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Occurrence of gibberellin-like substances in Norway spruce (*Picea abies* (L.) Karst.) and their possible relation to growth and flowering

Förekomst av gibberellinliknande substanser hos gran (Picea abies (L.) Karst.) och deras betydelse för tillväxt och blomning

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Abstract

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Growing shoots and young seedlings of Norway spruce (Picea abies) contain at least six different gibberellin-like substances; most probably none of them is identical to gibberellic acid or gibberellin A_1. The gibberellin-like substances are all active in a specially designed spruce seedling bioassay, as well as in a number of traditional gibberellin bioassays. Young spruce seedlings grown in nutrient solution are retarded by the substances Amo-1618, B-995, and CCC. Both root and shoot growth are affected. Gibberellic acid counteracts the effects of all the retardants on shoot growth, and of Amo-1618 on root growth, but has no effect when supplied alone. Application of the growth retardants B-995 and CCC to spruce grafts in a seed orchard results in decreased flowering. Preliminary results show, that flowering and non-flowering grafted spruce clones display remarkable differences in the dynamics of gibberellin-like substances; a high content of these substances during the supposed time of flower bud initiation is correlated to good flowering ability. All these findings are consistent with the hypothesis, that gibberellins are intimately involved in the process of flower bud induction. Literature dealing with the occurrence and effects of gibberellins within the class Coniferopsida is summarized.

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Contents

1 General introduction

Since the rediscovery of the gibberellins in the early 1950's (Mitchell and Angel, 129; Stodola *et al.*, 186) an ever increasing number of investigations on the occurrence and functions of gibberellins in plants have been published. In 1971 alone around 400 articles on this topic were issued. Gibberellins are now considered to be normally occurring hormonal regulators in higher plants (Cleland, 22; Paleg and West, 140), and there are also scattered reports on the existence of gibberellins or gibberellin-like substances in bacteria, algae, fungi, mosses and pteridophytes, as well as a considerably higher number of reports on physiological effects of gibberellins exogenously applied to such plants (Tamura *et al.*, 191).

In angiosperms, gibberellins are considered to be necessary for the normal elongation growth, and a lacking ability to biosynthesize gibberellins causes dwarfism, that can be overcome by an exogenous supply of gibberellin (Brian and Hemming, 12; Wittwer and Bukovac, 201). Gibberellins induce parthenocarpic fruit development (Wittwer *et al.*, 202; Crane, 24), they promote seed germination (Kahn *et al.*, 79, 80; Lona, 110) and the breaking of bud dormancy (Donoho and Walker, 37; Lona and Borghi, 113; Lona, 112; Eagles and Wareing, 43). They are also involved in the flowering process of many cold-requiring or long-day-requiring species (Bünsow and Harder, 17; Lona, 111; Lang, 104, 105, 106; Chailakhyan and Lozhnikova, 20). Gibberellins are used at a practical scale by horticulturists to promote flowering or to obtain parthenocarpic (seedless) fruits, and in the brewery industry to ensure germination of barley used for malting. Gibberellins have also been used for vernalization of seed of some cereal species, and other practical applications for gibberellins are under way (Stuart and Cathey, 189).

Since the structure of gibberellic acid was finally elucidated in 1959 (Cross *et al.*, 25, 26), many other different gibberellins have been found and identified, the number now being (at least) thirty-eight. It has been agreed (MacMillan and Takahashi, 115), that only fully identified naturally occurring substances, possessing *both* a chemical structure derived from gibbane (see figure 1) *and* some characteristic biological properties should be called a gibberellin and given a number. Most gibberellins only have numbers in the series A_1, A_2, A_3 .... A_n, but gibberellin A_3 is also often named "gibberellic acid" although in fact all identified gibberellins are carboxylic acids. Gibberellic acid is the commercially available compound that is used in most application experiments and as a reference in chromatography *etc.*

Substances with biological properties similar to gibberellic acid or other gibberellins but with unknown chemical structures are often found in plant extracts or exudates. As long as their chemical structures are not known, they should be referred to as "gibberellin-like substances" (Phinney and West, 154; Paleg and West, 140). Sometimes such a substance has chromatographic or other physical or chemical characteristics that are partly identical to the characteristics of some known gibberellin, and it is then of course possible (although not necessarily very probable) that this substance is really identical with that particular gibberellin. A safe identification can however be achieved only by means of mass spectrometry or infrared spectrophotometry of sufficiently pure substances, and often other complementary investigations are necessary as well.

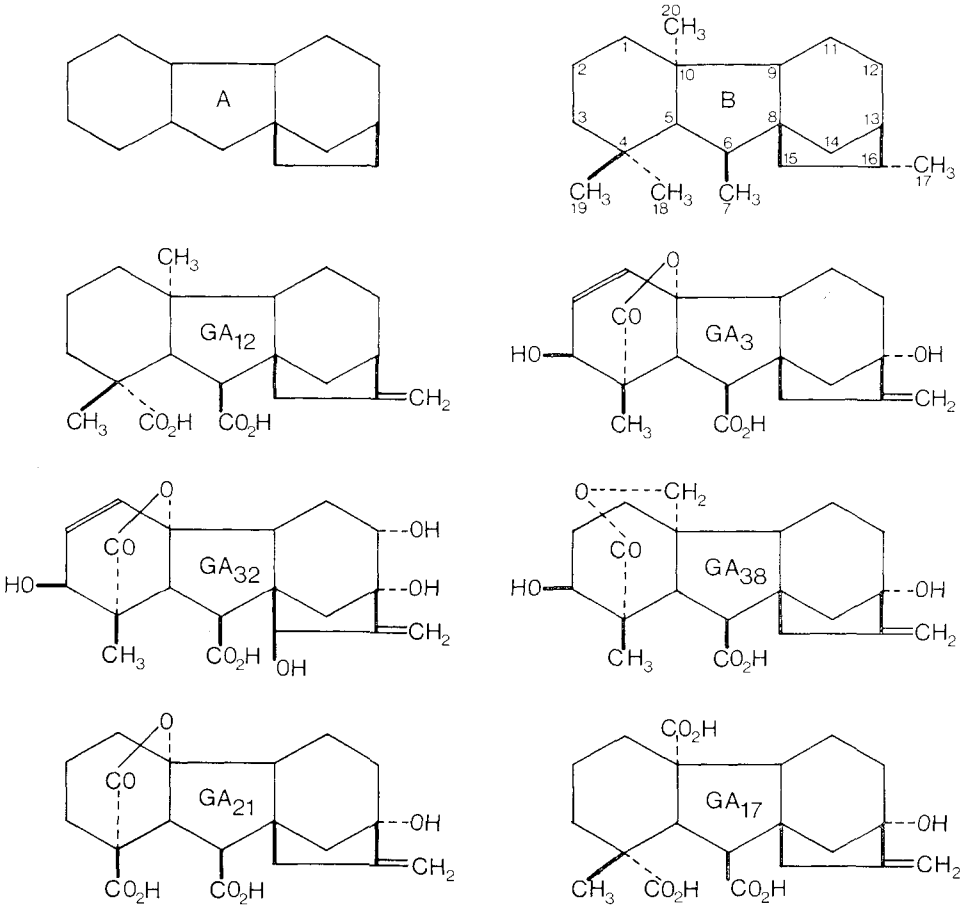


Figure 1. Chemical structures of gibbane (A), *ent*-gibberellane (B), and of some gibberellins.

There is a strong contrast between the very large number of investigations on gibberellins from angiosperms and the very limited number of similar investigations dealing with coniferous species. Gibberellin-like substances have so far been detected in only 21 coniferous species (see table 1). Furthermore, most investigations have been based on relatively crude techniques, that have neither been very successful in removing interfering substances nor have possessed very good resolving power. As a consequence, the yields of gibberellin activity have generally been low (see table 1), and little can be said regarding the nature of the gibberellin-like substances found. In just a few investigations (Kopcewicz, 94; Crozier *et al.*, 28) more elaborate methods have been used, and in these cases a number of gibberellin-like substances were found. These substances were also partly characterized, and by means of silicic acid partition column chromatography, various bioassays, and gas-liquid chromatography, gibberellic acid was tentatively identified as the main gibberellin in *Pseudotsuga menziesii* (Mirb.) Franco (Crozier *et al.*, 28). This is the only reasonably safe identification of a gibberellin from a coniferous species.

Also reports on effects of exogenous application of gibberellin to conifers are scarce. The first investigation of this kind (Marth *et al.*, 116, 117) reported positive results obtained by application of gibberellic acid as a spray (at the concentration

Table 1. Reports on occurrence of gibberellin-like substances in coniferous species.

Species	Year	References	Amount µg/kg f.w.
Pinaceae			
Larix kaempferi (Lamb.) Carr (= *L. leptolepis* Sieb. & Zucc.)	1965	64	
Larix decidua Mill.	1966	99, 124, 126	150
Pinus densiflora Sieb. & Zucc.	1969	67	16.3
Pinus elliottii Engelm.	1969	67	10.2
Pinus jeffreyi A. Murr.	1966	101, 102	
Pinus lambertiana Dougl.	1966	101, 102	4
Pinus ponderosa Dougl.	1966	101, 102	
Pinus radiata D. Don	1971	190	8
Pinus sibirica Mayr.	1972	155	
Pinus strobus L.	1969	67	1.9
Pinus sylvestris L.	1966	72, 73, 93—96, 98 99, 123—126	30
Pinus taeda L.	1969	67, 141	4.7
Pinus thunbergii Parl.	1966	67, 136	5.8
Pseudotsuga menziesii (Mirb.) Franco	1969	3, 27, 28	25
Taxodiaceae			
Cryptomeria japonica (L.f.) Don	1966	67, 130	3.2
Cunninghamia lanceolata (Lamb.) Hook.f.	1969	67	5.1
Metasequoia glyptostroboides Hu & Cheng	1969	67	2.4
Cupressaceae			
Chamaecyparis obtusa (Sieb. & Zucc.) Endl.	1969	67	0.3
Cupressus arizonica Greene	1968	3, 27, 169	200
Cupressus lusitanica Mill.	1968	145	
Juniperus chinensis L.	1962	81	

400 mg l^{-1}) or as a lanolin paste (at concentrations 0.25—1 %) to the species *Juniperus chinensis* L. cv. '*Pfitzeriana*', *Picea glauca* (Moench) Voss, *Pinus taeda* L. and *Pinus virginiana* Mill. The treatments gave increases in stem length over control as much as 50, 22, 40 and 68 %, respectively, although there was a certain variability of responses. On the other hand, *Pinus strobus* L. did not respond at all to gibberellin treatment. The number of plants that were treated in each case was not stated even in the more extensive of the two papers (Marth *et al.*, 117), and the experimental conditions were described very briefly. The conifers were said to be generally "less responsive than many other plants tested".

Many investigations during the following years with a number of coniferous species have given entirely negative results (early investigations are summarized by Westing, 199). With time, however, a few positive results of gibberellin application to conifers were also published. By now a still limited number of gibberellin effects have been reported, and as can be seen in table 2 they range from seed germination to flower bud initiation, from callus growth to apical dominance. It is of interest to note, that gibberellin stimulation of hypocotyl or stem growth has been observed very seldom, and when obtained at all such stimulations have generally been small as compared to the strong effects caused by gibberellin application to many angiosperms.

Stimulation of stem growth in intact plants is often considered to be the most typical physiological effect caused by gibberellins (Cleland, 22). Since conifers generally do not show this type of response, which is the most commonly studied, they have quite often been thought of as being indifferent to gibberellins. This opinion was also for a long time supported by the failure

Table 2. Reported effects of gibberellins applied to conifers.

Flower bud initiation	
Taxodiaceae:	
Cryptomeria japonica (L.f.) Don	58, 60—63, 66, 82—87, 171, 178
Metasequoia glyptostroboides Hu & Cheng	65, 66, 85, 178
Sequoia sempervirens (D. Don) Endl.	65, 85, 146
Sequoiadendron giganteum (Lindl.) Buchh.	146
Taxodium ascendens Brongn.	85
Taxodium distichum (L.) Rich.	85
Cupressaceae:	
Calocedrus decurrens (Torr.) Florin (= *Libocedrus decurrens* Torr.)	85
Chamaecyparis formosensis Matsumura	85
Chamaecyparis lawsoniana (A. Murr.) Parl.	9, 10, 59, 85
Chamaecyparis obtusa (Sieb. & Zucc.) Endl.	59, 68, 85
Chamaecyparis pisifera (Sieb. & Zucc.) Endl.	85
Cupressus arizonica Greene	9, 10, 142—145, 150, 151
Cupressus lusitanica Mill.	144, 145
Cupressus pygmaea (Lemm.) Sarg.	144, 145
Cupressus sempervirens L.	85
Juniperus communis L.	85
Juniperus virginiana L.	85
Thuja plicata D. Don	140, 142, 144, 145, 147, 148
Thuja standishii (Gord.) Carr.	85
Thujopsis dolabrata (L.f.) Sieb. & Zucc.	66, 85
All attempts to induce flowering by gibberellin applications in species belonging to the Pinaceae family have been resultless.	
Stimulation of seed germination and/or radicle growth	
Picea pungens Engelm.	51
Pinus densiflora Sieb. & Zucc.	171
Pinus lambertiana Dougl.	16
Pinus strobus L.	74
Pinus sylvestris L.	51, 91, 92, 97, 194
Pseudotsuga menziesii (Mirb.) Franco	161, 162
Cryptomeria japonica (L.f.) Don	171
Chamaecyparis obtusa (Sieb. & Zucc.) Endl.	171
Stimulation of hypocotyl and/or stem growth	
Larix decidua Mill.	122
Picea abies (L.) Karst. cv. '*Nidiformis*'	55
Picea glauca (Moench) Voss	116, 117
Picea glauca cv. '*Conica*'	55
Picea pungens Engelm.	51
Pinus strobus L.	133
Pinus sylvestris L.	51
Pinus taeda L.	7, 116, 117, 163
Pinus virginiana Mill.	116, 117
Juniperus chinensis L. cv. '*Pfitzeriana*'	116, 117
Thuja occidentalis L. cv. '*Hoveyi*'	120
Taxus sp.	32
Breaking of dormancy	
Pinus elliottii Engelm.	11
Hastening of autumn coloration and leaf-fall	
Taxodium distichum (L.) Rich.	14

Stimulation of pollen germination and/or pollen tube growth	
Pinus banksiana Lamb.	88, 89
Pseudotsuga menziesii (Mirb.) Franco	21
Increase of sugar content	
Pinus sylvestris L.	91, 92
Stimulation of phloem differentiation, cambial activity and/or formation of compression wood	
Larix decidua Mill.	198
Pinus radiata D. Don	197
Pinus strobus L.	33
Pinus sylvestris L.	70
Pseudotsuga menziesii (Mirb.) Franco	198
Stimulation of auxin transport	
Pinus sylvestris L.	70
Participation in apical dominance	
Pinus sylvestris L.	195
Sequoia sempervirens (D. Don) Endl.	167, 168
Cupressus arizonica Greene	152
Haploid parthenogenesis (seed development in unpollinated cones)	
Pseudotsuga menziesii (Mirb.) Franco	184
Tissue culture, stimulation of callus growth	
Cupressus funebris Endl.	187
Stimulation of amino acid release from the endosperm of germinating seed	
Pinus sylvestris L.	194
Stimulation of tryptophan decarboxylation in the endosperm of germinating seed	
Pinus sylvestris L.	194

of virtually all investigations aiming at the isolation of gibberellin-like substances from conifers (Westing, 199; Radley, 157). As has been mentioned already, a number of investigators have shown during the last six years the existence of such substances in a still limited but increasing number of coniferous species. Since their results often indicate the existence of gibberellin-like substances not identical to gibberellic acid, one may speculate that the poor results obtained by treatment with gibberellic acid simply reflects the fact that this particular gibberellin is not a relevant one.

Another explanation to the apparent insensitivity of coniferous species to gibberellins may be, that conifers are not very well suited as a standard experimental material in plant physiology laboratories. There may be also a certain lack of understanding among foresters and forestry organizations for the need of basic physiological research, and if plant physiologists are not positively encouraged to work with conifers they most naturally tend to concentrate their efforts on more suitable and more easily handled materials.

The most impressive effect caused by gibberellins on conifers is no doubt their ability to stimulate the initiation of flower

buds. This effect was actually detected already in 1958 by Japanese researchers (Kato *et al.*, 84, 85, 87; Kato, 82; Hashizume, 58, 59; Shidei *et al.*, 178), who found that a number of species belonging to the *Taxodiaceae* and *Cupressaceae* families could be forced to develop flower buds by gibberellin treatment (see table 2). These very spectacular and interesting results were however very little known outside Japan until 1965, when Pharis *et al.* (151) started research with the effects of gibberellin on the flowering of *Cupressus arizonica* Greene. As can be seen in table 2 a total of six *Taxodiaceae* and fourteen *Cupressaceae* species have been reported to respond positively to gibberellin treatment. All attempts to evoke the same response in species belonging to the *Pinaceae* family have been resultless, however.

The use of gibberellins to increase flowering or to induce precocious flowering may be of great advantage to the forest geneticists, who are often set in difficulties by poor flowering and are generally handicapped by the long juvenility period of most conifers (Gerhold, 49; Duffield, 38). It has been clearly shown that the flower development caused by gibberellin treatment does not differ principally from the normal flower bud development (Hashizume, 65; Owens and Pharis, 138, 139), that pollen produced in the male catkins is normal and fertile (Pharis *et al.*, 151), and that seeds developing in the female inflorescences become viable and give rise to normal seedlings (Hashizume, 61; Kato *et al.*, 86). Also the positive effect of gibberellins on seed germination is promising to the geneticists, since failures of seed development or germination often occur in crosses, especially in interspecific crosses among the genus *Pinus* (McWilliam, 121; Dogra, 36; Kriebel, 100; Krugman, 103). The genus *Picea* is not at all as well explored for the possibilities of interspecific crossing, but according to Mikkola (127) similar incompatibility barriers exist in this genus as well. The most promising use of gibberellins in forest genetics may be for the induction of haploid seed development, *i. e.* seed (and embryo) development in unpollinated cones, as has been reported to occur in *Pseudotsuga menziesii* (Mirb.) Franco (Stettler *et al.*, 184).

Norway spruce (*Picea abies* (L.) Karst.) is a species of very great importance to Swedish forestry. Clonal seed orchards of this species have been established to ensure the supply of high quality (genetically and also physiologically) seed for reforestation purposes, and also to enable the geneticists to run a crossing program including both Swedish and foreign provenances (Gustafsson, 52; Andersson, 2; Stern, 183; Johnsson, 76; Hadders and Samuelsson, 53). Both these aims of the spruce seed orchards are severely threatened by very poor and also markedly periodic flowering. It was therefore considered necessary to investigate the flowering physiology of this species, and since flowering in angiosperms and also sexual reproduction in most cryptogamous groups is generally supposed to be under the control of hormonal mechanisms (Chailakhyan, 19; Evans, 45; Raper, 159; Näf, 132; McMorris and Barksdale, 119; Arsenault *et al.*, 4; Yanagishima, 203; Ende and Stegwee, 44), the hormonal physiology of the spruce tree was considered to be a suitable starting point. This topic has been studied before by Steen (180, 181), who explored the occurrence of auxins and inhibitors. Gibberellins and cytokinins have not even been shown to occur in any one *Picea* species.

The limited resources of this project made it necessary to concentrate the study initially to just a single group of the plant hormones. The very promising results obtained with gibberellin treatment of a number of conifers made this group of regulatory substances a natural choice, and further support for this choice was given by the fact that flowering of many angiosperms (Lang, 106) as well as the sexual reproduction of a number of fern species (Schraudolf, 172, 173; Voeller, 196) is known to be directly or indirectly controlled by gibberellins. The purpose of the first part of the investigation was defined as the answering of three questions:

1) Are gibberellic acid and/or other gibberellin-like substances present in *Picea abies* (L.) Karst.?
2) Do gibberellic acid and/or the possible endogenous gibberellin-like substances have any kind of effect on *Picea abies*?
3) Can any kind of evidence be found that gibberellins are involved in the natural process of initiation of flower buds in *Picea abies*?

This publication describes how the problems were attacked and also gives the (positive) answers to the three questions. Finally, the possible further continuation of the project is briefly outlined.

2 Occurrence of gibberellin-like substances

2.1 **Introductory experiments**

2.1.1 *Introduction*

Attempts to isolate gibberellin-like substances from Norway spruce were initiated during the winter 1969—1970. Experiments with growth retardants (see below and also Dunberg and Eliasson, 40) had yielded results that could serve as the starting point. It was therefore considered suitable to use a similar experimental material as had been used in those investigations, *i.e.* spruce seedlings grown in nutrient solution in a controlled environment. Young actively growing seedlings could also be supposed to contain reasonably large amounts of gibberellin-like substances, so that a detection of such substances could be possible even with the use of fairly crude methods.

2.1.2 *Materials and methods*

Seedlings of Norway spruce (*Picea abies* (L.) Karst.) four months old and grown in nutrient solution (composition as shown in table 3 on page 35) in a growth chamber (16 h light, intensity 10 000 lux from cool white fluorescent tubes, spectral distribution shown in figure 2, temperature 23°C; and 8 h darkness, temperature 18°C; relative humidity constantly 65—75 %) were used for the extractions. Twenty grams of these seedlings, root and shoot, were homogenized in 200 ml of ice-cold absolute methanol and left in ice bath for two hours. The methanolic extract was then filtered off on a Büchner funnel, and the tissue debris was rinsed twice with fresh absolute methanol of room temperature (see figure 3). The three methanol volumes were combined, and the solvent was evaporated to dryness in a rotary evaporator at reduced pressure and 35°C.

The extract was then dissolved in 250 ml of phosphate buffer (0.5 M, pH 8.0) and partitioned three times with equal volumes of ethyl acetate (see figure 3). This neutral ethyl acetate fraction, containing most of the lipids, chlorophyll and other pigments, was discarded. The buffer fraction was acidified to pH 3.0 with 1 N hydrochloric acid and again partitioned three times with equal volumes of ethyl acetate. The buffer phase was then discarded. The acidic ethyl acetate fraction was retained, and the ethyl acetate was evaporated to dryness at reduced pressure and 35°C.

The remaining extract was then dissolved in a small volume of acetone and transferred to a piece of filter paper. The acetone was allowed to evaporate completely at room conditions, and the piece of filter paper with the extract adsorbed to it was placed on top of a glass column (25 mm inner diameter) filled to a height of 20 cm with silicic acid (Mallinckrodt Silica Gel 100—200 mesh) that had been hydrated with 0.5 M formic acid and then slurried in *n*-hexane (Powell and Tautvydas, 156). The column was eluted with 50 ml fractions of *n*-hexane—ethyl acetate, in which the percentage of ethyl acetate was increased stepwise from 0 to 72 % in steps of 6 % and then directly to 100 %, so that a total of 14 fractions were collected (see abscissa in figure 4). The fractions were immediately evaporated to dryness, and the residues were dissolved in acetone and transferred to pieces of filter paper, that after evaporation of the acetone were stored *in vacuo* at —20°C until bioassayed.

Fractions from the silicic acid column as well as entire unchromatographed acidic ethyl acetate fraction residues were bioassayed with the barley half-seed α-amylase release bioassay, performed as described by

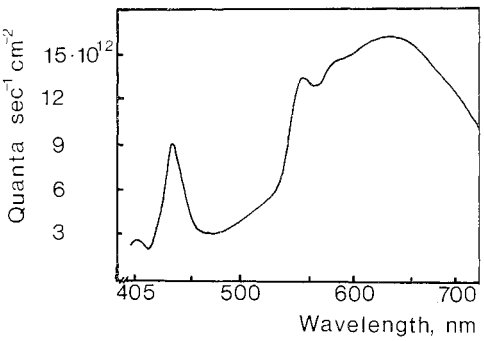


Figure 2. Spectral distribution of light in the growth chamber between 405 and 720 nm. The measurement was done at a point 70 cm below the light tubes. Due to the construction of the measuring equipment, the wavelength scale is not strictly linear.

Jones and Varner (78). A four-year old lot of barley seed, cv. *'Ingrid'* (purchased from W. Weibull AB, Landskrona, Sweden) was used. No antibiotics were added to the bioassay medium, but careful handling of all bioassay procedures prevented any visible microbial contamination.

A standard series of known amounts of gibberellic acid as well as a number of zero controls were run parallell to the extracts. The same kind and amount of filter paper was added to these vials also, and gibberellic acid was added by adsorption to filter paper. It is possible that this method for adding the extracts as well as gibberellic acid decreases the sensitivity of the bioassay. The theoretical maximum sensitivity, obtained by extrapolation of the dose-response curve of the standard series, was only around 2 ng per vial as compared to 0.1 ng per vial according to Jones and Varner (78). The smallest statistically significant response ($p \leq 0.01$) corresponded to around 6 ng of gibberellic acid per vial, with slight deviations from one bioassay run to another.

Since identical reagents, dilutions and reaction times and conditions were used in all cases, the response of the bioassay was expressed directly as the absorbance ("optical density") at 620 nm, measured in a Bausch and Lomb Spectronic 20 spectrophotometer. The decrease in absorbance is roughly proportional to the logarithm of gibberellin concentration, in accordance with the findings of Jones and Varner (78).

All solvents used were reagent grade or redistilled. Control chromatography with only the solvents proved that these did not affect the bioassay. Three chromatographed extracts and four unchromatographed were bioassayed.

2.1.3 *Results and discussion*

The results, shown in figure 4, were somewhat varying. Gibberellin activity was reproducibly found in fractions 12—14. It is difficult to judge, whether this zone contains one or two active substances, although the latter possibility seems more probable. In addition, a statistically significant activity was repeatedly found in fraction 3 or 4. Another repeatedly found peak of activity occurred in fraction 6 or 7, although in only one case this activity was statistically significant. It can be summarized, then, that the results indicate the occurrence of at least one and probably two highly polar gibberellin-like substances as well as one or perhaps two less polar. The four unchromatographed extracts were also active in the bioassay, and interpolations from the standard curves gave in these cases activities corresponding to 68, 160, 340 and 400 ng of gibberellic acid, respectively.

The partitioning procedure that has been used in this investigation is in accordance with methods that have been used and are still used by most researchers extracting gibberellin-like substances from plants. It has recently been noticed, however, that large amounts of the less polar gibberellins will partition into the neutral ethyl acetate fraction, and there either appear as "neutral gibberellins" or be discarded (Crozier *et al.*, 27, 29). From partition coefficients recently given by Durley and Pharis (42) it can be calculated, that almost all of the amounts of gibberellins A_9 and A_{12}, if present, must have been lost into the discarded neutral ethyl acetate phase, as well as around 66 % of gibberellin A_4 and

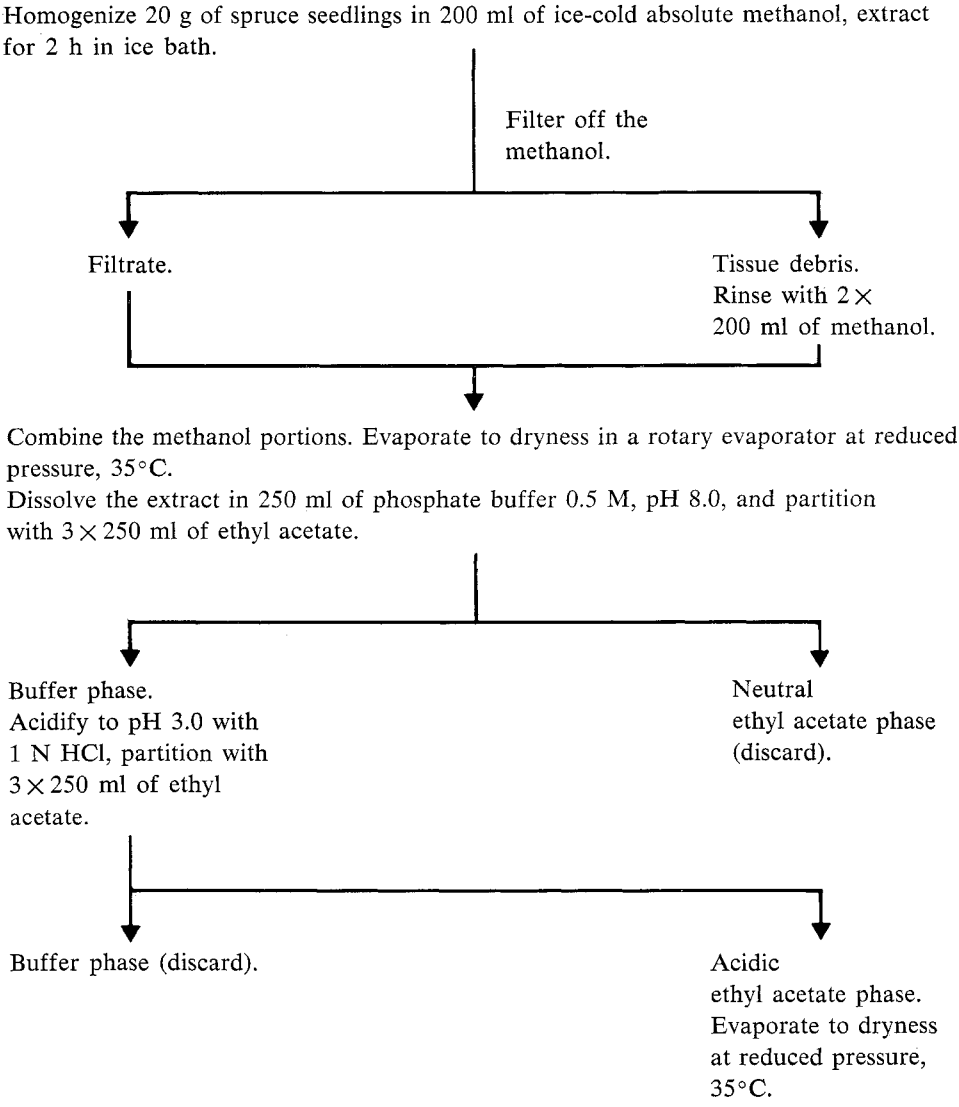


Figure 3. Flow diagram showing procedure for extraction and gross purification of gibberellin-like substances from spruce seedlings.

55 % of gibberellin A_7. It is also known (Crozier *et al.*, 30) that the barley half-seed bioassay, which has been used in this investigation, is insensitive to a number of gibberellins, or actually to *most* gibberellins. Therefore it is possible that other gibberellin-like substances may exist in spruce in addition to those two or three that have repeatedly been found in this investigation. It is also very probable, that inhibitory compounds in the crude extracts antagonize the promoting effects of gibberellin-like substances. To overcome this last-mentioned difficulty, other and more powerful methods of purification, preferably adapted especially to the chemistry of coniferous species (Norin, 135), must be developed and applied.

The total amount of gibberellin activity, calculated as gibberellic acid, varies be-

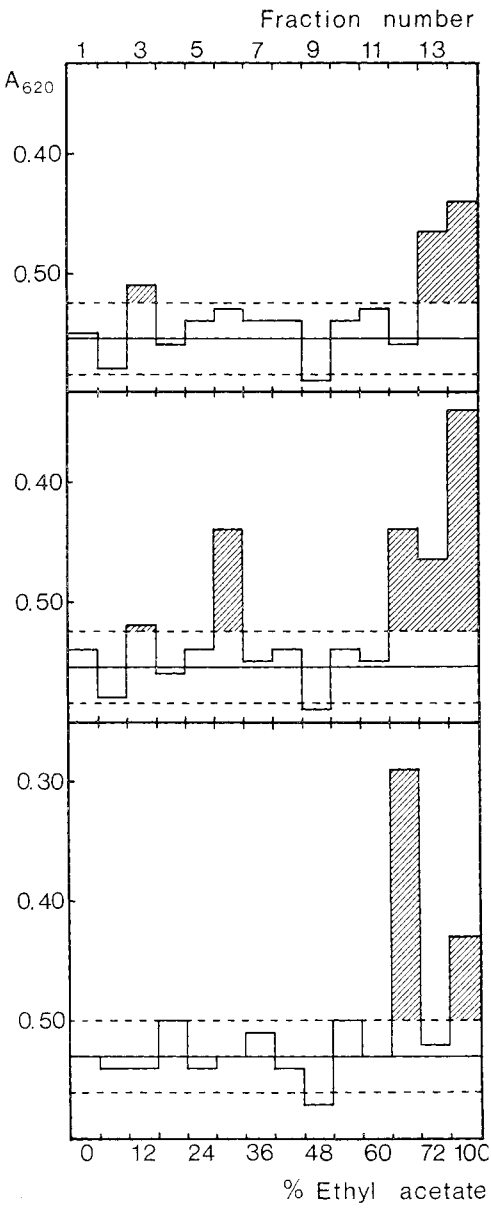


Figure 4. Gibberellin-like substances from four-month old spruce seedlings. The results of three different extractions (procedure described in figure 3) chromatographed on a silica gel partition column are shown.

Abscissa: Fractions from the silicic acid column.

Ordinate: Response of the barley half-seed bioassay (absorbance at 620 nm). Hatched horizontal lines give the least significant difference from the control at 1 % risk level.

tween 65 and 400 ng per 20 g fresh weight of tissue, or between 3.25 and 20 μg per kg. This great variation may in part be caused by imperfections of the partitioning and chromatography methods, but far more probable is the possibility mentioned above, that inhibitory compounds in the very crude extracts to a varying degree counteract the effects of the promoting substances. Probably then, even the highest value, 20 μg of gibberellin activity per kg, is actually an underestimate.

2.2 Further experiments: comparisons between juvenile and mature spruce shoots, and between shoot axes and needles

2.2.1 *Introduction*

Around the time when the introductory experiments just described were finished, several publications from a research group headed by Dr. R. P. Pharis at the University of Calgary, Canada, were issued (Crozier *et al.*, 27; Aoki *et al.*, 3). These investigations described the particular difficulties connected with the extraction of tissue from coniferous species, and also clearly demonstrated the poor efficiency of traditional methods for the extraction and detection of gibberellin-like substances when applied to such species: an ordinary extract from *Cupressus arizonica* Greene (crude acidic ethyl acetate-soluble fraction) that initially showed no gibberellin activity at all, was purified in a series of different methodological steps and finally displayed a major peak of activity corresponding to 1000 μg of gibberellic acid per kg dry weight or around 200 μg per kg fresh weight of tissue. These results were very promising, and the methods developed could equally well be applied to other coniferous species, like *Picea abies*. Contacts were therefore established with the Canadian group, and it was decided that the new methods should be tried in an investigation of gibberellin-like substances from Norway spruce. Since one of the main goals of the studies of gibberellin-like substances from

Picea abies was to explore the possible relation of these substances to flower bud initiation, the present investigation was designed as a comparison of the content of gibberellin-like substances between juvenile and mature trees. The idea was that the change from juvenility to maturity in this species may be related to some kind of changes (qualitative or quantitative) in the content of gibberellin-like substances. A comparison between shoot axes and the attached needles was also included. Needles contain substances (chlorophyll and other pigments, lipids *etc.*) that give rise to particular difficulties during the extraction and purification procedures, and it would be an advantage if they could be excluded from the extracted tissue. The possibility existed, it was thought, that even the new and much more efficient purification procedures did not entirely remove inhibitory compounds in the needles, and if so the detection of gibberellin-like activity in the needle extracts, and also in extracts from entire shoots, would be more difficult, and the detected amounts lower, than in extracts from shoot axes.

Collection of tissue and the initial extraction and purification steps up to the crude acidic ethyl acetate-soluble fraction were to be done at the Department of Botany, University of Stockholm. The final work (*i.e.* the main part of the investigation) was to be done during a three-month visit of the author as a guest researcher in Dr. R. P. Pharis' laboratory at the Department of Biology, University of Calgary, Canada.

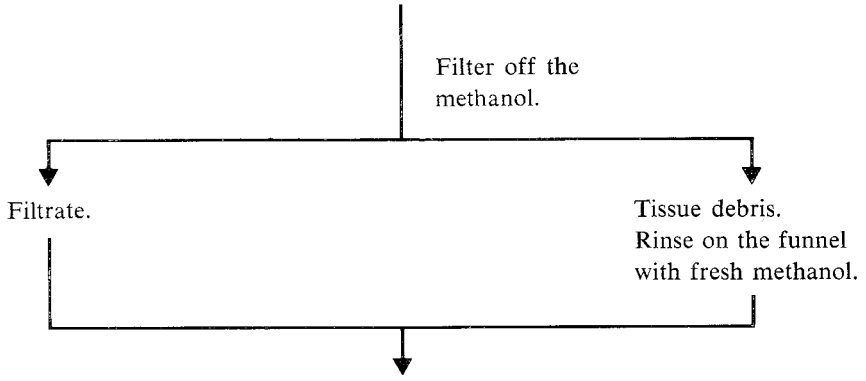
A brief report of this study has already been published elsewhere (Dunberg, 39).

2.2.2 *Materials and methods*

The entire growing shoots both from juvenile spruce (*Picea abies* (L.) Karst.) trees, age 10—15 years (estimated from the number of true branch whorls), and from the upper part of the crown of fully mature, cone-bearing trees, age about 80 years (counted on the stumps after the trees were felled), were collected in early June 1970. The date was within the period of very rapid shoot elongation, which lasts for only a few weeks, depending on temperature conditions (Romell, 166). The collected tissue was immediately frozen in dry ice and kept at —20°C until used for extractions. All extractions up to the acidic ethyl acetate-soluble stage (see below) were completed within three weeks after the date of collection.

The frozen tissue was homogenized in batches of 500—800 g (fresh weight) at 0°C in absolute methanol (5 × volume: weight) with an Ultra-Turrax. It was then extracted over night in ice bath and with continous stirring. The methanol was filtered off on a Büchner funnel, and the tissue debris was rinsed on the funnel with additional room-temperate absolute methanol until it was colourless. The total methanolic extract was evaporated to dryness in a rotary evaporator at reduced pressure and 35°C. The green, smeary residue was dissolved in phosphate buffer (0.5 M, pH 9.0), and the pH was adjusted to 9.0 with 6 N KOH. This final pH adjustment is necessary, since the extracts normally contain acidic substances that lower the pH value of the buffer; at pH 9.0 the buffering capacity is actually very small. The buffer-dissolved extract was partitioned four times with equal volumes of freshly redistilled diethyl ether (the first portion of ether was also used in small volumes alternating with the buffer to aid in dissolving the greasy extract, in this way the extract was dissolved almost completely). The partitioning had to be done very carefully, without vigorous shaking, to prevent the formation of emulsions, and to compensate for this it was extended to five minutes each time. Small emulsions, that sometimes occurred, disintegrated if the separatory funnel was laid flat (to increase the surface between the two phases) and placed in a refrigerator for about an hour. The ether fraction, containing almost all the chlorophyll and other pigments, was discarded (see figure 5). The buffer fraction was acidified to pH 3.0 with 6 N hydrochloric acid and partitioned six times with ethyl acetate (ethyl

Homogenize spruce tissue in ice-cold absolute methanol (5× v/w), leave overnight in ice bath with continuous stirring.



Combine the methanol portions. Evaporate to dryness at reduced pressure, 35°C

Dissolve the extract in phosphate buffer 0.5 M pH 9.0, and partition with equal volumes of freshly redistilled ether four times.

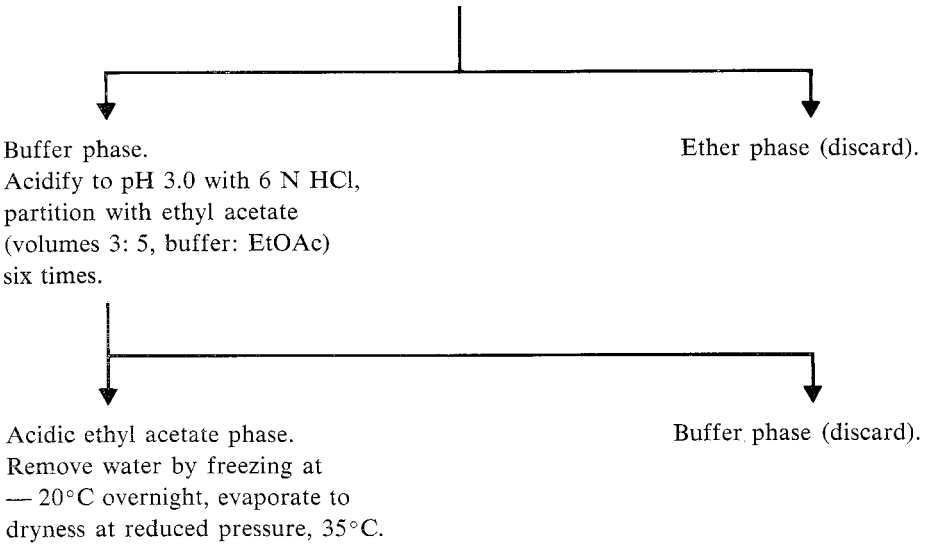


Figure 5. Flow diagram showing the improved procedures for extraction and gross purification of gibberellin-like substances from growing shoots of mature and juvenile Norway spruce.

acetate:buffer = 3: 5 by volume). The combined ethyl acetate phases were dried by freezing out the water at —20°C and then evaporated to dryness at reduced pressure and 35°C.

This part of the work was done at the Department of Botany, University of Stockholm. Extracts and author were then transported by air to the University of Calgary, Canada, where the work was done in the Laboratory of Plant Physiology.

The acidic ethyl acetate-soluble extract was first subjected to the "backwash" procedure described in figure 6. This procedure is designed to remove less-polar compounds without appreciable losses of the less polar gibberellins (see Crozier *et al.*, 27, 29 and above page 13). In the cal-

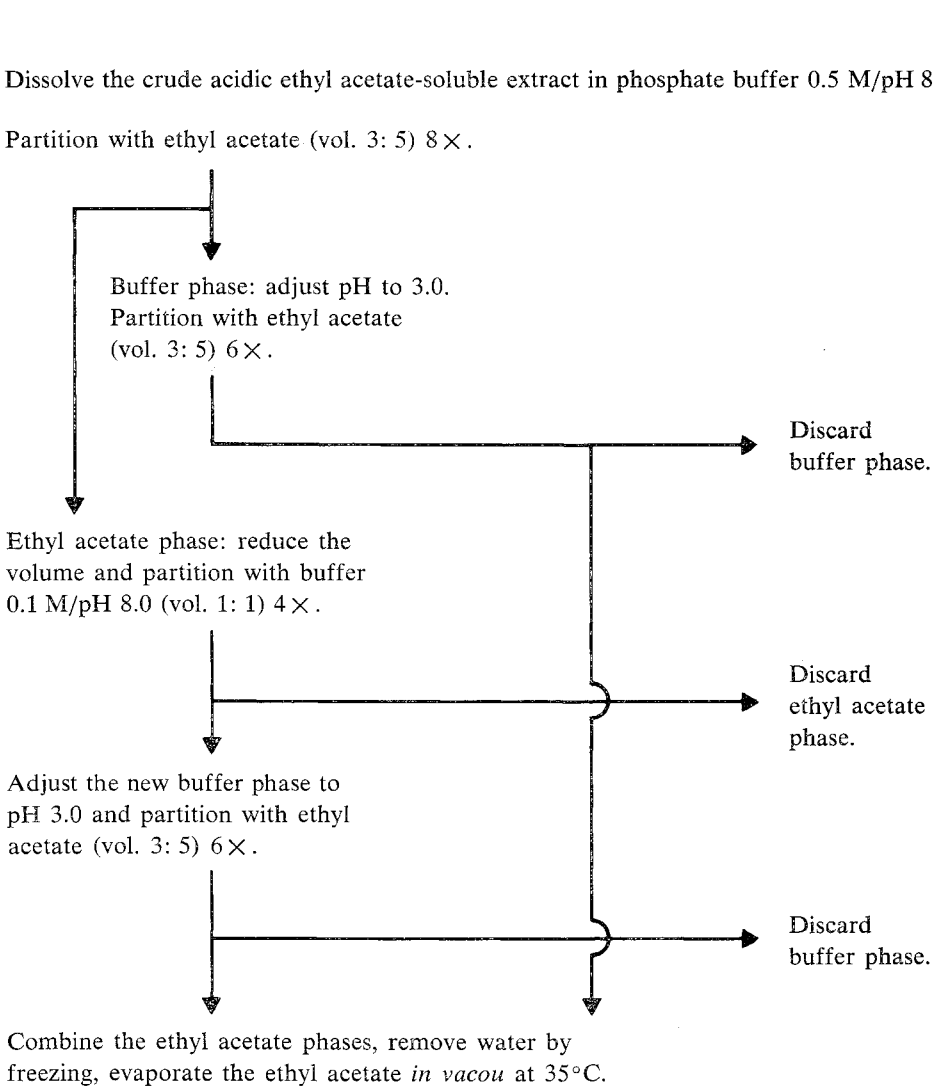


Figure 6. Outline of the backwash procedure used for further purification of gibberellin-like substances from Norway spruce.

culation of the details of this procedure, the partition coefficients of the gibberellins experimentally determined by Durley and Pharis (42) have been used. The purification efficiency of the backwash procedure, as measured by the decrease in dry weight of the extract, is around 50 %. Since mainly lipoid substances are removed, the risk for emulsion formation during the further work with the extract is greatly reduced. The disadvantages of the method are its great consumption of time and solvents.

The remaining extract residue after backwash was dissolved in a small volume of phosphate buffer (0.1 M, pH 8.0) and chromatographed on a 1.9×30 cm poly-*N*-vinylpyrrolidone (PVP) column (Glenn *et al.*, 50). PVP in the form of "Polyclar AT Powder" (from GAF Corporation) was used, and particles smaller than 150 μm were removed by use of a combined sieving and decanting procedure. The column was eluted with phosphate buffer (0.1 M, pH 8.0) by gravity flow, the flow rate was around 250 ml h^{-1}. The volume

50—230 ml was collected, acidified to pH 3.0 with 6 N hydrochloric acid and partitioned six times with 0.6 volumes of ethyl acetate. The combined ethyl acetate phases were dried by freezing out the water and evaporated to dryness as before. A second PVP column 1.9×120 cm was then run. The volume 225—625 ml was collected and treated similarly as before. The purification efficiency of each of these columns, as measured by the decrease in extract dry weight, varied between 70 and 90 %, the two columns in combination giving a reduction in extract dry weight of around 95 %. Phenolic compounds, that are abundant in conifers (Norin, 135) and strongly inhibit the bioassay responses, are effectively retained by the PVP matrix, presumably by the formation of hydrogen bonds (Loomis and Battaile, 114; Andersen and Sowers, 1). Gibberellins, on the other hand, pass through the column almost following the solvent front (Glenn *et al.*, 50). The PVP column technique is thus an efficient, selective, simple and swift method for purification of gibberellin extracts from conifers (and from other species as well). Some very powerful inhibitors, notably abscisic acid, are however eluted from the column in the same volume range as are the gibberellins.

Gibberellin-like substances in the purified extracts were separated by chromatography on a 1.3×19 cm silicic acid partition column eluted with a gradient of ethyl acetate in *n*-hexane. The method was originally described by Powell and Tautvydas (156) and has been developed further by Durley *et al.* (41). Twenty-five fractions of 20 ml were collected plus a final fraction of 40 ml of methanol. This chromatographic method does not involve extremes of pH or other variables and is thus relatively little hazardous to the gibberellins. It can be scaled up to process large preparative extracts. Resolution and recovery are much better than can be obtained by paper or thin layer chromatography, and the elution volumes are more reproducible than the very varying R_f values of the two latter methods. For special purposes the standard gradient used in this investigation (see Durley *et al.*, 41) can be modified at will to ensure maximum resolving power around those fractions where it is needed. Routine analyses can be greatly simplified by use of an automatic gradient mixer, that delivers a reproducible and easily defined gradient. Not even this column chromatography method does separate all the gibberellins, however, and especially a number of the less polar gibberellins appear as a group in the first few fractions.

Dwarf rice '*Tan-ginbozu*' microdrop bioassay (Murakami, 131), barley half-seed α-amylase release bioassay (Jones and Varner, 78) and lettuce hypocotyl bioassay (Frankland and Wareing, 46) were performed with modifications as described by Crozier *et al.* (30).

All solvents used were reagent grade or redistilled. Control chromatography with only the solvents proved that these did not affect the bioassays. Two extracts each were made from juvenile and mature tissue. In addition, one extract each was made from needles and from shoot axes, both from mature trees. The needles and shoot axes originally belonged together.

2.2.3 *Results and discussion*

The results are shown in figures 7—9. A number of gibberellin-like substances, ranging from fairly non-polar to very polar, could be detected by means of the dwarf rice bioassay. No obvious qualitative differences in the spectra of gibberellins from juvenile and mature trees are present (figure 7), rather all four extracts show very similar patterns of gibberellin activity. It is very difficult to estimate how many different active substances that are present. One can rather distinguish four more or less wide active zones. The first one comprises fractions 1 and (sometimes) 2, and is followed by a strongly inhibitory fraction. The second active zone includes fractions 5 to 10, and there is some reason to suggest the existence within it of two different gibberellin-like substances,

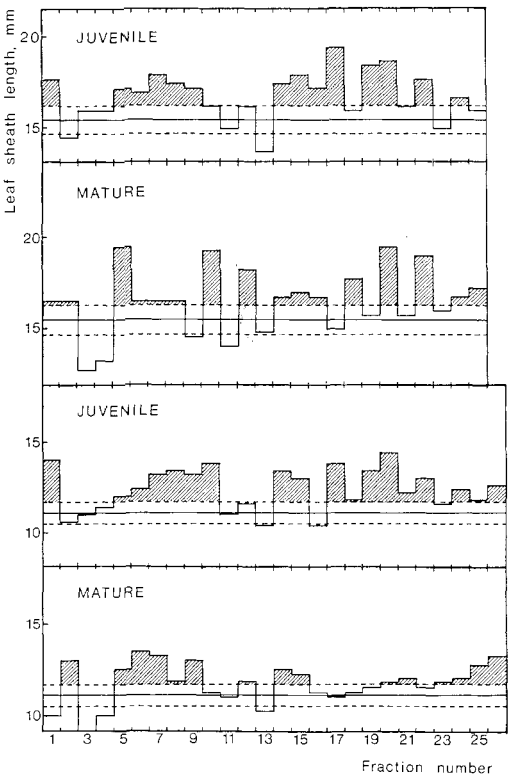


Figure 7. Gibberellin-like substances from growing shoots of juvenile and mature Norway spruce, as measured by the dwarf rice bioassay.

Abscissa: Fractions from the silicic acid column.

Ordinate: Response of the dwarf rice bioassay (leaf sheath length). Hatched horizontal lines give the least significant difference from the control at 1 % risk level.

one in fractions 5—6 and the other in fraction 9 (or 10). Fractions 11 and 13 are consistently inhibitory, and there is some evidence for a gibberellin-like substance inbetween, in fraction 12 (zone 3). The fourth active zone encloses the rest of the chromatogram, and it may contain as many as four or five active substances (the difference in fraction number collected was caused by a small change in the volume of each fraction). Another possibility is, that a few wide peaks of activity (the peak width increases with increasing elution volume) are split up by small amounts of strongly inhibitory substances, and that thus only a few gibberellin-like substances are actually present.

Quantitative differences in the content of gibberellin-like substances between juvenile and mature trees are not significant. Inhibitory substances, especially in the fractions 2—4, seem to be more abundant in mature trees. Pure abscisic acid is eluted in fraction 4 (Durley *et al.*, 41), and since this substance has been shown to occur in conifers (Milborrow, 128; Bonnet-Masimbert, 8; Lenton *et al.*, 108; Little *et al.*, 109; Jenkins and Shepherd, 75) and is not separated from the gibberellins by the purification procedures used in this investigation, the inhibitory activity in this zone may be at least in part caused by abscisic acid. Evidence suggesting the occurrence of abscisic acid in *Picea abies* has previously been published by Steen and Eliasson (182).

Qualitative differences in the spectra of gibberellins are apparent between extracts from shoot axes and from needles (figure 8—9). Fractions 13—15 of the shoot axes extract are active in all three bioassays, whereas these fractions of the needle extract are active only in the dwarf rice and perhaps slightly active in the lettuce bioassay. These fractions of the two extracts thus seem to contain different promoting substances with different bioassay specificities. It should be kept in mind, though, that the bioassays measure the balance between promoters (*i.e.* gibberellin-like substances) and inhibitors, and the needles may well contain larger amounts of inhibitors. The needles also seem to contain a greater number of gibberellin-like substances than the shoot axes. No gibberellin activity is detected in fractions 17—22 of the shoot axes extract, where two (or perhaps even three) peaks of activity occur in the needle extract, and also in the extracts of entire shoots.

There is only one extract each of shoot axes and of needles to compare, and the validity of the comparison should be judged upon that basis. The four extracts of entire shoots can also be used for comparison, however. Especially the needle

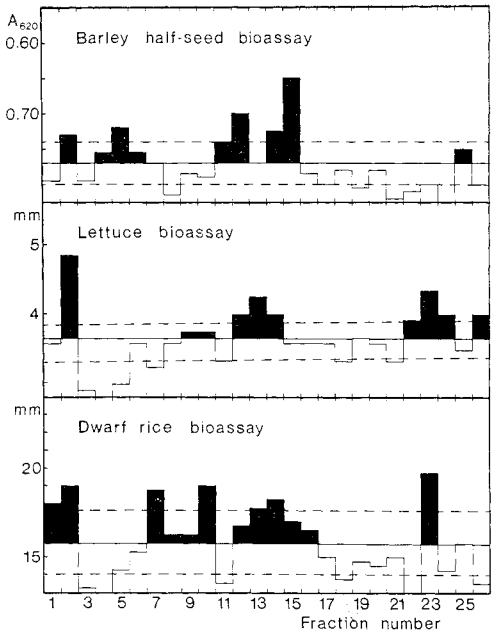


Figure 8. Gibberellin-like substances from shoot axes of mature growing shoots of Norway spruce, as measured by the barley half-seed (top), lettuce (middle) and dwarf rice (bottom) bioassays.

Abscissa: Fractions from the silicic acid column.

Ordinate: Bioassay response (absorbance at 620 nm, hypocotyl length, and leaf sheath length, respectively). Hatched horizontal lines give the least significant difference from the control at 1 % risk level.

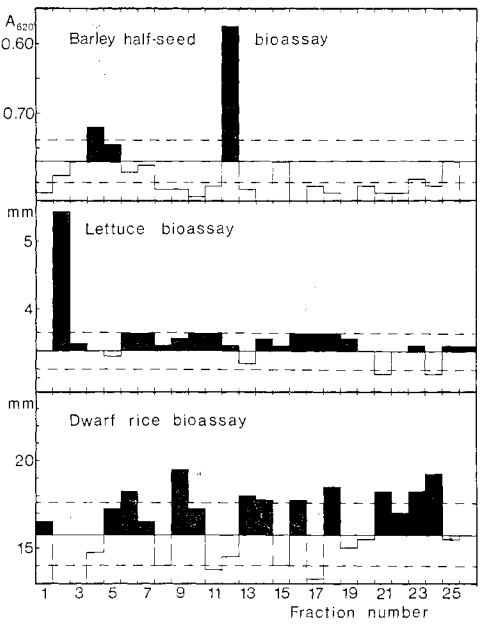


Figure 9. Gibberellin-like substances from needles of mature growing shoots of Norway spruce, as measured by the barley half-seed (top), lettuce (middle) and dwarf rice (bottom) bioassays.

Abscissa and ordinate as in figure 8.

extract, but also the shoot axes extract with the exception of fractions 17—22, are very similar to the extracts of entire shoots. If the two extracts of separate parts are superimposed, the similarity even increases.

The results of this investigation strongly support the presence of a number of gibberellin-like substances, at least four but presumably eight or nine, in growing shoots of *Picea abies*. This finding is in good agreement with the two most careful and extensive investigations of this kind that have been published previously: Kopcewicz (94) found seven different gibberellin-like substances in *Pinus sylvestris* L., and Crozier *et al.* (28) found at least five in *Pseudotsuga menziesii* (Mirb.) Franco.

It may seem as if the gibberellin relations of conifers were more complicated than those of angiosperms, but recent investigations using methods similar to those used in this study have shown, that the same complexity also exists in angiosperms, with a number of different gibberellin-like substances present (Crozier and Reid, 31; Crozier *et al.*, 29). Results presented here suggest, that the needles contain some more gibberellin-like substances in addition to those present in shoot axes. The physiological explanation for this is not clear, but it has been found that chloroplasts contain gibberellins (Stoddart, 185; Railton and Wareing, 158), although their function is not known. The needles also store waste products, and perhaps the comparably polar gibberellins present only in the needles are the inactivated end products of the gibberellin metabolism. This metabolism is known to procede from the most nonpolar to the most polar gibberellins,

by means of carboxylation and hydroxylation (Tamura *et al.*, 191).

An additional finding of this investigation is a strongly inhibitory zone with chromatographic properties similar to abscisic acid. While there were no qualitative or quantitative differences in the spectra of gibberellin-like substances between juvenile and mature spruce trees, mature trees contained larger amounts of this abscisic acid-like inhibitor. This is however not reason enough to speculate in the possible relation of this poorly defined inhibitor to the termination of the juvenile phase.

The total amounts of gibberellin-like activity, calculated as gibberellic acid equivalents, invariably exceed 20 μg per kg fresh weight of tissue, which was the maximum activity obtained in the previously described introductory experiments. It must be remembered, that large-scale extractions like these in the present investigation never can become highly efficient. It therefore seems probable that the amounts of gibberellin-like substances present in *Picea abies* do not differ from amounts commonly found in angiosperms.

2.3 Further attempts to characterize the gibberellin-like substances from spruce

2.3.1 *Introduction*

The results already obtained demonstrated beyond doubt the occurrence in *Picea abies* of a complex spectrum of different gibberellin-like substances. A number of questions remained unanswered, however. Very elaborate but also laborious methods had been used; since every step in the procedures introduced new hazards or at least uncertainties, a critical evaluation of the reproducibility of the obtained results seemed desirable. Before the methods were to be used for further qualitative or quantitative comparisons, or even for estimations of absolute concentrations of different gibberellin-like substances, their possible capacity for yielding such information had to be determined. A better physiological characterization of the different active substances, by means of various bioassays, was also desirable. Since nothing was known regarding the molecular identity of these substances, work aimed at the elucidation of this particular question, *e.g.* by means of infrared spectroscopy, ought to be initiated. Also, bioassay routines had to be developed at the Department of Botany, where no extensive work on gibberellins had been carried out before.

The purpose of this investigation was to evaluate the validity and reproducibility of the employed methods, and to determine the possibilities to apply them to qualitative or quantitative comparisons or to estimations of actual concentrations of gibberellin activity. At the same time bioassay routines were to be worked out and applied to spruce extracts for a more detailed physiological characterization of the active substances found, and a preliminary study of the infrared spectra of these substances was also to be undertaken.

2.3.2 *Materials and methods*

Growing shoots of Norway spruce (*Picea abies* (L.) Karst.) from field-grown trees of various age as well as from seedlings grown in nutrient solution in a growth chamber were used. All samples were either immediately frozen in dry ice and after that stored for maximally two weeks at —20°C (field-collected) or used directly for extractions (samples from growth chambers). Twenty gram fresh weight of tissue was chosen as the standard extraction amount. Procedures of extraction, partitioning, backwash and poly-*N*-vinylpyrrolidone (PVP) column chromatography were as described previously (Dunberg, 39 and page 16 above), with the slight exception that two identical PVP columns 1.9 × 30 cm were run. In each case the volume 50—230 ml was collected.

Gibberellin-like substances in the purified extracts were separated by chromatography on a column containing 10 g of silicic acid (Mallinckrodt Silica Gel, 100—

200 mesh), that had been hydrated with 6.25 ml of 0.5 M formic acid and thereafter slurried in *n*-hexane, poured into the column and allowed to settle by gravity flow only (Powell and Tautvydas, 156). The sample was dissolved in a minute volume of acetone and transferred to a circle of filter paper with a diameter slightly smaller than the inner diameter of the glass column. The acetone was allowed to evaporate under room conditions, and then the circle of filter paper with the sample adsorbed to it was carefully and gently placed flat on top of the column. This was eluted with 10 ml fractions of *n*-hexane—ethyl acetate, in which the percentage of ethyl acetate was stepwise increased from 0 to 100 %. A total of 23 fractions were collected plus a final fraction of 20 ml of methanol. This methanol fraction dissolved some of the silicic acid and also destroyed its hydration, so a new column had to be prepared for each sample. Pure gibberellic acid was eluted in fractions 16—19, with a maximum in fraction 18.

Fractions from the silicic acid column were tested for gibberellin activity in the barley half-seed α-amylase release bioassay, dock leaf (*Rumex*) senescence bioassay, dwarf rice '*Tan-ginbozu*' microdrop bioassay and cucumber hypocotyl bioassay. In addition, a specially designed spruce seedling hypocotyl bioassay was employed.

The barley half-seed α-amylase release bioassay was carried out according to principles described by Jones and Varner (78), but with some modifications caused by the practical situation. A one-year old lot of barley seed cv. '*Ingrid*' (purchased from W. Weibull AB, Landskrona, Sweden) was used. Another variety, '*Bonus*', was also tried but found less satisfactory. Seeds were manually cut in halves by help of a scalpel. The embryoless seed halves (endosperms) were then surface sterilized by soaking for 20 minutes in sodium hypochlorite solution (containing approximately 4 % of free chlorine), rinsed three times with large volumes of sterile distilled water, and placed to imbibe on several layers of moist, sterilized filter paper in large Petri dishes. After three days in darkness at +4°C the endosperm halves were fully imbibed, and the bioassay was started. Ten seed halves plus either sample fractions (corresponding to 0.2 g fresh weight of tissue), pure gibberellic acid in known amounts, or solvent blanks, all adsorbed to small pieces of filter paper, were added to autoclave sterilized 10 ml Erlenmeyer flasks containing 1.0×10^{-5} moles of $CaCl_2$, 1.0×10^{-6} moles of acetate buffer at pH 4.8 and 3.4×10^{-7} moles of streptomycine sulphate, all in a total volume of 1.0 ml of distilled water. The flasks were then placed on a gyrotory shaker (velocity 100 rpm) in darkness at 25°C for 72 hours. After that time 100 µl were taken by a microsyringe from the solution in each flask, diluted with distilled water to 1.0 ml, and pipetted into 1.0 ml of a solution containing 4.4×10^{-5} moles of KH_2PO_4, 2.0×10^{-6} moles of $CaCl_2$, and 1.50 mg of soluble starch. The enzyme reaction was allowed to run for 3.0 minutes at room temperature, and was then stopped by addition of 1.0 ml of acidic iodine reagent (60 mg of KI plus 6 mg of I_2 dissolved in 100 ml of 0.05 N hydrochloric acid; has to be prepared fresh for each bioassay). After dilution with 5 ml of distilled water, the absorbance ("optical density") at 620 nm was measured in a Bausch and Lomb Spectronic 20 spectrophotometer. Since the reaction conditions were invariably the same, the bioassay response was expressed directly as the absorbance at 620 nm. Typical dose-response curves for standard amounts of gibberellic acid are shown in figure 10.

The long incubation time of 72 hours was chosen, since it has been shown (Jones, 77) that this permits a wider range of gibberellins to be detected. With this long incubation time it became necessary to add streptomycine sulphate to the incubation medium, since microbial contamination otherwise occurred rather frequently. Preliminary extractions and bioassay runs showed, that very little gibberellin activity was detected in the spruce extracts by this

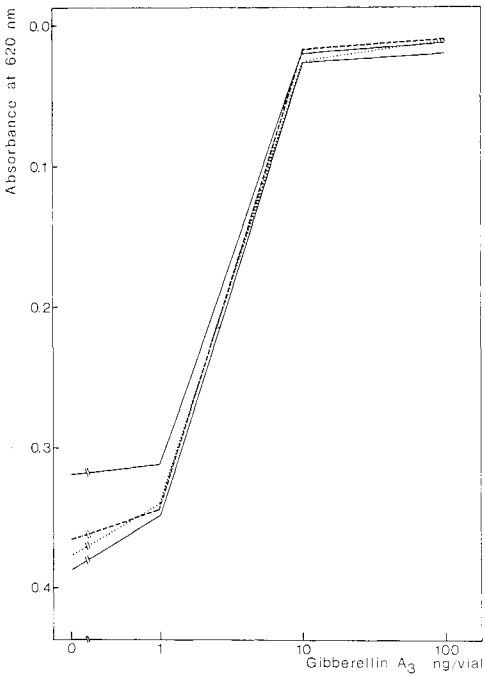


Figure 10. Dose-response curves of the barley half-seed α-amylase release bioassay. The results obtained in four different bioassay runs are shown. Reaction time and other parameters are as described in the text. Standard errors are too small to be shown in the figure.

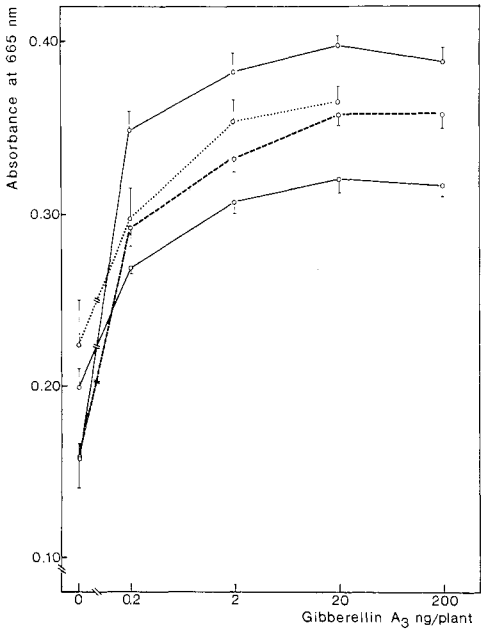


Figure 11. Dose-response curves of the dock leaf (*Rumex obtusifolius*) bioassay. The results obtained in four different bioassay runs are shown. Vertical bars indicate the standard errors.

bioassay. For the main investigation were therefore chosen enzyme reaction conditions that gave maximum sensitivity at the expense of the possibilities to discriminate between concentrations higher than 10 ng per vial (see figure 10). The theoretical maximum sensitivity, obtained by extrapolation of the dose-response curve of the standard series, varied between 0.5 and 0.8 ng of gibberellic acid per vial, which corresponds to a lowest detectable level of 2,5—4 μg per kg fresh weight of tissue.

The dock leaf (*Rumex obtusifolius* L.) senescence bioassay was carried out according to Whyte and Luckwill (200). Clonal material grown under a constant long-day light regime in a growth chamber was used. A sufficient number of suitably aged leaves were cut off and kept in darkness at room temperature with the leaf stalks submerged in water for 24 hours. Small leaf disks 7 mm in diameter were punched out by use of a cork borer (care was taken to avoid the large vessels) and collected in distilled water in a large Petri dish. To circles of filter paper with the diameter 25 mm, placed in slightly wider plastic dishes, were added either sample fractions (corresponding to 0.4 g fresh weight of tissue), pure gibberellic acid in known amounts, or solvent (95 % ethanol) only. After the solvent had evaporated, 0.3 ml of distilled water was added to each vial, and four leaf disks (chosen at random among disks from at least four different leaves collected in the same Petri dish) were placed with their morphological lower surface down on the moist circle of filter paper in each vial. All vials were then kept in darkness at 23 ± 2°C in a water-saturated atmosphere for seven days. The four leaf disks from each

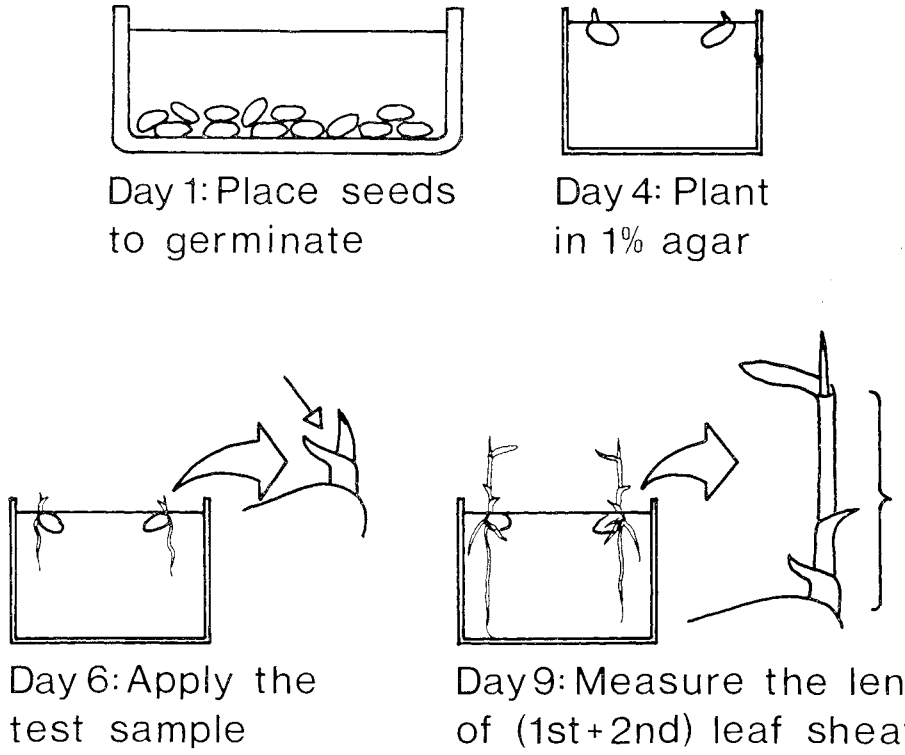


Figure 12. Brief pictorial description of the dwarf rice bioassay.

vial were thereafter placed in sealed test tubes containing 6.0 ml of absolute methanol to extract the remaining chlorophyll. After 24 hours, during which period of time all test tubes were stirred vigorously at least three times, the chlorophyll concentration was measured as the absorbance ("optical density") at 665 nm in a Bausch and Lomb Spectronic 20 spectrophotometer equipped with a special red light sensitive phototube. The bioassay response was expressed directly as the measured absorbance.

Typical dose-response curves for gibberellic acid are shown in figure 11. Even the smallest amount tested, 0.2 ng, caused a very strong response of the bioassay. The responses in the concentration range 2—200 ng per vial were most often not significantly different ($p \leq 0.01$), and this bioassay was therefore not suitable for quantitative estimates or comparisons. Since the bioassay response was not even roughly proportional to either the absolute concentration of gibberellic acid or the logarithm of the concentration, no theoretical maximum sensitivity could be estimated. If one assumes a maximum sensitivity of 0.05 ng (the maximum sensitivity reported by Whyte and Luckwill, 200, is as low as 0.01 ng), then this bioassay will detect, with the amounts of extract equivalents used in this investigation, amounts as small as 0.125 μg of gibberellin activity per kg fresh weight of tissue.

Dwarf rice *'Tan-ginbozu'* microdrop bioassay was performed according to the description given by Murakami (131). Seeds from harvests grown in growth chamber were germinated submerged in distilled water in constant conditions of 30°C, 7500 lux light intensity (from cool white fluorescent tubes only, the spectral distribution is shown in figure 2), and 85 % relative humidity. The water was changed daily. After 64 hours germinating

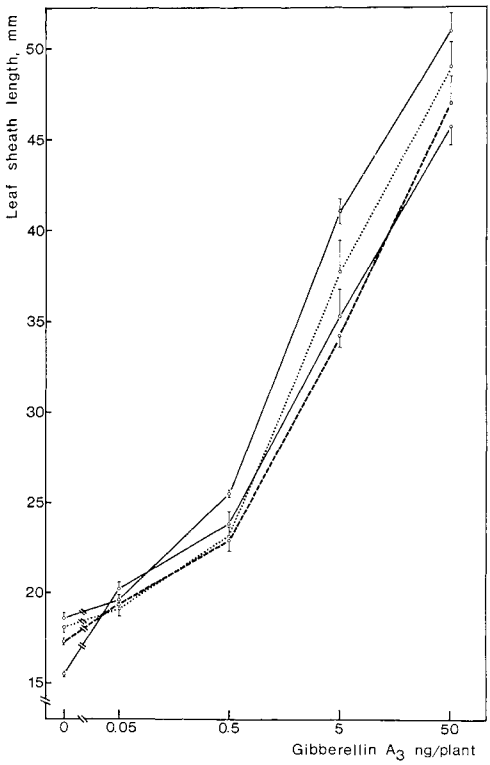


Figure 13. Dose-response curves of the dwarf rice bioassay. The results obtained in four different bioassay runs are shown. Vertical bars indicate the standard errors.

seedlings with a coleoptile length of around 2 mm were selected and planted in 1 % water agar in 25 ml plastic vials, eight seedlings per vial. The vials were placed back in the conditions described above, and the agar surface was kept covered with a thin layer of distilled water. After another 48 hours the coleoptiles were split up by the emerging fully grown first leaf, and these together formed a little "cup" into which a small amount of solution might be applied. Selection for uniformity now took place, leaving six seedlings per vial, and by means of a microsyringe 0.5 μl of 95 % ethanol containing either sample fractions (corresponding to 0.1 g fresh weight of tissue), pure gibberellic acid in known amounts, or solvent blanks, were added to the "cup" between the coleoptile and the first leaf. This was done in the laboratory, after the surface of the seedlings had been allowed to dry out for 30 minutes, and within 60 minutes the seedling vials were placed back in the growth chamber. Still 72 hours later the length of first plus second leaf sheath of each seedling was measured to the closest full mm by means of a transparent ruler. Sometimes a third leaf sheath had emerged when very high concentrations of gibberellic acid had been applied, and this was also included in the measure. Since there is always a risk that seedlings become damaged during planting and selection, or that the applied microdrop runs off the seedling, the shortest seedling in each vial was excluded, and the bioassay response was expressed as the mean length of the five remaining.

Typical dose-response curves for gibberellic acid are shown in figure 13. The bioassay response was approximately proportional to the logarithm of gibberellin concentration in the range 0.05—50 ng per seedling. Standard errors were very small, and thus the dwarf rice bioassay is particularly well suited for quantitative studies. Extrapolation of the dose-response curve gave a theoretical maximum sensitivity of 0.02—0.03 ng, corresponding to 0.2—0.3 μg of gibberellin activity per kg fresh weight of tissue.

The method followed for the cucumber hypocotyl bioassay was essentially that of Brian *et al.* (13). Cucumber seed, cv. *'Rhensk Druv'* (purchased from W. Weibull AB, Landskrona, Sweden) was germinated on moist filter paper in large Petri dishes, at 25°C in darkness. After 48 hours germinating seedlings with a root 10—15 mm in length were planted in 1 % water agar in 100 ml plastic beakers, twelve seedlings per beaker. The beakers were placed in plastic boxes covered with glass plates, and the whole assembly was kept at 20°C in diffuse white light for 24 hours. The seedcoats were then removed, and the seedlings were selected for uniformity, leaving ten seedlings per beaker. The surface of the cotyledons was allowed to dry out for 30 minutes, and then 2 μl drops of 95 %

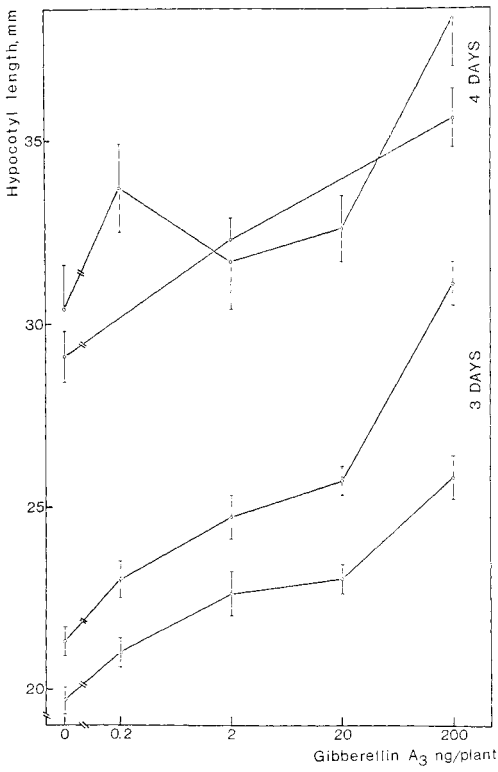


Figure 14. Dose-response curves of the cucumber bioassay. The upper two curves represent responses measured after four days; the lower two curves represent responses measured after three days. Vertical bars indicate the standard errors.

ethanol containing either sample fractions (corresponding to 0.4 g fresh weight of tissue), pure gibberellic acid in known amounts, or solvent blanks, were applied to one of the cotyledons by means of a microsyringe. Four days later the hypocotyl lengths of the seedlings were measured to the closest full mm by means of a transparent ruler. Individual seedlings sometimes occurred, that were very markedly taller or shorter than the others in the same vial. To increase the uniformity, the tallest and the two shortest seedlings in each beaker were excluded, and the bioassay response was expressed as the mean hypocotyl length of the seven remaining seedlings.

Typical dose-response curves for gibberellic acid are shown in figure 14. If the hypocotyls were measured after three days, as proposed by Brian *et al.* (13), there was a relatively good proportionality between gibberellin concentration and bioassay response. For practical reasons this measurement could not be done until after four days in this investigation, and for reasons that are not easily understood the proportionality was then lost. The bioassay was therefore no longer suitable for quantitative comparisons. Also, the standard errors increased, and even if the theoretical maximum sensitivity appeared to be unaffected (less than 0.2 ng of gibberellic acid per seedling), the smallest statistically significant ($p \leq 0.01$) response was sometimes not obtained by less than 20 ng of gibberellin. Concentrations as high as 1 μg of gibberellic acid per seedling caused a marked reduction in growth, as well as chlorosis and other abnormalities.

Since it was considered important to demonstrate, if possible, that the isolated gibberellin-like substances were biologically active in the species they originated from, a special spruce hypocotyl bioassay was designed. Hypocotyl growth of young seedlings was chosen as the experimental response for several reasons: enhancement of elongation growth of intact plants is often considered to be the most typical physiological effect of gibberellins (Cleland, 22), a similar test had been described previously (Kopcewicz, 94), and a number of uniform seedlings could easily be grown from seeds under standardized conditions and within reasonable time.

Seeds of Norway spruce (*Picea abies* (L.) Karst.) of a Central-Swedish provenance (60° lat. N.) were imbibed for 24 hours and placed on moist filter paper in large Petri dishes to germinate. After six days in continuous light (intensity 10,000 lux, spectral distribution shown in figure 2) and the temperature 20° C, seedlings with a root at least 2 mm long were selected and planted in 1 % water agar in 25 ml plastic vials, 12 seedlings in each vial. The vials were maintained in the same conditions as mentioned, and the

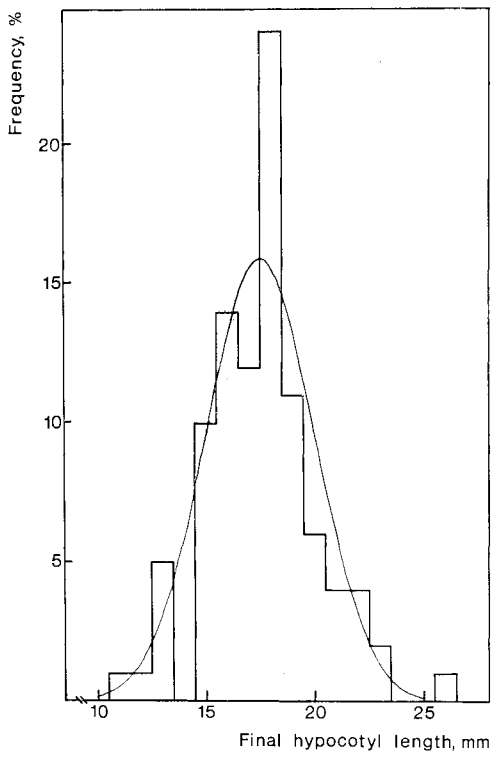


Figure 15. Example of spruce seedling population used for the spruce seedling bioassay, and the corresponding Gaussian ("normal") distribution. The population consists of one hundred control seedlings, not treated with plant extracts or gibberellin.

agar surface was kept moist by frequent watering with distilled water. Eight days later the seedlings were selected for uniformity, leaving seven seedlings per vial. To each of these, 5 μl of 95 % ethanol, containing either sample fractions (corresponding to 1.0 g fresh weight of tissue), or solvent blanks, were added in small droplets to the angles between the cotyledons and the epicotyl bud by means of a microsyringe. The seedlings were kept in the environmental conditions described for another seven days, and then the hypocotyl length of each seedling was measured to the closest full mm by means of a transparent ruler. Skilful handling was necessary to avoid that part of the 5 μl droplets ran off the seedlings at the application. Since this sometimes happened in spite of all precautions, the two shortest seedlings in each vial were excluded, and the bioassay response was given as the mean hypocotyl length of the five remaining seedlings.

This bioassay did not respond to gibberellic acid, and therefore no standard dose-response curves could be obtained. According to Kopcewicz (94), also gibberellins A_1, A_4, A_7, and A_{13} were without effect in a similar bioassay. This is furthermore in agreement with the general lack of success of most attempts to evoke this response in conifers by application of gibberellins. In spruce extracts, however, a number of active substances could be detected. Another finding was, that this bioassay seemed to be much less sensitive to inhibitory substances in spruce extracts than were all the other bioassays used in this investigation.

The distribution of final hypocotyl lengths among 100 control seedlings is shown in figure 15. The Gaussian ("normal") distribution with the same mean and variance is also shown. As one may have expected, the actual distribution is not significantly different from the "normal" distribution (as assayed by the X^2 method of statistical analysis), and thus ordinary statistical methods can be used for the evaluation of the responses of this bioassay.

The statistical significance of the results obtained by each individual extraction can easily be calculated (see *e.g.* figure 17), but that was not the main purpose of the present investigation. Rather the methodological and biological reliability and reproducibility was to be evaluated in some way or another. Therefore a total number of 20 extractions were carried out, and the mean response of each corresponding fraction from the silicic acid column was calculated for the different bioassays, as well as the mean values of the control. Since some variation in elution volume of the various peaks of activity occurs from one silicic acid column to the other, this method of calculation will eliminate small and

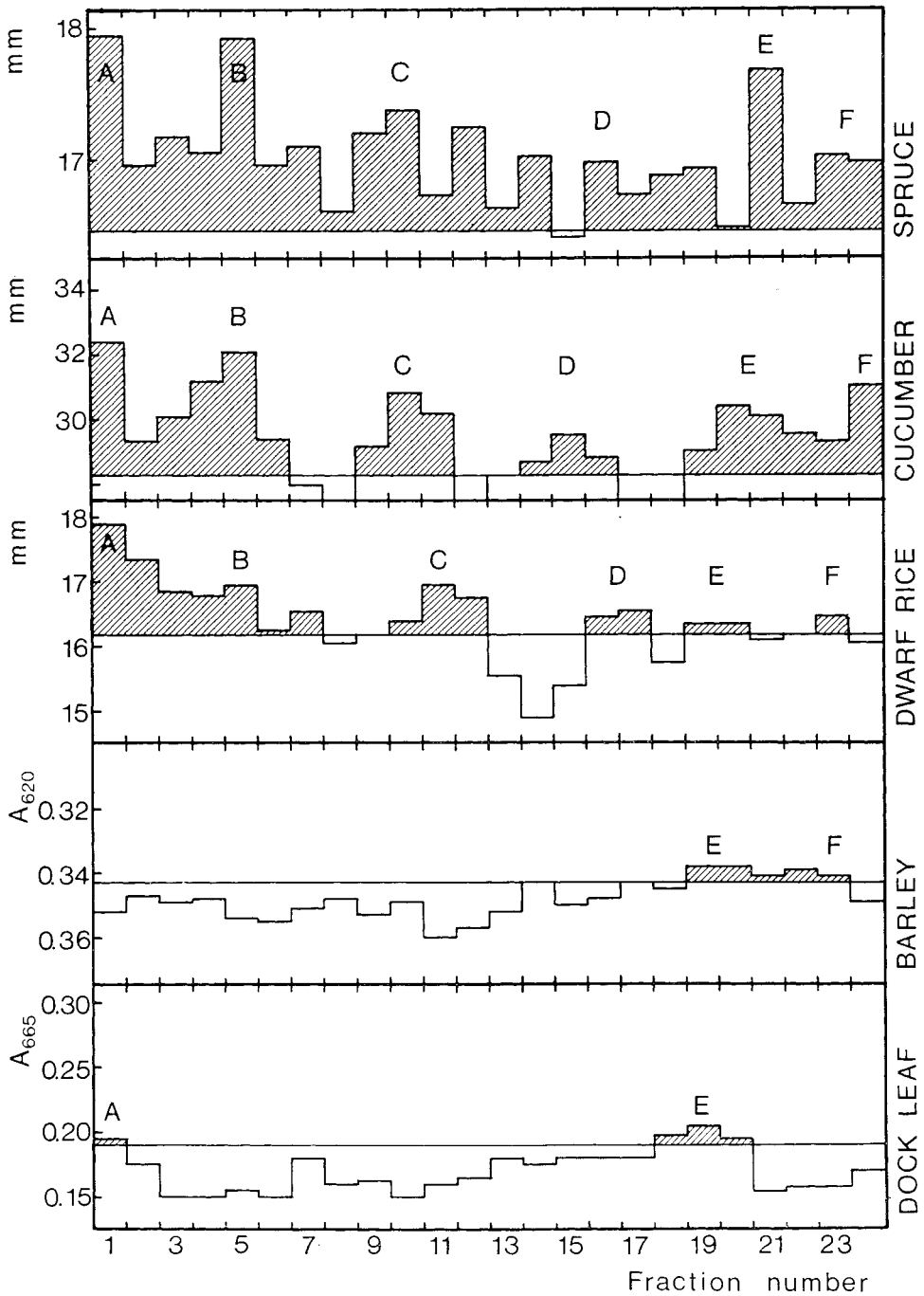


Figure 16. Mean response of 20 spruce extracts in five bioassays. For each of the 24 fractions obtained from the silicic acid partition column, the mean response in every particular bioassay of the 20 extractions was calculated.

Abscissa: Fractions from the silicic acid column.

Ordinate: Bioassay response (hypocotyl length in the spruce and cucumber bioassays, leaf sheath length in the dwarf rice bioassay, absorbancies at 620 nm and 665 nm, respectively, in the barley half-seed and dock leaf bioassays). Shaded areas indicate a mean bioassay response exceeding the mean of the controls. Active zones A—F are marked out in block letters.

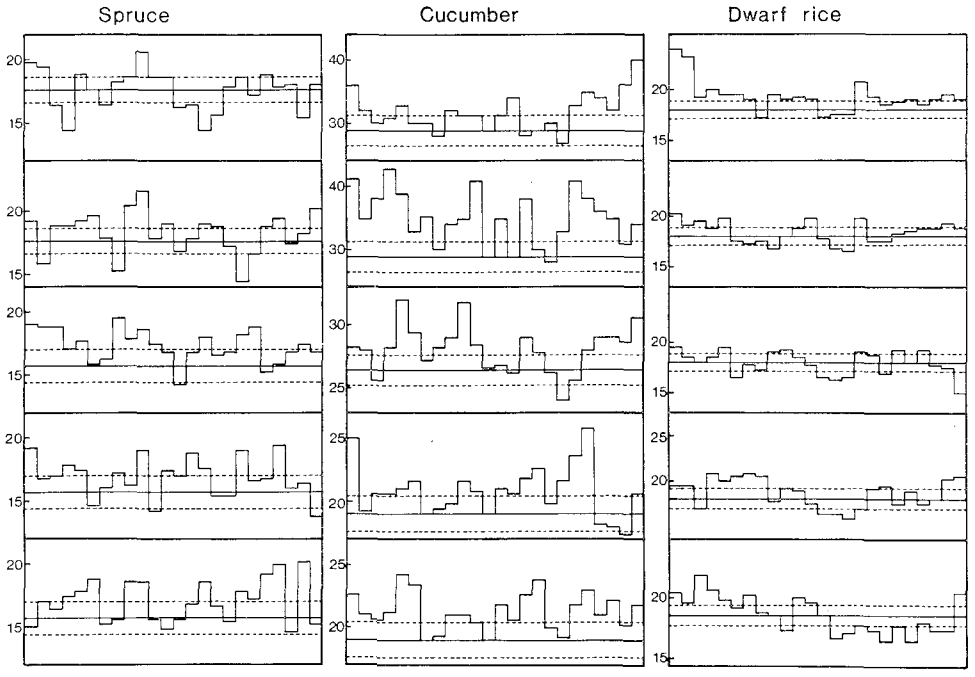


Figure 17. Responses in the spruce, cucumber, and dwarf rice bioassays of some individual extractions, included in the mean responses shown in figure 16.

Abscissa: Fractions from the silicic acid column.

Ordinate: Bioassay response (hypocotyl length, mm, in the spruce and cucumber bioassays, leaf sheath length, mm, in the dwarf rice bioassay). Hatched horizontal lines give the least significant differences from the control at 1 % risk level. Comparisons within the figure are to be made only vertically.

not reproducible random or "noise" peaks and leave significant zones of biological activity. The width of these zones will depend upon how varying the elution volume is. Peaks not separated by more than this variation will overlap and are not possible to resolve from each other, although they sometimes may show up in the results of individual extractions.

Although fractions collected from the silicic acid column are by no means pure substances, it was considered worthwhile to record the infrared spectra of the active fractions. These were obtained from an extract of 500 g of tissue, processed according to the standard methods outlined above (although all volumes and column sizes were scaled up), by use of the KBr disk technique and a Perkin-Elmer Model 137E Infracord double-beam spectrophotometer.

2.3.3 *Results*

The mean biological activity of the twenty extracts in five bioassays is shown in figure 16. Six corresponding, more or less distinct zones of activity can be seen in the spruce, cucumber, and dwarf rice bioassays. In figure 16 they are denoted by the letters A through F. The fraction of maximal activity is not always the same in all three bioassays. This is however not very surprising, since it is well known that even in the same extract different bioassays may give their greatest response to the same compound in different chromatographic fractions. Most often a substance is distributed over several fractions, in some of which perhaps also various inhibitory compounds occur. The bioassays measure some kind of resulting difference between promotion and inhibition, and they all do so in their own particular way. The spread of

peaks will principally become more pronounced with increased elution volume or if the elution gradient flattens out, and this is in agreement with the results presented here: the discrepancies between the three bioassays are largest in fractions 12—20.

If the principal difficulties described above, that are inherent parts of all bioassay investigations, are considered, one is rather impressed by the great similarities between the responses in these three bioassays. The differences in the fractions 12—20 (active zones D and E) may be caused by the presence in this part of the chromatograms of three or even four active substances, that are not separable because they overlap in varying patterns in different individual extractions. As can be seen in figure 17, individual extracts sometimes show three discrete peaks of activity in this area.

A strong contrast to the wide spectrum of gibberellin-like substances detected by these three bioassays are the responses obtained in the dock leaf and barley half-seed bioassays. A general finding is, that these two bioassays seem to be very sensitive to inhibitory compounds present in the spruce extracts. The dock leaf bioassay detects peaks A and E. The barley half-seed bioassay indicates a slight activity in the area of highly polar compounds, *i.e.* in zones E and F. The activity in the F zone is however not always present and may in fact have been caused rather by tailing of compound(s) E.

Infrared spectra of peaks A—F are shown in figure 18. It must be noticed, that bioassay data as well as other evidence show that none of these peaks contains a single substance, and the validity of these spectra is therefore questionable. Anyhow, the infrared spectra of peaks B through F are similar to the spectrum of pure gibberellic acid, and to published spectra of other gibberellins (Tamura *et al.*, 191). It is thus possible that the biologically active substances present in these peaks are really true gibberellins. The absorbancy peak at 3.1 μm in the infrared spectra indicates an increasing hydroxylation with increasing

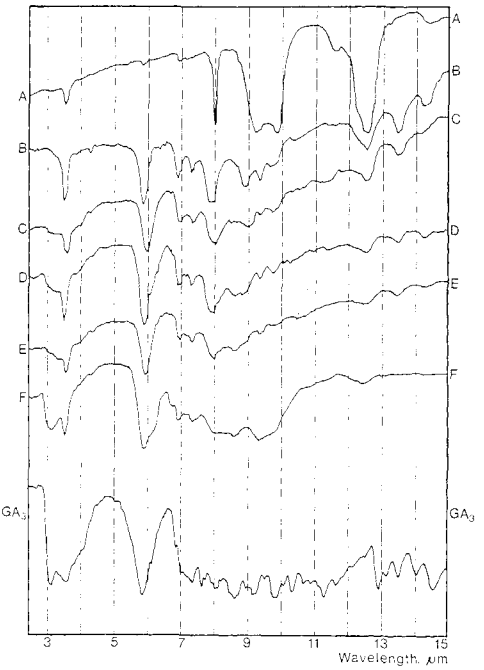


Figure 18. Infrared spectra (KBr disk technique) of active fractions A—F of a spruce extract, and of gibberellic acid (GA_3).

fraction number, as was expected. Peak A, however, shows a completely different spectrum. Obviously the main compound in peak A is not a gibberellin. Neither is it very probable, that the great biological activity of this fraction can have been caused by a gibberellin present in amounts so small that it does not show up in the infrared spectrum.

2.3.4 *Discussion*

The results of this investigation not only show conclusively the occurrence in *Picea abies* of a wide spectrum of different gibberellin-like substances, but also demonstrate the good reproducibility of the methods used. This reproducibility is not only qualitative: extractions of various amounts of identical tissue that are in progress have shown a reasonably good quantitative reproducibility as well. The main problem in quantitative comparisons may be the particular properties and limitations of the bio-

assays, as will be discussed below. The results presented here agree well with results obtained previously with similar methods but in another laboratory (Dunberg, 39, and page 19 above). They also give a first hint towards the possible true gibberellin identity of the active substances, as indicated by their infrared spectra. Similar spectra were obtained and the same cautious conclusions were drawn by Kopcewicz (94) in his work with *Pinus sylvestris* L.

The gibberellin-like substances isolated from *Picea abies* are active not only in the cucumber and dwarf rice bioassays, but also in the specially designed spruce seedling hypocotyl bioassay. This latter bioassay does not respond to gibberellic acid, and a similar bioassay was insensitive also to gibberellins A_1, A_4, A_7, and A_{13} (Kopcewicz, 94). These findings suggest, that the endogenous gibberellin-like substances in spruce are not identical to known gibberellins. Further support for this hypothesis is given by the fact, that spruce trees or seedlings do not react in any measurable way to exogenously applied gibberellins. The only exceptions are spruce seedlings previously treated with growth retardants (Dunberg and Eliasson, 40), and a dwarf variety (*Picea abies* (L.) Karst. cv. '*Nidiformis*') (Hansen, 55), and these two exceptions will be discussed in the following chapter (see page 41).

The particular characteristics of peak A may merit some discussion. This peak is eluted from the silicic acid column by pure *n*-hexane, and must thus be less polar (at the low pH value maintained during the chromatography) than all the known gibberellins. The infrared spectrum of this peak shows no similarities to gibberellin spectra, in fact there is no evidence for the occurrence of a carboxyl or other C=O bond. The biological activity is nevertheless very high. From the partitioning schemes in figures 5 and 6 it is evident, that this substance is not soluble in diethyl ether at pH 9.0 or in ethyl acetate at pH 8.0. Work now in progress also shows it is not soluble in petroleum ether at pH 8.0. The identity of this compound remains unknown, but its high biological potency seems to merit further studies on it.

Another question of special interest is the possible occurrence of gibberellic acid. This compound has been shown to be the main gibberellin in Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Crozier *et al.*, 28) and perhaps also in Arizona cypress (*Cupressus arizonica* Greene) (Ruddat *et al.*, 169). The possibility that gibberellic acid occurred in Norway spruce as well was one of the working hypotheses of this investigation, but the results do not support it. Both the dwarf rice, the barley half-seed, and especially the dock leaf bioassays are extremely sensitive to gibberellic acid (Crozier *et al.*, 30; Whyte and Luckwill, 200). Very little activity was detected around fraction 18 with these bioassays, so if gibberellic acid is at all present it is by no means a main gibberellin in *Picea abies*. Judging from the dock leaf bioassay the amount of gibberellic acid must be less than 5 μg per kg fresh weight of tissue, and probably less than 0.125 μg.

The strong differences in results obtained by different bioassays remain to be explained. It has been pointed out already, that the fractions from the silicic acid column are not pure substances, and that inhibitory compounds may be present. The extracts have been extensively purified before the final chromatography step, but some potent inhibitors, notably abscisic acid, are known to pass through all purification steps along with the gibberellins. Conifer tissue is extremely rich in terpenoid substances (Norin, 135) that may have chemical properties similar to gibberellins (see *e.g.* figure 26 for chemical structures of gibberellins and some other diterpenes), and these substances are often strongly inhibitory in the bioassays. Some of the most sensitive bioassays are unfortunately also very sensitive to inhibitors. This is obviously true for the dwarf rice and dock leaf bioassays, and perhaps also for the barley half-seed bioassay. On the other hand, the cucumber bioassay and especially the spruce bioassay are much less sensitive to inhibitors, and although they are perhaps not

maximally sensitive to gibberellins in terms of standard dose-response curves (this is true at least for the cucumber bioassay), they may well turn out to be more reliable and valuable than the others when applied to work with plant extracts. In this investigation very small amounts of extracts have been applied to the bioassays, since it has been found that applications of strongly increased amounts of extracts often give about the same or even lower responses. If quantitative comparisons are to be made, then the extract concentration must be kept comparably low. Otherwise the bioassays tend to measure inhibitors rather than promoters.

The barley half-seed bioassay, when used in a previous investigation (see page 13), did detect some gibberellin-like activity. It has been found in this investigation, that such activity may be detected in extracts of young seedlings, but not in extracts of older trees. Probably this finding reflects a higher content of inhibitory substances in the latter tissue. The chromatographic properties of the active substances found in the previous investigation (see figure 4) and in this one are similar.

There may exist still other gibberellin-like substances in *Picea abies* in addition to those detected in the present investigation. Both the neutral ether fraction and the buffer fraction (see figure 5) may contain such substances. Especially the buffer fraction ought to be investigated, since a number of gibberellin glucosides are known (Schreiber *et al.*, 174, 175; Sembdner *et al.*, 176; Tamura *et al.*, 192; Yokota *et al.*, 204, 205; Hiraga *et al.*, 71), and they will all appear in that fraction. Also the recently detected gibberellin A_{32} (see figure 1) is so highly polar that it remains in this fraction and is not partitioned into the acidic ethyl acetate fraction (Coombe, 23). Some very polar compounds have been detected in the present investigation, and these may in part have been lost when the buffer fraction was discarded. The physical and chemical properties of the known gibberellins are so different, that the same general methods can no longer be applied to all of them. By selecting some particular methods then, one will also define the quality and the limits of the investigation. But with the developing knowledge of partition coefficients, R_f values, gas-liquid chromatography retention times and other relevant data of most of the characterized gibberellins, poor methods should no longer be allowed to draw the limits more narrowly than is necessary.

3 Effects of growth retardants on spruce seedlings

3.1.1 *Introduction*

As mentioned earlier, most investigators trying to establish effects of gibberellins on the elongation growth of conifers have had no success. These negative findings do not exclude the possibility, however, that endogenous gibberellins are normally functioning in the growth regulation of coniferous species, since the negative results obtained with gibberellic acid and a few other gibberellins may mean only, that these particular gibberellins are not the appropriate ones. An investigation of the effects of all the known gibberellins is however practically impossible (some of them have not yet been isolated in amounts large enough for such an experiment). Also, the possibility obviously exists, that the endogenous gibberellins of the tested species are different from all the characterized gibberellins. In the present investigation methods were therefore looked for, that could indirectly show the involvement of endogenous gibberellins in the growth-regulating processes, irrespective of the details of chemical structure of these gibberellins.

Growth retardants (for a review of early history, chemistry, and biological effects as well as practical applications, see Cathey, 18) are substances with widely different chemical structures that cause dwarfism in plants similar to the state of growth resulting from a lacking ability to biosynthesize gibberellins. Furthermore, the effects of the retardants on plant growth can usually be counteracted by application of gibberellin (Lang, 107), and the inhibitory effect of growth retarding chemicals is often closely correlated to the ability of these substances to inhibit the production of gibberellins in the fungus *Gibberella* (Kende *et al.*, 90; Ninnemann *et al.*, 134; Harada and Lang, 56). For most of the commonly used growth retardants even the particular steps in the biosynthetical pathway leading to gibberellins that are blocked by the retardants have been found (Dennis *et al.*, 34; Shechter and West, 177; Robinson and West, 164, 165), and these steps are shown in figure 26. The effects of growth retardants are therefore interpreted as effects of totally or partially blocked gibberellin biosynthesis (Lang, 107). Since the biosynthesis of all the different gibberellins is equally affected, growth retardants were considered to be an ideal tool for a study of gibberellin participation in growth regulation in *Picea abies*.

There are very few reports of effects of growth retardants on conifers. Actually Cathey (18) stated that growth retardants were inactive on gymnosperms. However, Asher (5) found that seedlings of slash pine (*Pinus elliottii* var. *elliottii* Engelm.) were sensitive to the growth retardant CCC, and Pharis *et al.* (152) presented data indicating that shoot elongation of *Cupressus arizonica* Greene was suppressed by the growth retardants Amo-1618 and B-995. In a more extensive investigation, Pharis *et al.* (153) showed that the three species *Cupressus arizonica* Greene, *Sequoia sempervirens* (D. Don) Endl., and *Pinus coulteri* D. Don all were sensitive to B-995. CCC had no effect on the two species that were tested. Amo-1618 and Phosphon-D were effective in some cases, but in other cases not. Obviously there are great differences both between species and between various growth retardants. The only conclusion that can be drawn is, that at least some conifers respond to certain growth retardants. Pharis *et al.* (153) also found that the effects of growth retardants could be prevented or at least decreased by si-

Table 3. Composition of nutrient solution used for growing spruce seedlings.

NH_4NO_3	40.0 mg l^{-1}
KH_2PO_4	43.9
$MgSO_4$ 7 H_2O	49.2
$Ca(NO_3)_2$ 4 H_2O	35.3
$FeCl_3$ 6 H_2O	1.4
H_3BO_3	0.29
$MnCl_2$ 4 H_2O	0.18
$ZnCl_2$	0.012
$CuCl_2$ 2 H_2O	0.015
$NaMoO_4$ 2 H_2O	0.0022
N	1.30 millimoles l^{-1}
K	0.32
P	0.32
Mg	0.20
S	0.20
Ca	0.15
Cl	0.018
Fe	0.0052
B	0.0047
Mn	0.00091
Zn	0.000088
Cu	0.000088
Na	0.000010
Mo	0.000010

multaneous application of gibberellic acid. This is consistent with the hypothesis that growth retardants act through inhibition of the gibberellin biosynthesis (Cathey, 18; Lang, 107).

This investigation deals with the effects obtained by treatment of young seedlings of Norway spruce with growth retardants and gibberellic acid. A short report of the investigation has also been published elsewhere (Dunberg and Eliasson, 40).

Abbreviations:

Amo-1618: 4-hydroxyl-5-isopropyl-2-methylphenyl trimethylammonium chloride, 1-piperidine carboxylate
B-995: *N*-dimethylamino succinamic acid
CCC: (2-chloroethyl)trimethylammonium chloride
Phosphon-D: 2,4-dichlorobenzyl-tributyl-phosphonium chloride

3.1.2 *Materials and methods*

Spruce seeds (*Picea abies* (L.) Karst.) of a Central-Swedish provenance were imbibed

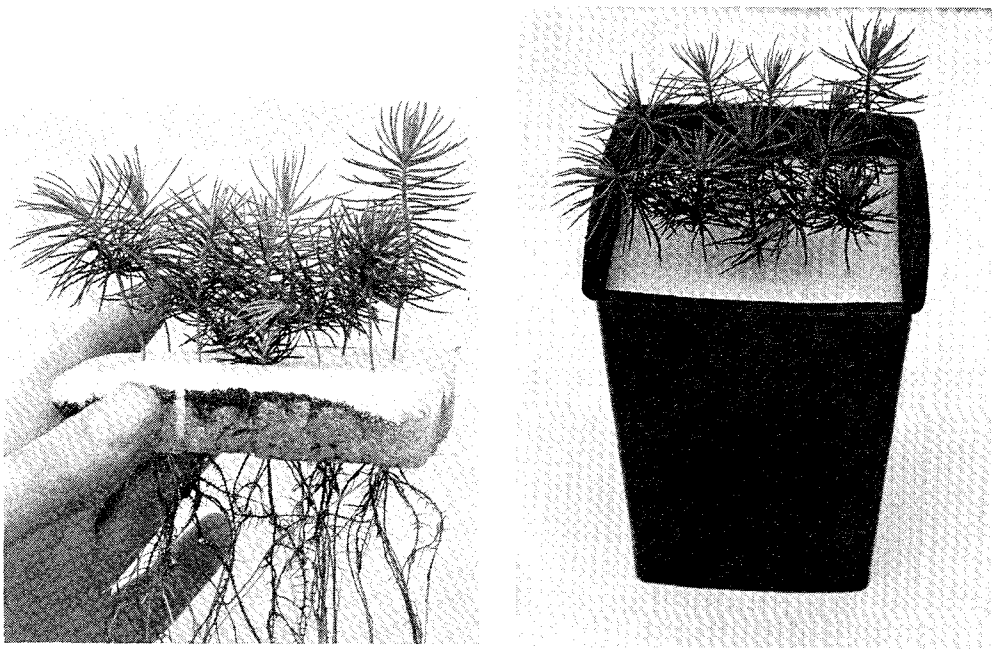


Figure 19. Method for cultivation of spruce seedlings. Left: Ten seedlings attached on a disk of plastic foam. Right: Entire disk placed in a one liter plastic pot filled with nutrient solution.

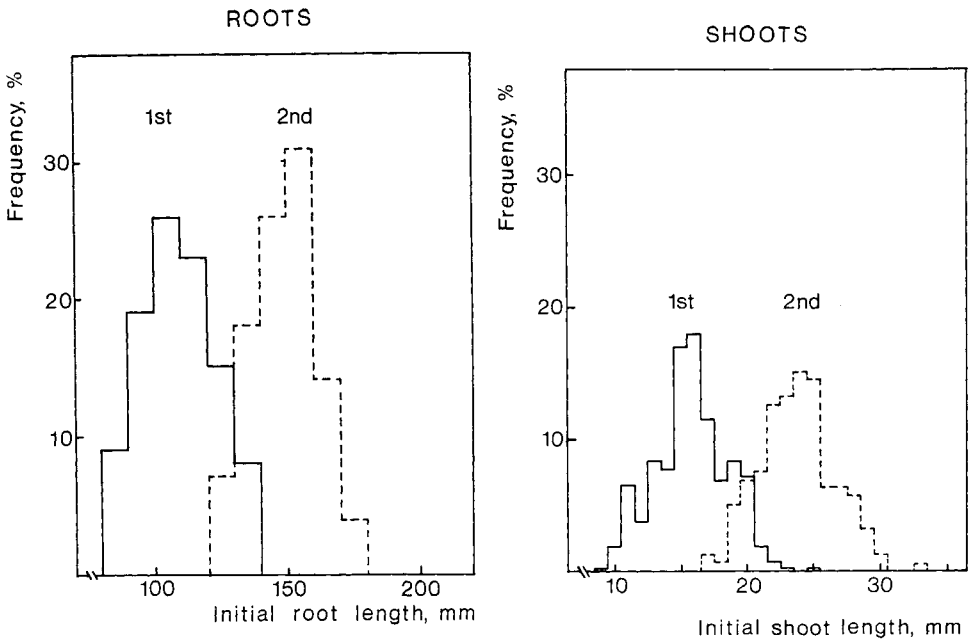


Figure 20. Distribution of initial root (left) and shoot (right) lengths among spruce seedlings used in the first and second experimental series.

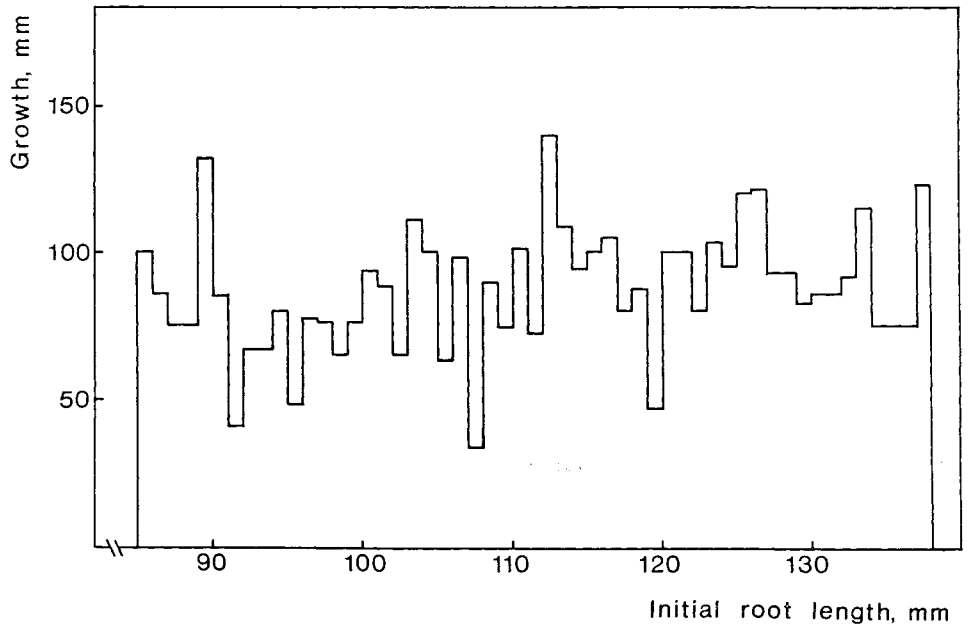


Figure 21. Effect of initial length upon the growth of roots of control spruce seedlings, first experimental series.

for 24 hours and sown in moist vermiculite. They were placed in a growth chamber (16 h light, 10 000 lux from cool white fluorescent tubes, spectral distribution shown in figure 2, temperature 23°C; and 8 h darkness, temperature 18°C; relative humidity constantly 65—75 %) and kept there for the duration of the experiment.

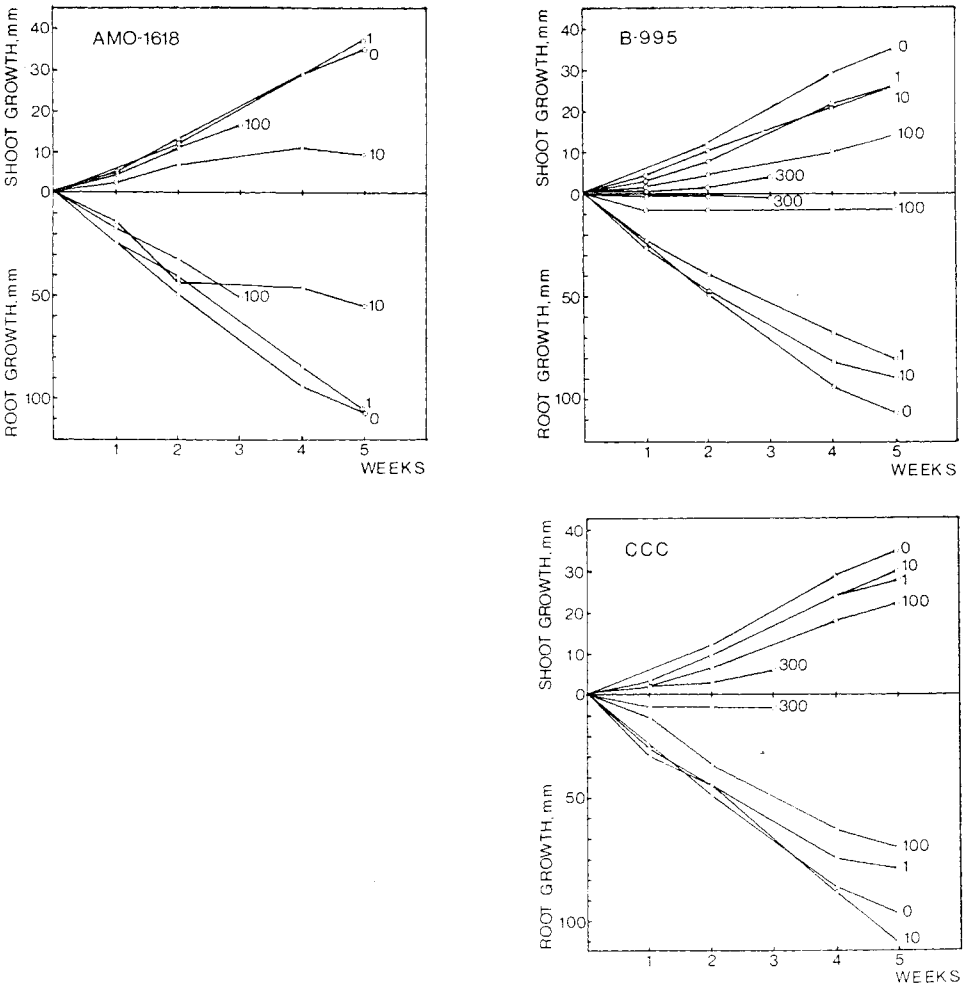


Figure 22. Effects of growth retardants on shoot and root growth of spruce seedlings. First series, initial age of seedlings five weeks. Concentrations of retardants are given in mg l^{-1}.

Abscissa: Time from beginning of the experiment.

Ordinate: Shoot growth (from zero and upwards) and root growth (from zero and downwards).

After two weeks the seedlings were transferred to disks of plastic foam, floating on nutrient solution (the composition is shown in table 3) in black one-liter plastic pots. Air was constantly led through the solution, and it was changed weekly. After this time the pH of the solution was slightly lowered. The method for cultivation of seedlings is shown in figure 19.

At the age of five weeks (first series) or six weeks (second series) seedlings were selected for even root and shoot length and distributed at random, 10 seedlings per treatment. In the first series there were 19 different experimental treatments and 13 replicates of the untreated control. In the second series there were two replicates each of five experimental treatments and six replicates of the control. The distribution of initial root and shoot lengths in the material used for the two experimental series are shown in figure 20.

Root and shoot lengths of each seedling were measured at the beginning of the experiment and then weekly, and record was kept of the elongation of each individual

seedling. The average elongation of seven seedlings was used for the calculations, excluding the one exhibiting the best growth and the two exhibiting the poorest growth. The reasons for doing so were, that seedlings were sometimes damaged during the measurements or the exchange of nutrient solution, and on the other hand a few individual seedlings demonstrated a very strong growth far outside the range of all others. The effect of initial length upon growth was checked and considered not significant (see figure 21). At the end of the experiment fresh and dry weights were also recorded.

A small experiment was also carried out with five-year old potted spruce plants (height around 70 cm). Plants with non-dormant winter buds were brought into the climate chamber (environmental conditions as described above), and after the shoot elongation had started they were sprayed with commercial preparations of B-995 (Alar-85, Uniroyal Company; B-nine, United States Rubber Company) or CCC (Cycocel, American Cyanamide Company). Both substances were applied as 25 ml aqueous sprays in the concentrations 0.2 % and 1.0 % of active substance. The application was repeated weekly. Three plants were used for each experimental treatment. The lengths of the ten uppermost terminal shoots were measured weekly, and the mean lengths for each treatment was calculated. Other visible effects of the treatments were also recorded.

3.1.3 *Results*

The results of the first series (initial age of seedlings five weeks) are shown in figure 22 and in tables 4 and 5.

Amo-1618 in the concentrations 100 and 10 mg 1^{-1} significantly retarded both shoot and root growth, but 1 mg 1^{-1} had no effect. No symptoms of toxicity could be detected. Fresh and dry weights were significantly increased by 1 mg 1^{-1} of this retardant.

B-995 was clearly toxic at 300 mg 1^{-1}. Roots never elongated but became black and necrotic. Shoots grew extremely poorly, first became chlorotic and later turned brown. The concentrations 100, 10, and 1 mg 1^{-1} retarded shoot and root growth, the higher concentration was probably still toxic to the roots. Fresh weight was decreased by 10 mg 1^{-1}, fresh and dry weights were decreased by 100 mg 1^{-1} and 1 mg 1^{-1}.

Table 4. Shoot and root growth of seedlings treated simultaneously with growth retardants and gibberellic acid. First series, initial age of seedlings five weeks.

Treatment mg/l		Increase in length, % of control	
		Shoots	Roots
Amo-1618	10	25	52
Amo-1618	10 + GA 0.1	82	109
Amo-1618	10 + GA 10	142	106
B-995	100	40	8
B-995	100 + GA 0.1	31	5
B-995	100 + GA 10	75	13
CCC	100	62	70
CCC	100 + GA 0.1	59	82
CCC	100 + GA 10	100	67

CCC appeared to be toxic at 300 mg 1^{-1}, but not as strongly as was B-995. Shoots became chlorotic but never turned brown. Shoot growth was retarded by 100, 10, and 1 mg 1^{-1}, root growth by 100 and 1 mg 1^{-1}. Only 100 mg 1^{-1} decreased fresh and dry weights.

Gibberellic acid applied together with the retardants restored at least partially and in some cases totally the normal growth of shoots but not of roots. The effects on root growth of Amo-1618 in the concentration 10 mg 1^{-1} were however also counteracted by gibberellic acid. The restoration of shoot growth was obviously dependent upon both the degree of retardation and on gibberellin concentration: a concentration of 0.1 mg 1^{-1} of gibberellic acid was sufficient to overcome in part the retardation caused by 10 mg 1^{-1} of Amo-1618, and 10 mg 1^{-1} of gibberellin applied together with this concentration of retardant gave a growth that was not only restored but enhanced

Table 5. Fresh and dry weights of seedlings treated with growth retardants and gibberellic acid. First series, initial age of seedlings five weeks. Values denote per cent of untreated control seedlings.

Treatment mg/l		Fresh weight %	Dry weight %
Amo-1618	10	50 s	65 s
Amo-1618	10 + GA 0.1	74 s	99
Amo-1618	1	111 s	109 s
B-995	100	60 s	77 s
B-995	100 + GA 0.1	33 s	58 s
B-995	10	92 s	103
B-995	1	76 s	93 s
CCC	100	76 s	80 s
CCC	100 + GA 0.1	56 s	72 s
CCC	10	104	102
CCC	1	104	99
GA	0.1	91 s	92 s

s = value differs significantly from the control at 5 % risk level.

Table 6. Fresh and dry weights and elongation growth of seedlings treated with growth retardants. Second series, initial age of seedlings six weeks. Values denote per cent of untreated control seedlings.

Treatment mg/l		Parts of plants	Fresh weight %	Dry weight %	Increase in length %
Amo-1618	30	roots	93	91 s	106
		shoots	107 s	117 s	97
		total	101	109 s	—
B-995	100	roots	51 s	71 s	6 s
		shoots	83 s	111 s	46 s
		total	68 s	98	—
B-995	30	roots	77 s	84 s	38 s
		shoots	96	111 s	64 s
		total	88 s	103	—
CCC	100	roots	93	93	88
		shoots	87 s	96	77 s
		total	90 s	95 s	—
CCC	30	roots	86 s	100	94
		shoots	90 s	101	76 s
		total	88 s	100	—

s = value differs significantly from the control at 5 % risk level.

over the control, but 10 mg l^{-1} was necessary to counteract the effects of 100 mg l^{-1} of CCC, and even this higher concentration of gibberellin was insufficient for a total restoration of normal growth of plants treated with 100 mg l^{-1} of B-995.

Gibberellic acid also fully restored dry weight and partially restored fresh weight of plants treated with 10 mg l^{-1} of Amo-1618. When gibberellic acid was applied together with 100 mg l^{-1} of B-995 or CCC, fresh and dry weights decreased even more than in seedlings treated with the retardants alone.

The results of the second series (initial age of seedlings six weeks) are shown in table 6.

Amo-1618 in the concentration 30 mg l^{-1} did not affect shoot or root elongation. Fresh and dry weights of shoots, however, were increased, and dry weight of roots was decreased.

B-995 retarded both shoot and root growth in the two concentrations used. Fresh weights were decreased. Dry weight of roots was also decreased, but dry weight of shoots was significantly increased.

CCC retarded shoot growth, the higher concentration also root growth. Fresh weights were decreased. Dry weights were decreased by 100 mg l^{-1} but were unaffected by 30 mg l^{-1}.

The effects of all the retardants were weaker in the second series than in the first.

The effects of the commercial preparations sprayed onto five-year old potted spruce plants were similar. Both preparations of B-995 shortened the developing shoots, and did so even at the lower concentration of 0.2 %. No damage was done to the plants, except from a drying out of a small number of new needles caused by the stronger concentration of B-nine. The CCC preparation also shortened the developing shoots, at least in the stronger concentration (see figure 23). This concentration caused yellowing of about 80 % of the new needles. The lower concentration also caused some yellowing, but the damage was only marginal.

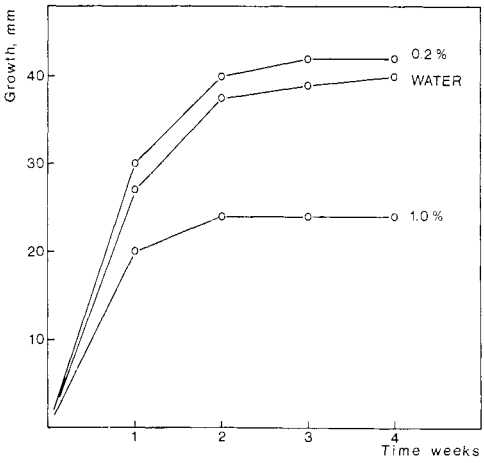


Figure 23. Effects of spray application of the growth retardant CCC (Cycocel) to the growing shoots of five-year old spruce plants. The retardant was applied at bud break and thereafter once a week for five weeks. Each point represents the mean of 30 measurements.

3.1.4 *Discussion*

Although all results are not shown here, the experiments with seedlings grown in nutrient solution have been repeated several times with essentially the same results. The quantitative responses to the retardants have been somewhat varying from one experiment to the other, and these two experimental series are representative for the observed variation.

All three growth retardants used retard the growth of spruce seedlings. The fact that this effect can be at least partly counteracted by gibberellic acid indicates, that the action is upon gibberellin biosynthesis, as is generally believed (Cathey, 18; Lang, 107). However, the possibility that growth retardants at least in some species may influence growth through other mechanisms like influence on auxin catabolism (Halevy, 54; Gaspar and Lacoppe, 47) has not been excluded. Such a mode of action may be likely in the case of B-995 (Gastro, 48), as there is still no evidence that B-995 affects the gibberellin biosynthesis (Lang, 107). It is also evident that high concentrations of the retardants exert toxic effects that cannot be explained by blocking of gibberellin biosynthesis. More unspecific effects like uncoupling of oxidative phosphorylation (Heatherbell *et al.*, 69) or inhibition of protein synthesis (Berry and Smith, 6) may account for these symptoms.

The retardation of root growth caused by B-995 and CCC could not be counteracted by gibberellic acid. Not only the length of the roots but also their dry weights were decreased by high concentrations of these retardants. It is probable that these effects on root growth are due to unspecific toxic actions. These toxic effects seem to decrease with increasing plant age. Amo-1618 possibly acts in another way, since root growth of plants retarded by Amo-1618 could be restored by gibberellic acid.

At high concentrations of B-995 and CCC the shoot growth certainly was detrimentally influenced by decreased root functions. Thus the effects on shoot growth at these concentrations are at least two-fold: specific retardant effects, perhaps involving blocked gibberellin biosynthesis, and indirect effects due to intoxication of the roots. If gibberellins are produced predominantly in the roots (Sitton *et al.*, 179; Reid *et al.*, 160; Lang, 107) or are transformed there (Crozier and Reid, 31), then this intoxication would affect the gibberellin content in an indirect and unspecific way.

Other unspecific effects of the growth retardants may well occur, and care should be taken when drawing conclusions from experiments of this kind. Although the results presented here are consistent with the concept that growth retardants affect the gibberellin level in the plant, this should be proven by extraction of gibberellins from treated and untreated plants. This work is in progress; preliminary results so far show no decrease in gibberellin content in retardant-treated seedlings. During this work it has been observed, however, that gibberellin extracts from retardant-treated plants become much cleaner than extracts from control plants. At the same time as this finding casts some doubt on the absolute specificity of the biosynthetic blockage

caused by growth retardants, it suggests that decreased amounts of inhibitory substances may outbalance the decreased gibberellin content when biological activity is measured in the bioassays, and that this explains why no decrease in gibberellin content is detected.

One question remains to be answered: why do the spruce seedlings respond to gibberellic acid only in connection with treatment with growth retardants? If the concentration of gibberellins in young spruce seedlings is high enough to be saturating, then no further response can be obtained by application of exogenous gibberellin. But if the initially saturating concentration is lowered, *e.g.* by a blocking of the gibberellin biosynthesis, then the gibberellin concentration will be growth-limiting, and application of exogenous gibberellin will result in increased growth. This explanation is not very convincing, though, since most angiosperm species, even if they are already growing vigorously, respond to additional gibberellin. An endogenous concentration of gibberellin high enough to be saturating is probably never normally present. Also, *Picea abies* most probably does not normally contain gibberellic acid (see page 32 above). Another and perhaps more reasonable explanation is, that the added gibberellic acid can be converted to other, biologically active gibberellins by the endogenous enzyme mechanisms, although the affinity of gibberellic acid molecules to some key enzyme is very low. Normally then, the gibberellic acid molecules cannot compete successfully with the molecules that are the normal substrate of this enzyme, but if such molecules are absent, and at high concentrations of gibberellic acid, that substance may enter the enzymic machinery instead and become converted to some active gibberellin.

It has been reported previously (Hansen, 55) that the dwarf variety *Picea abies* (L.) Karst. cv. '*Nidiformis*' responded to exogenously applied gibberellic acid. Shoot length and needle length were increased, but the number of laterals formed was decreased. Possibly this variety is characterized by a lower than normal rate of gibberellin production, and then it is principally comparable to spruce seedlings treated with growth retardants. This variety, and perhaps other dwarf varieties as well, seems to be a very promising material for a further study of the gibberellin physiology of conifers.

Growth retardants may not be absolutely specific inhibitors of gibberellin biosynthesis (as findings presented above indicate), but most probably their inhibitory effects are restricted to the biosynthesis of diterpenes, as caused by the blocked cyclization of *trans*-geranyl-geranyl pyrophosphate (see figure 26). The results obtained in this investigation are therefore evidence for the diterpenoid identity of the gibberellin-like substances in *Picea abies*. The facts that the growth retardants decreased the growth of the seedlings, and that gibberellic acid counteracted the effects of all the retardants, at least partially, clearly show that gibberellins take part in the natural regulation of growth in this species.

4 Possible relation of gibberellin-like substances in spruce to flowering

4.1 Application of growth retardants to spruce grafts

4.1.1 *Introduction*

It had been conclusively shown already, that gibberellin-like substances were present in *Picea abies,* and that such substances were functioning within the natural regulation of hypocotyl and shoot growth of this species. The remaining question was then: are gibberellin-like substances in any way responsible for or at least involved in the natural process of flower bud initiation? Since a series of experiments with spray application of various substances to spruce grafts in seed orchards was planned to take place (as a rather "trial and error"-looking attempt to find immediate methods to increase the flowering and seed production in these seed orchards), it was decided that also applications of the growth retardants B-995 and CCC should be included in this series of experiments. These two growth retardants were chosen because they were available as reasonably inexpensive commercial preparations, and they were also known not to accumulate in the soil (Cathey, 18). Since the effects of these two substances could probably be interpreted as effects of gibberellin deficiency (Lang, 107; Dunberg and Eliasson, 40, and pages 34—41 above), any negative effect on flowering could be considered as an indirect evidence for the participation of gibberellins in the flowering process.

A positive effect on flower initiation caused by B-995 and CCC has been reported for *Rhododendron* (Stuart, 188), *Ilex* (Marth, 1964, *in* Cathey, 18), and *Camellia* (Stuart, 1962, *in* Cathey, 18), and also for apple and cherry (Batjer and Williams, 1963, *in* Cathey, 18). As discussed by Cathey (18), this effect may be an indirect one resulting from the lowered vegetative growth. Growth retardants had been shown to decrease the vegetative growth of spruce seedlings (Dunberg and Eliasson, 40) and also of five-year old spruce plants (see page 39 above), so there was a possibility that they could positively affect the flowering of this species also, although it was not considered very probable. In that case, however, the hypothesis that gibberellins were involved in the flowering process and positively affected it could no longer be supported.

4.1.2 *Materials and methods*

Twenty-five grafts from each of five clones of *Picea abies* (L.) Karst. growing in a seed orchard at Skara in the southern part of Sweden (50° lat. N.) were selected for the experiment (clone numbers: S 3355, W 2012, W 3008, Y 2014, Z 3010). Height, growth habit and flowering were recorded, and the grafts were divided into five equal groups by partial randomization: five grafts from the same clone as equal as possible in the recorded characteristics were first selected, and these five grafts were then distributed at random among the five experimental treatments. These treatments were as follows:

B-995 1.0 % in aqueous solution
B-995 0.2 % in aqueous solution
CCC 0.5 % in aqueous solution
CCC 0.2 % in aqueous solution
Control, water only

The solutions were made from commercial preparations of B-995 (Alar-85, Uniroyal Company) and CCC (Cycocel, American Cyanamide Company) by dilution with tap water. The concentrations given above refer

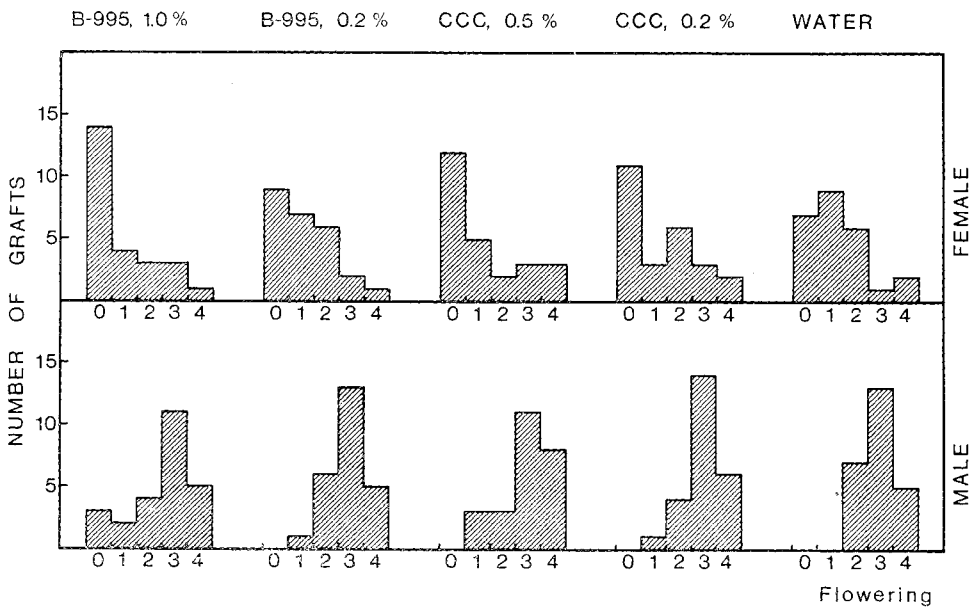


Figure 24. Effects on female and male flowering in the following year of spray application of the growth retardants B-995 or CCC to Norway spruce grafts in a seed orchard. Twenty-five grafts (five from each of five different clones) were treated in each case. Female flowering was recorded as the number of developing cones, but was for convenience grouped in the four classes 1—4; male flowering was estimated by eye and classified according to a relative scale 1—4 (see text). Zero means no flowering.

to the content of active substance. The substances were sprayed to the shoots on May 18, May 31, June 17, June 30, and July 15 in the year 1969. This repeated treatment was considered to be necessary, since the exact time intervals for the initiation and development of flower buds were not known. The first treatment consisted of 50 ml of solution, all other treatments (May 31—July 15) consisted of 100 ml of solution. The spraying was directed towards the developing buds or shoots. The method of application was probably rather inefficient, since on some of the application dates the weather was a little windy. Generally however, care was taken to select days with little wind, no rainfall within the period of two days before the treatment and no risk for it during the 24 hours immediately following.

In May 1970 the flowering of the grafts was recorded. The number of cones was counted and for further calculations divided into five groups as follows:

0 = no cones
1 = 1—5 cones
2 = 6—25 cones
3 = 26—50 cones
4 = more than 50 cones

Male flowering was estimated by eye and classified according to a five-graded relative scale:

0 = no flowering
1 = very sparse flowering
2 = sparse flowering
3 = average flowering
4 = heavy flowering

The lengths of the two stem segments formed in 1969 (the year of application) and in 1970 were also recorded.

4.1.3 *Results and discussion*

The results are shown in figure 24 and in table 7. None of the retardants did affect

Table 7. Growth of spruce grafts sprayed with retardants B-995 or CCC.

Retardant applied, concentration	Growth in year of application, cm	Growth in subsequent year, cm
B-995, 1.0 %	33.5 ± 3.6	33.5 ± 3.9
B-995, 0.2 %	40.0 ± 4.7	40.2 ± 4.5
CCC, 0.5 %	36.6 ± 5.0	36.6 ± 4.1
CCC, 0.2 %	33.1 ± 3.6	37.4 ± 4.5
Control	35.1 ± 3.6	35.1 ± 3.5

No differences are statistically significant.

the elongation growth of the grafts, neither in the year of application nor in the following year. Previous experiments have shown (see page 39), that five-year old spruce plants are sensitive to the same concentrations of the same two commercial preparations of growth retardants as have been used in the present investigation, and the lack of effect on elongation growth of the retardants is therefore probably an indication of poor efficiency of the application method when used in open-air conditions. A few cases of yellowing of the young needles caused by 0.5 % of CCC could be observed, but the damage was negligible. This also indicates that the application method was unefficient, since comparable experiments with five-year old plants gave much stronger effects.

Both retardants caused a decrease of both female and male flowering. The effect was more pronounced at the higher concentration, and B-995 appeared to be more effective than CCC. The actual number of cones per graft varied from zero to around 150, and it was therefore necessary to group the actual counts together into five classes before any meaningful comparisons could be made. The class limits have been chosen rather arbitrarily from a graph showing the distribution of the whole experimental material, but with the idea that the cone counts showed a certain similarity to a Poisson distribution, and that this similarity should not be totally lost because of the grouping. Anyhow, calculations have shown that the class number and the limits of each class may be chosen differently without any important differences in the final picture.

The results of field experiments are strongly dependent upon uncontrolled environmental conditions. Before any generalizing conclusions can be drawn, the experiments have to be repeated a number of times. The experiment described here was repeated in 1971, but a general lack of flowering in 1972 made this work useless. The generality of the results presented here therefore remains to be proven. A more efficient method of application seems desirable also. Anyhow, the results give a first indirect evidence for the participation of endogenous gibberellins in the natural process of flower bud initiation in *Picea abies*.

4.2 **Dynamics of gibberellin-like substances in spruce clones with different flowering ability**

4.2.1 *Introduction*

Exogenously applied gibberellin induces the development of flower buds in a number of coniferous species belonging to the *Taxodiaceae* and *Cupressaceae* families (see table 2, page 8). So far, however, no single species belonging to the *Pinaceae* family has been reported to respond similarly. One possible explanation to this difference is, that the flowering physiology of the different conifer families is not identical, and that gibberellins are involved in the flowering physiology of species belonging to some families but not in species belonging to the *Pinaceae* family. Another equally possible explanation is, that there are differences among the families in the identity of the endogenous gibberellins, or in the gibberellin metabolism. The success of gibberellin application may be only a problem of selecting the "right" gibberellin. Except from a recent tentative identification of gibberellic acid as the main gibberellin in Douglas fir (Crozier *et al.*, 28), very little is however known regarding the identity of gibberellins present in conifers, so there is little hope that the appropriate gibberellin can be found very soon.

Experiments with growth retardants (see page 43 above) gave some indirect indications, that endogenous gibberellins participate in the natural process of flower bud initiation in *Picea abies*. Another and more direct approach to the problem would be to study the dynamics of gibberellin-like substances in flowering and not flowering trees during the time of flower bud initiation and differentiation. If gibberellins are involved in these processes one would expect to find differences in the content of gibberellin-like substances that could be correlated to differences in the flowering ability.

Spruce clones of Polish origin are grown as grafts in some seed orchards in the southern part of Sweden, and also outside Uppsala. At least on this latter locality they do not flower, however. The seed orchard also comprises a number of grafted Swedish clones with very varying flowering ability. This seed orchard was selected for a study of the kind mentioned above. The use of clonal material eliminates the considerable genetical variation within the provenances, and also the environmental conditions are similar to all grafts in the same seed orchard. Since the grafts were not taller than maximally two meters, the collection of material for extractions was greatly simplified.

4.2.2 *Materials and methods*

During the time May 15—July 10, 1971, bursting buds and growing shoots of *Picea abies* (L.) Karst. were collected at four occasions from three clones growing in a seed orchard outside Uppsala, Sweden (60° lat. N.):

S 6016 with good male and female flowering
T 2001 with good male but poor female flowering
PL 7014 with no male or female flowering

The first two clones are from about the same latitude in Sweden as Uppsala. The third clone is Polish (from around 54° lat. N.). All Polish clones flower very poorly or not at all at Uppsala.

From the collected tissue an amount of 100 g was extracted and processed as described on page 22 (partitioning, backwash, and repeated PVP column chromatography). Gibberellin-like substances in the purified extracts were separated by chromatography on a column consisting of 10.6 g of silicic acid (Woelm Silica Gel for partition chromatography, contains 20 % water) according to the method of Powell and Tautvydas (156) and Durley *et al.* (41). The column was eluted with a gradient of ethyl acetate in *n*-hexane, obtained from a Buchler "Varigrad" automatic gradient mixer, containing 99.0 ml of *n*-hexane in chamber 1 and 72.5 ml of ethyl acetate in chambers 2 and 3. A total of 23 fractions slightly larger than 10 ml were collected plus a final fraction consisting of 20 ml of methanol.

The gibberellin activity of each fraction was measured at 100-fold dilution by the dwarf rice '*Tan-ginbozu*' microdrop bioassay (Murakami, 131) as described on page 25. The total gibberellin activity of each extract was obtained by addition of the activities above the control level for all 24 fractions. A standard dose-response curve for pure gibberellic acid was obtained in each bioassay run, and this was used for quantification. All collections from the same date were measured in the same bioassay run, although the great reproducibility of the dwarf rice bioassay makes it possible to compare, by help of the standard dose-response curves, extracts bioassayed at different occasions.

4.2.3 *Results and discussion*

The results are shown in figure 25. There is a very strong similarity between the two flowering clones and a just as strong difference between these and the Polish clone that does not flower. A second Polish clone shows a principally similar pattern of gibberellin dynamics, but since it was in part extracted according to different methods (buffer extraction), these results are not included in figure 25. There were certain

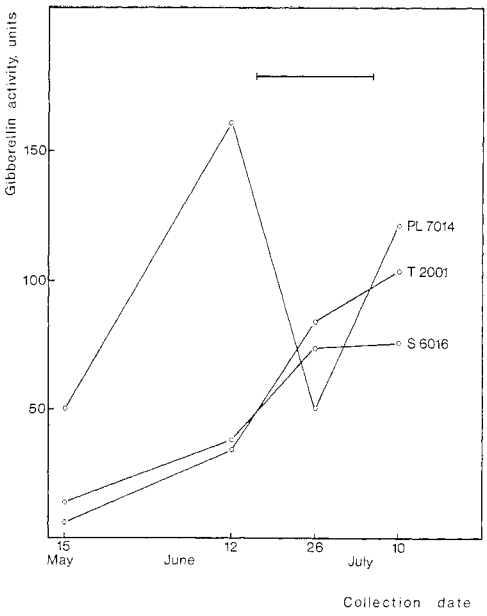


Figure 25. Dynamics of gibberellin-like substances (measured by the dwarf rice bioassay) in three clones of Norway spruce. The horizontal bar in the upper part of the figure indicates the period during which an increase in temperature leads to increased flowering the following year (Brøndbo, 15). During this period the biochemical and physiological processes leading to flower bud differentiation may be supposed to take place.

Each point represents a single measurement.

qualitative differences in the spectrum of gibberellin-like substances between the various clones as well, but it seems premature to present those data before they have been verified in a repeated investigation.

It has been shown (Brøndbo, 15), that a high temperature around the grafts during the time June 21—July 5, but not later, resulted in a considerable increase in the number of flower buds that developed. Microscopy studies of the bud development in *Picea abies* have shown, that vegetative and floral primordia can be distinguished from each other in early July (Dr. L. Brunkener, personal communication). It is thus probable, that the induction of flowering takes place in late June, perhaps as a response to the culmination and decrease in daylength. During this time the gibberellin activity steadily increases in the two Swedish clones, while during the same time the activity drops sharply in the Polish clone. There is indeed a good correlation, then, between the gibberellin activity during the probable time of flower bud initiation on one hand and the clonal flowering ability on the other.

It is not yet possible to judge upon the general validity of the results obtained in this investigation. Spruce clones that have been moved almost 700 km northwards from their native locality are obviously very poorly adapted to the changed photoperiodic conditions. The differences in gibberellin dynamics between Swedish and Polish clones grown outside Uppsala may be a reflection of disturbancies in the adaptation of the latter clones to the prevailing photoperiod, and the absence of flowering in the Polish clones may depend upon the same photoperiodic disturbancies. The fact that the flowering ability is correlated to the gibberellin activity during some period of time, however critical this period may be, must not necessarily mean that the flowering is directly dependent upon or controlled by gibberellins. Most probably, however, the flower initiation is in some way ultimately controlled photoperiodically, and gibberellins may then be the connecting link therebetween, since it is known that the content of gibberellins in many woody species is controlled by the photoperiod (Digby and Wareing, 35). In view of this, the results of the present investigation are a further indication that gibberellins *are* in some way involved in the flowering physiology of *Picea abies*.

5 General comments

Summing up all pieces of information gained in this work, one can with confidence give positive answers to the three questions put forward in the general introduction:

1) Gibberellin-like substances are present in *Picea abies*. In fact, there is a complex spectrum of biologically active substances, at least six different ones but perhaps as many as eight or nine. This has been shown in a very extensive investigation, using methods for purification and detection far more elaborate and efficient than those presently in frequent use. If the extract concentrations are kept fairly low it is even possible to carry out semi-quantitative estimates of gibberellin contents in various tissues. Judging from bioassay data and from the general lack of response of spruce plants to the application of gibberellic acid, it is not probable that this particular compound (or the chromatographically indistinguishable gibberellin A_1) is present in *Picea abies*. It is probable, though, that the biologically active compounds isolated from Norway spruce are true gibberellins, with a gibbane carbon configuration, since application of growth retardants obviously prevents their biosynthesis, and also preliminary data from infrared spectroscopy support this conclusion.

2) Gibberellins possess growth-regulatory properties in the spruce plant. They are necessary for the normal elongation growth, just as they are well known to be in the far more frequently studied angiosperms. Possibly some dwarf cultivars of *Picea abies* and related species have a reduced biosynthesis of gibberellins, and thus such cultivars may serve as a suitable material for the study of gibberellin physiology in conifers.

3) There are several pieces of evidence suggesting that gibberellins are directly or indirectly related to the process of flower initiation in Norway spruce. Application of growth retardants decreases the flowering, presumably by decreasing the biosynthesis of gibberellins. Furthermore, a relatively high level of gibberellins during the time of flower initiation is correlated with a relatively high clonal flowering ability. It is however not yet possible to determine by what mechanism gibberellins affect the flower initiation process, and the relation may therefore be only an indirect one.

The flowering physiology and biology of *Picea abies* is profoundly affected by environmental factors like temperature and rainfall (Tirén, 187; Sarvas, 164). It has also been suggested (Tirén, 187), that a strong interrelation exists between flowering and the vegetative development of the shoot system, and that this "bud reduction effect" may be the main reason for the markedly periodic flowering of Norway spruce. This concept is strongly supported by the results of an investigation (Dunberg and Eriksson, unpublished) in which the flowering and vegetative development of individual spruce grafts was followed in detail during three years. But although there may be good reason to keep in mind the possible limitations in flowering intensity, and thus in seed production in the seed orchards, that may be caused by morphological and environmental factors, there is good reason to believe that the flowering of *Picea abies* could be stimulated by application of gibberellin, provided the proper gibberellin were available. In Japan, gibberellin is already used at a practical scale to increase the flowering and shorten the juvenility period of sugi (*Cryptomeria japonica*) (Ohba, 137) and thus make possible the running of a breeding program involving several genera-

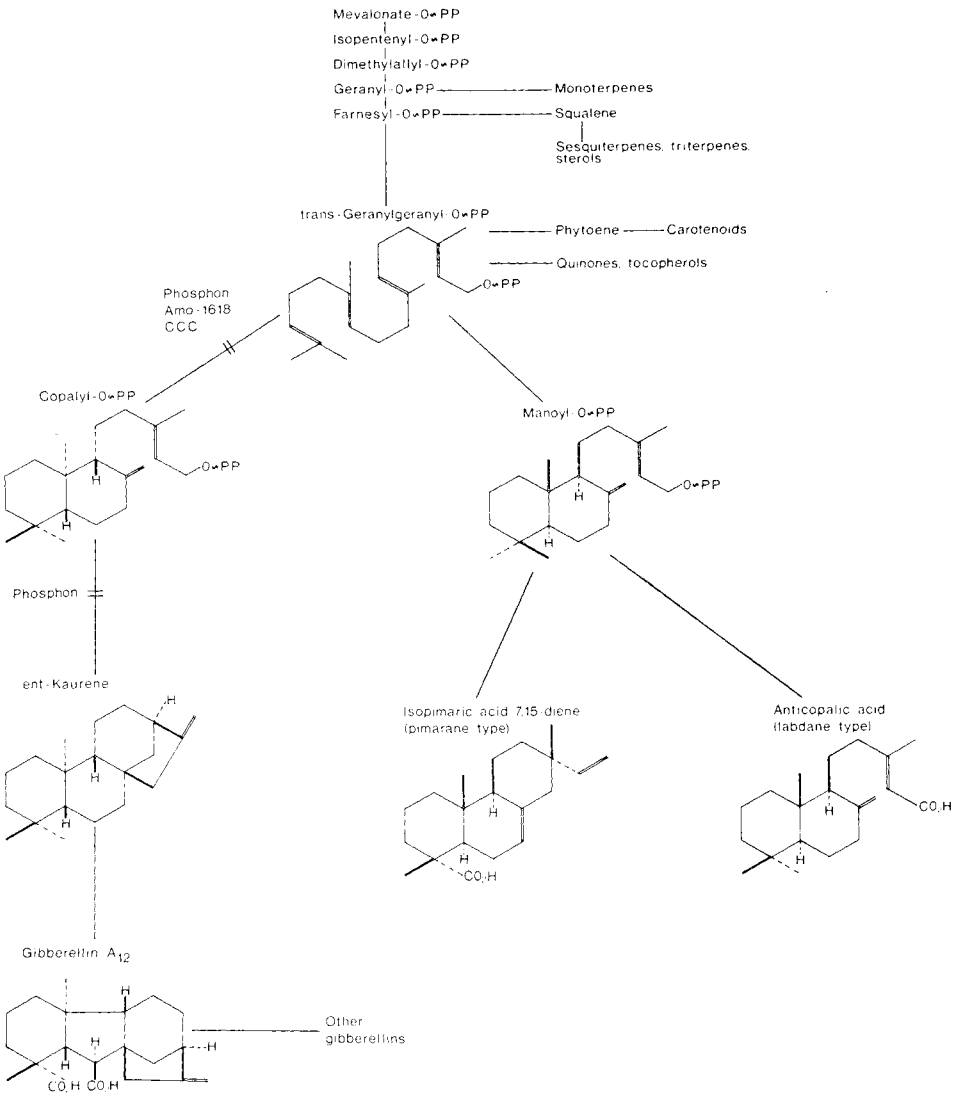


Figure 26. Biosynthesis of gibberellin and other diterpenes. Enzymic steps known to be specifically blocked by growth retardants are indicated.

tions within a reasonably short time. The immense importance to forest genetics of the introduction of breeding programs instead of mass selection is obvious.

The problem, then, is to identify and make available the proper gibberellin. It is thus very desirable that the gibberellins isolated from Norway spruce are identified. This can be done by mass spectrometry and/or infrared spectrophotometry, but these methods can be used only if a sufficient amount of a sufficiently pure sample is available. Work to obtain such a sample is under way, although the problems caused by the abundance of other terpenoid substances in Norway spruce make the work difficult and tedious. Diterpenes with molecular structures and therefore also chemical properties similar to gibberellins are common in all conifers (see figure 26), and since they are often growth-inhibiting they may prevent the detection of gibberellins

and other growth-promoting substances. New methods for purification of the extracts have to be worked out and tested, and because of the absence of chemical or physical methods of detection that are sensitive enough to react to nanogram quantities of gibberellins, time-consuming bioassays remain the only practically useful means of detection. If only the spruce gibberellins were identified, however, one could for future work select methods directly related to the by then known details of chemical and physical properties of those particular gibberellins, and this would greatly simplify the problems. Also, if the spruce gibberellins were identical with or structurally very similar to some already known gibberellins, other sources could be chosen for large-scale isolation, or readily available gibberellins could be chemically modified. If the biosynthetic pathway leading to physiologically active gibberellins in spruce were known, precursors could be applied instead of the final products, with the same physiological effects.

It is probable that other growth regulators, like auxins and cytokinins, and also photoperiod and heat sum affect the process of flowering, presumably in more or less intimate interplay with the gibberellins. Before the second generation of seed orchards is to be established it is desirable that also these problems are taken into study, since photoperiod-sensitive mechanisms in the plant are affected by the geographical localization of the seed orchards. In addition to studies of hormonal physiology, also histochemical and ultrastructural work would be of great value, *e.g.* to obtain a better knowledge concerning the time-course of differentiation and development of the flower buds. As a whole, this field of research is very little explored, and a continued and extended study of the physiology of hormonal regulation and differentiation in Norway spruce and other conifers would yield results not only of theoretical interest but also of direct practical and economical importance to forest genetics and to forestry in general.

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Sammanfattning

Ända sedan gibberellinerna återupptäcktes i början av 1950-talet har ett ständigt ökande antal uppsatser behandlat förekomsten och funktionen av denna hormongrupp hos högre växter. Hos angiospermer anses gibberelliner vara naturligt förekommande hormoner som är nödvändiga för den normala sträckningstillväxten. Vidare inducerar gibberelliner partenokarpisk fruktsättning, de befrämjar frögroning och brytande av knoppvila. De är dessutom inblandade i blomningsprocessen hos många arter som normalt behöver en köldperiod eller korta dagar för att blomma. Gibberelliner används i praktisk skala t.ex. av trädgårdsodlare, i bryggerier (för att erhålla en bättre och jämnare mältning), och för vernalisering av vissa sädesslag.

Man har funnit och identifierat 38 olika gibberelliner, som alla har en gemensam kemisk grundstruktur och dessutom har biologiska effekter som är typiska för denna hormongrupp. Gibberelliner benämns med nummer i serien A_1, A_2, A_3 ... A_n, men gibberellin A_3 kallas också oftast "gibberellinsyra", trots att alla gibberelliner är karboxylsyror. Gibberellinsyra är den kommersiellt tillgängliga substans som används i de flesta försök med gibberelliner.

Mycket få undersökningar har gjorts rörande förekomst och funktion av gibberelliner hos barrträd. Delvis beror detta på, att tidiga undersökningar gav negativa resultat och klart visade att de tillgängliga metoderna var undermåliga vid arbete med barrträd, och delvis beror det säkerligen på, att man inom det praktiska skogsbrukets organisationer ännu inte (som t.ex. inom jordbruk och trädgårdsbruk) på allvar har insett betydelsen av växtfysiologisk grundforskning. Den mest slående effekten av gibberelliner på barrträd är utan tvekan deras förmåga att stimulera blomningen, en effekt som tycks kunna framkallas hos praktiskt taget alla arter inom familjerna Taxodiaceae och Cupressaceae. Hittills har emellertid alla försök att åstadkomma samma effekt hos arter inom familjen Pinaceae (dit alla våra ekonomiskt viktiga barrträd hör) varit resultatlösa. Metoder att öka blomningen eller åstadkomma tidigare blomning skulle vara av enorm betydelse för skogsträdsförädlingen. Det har kunnat visas, att blomutvecklingen orsakad av gibberellinbehandling inte skiljer sig från den normala, att bildat pollen är normalt och funktionsdugligt, och att bildade frön är livsdugliga och ger upphov till normala plantor. Gibberelliner kan också komma att få betydelse för möjliggörandet av artkorsningar och för framställandet av haploida plantor.

De fröplantager med gran som anlagts skall tjänstgöra både som frökällor för skogsbrukets behov och som experimentenheter för skogsgenetiskt forskningsarbete. Båda dessa funktioner hotas starkt av granplantagernas hittillsvarande dåliga och periodiska blomning. Det forskningsprojekt varav här redovisat arbete är en del har initierats som ett försök att finna de fysiologiska mekanismer som reglerar blomning en, och även om behovet av hormonfysiologisk grundforskning från början var up penbart, så bestämdes projektets upplägg ning till stor del av önskemålet att relati snabbt finna praktiskt tillämpbara metod att öka blomningen i granfröplantagern Eftersom blomning hos angiospermer ans vara reglerad av hormonella mekanism där gibberelliner spelar en viktig roll, c eftersom ett stort antal barrträdsarter k induceras att blomma genom behandl med gibberelliner, så var det naturligt inledningsvis ägna denna hormongrupp ciellt intresse. Målet för denna del av

jektet formulerades som besvarandet av tre konkreta frågor:

1) Finns det gibberellinsyra eller andra gibberelliner hos gran?
2) Har gibberellinsyra eller eventuella naturligt förekommande grangibberelliner något slags mätbar effekt på granplantor?
3) Kan man på något sätt visa eller göra troligt att gibberelliner spelar en roll i den naturliga blomningsprocessen hos gran?

Denna avhandling visar, att alla tre frågorna kan besvaras jakande.

Att påvisa förekomsten av gibberelliner hos gran visade sig vara mycket svårt, huvudsakligen beroende på avsaknaden av effektiva metoder att separera gibberelliner från andra kemiska beståndsdelar. Dylika metoder måste därför utarbetas, och av stor betydelse var här författarens vistelse vid universitetet i Calgary, Canada, där en stor och kunnig forskargrupp arbetade på samma problem. Med hjälp av metanolextraktion, flera olika utskakningsförfaranden, upprepad kromatografering av extrakten på polyvinylpyrrolidon-kolonn och separering av olika gibberelliner genom kromatografering på kiselsyrakolonn, var det slutligen möjligt att med hjälp av biologiska test påvisa förekomsten av ett helt spektrum av s.k. gibberellinliknande substanser, dvs. ämnen med typiska gibberellineffekter men med tillsvidare okänd kemisk struktur. Denna del av arbetet utvidgades ytterligare med en undersökning av aktiviteten av de isolerade gibberellinliknande substanserna i ytterligare ett antal biologiska test, inkluderande ett test med små granplantor framtaget speciellt för detta projekt. Ett försök gjordes även att med hjälp av infraröd-spektrofotometri analysera den kemiska strukturen hos de aktiva substanserna. De uppnådda resultaten var givetvis inte tillräckligt noggranna för att en säker identifikation skulle vara möjlig, men de motsade åtminstone inte möjligheten, att de isolerade aktiva substanserna skulle vara verkliga gibberelliner.

Samtliga isolerade aktiva substanser hade en sträckningsbefrämjande effekt på unga granplantor. Gibberellinsyra var däremot verkningslös. Samtidigt som dessa resultat visar, att de gibberellinliknande substanser som kan isoleras från gran även har fysiologiska effekter på denna art, visar de att gibberellinsyra sannolikt inte förekommer som ett fysiologiskt aktivt hormon hos gran. Eftersom gibberellinsyra är det enda kommersiellt tillgängliga gibberellinet, innebär detta att det är svårt att påvisa effekter av gibberelliner på granens tillväxt eller blomning. En alternativ väg att indirekt påvisa sådana effekter är att tillföra s.k. tillväxtretardenter, dvs. substanser som blockerar biosyntesen av gibberelliner i den behandlade växten. Försök med unga granplantor odlade i näringslösning visade, att de tre tillväxtretardenterna Amo-1618, B-995 och CCC samtliga hämmade tillväxten. Gibberellinsyra kunde helt eller delvis återställa normal tillväxt hos de retardentbehandlade plantorna. Eftersom tillförsel av gibberellinsyra till normala plantor inte medför några mätbara effekter, kan detta resultat synas svårtolkat. En möjlig förklaring är, att granar redan normalt har en optimal gibberellinnivå, och att ytterligare tillförsel därför är utan verkan. Mer troligt är dock, att gibberellinsyra i sig inte har någon verkan, men att denna substans vid hormonbrist i växten kan metaboliseras till verksamma gibberelliner. Vilken av dessa förklaringar man än väljer, så visar försöket att gibberelliner produceras i växten och är nödvändiga för en normal sträckningstillväxt, helt i linje med förhållandet hos angiospermer. Försöket visar dessutom, att tillförsel av retardenter är en metod som kan användas för att påvisa fysiologiska funktioner av gibberelliner hos gran, oberoende av dessa gibberelliners kemiska detaljstruktur.

Granympar i en fröplantage sprayades med tillväxtretardenterna B-995 eller CCC vid fem olika tillfällen under tiden maj—juli. Av varje retardent prövades två koncentrationer, som i tidigare laboratorieförsök visat sig ej ge skador på plantorna. Dessutom ingick i försöket en kontrollgrupp, som ej behandlades. Året därpå re-

gistrerades blomningen hos de behandlade ymparna och hos kontrollymparna. Båda retardenterna orsakade en minskning av både han- och honblomningen, och denna effekt var mer uttalad vid den högre koncentrationen. De tillförda doserna gav däremot inga effekter på den vegetativa tillväxten, vilket visar att de varit relativt låga. Resultatet är en första indikation på, att gibberelliner är inblandade i blomningen hos gran.

Ytterligare en metod att påvisa gibberellinernas betydelse för blomningen prövades. Växande skott från tre olika grankloner med varierande blomningsförmåga insamlades vid fyra tillfällen under tiden 15 maj—10 juli, och med hjälp av ett biologiskt test mättes gibberellinaktiviteten hos de olika klonerna vid de olika tidpunkterna. Det visade sig, att det förelåg ett positivt samband mellan gibberellinaktiviteten under den tidsperiod när blomningsinduceringen förmodas ske och blomningsförmågan. Även detta resultat stöder därför antagandet, att gibberelliner spelar en viktig roll vid blomningsinduceringen hos gran.

Sammanfattningsvis har detta arbete visat, att det finns gibberelliner hos gran, men att inget av dessa kan förmodas vara identiskt med gibberellinsyra, att gibberelliner är nödvändiga för den normala sträckningstillväxten, och att de med stor sannolikhet också är direkt inblandade i blomningsinduceringen. Även om andra faktorer påverkar blomningens intensitet, och avkastningen i fröplantagerna därför i praktiken kan komma att begränsas av dessa, så är det uppenbart att blomningen hos gran skulle kunna stimuleras genom gibberellintillförsel, bara man hade tillgång till det relevanta gibberellinet. Det är därför önskvärt, att granens gibberelliner identifieras, och att metoder utarbetas för framställning i tillräckligt stor skala, t.ex. genom modifiering av gibberellinsyra med kemiska metoder. Det är troligt att andra hormongrupper, t.ex. auxiner och cytokininer, och även fotoperioden och värmeklimatet på växtplatsen påverkar blomningsförloppet i mer eller mindre intim samverkan med gibberellinerna. Innan anläggandet av nästa plantagegeneration påbörjas är det därför önskvärt att även dessa problem blir föremål för studium, eftersom exempelvis fotoperiodkänsliga mekanismer i växten påverkas av den geografiska lokaliseringen av fröplantagerna. Förutom hormonfysiologisk forskning skulle här histokemiska och ultrastrukturella studier vara av stort värde.

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