation of quantitative growth was not accompanied by the occurrence of a higher percentage of shoots with an extended apical meristem (Figures 3 and 6, Tables 2 and 3), except in the case of P2 shoots (Figure 5). GA_3 thus seemed capable of changing the growth strategy at the top of the tree into a more space-exploring development; the energy for this process may have been obtained at the expense of the growth of the axillary buds.

If GA_3 was actually absorbed by the roots and if it directly influenced the physiology of the buds then a relation with the state of dormancy cannot be excluded.

IAA in vivo and in vitro When IAA was applied to the apex of the tree the extension growth of shoots in vitro from buds on the highest branches after topping (P3, P7) was stimulated. Another IAA treatment in vitro eliminated this stimulation, perhaps as a result of overstimulation (Figure 3). In the same shoots more apical meristem extension occurred after the combined in vivo - in vitro IAA treatments. However, the same stimulation in P3 shoots was also observed after isolation from T1 trees: the IAA treatment had no effect. Shoots from buds on the lower branches reacted differently to the combined IAA treatments: fewer P4 and P8 shoots reached the CEF classes after IAA treatment of the trees compared with the corresponding shoots from intact trees. The combined IAA treatments boosted apical meristem extension to a high level exceeding that of shoots from T1 trees. It can be concluded that in vivo IAA treatment of the trees counteracted the influence of IAA in vitro on the apical meristem extension of P4 shoots: IAA in vitro inhibited the apical meristem extension of P4 shoots from T1 trees but stimulated it in those from T2 trees treated with IAA. Applying IAA in vivo increased the potential quantitative growth of buds on the middle (in this case upper) branches of topped trees.

GA_3/IAA in vivo - IAA in vitro Applying both GA_3 to the roots and IAA to the apex in vivo stimulated the extension growth of the remaining terminal buds (P3, P4) of T4 trees in vitro and inhibited that of the accompanying P7 and P8 buds (Table 2); the same was true for the extension of the apical meristem of these shoots. The influence of the two-side growth regulator treatment in vivo on extension growth in vitro in most cases depended on the stimulation of IAA in vitro; however, it directly stimulated apical meristem extension. As already concluded for the influence of GA_3 on T3 trees, in the case of T4 trees treated with GA_3 and IAA, the shoot initials from the uppermost terminal positions were also stimulated at the expense of those from the accompanying axillary positions.

In vivo - in vitro comparability The growth of the shoot initials in vitro always seemed to be slower than in situ. David (1982) concluded from comparable results from Boulay (1979) with Douglas fir that the in vitro system was not sufficiently optimalized to replace the root system. The present experiments show that the development of the shoot initials depend on the physiological condition of the tree; endogenous levels of growth regulators can quantitatively adjust the original growth strategy according to Massart's model (Edelin, 1977). Furthermore, the

tree seems to supply nutrients and growth regulators to the various buds in doses that depend on the physiological state of the tree (Evers, 1981b; 1982a; 1982b). The in vitro system therefore has to replace a balanced transport of nutrients, growth regulators, etc. through the branches that meets the specific demands of the buds which in their turn at least partly depend on the topophysical position of these buds. Since in experiments the "black box" approach was used, the conclusions on the action and levels of endogenous growth regulators must be tentative. In some cases the endogenous levels in the tree and/or the shoot initials may have been sufficient and in other cases a supplement to these growth regulator levels may have been necessary (Zaerr and Mapes, 1982). The relations between exogenous (in vitro) and endogenous levels of growth regulators in explants and between exogenous and endogenous levels in trees are not known. Applying BAP exogenously to the tree may have acted directly on the shoot initials e.g. by influencing IAA production (Hecht, 1980) or indirectly e.g. by changing the ratio of auxin to cytokinin in some of the branches. The effect of applying IAA exogenously in vivo and/or in vitro on the morphogenesis of shoot initials in vitro may have depended upon the endogenous levels of IAA oxidases (Johnson and Carlson, 1977; 1979). Since GA_3 applied in vivo influenced extension growth only in the presence of IAA in vitro, it cannot be excluded that GA_3 acted upon the biosynthesis of IAA and thus upon the auxin/cytokinin ratios of the different buds, thereby enabling IAA to have an effect in vitro.

5 ACKNOWLEDGEMENTS

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Growth and morphogenesis of shoot initials of *Douglas fir, Pseudotsuga menziesii (Mirb.) Franco,* in vitro

VI Comparison of growth and development in vivo and in vitro

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CONTENTS

SUMMARY

The flushing of buds in 10 topophysical positions on 2-year-old Douglas fir trees was compared simultaneously with the development in vitro of shoot initials isolated from buds in the corresponding positions.
Buds on the lower branches flushed earlier than those on the upper branches; axillary buds lower on the stem flushed later than those high on the stem. Since the in vitro development of shoot initials from all positions started at the same time, it was difficult to make a direct comparison with the in vivo situation.
Buds in vitro grew much more slowly than buds flushed in vivo, however, the difference depended on the topophysical position. Six weeks after the mean date of the first flushing bud (any position) the mean length of expanding shoots on the lower branches was greater than that of shoots on the highest branches; this difference was also observed between shoots from buds in the corresponding positions that had been cultured for 6 weeks in vitro. However, the shoots on the highest branches continued to grow after the six week period after the mean first bud of the tree flush moment, and this obscured the differences between the buds on the branches. The rate of extension growth (in $mm.day^{-1}$) of terminal buds on branches was most rapid in buds from the upper branches; of the axillary buds, those from the lower branches had the most rapid rate of extension growth. The differences between the growth rate in vivo and in vitro in buds in the uppermost positions were more substantial than that in buds on the branches. The disparity between growth in vitro and in vivo was least in bud positions near the base of the tree; this may have been due to the fact that exclusively in vitro grown shoots from these positions often displayed apical meristem extension.
Another difficulty in comparing in vivo and in vitro development of the shoots was that the buds on the branches developed a dorsiventral structure whereas the explants in vitro maintained a radial symmetry.

Key words:
Pseudotsuga menziesii - in vitro culture - bud flushing - topophysical position - shoot initials - stage of development.

1 INTRODUCTION

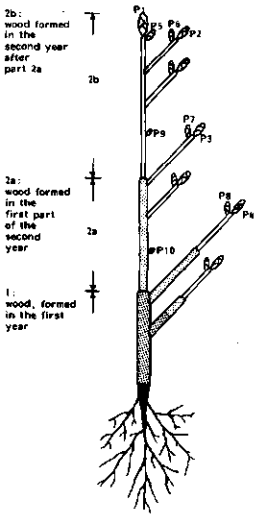
Growth and its coordination in trees is complex (Wareing, 1970) and is far from being completely understood. The results of experiments set up to reveal aspects of the strategies in growth coordination in trees are often difficult to interprete because of the variation over the season and because of topophysis (Kozlowski, 1971). Since this coordination largely depends on basic principles such as specific architecture (Nozeran et al., 1971; Hallé et al., 1978) and juvenility (Wareing, 1959; Borchert, 1976) it is difficult to observe endogenous regulation of growth exogenously. The best way to study growth coordination is the culturing of shoot apical meristems outside the complex of endogenous physiological gradients in the tree (Borchert, 1976).

In earlier studies (Evers, 1981a; 1981b; 1982a; 1982b; 1983) shoot initials of Douglas fir were cultured in vitro so that the potential quantitative and qualitative growth of the coming season in the trees could be ascertained. Shoot initials were isolated from various topophysical positions on trees that had been pre-conditioned in various ways. It appeared that each of the shoot types had its own characteristic photosynthesis and required its own medium, depending on the time in the season. From these results, conclusions were drawn about the potential in vivo development of the vegetative buds under defined conditions. However, it was not known whether in vitro and in vivo development of the shoots could be (in)directly compared. Therefore, in the present experiments, bud flushing in vivo was studied simultaneously with the in vitro development of shoot initials from comparable buds.

2 MATERIALS AND METHODS

Plant material and pre-treatments A total of 270 two-year-old Pseudotsuga menziesii (Mirb.) Franco provenance Arlington trees were selected on 14 April 1982, according to the system described in an earlier paper (Evers, 1981b; Figure 1); 120 trees were used in the in vivo experiment and 150 in the in vitro experiment.

Trees for the in vivo experiment were at random placed in the greenhouse at 25° ± 3° C in 6 x 6 cm pots on the day of their selection, the spacing of the pots was 15 x 15 cm.



A. Terminal positions:
Position 1(P1): Terminal buds of the tree
" 2(P2): " buds of the highest branch on stem part 2b
" 3(P3): " buds of the highest branch on stem part 2a
" 4(P4): " buds of the highest branch on stem part 1

B. Axillary positions close to terminal buds:
Position 5(P5): Axillary buds close to the P1 buds
" 6(P6): " " " " " P2 buds
" 7(P7): " " " " " P3 buds
" 8(P8): " " " " " P4 buds

C. Positions on the stem:
Positons 9(P9) : Axillary buds on the stem part 2b, closest to part 2a (Figure 2)
" 10(P10) : Axillary buds on the stem part 2a, closest to part 1

Figure 1. Positions of buds in selected 2-year-old Douglas fir trees (1a1), displaying an architecture according to Massart's model (Edelin, 1977; Evers, 1981b).

Subsequently, bud flushing of 100 trees was studied; the remaining 20 trees were used to replace trees that dried out and those with buds that remained dormant in any position. All trees were kept in the greenhouse until the last bud of the last tree had flushed which lasted approximately 2½ months. The trees were watered in the pot and sprayed twice daily.

The trees for the in vitro experiment were packed in plastic bags and stored in the dark at 4° C from the second until the twelfth day after selection until the buds were excised.

In vivo procedures To mark which buds from the 10 positions in the trees were to be measured, small clothes pegs of different colours were attached to the branch or nearest needle. For each tree a separate chart was used to note the days on which the buds flushed and to record extension growth and diameter growth. Firstly, the day on which the first bud irrespective of position of each of the 100 trees flushed was determined; the mean first flush of all trees will henceforth be referred to as Ta. This averaged day Ta is point 0 at the time scale in Figure 2. Secondly, the day on which the first buds from each of the positions of each tree started to flush was determined: this resulted in a mean first flush moment for each position, occurring between 0.2 and 13.7 days after the Ta of the trees. The length and diameter of these first expanding buds from each position on each of the 100 trees were measured with a marking gauche 6 weeks after the moment the first bud on these trees flushed, irrespective of position. The period of 6 weeks was chosen because the in vitro experiments always lasted that long. The growth of the same expanding buds was determined again 6 weeks after the moment the first bud of each position on each tree flushed, resulting in 10 new days of measurement for each tree: averaged 42.2 to 55.7 days after Ta. The mean moments of flushing and extension growth and diameter growth on these days were calculated. The mean growth of buds in the 10 positions was also determined 6 weeks after the start of the experiment (transfer to greenhouse); this was done by substracting the period from this transfer until Ta from the Ta's of each position and extrapolating growth at these new moments. Growth had to be assumed linear since no measurements were carried out in the 42 days after flushing. The differences in the mean growth of expanding buds in these 3 periods were tested statistically. Finally, the percentage of the expanding buds that stopped growing before the 42nd day after Ta was determined by comparing the length and diameter of the buds at this moment with their length and diameter on the 42nd day after the Ta mean first flush moment of each position.

In vitro procedures The optimalized media for shoot initials from each of the 10 positions in April (Evers, 1981b), the sterilization and preparation of shoot initials (Evers, 1981a) and the culture room (Evers, 1982b) were described in earlier papers All shoots were cultured at a light intensity of 22 Wm^{-2} during a 16-hour light period and at a constant temperature of 25° C. Ten shoot initials, one of each position, were isolated from each of the 150 trees resulting in a total of 1500 cultured shoots. It took 10 days (from the second to the 12th day after selection of trees) to inoculate the shoots; every day, 150 shoots (15 per position) were inoculated. The second shoot per group of three from each position was only used if the first

or the third shoot became infected. After 6 weeks in culture the length and diameter of 100 shoots per position were measured, using millimetre paper. The mean extension growth and diameter growth per position were calculated and the differences between the positions were determined. Furthermore, the growth in vitro of the shoots was compared with the growth of the expanding buds in vivo. The expanding buds in vivo did not extend their apical meristem in the 6 weeks of the experiment. Most of the shoots in vitro, however, showed rhythmic growth or free growth (Evers, 1981a); the percentages of these shoots from each of the positions were also determined. The differences between the positions in the occurrence of shoots in the various stages of development were tested using the chi-square method. The analysis of variance showed that the factor 'position' was very significant. The contrasts in growth between the positions were determined for their significance using the Students t-test.

3 RESULTS

3.1. Bud flushing in vivo

First flushing bud The word bud is exclusively used in the in vivo experiment. It took 8.2 days to reach point Ta, i.e. the mean number of days in the greenhouse until the first bud on the trees flushed. This period ranged from 1 up to 19 days with a peak at 9 days: 46% of the trees flushed for the first time on day 9. The day 6 weeks after the start of the experiment was determined at 42 - 8.2 = 33.8 days. Usually the buds on the lowest branches flushed first; this was clearly shown when, for each of the 10 positions the mean numbers of days were calculated from Ta until one bud flushed (Figure 2). In 92 trees the P4 buds were the first to flush. Buds on the middle branches (P3, P7) flushed later and those from the upper branches (P2, P6) flushed last. The P6 buds flushed later than any other bud position in the trees. Flushing in terminal buds continued in a acropetal direction (P4, P3, P2, P1); in axillary buds on the stem it continued in a basipetal direction (P5, P9, P10). P1 buds and P10 buds flushed simultaneously, as did P2 buds and P9 buds. Considerable differences were observed in the time the trees needed to flush at least one bud from every position. The fastest tree had these 10 buds flushing in only 2 days, but the slowest in 59 days; the mean number of days was 19.8. However, in most trees (79%), the flushing of one bud from positions P1 up to P10 was achieved in less than 29 days.

Extension growth The differences in Ta between the shoots did not account for the differences in extension growth of the buds from the various positions (Figure 2). At 42 days after Ta groups of positions could be distinguished according to their extension growth:

- the uppermost positions (P1, P5),
- the terminal positions and P9 buds (P2, P3, P4, P9),
- the axillary positions on the branches and P10 buds (P6, P7, P8, P10).

The same grouping was apparent at the extrapolated point Tb-Ta. Until Tb the terminal buds of the trees grew the fastest, closely followed by the P5 buds. However, the extension growth in $mm.day^{-1}$ differed considerably. At 42 days after Ta growth of P1 and P5 buds slowed down to a similar rate; 9% and 18% of the P1 and P5 buds, respectively, had already terminated growth at Tb. In the second, slower-growing group of positions of buds (P2, P3, P4, P9) the rank order in length at 42 days after Ta was not accompanied by comparable differences in extension per day. The P4 buds started to flush early, but the mean extrapolated growth rate of P3 buds was faster: the difference in length decreased, which was the result

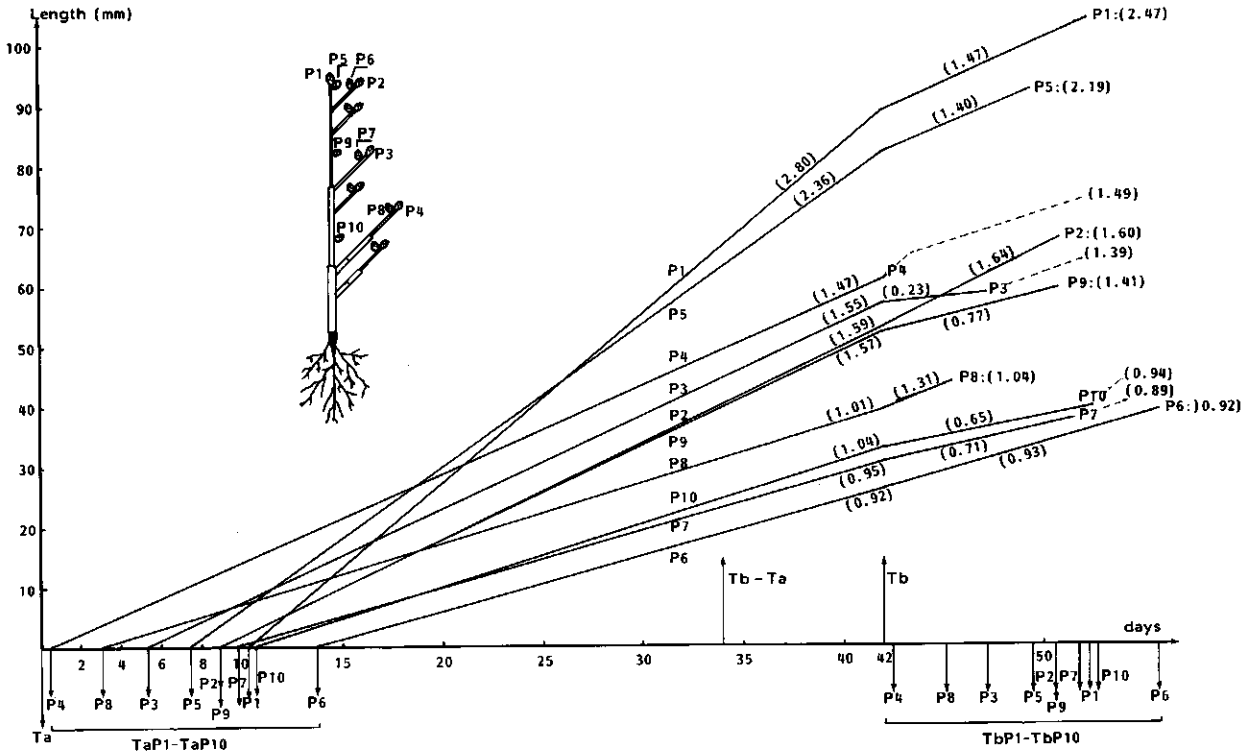


Figure 2. Extension growth of expanding buds in 10 positions on the trees, 6 weeks (42 days) after the trees had been transferred to the greenhouse (Tb-Ta), 6 weeks after the mean day on which the first bud on all trees flushed (Tb) and 6 weeks after the mean day on which the first bud in that position flushed (TbP1-TbP10). Ta: mean time of flushing of the first bud on all trees. Tb: 6 weeks after Ta. TaP1-TaP10: mean start of flushing of buds from positions P1 to P10 (Figure 1). TbP1-TbP10: 6 weeks after TaP1-TaP10. In brackets: growth in mm.day^{-1} until Tb (left), from Tb until TbP1-TbP10 (centre) and from TaP1-TaP10 until TbP1-TbP10 (right).

of the early start of P4 buds until the 42nd day after Ta. However, 39% of the P3 shoots had stopped growing at this day, 40% of the P3 shoots grew less than 5 mm and 98% of the P4 buds did not continue to grow out after this point. The P3 shoots therefore did not become longer than the P4 shoots. At the beginning of the experiment and at the 42nd day after Ta there was no difference in growth between P2 and P9 buds. The original difference between these buds and P3 buds as a result of the earlier flushing of the latter buds still existed after 6 weeks because the rate of extension growth per day of these buds was the same. At this point, only 11% of the P2 buds and 21% of the P9 shoots had stopped growing. The growth rate of P9 shoots slowed down after 6 weeks but not to the level of P3 buds: the P9 buds reached the same length as P3 buds. After 6 weeks, P2 buds continued to grow, even at a slightly faster rate than before this point, becoming longer than P3 and P4 buds and achieving the fastest growth rate of buds in this group of positions during the 6 week period after the Ta of each position. The overall growth rate of P4 buds in this period was faster than that of P3 buds and P9 buds. After 6 weeks P3 and P9 buds grew more slowly, but this deceleration in growth had probably taken place before this point in the P4 buds. In the last and also smallest group of positions there was a great difference in length between P8 buds and P10 buds and between P7 buds and P6 buds, which resulted from the difference in the start of flushing. After 6 weeks, P6 buds continued to grow at the same rate, P7 buds slowed down and P10 buds slowed down even further: at the end of the experiment the P6, P7 and P10 buds had the same length. P8 buds grew further at a more rapid rate, resulting in the fastest extension growth of this group in spite of the fact that 42% of the buds had stopped growing after 6 weeks; the corresponding values for P6, P7 and P10 buds that had terminated growth after 6 weeks were 14%, 32% and 28%, respectively. It can be concluded that the rank order in length of terminal buds (P4, P3, P2) and accompanying axillary buds (P8, P7, P6) at the 42nd day was not the same as the rank order of growth rate per day; some of the differences in absolute length disappeared after the 42nd day because the extension growth of P2 buds and P6 buds did not slow down at the end of the 6 week period after the Ta of each position. The 42nd day after Ta proved to be not a suitable moment to measure differences in growth between buds from the various positions because their actual growth period was not constant.

Diameter growth The differences in diameter growth (including needle growth) between the positions differed considerably from the differences in extension growth (compare Figures 2 and 3). After point Tb, diameter growth slowed

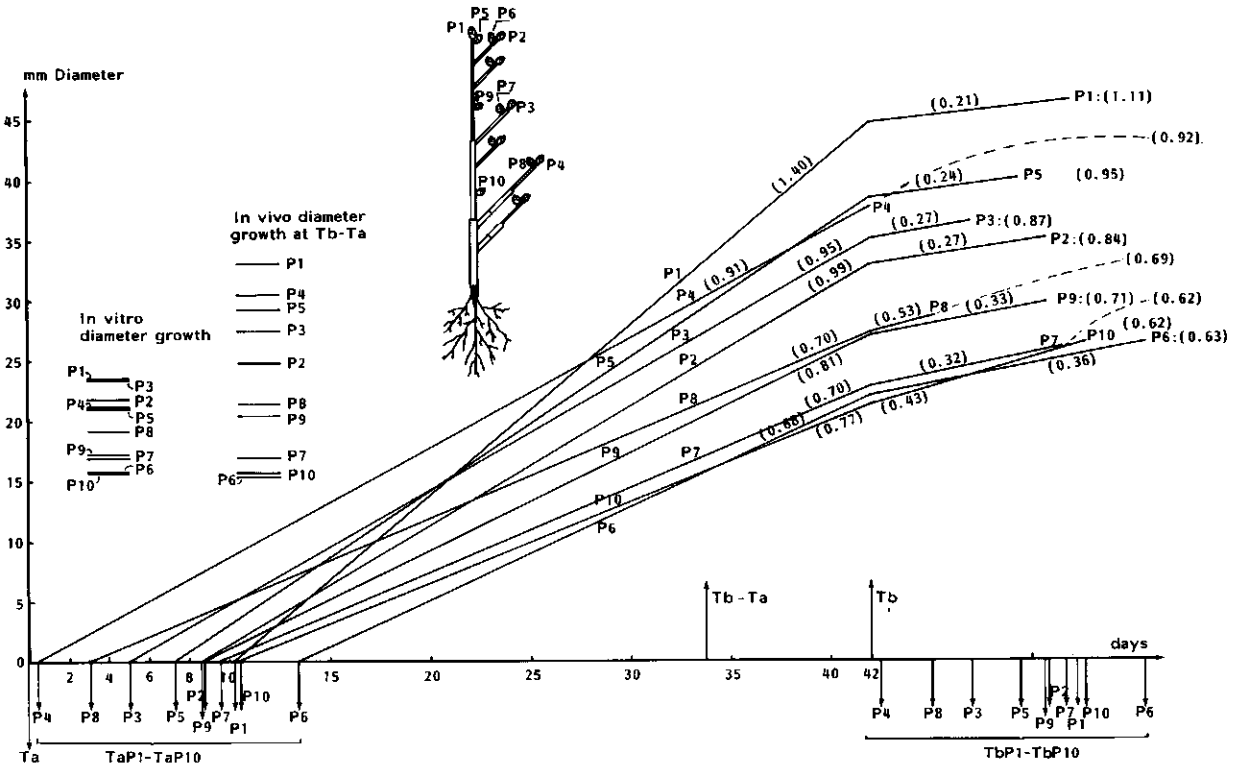


Figure 3. Diameter growth of expanding buds in 10 positions on the tree, 6 weeks (42 days) after the trees had been transferred to the greenhouse (Tb-Ta), 6 weeks after the mean day on which the first bud on all trees flushed (Tb) and 6 weeks after the mean day on which the first bud in that position flushed (TbP1-TbP10). Ta: mean time of flushing of the first bud on all trees. Tb: 6 weeks after Ta. TaP1-TaP10: mean start of flushing of buds from positoins P1 to P10. TbP1-TbP10: 6 weeks after TaP1-TaP10. In brackets: growth in mm.day^{-1} until Tb (left), from Tb until TbP1-TbP10 (centre) and from TaP1-TaP10 until TbP1-TbP10 (right).

down in buds from all positions, except perhaps in P4 buds. Again, P1 buds grew faster than buds from any other position. Notwithstanding the later start of flushing of P5 buds these buds achieved the same diameter as P4 buds, but they could not surpass the P4 buds. The rank order in diameter growth of buds from terminal positions (P4, P3, P2) at Tb was maintained until the end of the experiment. As in the case of extension growth, the rank order in absolute diameter was the reverse of the rank order in growth per day. At TbM there was no difference in the growth per day or in the diameter between P4 buds and P3 buds, or between P3 buds and P2 buds. In the Ta-Tb period the diameter growth of P8 buds and P9 buds was the same, but before Tb the P9 buds had a faster growth rate. The slowest growing buds (P6, P7, P10) all had the same growth rate in the Ta-Tb period and reached the same diameter. Before Tb, the P6 buds had a faster growth rate than the P7 buds and P10 buds.

3.2. Shoot development in vitro

Extension growth The word shoot was exclusively used in the in vitro experiment. Shoots from P1 and P4 buds were the fastest growing explants during the 6 weeks in vitro (Figure 4).
The lower down the tree the shoots from terminal buds (P2<P3<P4) or from axillary buds on the branches (P6<P7<P8) had been excised, the faster was extension growth of the shoots in vitro. The shoots from axillary buds were always smaller than those from terminal buds, except in the case of P2 shoots and P8 shoots: they were the same length. The P5 shoots grew as long as P2 shoots and P3 shoots. P9 shoots grew as fast as P7 shoots and P10 shoots as fast as P6 shoots.

Diameter growth P1 shoots and P3 shoots showed the fastest diameter growth in vitro (Figure 3). In all the shoots in a slower-developing group (P2, P4, P5) the diameter growth was the same. The rank order in diameter growth of axillary shoots from the branches (P6<P7<P8) was the same as that for extension growth. As shown in the data on the extension growth, the diameter growth of P9 shoots was the same as that of P7 shoots, and that of P10 shoots was the same as P6 shoots.

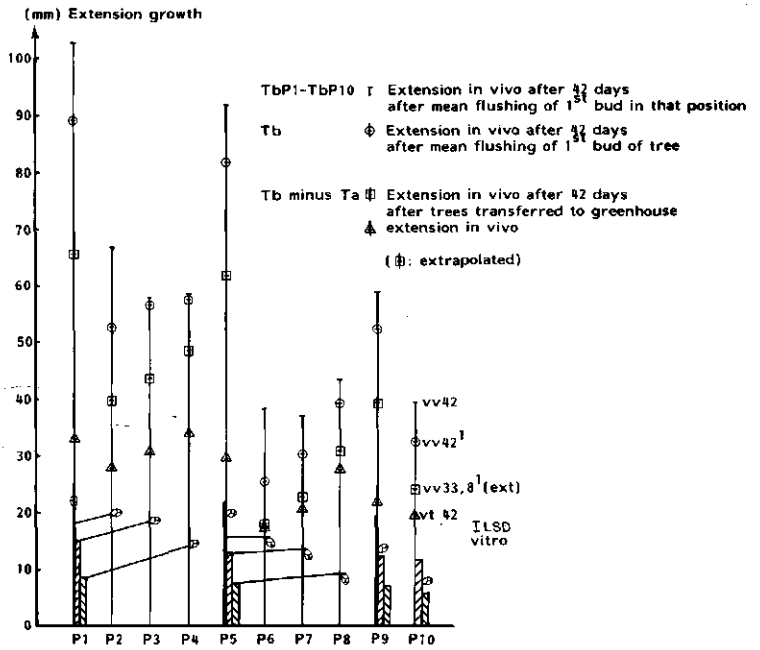


Figure 4. Comparison of extension growth of shoots from buds in 10 positions in vitro whit that of the corresponding shoots on the tree at Tb minus Ta, at Tb and at TbP1-TbP10. vv = in vivo. vt = in vitro. For further explanation see text.

Stages of development The stages of development that were reached by the shoots in vitro are given in table 1. In shoots isolated from buds in the lower parts of the tree (P4, P8, P10) the extension of the apical meristem (C+E+F) was most marked; this gradually decreased the higher up the tree the bud had been excised (P3+P7+ P9 > P2+P6 > P1+P5. This contrast was especially clear when shoots from buds in the highest positions (P1, P5) were compared with those from buds in the most basal positions (P8, P10). Most free growth occurred in P8 shoots and P10 shoots; most rhythmic growth occurred in P4 shoots.

Table 1. Numbers of shoots reaching the stages of development B-F, originating from buds in 10 positions. B: no apical development. D: no apical meristem extension. C: rhythmic growth on non elongated shoot. E: rhythmic growth. F: free growth; + = more, - = less than expected from the chi-square test.

Stage	Positions										
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Total
B	6	10	3	2	13	4	3	0	0	0	41
D	45+	36	30	21	41	43+	34	17-	32	18-	317
B+D	51+	46	33	23	54+	47	37	17-	32	18-	358
C	0	1	0	1	0	1	1	1	2	2	9
E	12	12	18	22+	15	9	11	18	11	14	142
C+E	12	13	18	23+	15	10	12	19	13	16	151
F	37-	41	49	54	31-	43	51	64+	55	66+	491
C+E+F	49	54	67	77	46	53	63	83	68	82	642
Total	100	100	100	100	100	100	100	100	100	100	1000

3.3. Comparison of growth and development in vivo and in vitro

Extension growth As expected, the growth of shoot initials in vitro was much slower than that of the corresponding shoots in vivo (Figure 4). As Table 2 shows, only in P6 shoots was the extrapolated growth at 42 days after transfer to the greenhouse the same as the growth of corresponding shoots in vitro.

Table 2. Extension growth of shoots in vitro as a percentage of the growth in vivo at 3 points of measurement. Ta = mean first flush moment.

In vivo period: 6 weeks after	Positions										
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean
Ta of each position	32.6	42.5	53.4	54.9	32.5	46.4	55.4	63.5	37.4	49.3	46.8
Ta of the trees	38.1	54.1	54.5	56.2	36.3	69.2	67.9	69.9	42.1	59.7	54.8
Start experiment (extrapolated)	51.3	71.5	70.6	70.5	48.1	99.6	91.7	90.1	56.1	74.7	72.4

When in vivo and in vitro growth were compared at the 42nd day after fransfer to the greenhouse and at the same period after Ta the growth of the shoots from axillary buds on the branches were the most efficient of all the shoots in vitro, whereas shoots from buds excised from the terminal part of the tree (P1, P5) seemed to do least well in vitro. There were no differences in extension growth in vitro between shoots from axillary origin or between the shoots from the terminal positions on the branches. At the 42nd day after the Ta's of each position this situation had changed: there was a vertical gradient in efficiency of extension growth in vitro along the bud positions: P1/P5<P2/P6<P3/P7<P4/P8. The extension growth was greater the lower the shoots had been isolated in vitro from the branches, and this

coincided with a decreasing difference in growth in vivo.
The major difficulty in comparing extension growth in vivo and in vitro was that the in vitro culture of shoots was started when the buds in the various positions in vivo were at different stages of morphogenetic preparation ("swelling"). However, the in vivo rank order in growth at Tb of P2-P3-P4 and P6-P7-P8 was also found in vitro, and the difference in growth between axillary shoots and terminal shoots was maintained in vitro.
Shoots from the highest positions in the tree grew in vitro at a rate similar to that of shoots from the terminal positions from the branches; on the tree the P1 buds and P5 buds grew the fastest. Also, the buds on the stem (P9, P10) had the same growth rate as P2 buds and P7 buds, respectively, but the shoots in vitro from stem buds grew slower than shoots from the latter buds. The development of the shoots in vivo and in vitro could not be compared for more than 6 weeks because the growth of all shoots in vitro declined after this period, often because of basal necrosis and the exhaustion of the medium. When the in vitro shoots were subcultured, the base had to be cut again; furthermore, a lag in growth occurred. In vivo, the P2, P6 and P8 buds continued to grow after all the 6-week periods; the growth of buds from all the other positions tailed off after 6 weeks after Ta.

Diameter growth At the 42nd day after the start of the experiment the extrapolated diameter growth of P6, P7 and P10 buds in vivo was the same as that of comparable shoots in vitro (Figure 3). The rank order of increasing diameter growth of buds in vivo (P2-P3-P4) did not occur when shoots from these buds were cultured in vitro however, the rank order of growth of shoots from the axillary positions on the branches in vivo (P6-P7-P8) was maintained in vitro. The diameter growth in vitro expressed as a percentage of diameter growth in vivo was always higher than the corresponding percentage for extension growth.

Surface area The needles in vivo were oriented in a 2-dimensional plane (except for P1) and therefore the length x the diameter (including the needles) gave a rough idea of the space explored by the buds in the 10 positions. At the 42nd day after Ta the rank order in the surface area (= length x diameter) of the shoots from the various positions was the same as in the extension growth. These surfaces were difficult to compare with any of the in vitro parameters of development because shoots in vitro showed radial symmetry, thus giving rise to a more cylindrical shape. The surface area of such an in vitro developed imaginary cylinder gave a better impression of the in vitro development of the shoots than only the extension growth. At the 42nd day after the Ta of each position the surface area of the shoots

in vivo only exceeded the surface area of the cylinder in P1, P5 and P9 shoots that had developed in vitro; however, the surface area in vivo had a top and a bottom side.

Stages of development The most difficult parameter in the in vivo - in vitro comparison of the shoots was the stage of development. All buds in vivo developed their primordia into needles, extended the primordial axis and formed a terminal bud. This stage D was reached by about 1/3 of the shoots in vitro, but an apical bud rarely developed. In 64% of the shoots in vitro, the apical meristem extended: an important part of the length achieved in vitro resulted from the extension of a newly formed bud or from free growth. More than one period of rhythmic growth was not observed in vivo; free growth was unlikely to occur as judged visually in the shoots on the tree but cannot be discounted. It can be concluded that the ultimate length of the shoots in vitro was strongly influenced by the extension of the apical meristem and that this phenomenon differed between the bud positions.

4 DISCUSSION

As expected, the growth in vitro of shoot initials from buds in various topophysical positions did not reflect all the properties of the architectural model (Edelin, 1977). In building the architecture of Massart's model, plagiotropic growth plays an important role, but during the in vitro experiment this type of differentiation hardly occurred.

The question remains, whether plagiotropic growth is a result of the differentiation of and control by the apical meristem of the bud or is also controlled by the physiology of the whole shoot initial, branch or tree. It is assumed that the lack of plagiotropic differentiation is not caused by dedifferentiation; this normally occurs after many subcultures (Borchert, 1976).

Plagiotropy on Douglas fir branches moreover is secondarily achieved by torsion of petioles and differential development of the sizes of dorsal, lateral and ventral needles. Phyllotaxis of the needle initials is a spiral in all meristems. This also points in the direction of plagiotropy caused by correlations outside the bud.

It can therefore be concluded that the development in vitro only reflected the bud's quantitative potential for growth as well as it reflected the potential stage of development that the bud could reach except the plagiotropic differentiation. Growth in vitro was always slower than in vivo suggesting that the conditions for the development of the shoots in vitro were far from optimal. It was not useful to compare the growth per day of the shoots in vivo and in vitro: the differences between the positions in this parameter of growth in vitro were the same as the difference in mean length of the shoots after 6 weeks in culture. In the comparison between in vivo and in vitro development of the shoots, the role of the period between the transfer to the greenhouse and Ta remained obscure: it was not clear whether the period between the start of the experiment and the mean day on which the first bud flushed on all trees somehow occurred during the first days of the development in vitro. If such a lag did occur in vitro, then the 6 weeks in culture of the shoots cannot be compared with flushing of the buds in the Tb-Ta period. Furthermore, a Ta period could not be added to the number of days in culture, because so many shoots stopped growing after approximately 6 weeks, mostly because of basal necrosis. It is possible that some of the ratios in development between the buds in the various positions of the tree (i.e. in vivo) changed in this last period before flushing. If the in vitro experiment could have been started at the moment of first flushing of all trees then the difference between in vivo and in vitro development would probably have been smaller. Since there was some doubt about the validity of comparing the in vivo and in vitro developmental periods of the shoots more growth

measurements during development would have been very useful. The growth of the shoots in vivo during the 6 weeks after the mean first flush moment seemed to be the best standard to compare with the development in vitro, since it was in that period that the differences in activation between the positions were introduced in both experiments. However, the difficulty remained that some of the shoots (P2, P6, P8) demonstrated an important part of their development in vivo after the 42nd day after Ta while others (P3, P4) had terminated development even before this day. It is unlikely that the condition of the shoots at the start of the in vivo and in vitro experiments determined all parameters of the shoot's subsequent developmen and the contrasts described in the results. During the in vitro development, some stages of development occurred (Evers, 1981b) while in vivo there was only one qualitative type (D) of morphogenesis. A better strategy for comparing in vitro and in vivo development would therefore have been to select in vitro only the D shoots and compare them with the D stage in vivo, or only to compare the experiments before the shoots began to differentiate in various stages in vitro.

The basis in comparing the in vivo and in vitro development was the declining length of shoots from the terminal positions from the lower towards the upper branches (P4>P3>P2) as well as those from the axillary positions from the same branches (P8>P7>P6). For shoots from the axillary positions, this decline also held true for the growth rate per day. This overall picture of development was complicated by the P2 shoots, because they grew during a longer period than the other shoots and had a faster growth rate. Perhaps the P2 shoots benifitted more from the extreme climate in the greenhouse: 25° C day and night; furthermore, the P2 shoots were the last shoots to flush from the terminal positions on branches and thus during the pre-flushing period they experienced this temperature the longest. The in vitro and the in vivo experiments were both carried out at 25° C to improve the comparability; however, the temperature in the greenhouse not only influenced bud flushing but also many other processes in the tree during the outgrowth of the shoots. The trees in the greenhouse seemed to have longer branches at their tops than in the field (unpublished results), but this assessment was only based on a superficial visual judgement of low accuracy. If the in vitro development of the shoots had been compared with flushing in the field then in the latter case growth would only rarely have occurred at 25° C. The in vivo growth rate per day of P4 shoots was lower than that of P2 shoots but perhaps not during the whole experiment: since P4 shoots started flushing so soon, their maximum growth rate was probably higher than that of P2 shoots at the beginning of the experiment. However since the growth rate of P2 shoots after 6 weeks did not decline, it must be assumed that the actual growth period of P2 shoots in vivo was longer than that of P4 shoots:

this might have been the result of the climate in the greenhouse. Another difference between the P2 shoots and P4 shoots was their potential to extend the apical meristem, as was shown in vitro: many more P4 shoots demonstrated free or rhythmic growth. During the last weeks in vitro the P4 shoots spent more energy on extending the apical meristem; this might have interfered with the difference in extension growth between P2 shoots and P4 shoots in vitro and partly explains the different behaviour of the 2 shoot types in vivo and in vitro. When shoots were isolated in vitro from the more basal positions in the tree the growth differed less from that in vivo than in shoots isolated from positions higher up the tree. It seemed obvious that this smaller difference with the in vivo growth was also partly caused by the occurrence of more apical meristem extensions in the shoots from basal positions. The P1 shoots and P5 shoots showed the greatest difference between in vivo and in vitro growth; the in vitro conditions do not imitate natural supply well especially for P1 and P5 shoots that had been isolated from trees that were in a physiological condition that was not optimal to start development in vitro.

The diameter growth in vitro seemed to be influenced by the diameter of the culture tubes. The mean diameter growth in vitro of shoots from all the positions never exceeded 24 mm, the diameter of the tubes.

The attempts to find better parameters of growth for comparing in vitro with in vivo development were not very successful, because plagiotropic growth with its dorsiventral orientation of the needles on the branch obscured these calculations: the orientation of the needles in vitro was very different from the development of the plagiotropic branches in vivo.

It can be concluded that the in vitro behaviour of the shoots gives much information on the potential for development after flushing on the tree but the findings can neither be generalized for trees in all physiological conditions, nor do they cover all phenomena of differentiation in vivo.

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URRICULUM VITAE

Peter Evers werd geboren op 20 maart 1953 te Utrecht. Na het behalen van et HAVO-diploma aan de St. Gregorius scholengemeenschap te Utrecht in 1970 egon hij in september van dat jaar met zijn MO Biologie studie aan de Rijksuni- ersiteit Utrecht. Na het behalen van het MO-diploma in januari 1976 vervolgde ij de biologie studie met het doctoraal programma. In november 1976 slaagde hij oor het doctoraal examen met als hoofdvak histologie-elektronenmicroscopie, als ijvakken endocrinologie en plantenfysiologie en als nevenrichting vakdidactiek. et hoofdvak betrof een histologisch onderzoek aan de epifyse van de Axolotl lsmede een scriptie over de blood-brain barrier.
an december 1976 tot juli 1978 was hij als erkend gewetensbezwaarde werkzaam ij het Rijksinstituut voor onderzoek in de bos- en landschapbouw "De Dorschkamp", edetacheerd bij de afdeling weefselkweek van de vakgroep Tuinbouwplantenteelt an de Landbouwhogeschool. Deze aanstelling werd door De Dorschkamp met een alf jaar verlengd. Van januari 1979 tot januari 1980 verrichtte hij een pilot study ij de vakgroepen Bosteelt en Tuinbouwplantenteelt van de LH, gedetacheerd bij e Dorschkamp. Deze pilot study mondde uit in een onderzoeksassistentschap bij ezelfde vakgroepen. Hij werd wederom gedetacheerd bij De Dorschkamp. Dit uurde van januari 1980 tot februari 1983. Vanaf februari 1983 is hij als weten- chappelijk ambtenaar verbonden aan De Dorschkamp.