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Cytokinin Profiles in the Conifer Tree *Abies nordmanniana*: Whole-Plant Relations in Year-Round Perspective

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Abstract Conifer trees are routinely manipulated hormonally to increase flowering, branching, or adjust crown shape for production purposes. This survey of internal cytokinin levels provides a background for such treatments in *Abies nordmanniana*, a tree of great economic interest. Reference points in the crown and root system were sampled destructively in 4- and 6-year-old trees and analyzed for a range of cytokinins by LC-MS/MS. No seasonal patterns were detected in the root samples, and a major portion of cytokinin was in conjugated forms. Dramatic and consistent seasonal changes occurred in the crown, at levels 17–65 times higher than in the root. Predominant among crown cytokinins was ZR, except in the needles where IPR was also prominent. Within the crown, cytokinin profiles in different organs differed consistently. The leader bud showed a pronounced mid-June minimum, and a maximum later in summer. Subapical buds showed the same June minimum but peaked in mid autumn at a much lower level. Maxima in these buds were preceded by peaks in the subapical stem. Parallel patterns were observed in homologous tissues on branches.This pattern is consistent with two surges beginning in the uppermost stem tissues leading to subsequent accumulation or stimulated production within the buds. Strong differential hormonal profiles between adjacent buds with different fates agree with recent evidence of localized cytokinin production. The data suggest a reduced role of root-derived cytokinins in crown development. Practical cytokinin treatments for crown-shape regulation require close attention to dosage as well as precise timing and positioning.

Keywords Bud physiology · Cytokinin synthesis · Positional information · Root function · Root-shoot relation · Tree physiology

Abbreviations

CK	Total cytokinin
DHZ	Dihydrozeatin
DHZ9G	Dihydrozeatin-N9-glucoside
DHZR	Dihydrozeatin riboside
IP	Isopentenyladenine
IPR	Isopentenyladenosine
IPRP	Isopentenyladenosine 5′-monophosphate
LC-MS/MS	Liquid chromatography-mass spectrometry
Z	Trans-zeatin
Z7G	Zeatin-N7-glucoside
Z9G	Zeatin-N9-glucoside
ZOG	Trans-zeatin-O-glucoside
ZR	Trans-zeatin-riboside
ZROG	Trans-zeatin-riboside-O-glucoside
ZRP	Trans-zeatin-riboside 5′-monophosphate (cytokinin abbreviations in accordance with Lexa and others 2003)
Z-group	Z + ZR + ZRP

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DHZ-group	DHZ + DHZR (DHZRP not found)
IP-group	IP, IPR, IPRP
Free bases	Z + DHZ + IP
Ribosides	ZR + DHZR + IPR
Ribotides	ZRP + IPRP
O-glucosides	ZOG + ZROG
N-glucosides	Z7G + Z9G + DHZ9G

Introduction

Woody plants typically have a long life span, seasonally rhythmic growth and development, and long physical distances between meristems. Classic theories of growth regulation imply that the main cytokinin production occurs in root tips (for example, Little and Pharis 1995), and thus is translocated via the xylem over great distances, especially when the plant in question is a tall tree. Recent studies, however, show widespread synthesis of cytokinins in various plant organs (Nordström and others 2004; Tanaka and others 2006) much more in harmony with the accumulating evidence of cytokinin synthesis or cytokinin retention in plant tissues deprived of root connections (for example, Vonk 1979; Wang and Wareing 1979; Cline and others 1997, Chatfield and others 2000). In that light it is also easy to explain why graftings or local gene activation of cytokinin over- or underproducing mutants may fail to be overruled by the cytokinin wild type expressed in the root parts (Faiss and others 1997). "The idea that CK [cytokinins] and auxin are synthesized only in root tips and shoot apices, respectively, is now overturned" (Sakakibara 2006). Decentralized synthesis would presumably require less translocation through the plant body and enable more local stability in concentrations (Zhao 2008).

We find that there is a lack of comparable data on the distribution of endogenous phytohormones in various parts of a large perennial plant over the seasons. Most such studies in woody plants have been carried out on xylem exudates, yielding information about what is in transport rather than local levels (for example, Fußeder and others 1992 and references therein). Although compartmentation and concentration gradients among tissues within a plant organ (for example, Uggla and others 1998) obviously would make analyses at organ level less meaningful, external applications are mostly carried out at the organ level. Dosing in such experiments, whether for scientific or practical purposes, outside of the range of natural concentrations may seriously perturb the processes they are intended to generate or affect (for example, Ljung and others 2004). Several tree growth problems that remain largely unclarified are probably hormonally regulated. These include *aging* which practically prevents vegetative propagation from anything but the youngest trees (for example, Malabadi and others 2004), *plagiotropy* which limits the use of rooted cuttings and grafts, and *apical control* phenomena that govern crown shape (for example, Cline 2000; Veierskov and others 2007). Control of flowering and of excessive leader growth in conifers is currently successfully achieved through hormonal treatments (Smith 1998; Landgren and others 2008).

This study is thus a mapping in time and space of local cytokinin levels in *A. nordmanniana*, a conifer whose ornamental crown shape makes it economically important for Christmas tree production. Repetitive and predictable branching patterns in this tree enable us to easily identify and compare homologous points, within and among trees and over time. The main framework of the crown consists of an orthotropic leader stem with a tiered arrangement of plagiotropic branches in whorls. The branch whorl arises as a dense spiral of vigorous lateral buds that are crowded below the leader bud, normally in a number of from four to six "whorl buds." A similar aggregation of subterminal lateral buds surrounds the terminal bud of the branches (Fig. 1), but here the arrangement of buds is plagiotropic (Veierskov and others 2007).

Meristem activity in the crown is subjected to a strong seasonal rhythm. Bud burst in Denmark occurs in mid-May, and the final length of the shoots is attained in the second half of July to early August, about 70 days after bud burst. When the shoots are in early expansion, development of new buds at the shoot tip begins, with scale leaf development and segregation of subterminal lateral shoot meristems in their axils. Scale leaves continue to form until midsummer when a rather abrupt change of meristem shape is accompanied by the initiation of the first needle

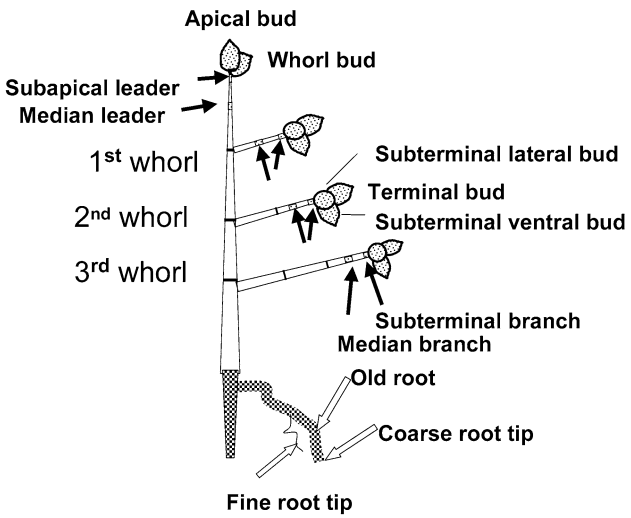

Fig. 1 Diagram of sampling points (stippled buds, axial samples indicated by *arrows*) shown in 5-year-old *Abies nordmanniana*, corresponding to trees of the second series at the end of the study

primordia. This organogenesis continues until the onset of winter dormancy in October when the buds contain all leaf primordia for the following spring (unpublished data). Fully preformed buds are general to many members of Pinaceae (for example, Owens and Molder 1973).

In contrast to the crown, conifer roots have no inherent seasonal dormancy and will grow at temperatures above 5°C (Lopushinsky and Max 1990). The species of *Abies* under Danish conditions are notable for the low minimum temperatures at which root tips are still turgid and white (+2°C; Nørgaard Nielsen, unpublished). Conifer root systems are composed of (a) large, fast-growing, and thick roots (2-4 mm) that constitute the main root axes, (b) slender roots (0.8-2 mm) that grow only 1-2 mm daily, and (c) short-lived feeder roots, often dichotomously branched and mycorrhized (Orlov 1980).

Materials and Methods

Plants

After four seasons in a seedling bed, trees of *Abies nordmanniana* Spach. were planted in 10-L containers in autumn and grown year-round under field conditions (Veierskov and others 2007). The pots were placed in outer pots sunken into the ground (double-pot system), enabling the root system to grow under ambient soil temperatures. The peat-based potting mixture was originally poor in microorganisms and there were no attempts to control new colonization during culture that lasted several years. Almost no mycorrhization (ecto-) was observed, however, and feeder roots were hardly developing. This is to be expected for a conifer in a peat-based substrate that is amply nitrogen-fertilized (Gagnon and others 1995). Growth conditions were uniform for all trees. Watering was adapted to the natural precipitation whereas nutrients were supplied according to the seasonal requirements of the trees to maintain strong growth and avoid stress conditions (U. Gejl, unpublished).

To reduce individual variation among the study trees, they were selected from a large population using morphologic criteria and time of bud burst. At each sampling session five study trees were picked at random and sampled destructively. Trees going into their seventh growth season (from seed) were sampled at regular intervals from June 2003 to April 2004 (first series) and trees going into their fifth growth season were sampled from June 2004 to May 2005 (second series). The first series initially carried four branch whorls and were 0.6-0.8 m tall, the second series had two branch whorls and were 0.3-0.5 m tall. Material was collected at the cultivation site at noon ± 1 h. Samples were kept on ice for 3-4 h and subsequently stored in an ultradeep freezer (−80°C) until extraction and analysis. In the first series the samples were pooled from five trees according to sample type; in the second series material from individual trees was analyzed separately at selected sampling times. Sample weights ranged from 6.8 mg for the smallest and youngest buds to 1.5 g for the largest, and root samples ranged from 75 to 900 mg.

Root tips were easily distinguished into coarse (up to 4 mm diameter) and slender types (<1 mm), presumably corresponding to the two main types of roots, (a) and (b) described above (Orlov 1980). From root tips appearing at the lump surface toward the sides of the pot, we excised the terminal 8-10 mm of both coarse and fine roots from each tree, three and six tips, respectively. We also sampled a 8-10-mm-long piece approximately 100 mm behind the tip of the coarse roots ("mature roots"), three from each tree (Fig. 1). These pieces were wiry but secondary thickening had not yet begun to increase the diameter noticeably, and the roots were still unbranched. Light and turgid root tips were to be found at all times, but at a minimum during a brief time while shoots were expanding in early June. This is a pattern known from other trees (for example, O'Hare and Turnbull 2004).

From the crown we sampled buds, transverse sections of the young leader shoot and branches, and the evergreen needles going through their first season from bud burst. We sampled the apical bud (leader bud), the subapical buds ("whorl buds"), terminal and subterminal buds on the branch whorls, only two uppermost whorls being sampled in the first series, and all three whorls in the second series (Fig. 1). Just after bud break the shoot tip samples constituted the extreme tip above the expanding leaf primordia and comprised the hardly differentiated apical/terminal bud, whorl/subterminal buds, and uppermost stem tissue, all of which were analyzed together. Already on the second sampling these parts were distinguishable entities. Bud samples comprised the shoot apical meristem with scale leaves and needle primordia attached. Stem cross sections contained vascular as well as cortical and medullary tissues. The time series of crown samples represented a maturation of tissues from bud break to the following spring.

In mid-May a diurnal analysis was performed of the second series, fine root tips, and one defined bud type (horizontal subterminal bud from branches in the uppermost branch whorl) which were being sampled repeatedly from the same five trees nine times around the clock. These trees were removed from their outer pots during the sampling day. For analysis, individual trees were pooled into two groups (2 and 3 trees, respectively).

Chemical Analyses

High-pressure liquid chromotography and electrospray tandem mass spectrometry provided simultaneous

identification and quantification of numerous cytokinins (Table 1). The samples were ground in liquid N_2. A mixture of 5 ml isopropanol/acetic acid (99:1 v/v) and 500 ml of a solution containing 50–300 ng of the deuterated internal standards was added [procedure slightly modified from Chiwocha and others (2003)]. The mixture was left overnight at 5°C and the extract subsequently was passed through a solid-phase extraction column [Isolute C18, 100 mg, 10 ml (International Sorbent Technology Limited, Mid Glamorgan, UK)] that had been activated with methanol and isopropanol/acetic acid (99:1 v/v). The extracts were evaporated to dryness in vacuum on a MaxiDry Plus vacuum centrifuge (Heto Holten, Denmark) and redissolved in 400 µl acetonitril/formic acid/water (20/1/79 v/v/v).

Cytokinins were separated on a Zorbax XDB column (3.5 µm, 2.1 × 150 mm) using a linear gradient between A (0.1% acetic acid with pH adjusted to 8.0 with NH_3) and B (methanol). Profile was 23% B to 57% B in 15 min. A Quattro LC (Micromass, Manchester, UK) equipped with an HPLC (Agilent, Waldberon, Germany) was used. The settings for the electrospray were as follows: desolvation gas (N_2) 550 L h^{-1} at 350°C, capillary voltage 3.0 kV, and source temperature 120°C. For the MRM method, argon at 2.5 mTorr was used for collision. The cone voltage and the collision energy were set individually for each of the cytokinins (settings in Table 1). Quantification was carried out with deuterium-labeled cytokinins and application of linear regression to the response factor versus concentration data.

The material was analyzed in large batches spanning several collection dates. Data from samples of the first 3 months of the first series of trees varied greatly, which was ascribed to initial problems with consistency in the sampling procedure. These data were discarded in the subsequent data analyses. Of the remaining 332 samples, 6 were discarded on account of outlier values and/or possible misidentification.

Table 1 MRM conditions for the different cytokinins

	IS	MRM segment	M^{+1}	Fragment	Cone (V)	Collision (V)	Rt (min)	LOD (fmol)
ZRP		I	431.8	219.8	30	20	6.30	12.0
DZRP		I	436.9	225.0	30	20	6.20	
Z7G		I	381.9	219.9	30	21	6.29	0.3
DZ7G		I	387.1	224.9	30	21	6.39	
Z9G		I	381.9	219.9	30	21	7.50	0.1
DZ9G		I	387.1	224.9	30	21	7.40	
ZOG		I	381.9	219.9	30	21	8.15	2.0
DZOG		I	387.1	224.9	30	21	7.93	
DHZ9G	DZ9G	I	383.9	221.9	30	25	8.08	0.4
Z		II	219.9	135.9	30	20	9.58	1.0
DZ		II	224.8	136.9	30	20	9.44	
ZROG		II	514.2	382.2	30	20	10.07	3.0
DZROG		II	519.0	387.0	30	20	9.95	
ZR		II	352.0	219.9	30	20	11.60	0.4
DZR		II	357.0	225.0	30	20	11.63	
DHZ		II	222.0	135.9	30	22	10.21	1.9
DDHZ		II	224.9	135.8	30	22	10.12	
DHZR		II	354.0	221.9	30	20	12.60	1.3
DDHZR		II	357.0	225.0	30	20	12.52	
IPRP		III	415.6	203.8	30	20	14.70	4.0
DIPRP		III	421.9	209.9	30	20	14.50	
IP		IV	203.9	135.8	26	19	19.80	0.9
DIP		IV	209.9	136.9	27	19	19.74	
IPR		IV	335.8	203.8	30	20	20.41	0.7
DIPR		IV	341.9	209.8	26	20	20.30	

Initial letter D represents the deuterated forms used as internal standard. If other internal standards have been used, it is marked in the column IS. The LOD refers to a signal to noise ratio of 3 and is the absolute amount injected on the column

M^{+1} signifies molecular mass of parent ion

Rt = Retension time

Data Processing

Phytohormone results were transformed in various ways to study the distribution of data. Numerous cytokinin values among the root samples were below detection level and thus were recorded as 0.00 pmol g^{-1} FW. Zero values present a special challenge because no transformation can adjust the data to meet assumptions for parametric tests (Delucchi and Bostrom 2004). As a technical precaution, the whole data set was raised by 0.001 pmol to avoid pure zero values before further calculation. In many cases data deviated from normal distribution. Where transformation enabled the data to fulfill the normality test, transformed data were analyzed. If rejection or acceptance of zero hypothesis was not affected by transformation, tests on raw data are shown.

All analyses were carried out on pooled five-tree values at a given date in series 1 and series 2, or mean values of five samples, and in the diurnal study on means of two- or three-tree values. A linear mixed model was used for series 1 and 2:

$$Y = \mu + t + o(t) + D + D \times t + e \quad (1)$$

where Y is the observed value for a given trait, μ is the overall mean, t is the fixed effect of type of material, that is, root or crown, $o(t)$ is the fixed effect of organ within type of material (buds, branch, or root samples), D is the random effect of sample date, $D \times t$ is the random effect of interaction between type of material and date of sampling, and e is the error term, whose distribution is assumed to be normal, independent, and identically distributed (NIID). In series 2 a separate analysis was carried out on crown organs alone:

$$Y = \mu + o + D + D \times o + e \quad (1a)$$

Analyses were carried out using SAS v9.1 (SAS Institute, Chicago, IL, USA) using the procedures proc MIXED, and predicted values (BLUP) of random effects (not transformed values) were used for presentation of seasonal variation.

Tests of random effects in model 1 and 1a were based on comparisons of models with and without a random effect, testing the difference between 2 × log ratio using a χ^2 test with one degree of freedom.

For the diurnal data a repeated-measures model was used for the two types of organs (root tip and one type of bud) using the SAS v9.1 procedure GLM option Repeated:

$$Y = \mu + c + o + c \times o + e \quad (2)$$

where c is the fixed effect of time of day, o is the type of organ and $c \times o$ is the interaction between time of day and type of organ.

Results

Diurnal Data

In tissue from fine root tips and one bud type that was sampled during a spring day just before bud burst, we found no diurnal trend in total cytokinin and no interaction between time of day and organ type (Table 2). This also applied to the various cytokinin conjugated forms and groupings. The two tissue types differed significantly in total cytokinin concentrations on a fresh weight basis, the concentrations in buds being about 17 times higher (Table 2), and the cytokinin composition differed considerably. N-glucosides (Z7G, Z9G) totally constituted more than 50% of the root cytokinin content but were only sporadically present in buds (Table 2). In contrast, O-glucosides were noted in about equal average proportions in both tissues. Ribosides, mainly ZR, constituted less than 2% in the root tips but more than half of the cytokinin pool

Table 2 Test for diurnal trends in cytokinin groups

	LS mean[a]		Probability levels[b] (p)		
	Fine root tips	Lateral bud	Root vs. bud	Diurnal time	Organ × time
Total cytokinin	5.96	99.70	0.009	0.844	0.505
N-glucosides	3.22	0.30	0.092	0.709	0.670
O-glucosides	2.21	26.47	0.021	0.693	0.688
Z-group	0.31	55.83	0.012	0.746	0.825
DHZ-group	0.00	4.84	<0.001	0.415	0.415
IP-group	0.22	12.27	0.008	0.121	0.211
Free bases	0.11	3.99	0.053	0.196	0.135
Ribosides	0.07	55.07	0.010	0.920	0.923
Ribotides	0.36	13.88	0.010	0.116	0.484

[a] Means (least square means from ANOVA) in pmol g^{-1} FW

[b] Probability tests were carried out with untransformed data based on repeated-measurements ANOVA analysis

in buds. The DHZ-group was detected only in the buds. The IP-group at this point was found in all bud samples, whereas the presence in root tips was irregular.

Annual Data

Total cytokinin concentration in crown samples rose significantly from July 1 through autumn in the second series of trees, and this was essentially confirmed by the less extensive sampling in the first series of trees (Fig. 2). Thus, the same general trend and levels appeared in two different ages of young trees and through two different years. No seasonal trend could be demonstrated in the root samples.

With respect to annual average of total cytokinin concentrations and to most cytokinin groupings, crown reference points were very significantly higher than root samples (Table 3, column 3). Cytokinins referable to the Z-group and the DHZ-group were more prevalent in shoot samples in relation to the total cytokinin pool, and those referable to the IP-group more so in the root (Table 3, columns 1 and 2). Different sample types within crown/root differed significantly with respect to total cytokinin and all cytokinin groupings (column 4). There was a significant effect of season on total cytokinin and several cytokinin groupings (sampling date, column 5), but not the DHZ-group, IP-group, and the glucosides. However, interaction between date and sample type was prevalent (column 6), meaning that sample types generally differed in cytokinin dynamics as well as levels over the year, with the exception of the Z-group, ribosides, and ribotides.

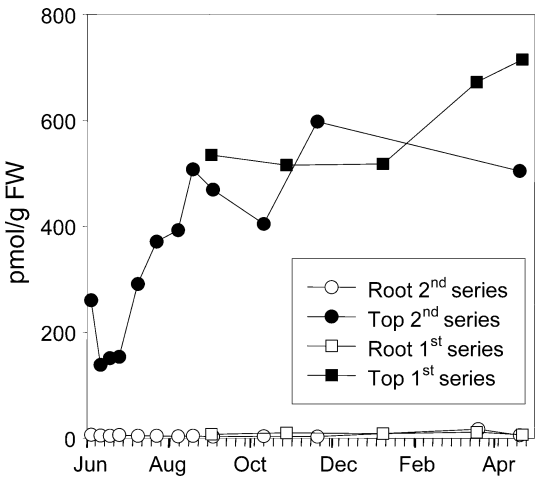

Fig. 2 Total cytokinin concentration (pmol g^{-1} FW) in *Abies nordmanniana* over the year – predicted (BLUP) values of root and crown samples at each sample date based on a mixed-model analysis. Root samples comprise two types of root tips and cross sections of mature root, and crown samples comprise a set of bud types, stem cross sections, and needles. Abscissa represents season as well as a maturation process in the crown samples. Long ticks represent the first of the month in question, the short ticks show week intervals. *Square symbols*: first series, 6-year-old trees; *circular symbols*: second series, 4-year-old trees. *Open symbols*: root samples; *filled symbols*: crown

Among points in the crown we also found numerous significant differences in cytokinin annual means (Table 4, column 1), with only glucosides not varying demonstrably from point to point. Seasonal effects among crown points were significant in all cytokinin groupings mentioned (Table 4, column 2).

Young needles began growth with a relatively large contribution of O-glucosides, to be followed during summer by increases in Z- and IP-ribosides (Fig. 3a, b). A seemingly large late summer increase in N-glucosides consisted of both Z9G and DHZ9G and thus could be real despite being supported by only one point in time. Overall, there was an increase in needle cytokinins during their first season of growth until the beginning of the next.

The leader bud showed the greatest temporal dynamics and the highest maximum contents (Fig. 4a, b). The basic features seem to be at a minimum in mid-June, followed by a pronounced peak in late July and a subsequent weaker one in late August (data from the first series suggest that the late peak might be more pronounced, Fig. 4b). The main cytokinin form was ZR throughout.

Whorl buds showed the same minimum in mid-June (Fig. 5a, b) but rose much less than the adjacent leader bud and reached a maximum in autumn. The main cytokinin was ZR as in the leader bud, but with ribotides apparently relatively more abundant.

The stem tissue immediately beneath the buds mentioned above showed a series of peaks during summer, followed by a rise during autumn and all through winter (Fig. 6a, b), the dynamics relying almost entirely on changing levels of ZR. In samples from the median part of the leader shoot there were faint fluctuations similar to those in the subapical part but apparently slightly delayed (only free bases plus ribosides shown, Fig. 7b). As the sum of free bases and ribosides was plotted for all parts of the tree crown, it was seen that the peaks in the subapical leader tissues (and faintly in the median leader) preceded those of the leader bud by 2–3 weeks (Fig. 7a, b).

Roughly the same phenomenon was seen at the end of whorl branches, with the peaks in the terminal bud coinciding in time with those of the leader bud but maximum values about four times lower (Fig. 7c, d). Concentration peaks in branch end tissues likewise preceded those in the buds, but at levels about half of those obtained in the leader. Similar to the median leader, the median part of the branch showed much lower levels than the distal part, with a suggestion of the same maxima slightly delayed.

The cytokinin profile of the terminal branch bud developed a pronounced maximum in the IP-group following the second maximum in the Z-group (Fig. 8a). This maximum consisted of mainly IPRP (data not shown).

Table 3 Annual means of cytokinin groups (least square means from mixed-model analysis – untransformed data) and comparison of sample type

	Type (LS mean)		Fixed effects (*p*)		Random effects (*p*)	
	Root	Crown	Root vs. crown	Samples within root/crown	Sampling date	Date × type
Total cytokinin	5.4	353.3	<0.001	<0.001	0.043	0.048
N-glucosides	1.9	77.7	<0.001	0.022	0.089	0.051
O-glucosides	0.4	32.2	<0.001	<0.001	0.655	0.001
Z-group	1.4	182.1	<0.001	<0.001	0.046	0.317
DHZ-group	0.0	15.2	<0.001	<0.001	1.000	<0.001
IP-group	1.7	32.4	<0.001	<0.001	0.655	<0.001
Free bases	0.2	6.0	<0.001	0.001	<0.001	<0.001
Ribosides	2.5	173.5	<0.001	<0.001	<0.001	1.000
Ribotides	0.5	53.5	<0.001	<0.001	<0.001	1.000

Means in pmol g^{-1} FW. Root = samples including fine and coarse tips as well as mature root. Crown = a selection of buds, young stem, and needles (compare with Fig. 1). Probability tests were carried out on ln-transformed data to improve normal distribution in residuals. Significance in column 3 signifies differences between root and crown samples, and significance in column 4 differences between type of samples within the crown or root system. Column 5 shows significant effects of season and column 6 shows whether root and crown react differently with respect to season

Table 4 Sample type variation within the crown

	Probability levels	
	Organ within crown	Date
Total cytokinin	<0.001	<0.001
N-glucosides	0.569	<0.001
O-glucosides	0.116	<0.001
Z-group	<0.001	0.010
DHZ-group	<0.001	<0.001
IP-group	<0.001	<0.001
Free bases	<0.001	<0.001
Ribosides	<0.001	0.001
Ribotides	0.005	<0.001

Comparison of annual concentration means of cytokinin groupings (least square means) and temporal variation. Probability levels from two-way ANOVA of untransformed data

From midwinter onward N-glucosides became prevalent. The subterminal lateral bud showed a similar pattern (Fig. 8b) but with much lower fluctuations in the Z-group and a temporary rise in first N-glucosides and O-glucosides before the midautumn maximum in IPRP. This lateral bud pattern reappeared in other subterminal branch buds analyzed (data not shown).

Free bases constituted a very small fraction of the cytokinin pool throughout. They generally increased slightly in autumn, remained stable through autumn and winter in maturing tissues, and in some cases appeared to increase further some time in early spring (Figs. 3b, 4, 5, 6b).

A comparison of root sample types indicated that mature root samples had higher concentrations of total cytokinin than the samples containing root tips but had lower concentration of ribotides. Differences were very significant for N- and O-glucosides and less so for ribotides (Table 5), the absolute values being quite small, however. An annual yearly variation was indicated for N-glucosides and free bases, but again at a very low level.

Discussion

Because no significant diurnal variation could be demonstrated, the samples taken within a specified time interval presumably are representative for that particular day. Lack of a diurnal pattern in cytokinin concentration in both root tips and buds of *A. nordmanniana* may appear surprising. Diurnal rhythms in endogenous cytokinins have been demonstrated by others in leaves (Hewett and Wareing 1974; Novakova and others 2005) and xylem sap (Machácková and others 1996). However, the temporal relationship between cytokinin fluctuations and time of day is not quite consistent in various studies and often lacks statistical testing. Fußeder and others (1992), analyzing xylem sap in almond trees under various water stress situations, demonstrated diurnal cytokinin patterns in only about one fourth of the cycles analyzed. This suggests that diurnal cycling was not very pronounced.

The analyses from points within the crown confirmed the expectation of strong local concentration differences. The results also showed that concentration patterns over time in homologous organs are similar (for example, Figs. 7, 8a, b). Perhaps significantly, the leader bud, outstanding by being the only orthotropic bud on the tree,

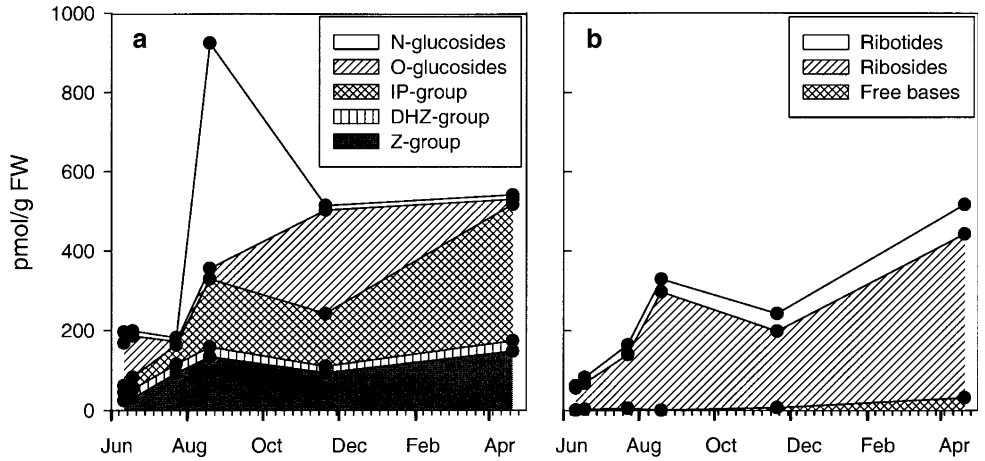

Fig. 3 Needle cytokinin composition over time. Time axis as in Fig. 2. Cumulative data, i.e., distance between plot lines shows the concentration of compound group in question at the time in question. **a** Cytokinin groupings explained under Abbreviations. N-glucosides comprise Z7G and Z9G mainly. **b** Distribution of the same data grouped as free bases, ribosides, and ribotides, glucosides omitted. Each data point is based on 1–3 samples of 70–100 mg needles. Data from series 2 only

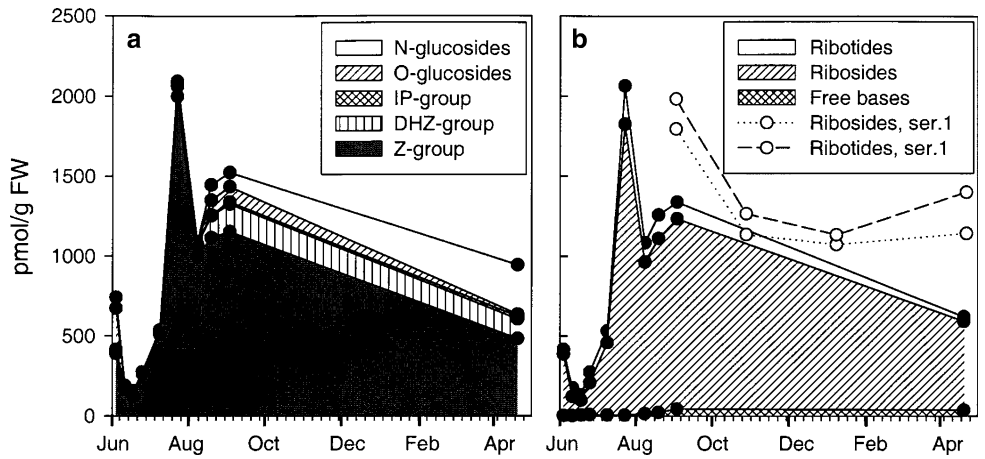

Fig. 4 Leader bud cytokinin composition over time. Time axis as in Fig. 2. Cumulative data, i.e., distance between plot lines shows the concentration of compound group in question at the time in question. Cytokinin groupings in **a** and **b** defined in Abbreviations. Data points based on buds from five trees lumped or a mean of 4–5 trees. Data from series 2. Supplementary data from series 1 shown as *open symbols* and *stippled lines* in **b**

surpassed all other crown parts analyzed with respect to cytokinin concentrations (Figs. 4, 7) and reached levels that must be considered unusually high for plant tissues. In general, the fate of buds seemed to be reflected in their cytokinin profiles, a result that confirms and extends previous studies in conifers (Bollmark and others 1995; Chen and others 1996).

Temporal resolution was not the same for all organs analyzed but the graphs largely confirmed the common temporal trends. Temporal resolution is too low to ascertain the exact time of concentration extremes, but the minimum in mid-June and subsequent maxima in shoot ends followed by maxima in buds (Fig. 7) all seem well corroborated.

The similar sequence of maxima in both leader tip and branch ends (Fig. 7) is consistent with two surges in cytokinin content beginning in the distal part of the shoot, with these surges being separated by about 4 weeks and leading to increases in the buds about 2 weeks subsequently. Because the median part of the shoots appears to show a fainter, delayed reaction, the signal is probably not ascending from below. Supportive data were obtained by Zaerr and Bonnet-Masimbert (1987) and Pilate and others (1990) who found lower cytokinin content in the basal and median parts of conifer shoots than in the apical part at selected points in time. This interpretation resembles the model described for bud activation in shoot tips that are released from apical dominance by decapitation (Shimiza-

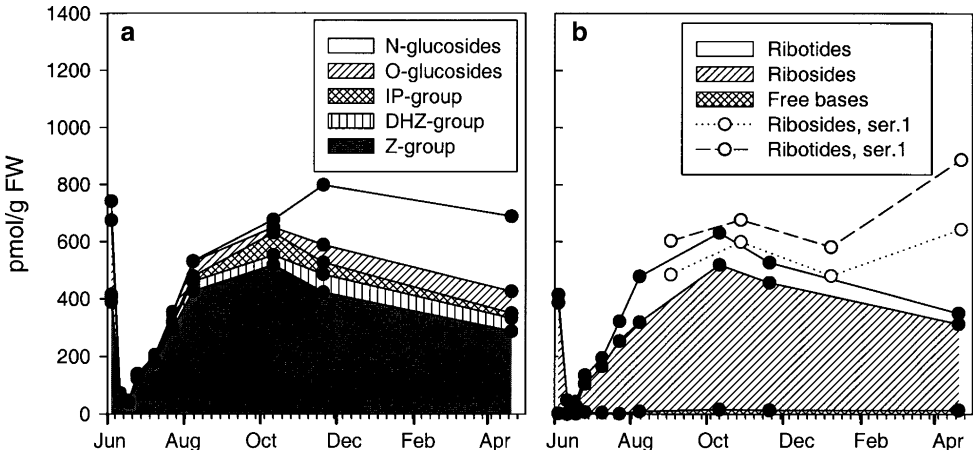

Fig. 5 Subapical buds, "whorl buds," cytokinin composition over time. Time axis as in Fig. 2. Cumulative data, i.e., distance between plot lines shows the concentration of compound group in question at the time in question. Cytokinin groupings in **a** and **b** explained in Abbreviations. Data points based on buds from either five trees lumped, a mean of five individually analyzed trees, or more. Data from series 2. Supplementary data from series 1 shown as *open symbols* and *stippled lines* in **b**

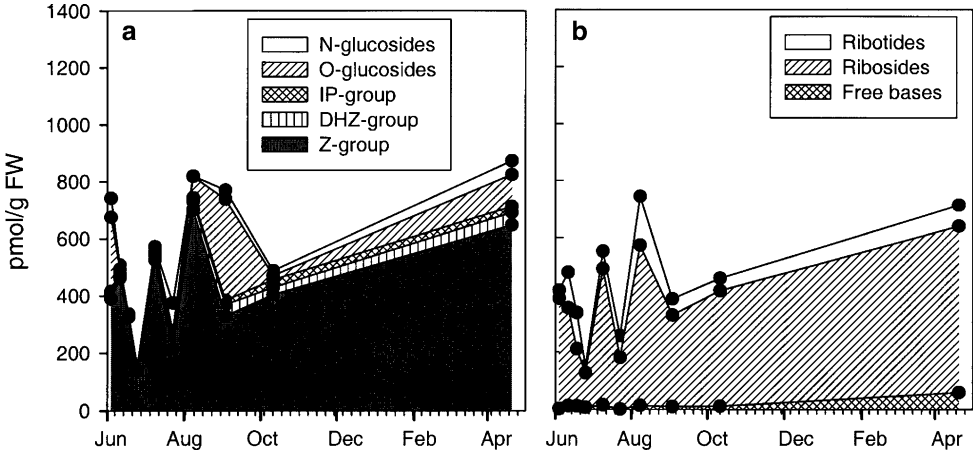

Fig. 6 Subapical leader stem, cytokinin composition over time. Time axis as in Fig. 2. Cumulative data, i.e., distance between plot lines, shows the concentration of compound group in question at the time in question. Cytokinin groupings in **a** and **b** explained in Abbreviations. Data points based on samples from five trees lumped, from series 2 only

Sato and Mori 2001), which is supported by demonstration of cytokinin synthetic activity in stem tissues beneath activated buds (Tanaka and others 2006). Our data are thus consistent with production sites within the stem as well as probably within buds. Buds, or the shoot apical meristems contained within them, have long been acknowledged as cytokinin production sites (Guivarc'h and others 2002).

This information could provide a basis for functional studies of apical control and allocation between growth points in conifers. The exact timing of events would, most probably, vary with environmental conditions of the year in question and possibly also change with the age of the tree. The data might suggest that the older trees of the first series maintained somewhat higher concentrations (Figs. 2, 4, 5), but a difference in season and thus in growth conditions between the two data series could also have influenced the levels found.

The June minimum in buds and stems coincides with the period of maximum shoot expansion. This might correspond to the cytokinin decrease in the days following bud break that was observed by Tromp and Ovaa (1994) in xylem saps of *Malus*, and in tobacco buds during a pause in organogenesis observed by Dewitte and others (1999). The first surge in the buds roughly coincides with when the shoot apical meristems switch from bud scale to needle production. The second surge in the buds might correspond to a point of maximum developmental activity of meristems but a temporal connection with any particular developmental event is not obvious. The IPRP accumulation in branch buds (Fig. 8a, b) in early October coincides in time with cessation of organogenesis.

An abrupt reduction in the content of natural growth regulators in buds of woody plants at the beginning of winter dormancy is known from other trees (Kefeli and others 2003) but could not be confirmed in *A. nordmanniana.* A comparison of summer and winter tissues in another evergreen conifer, *Pinus sylvestris,* also failed to show any winter decline in stem tissues (Moritz and Sundberg 1996). Winter cytokinin decrease and spring increase could be a characteristic of the xylem sap flow, as indicated by the data of Doumas and Zaerr (1988), and particularly of deciduous trees such as *Salix*, *Populus*, and *Malus* (Hewittt and Wareing 1974; Alvim and others 1976; Tromp and Ovaa 1994).

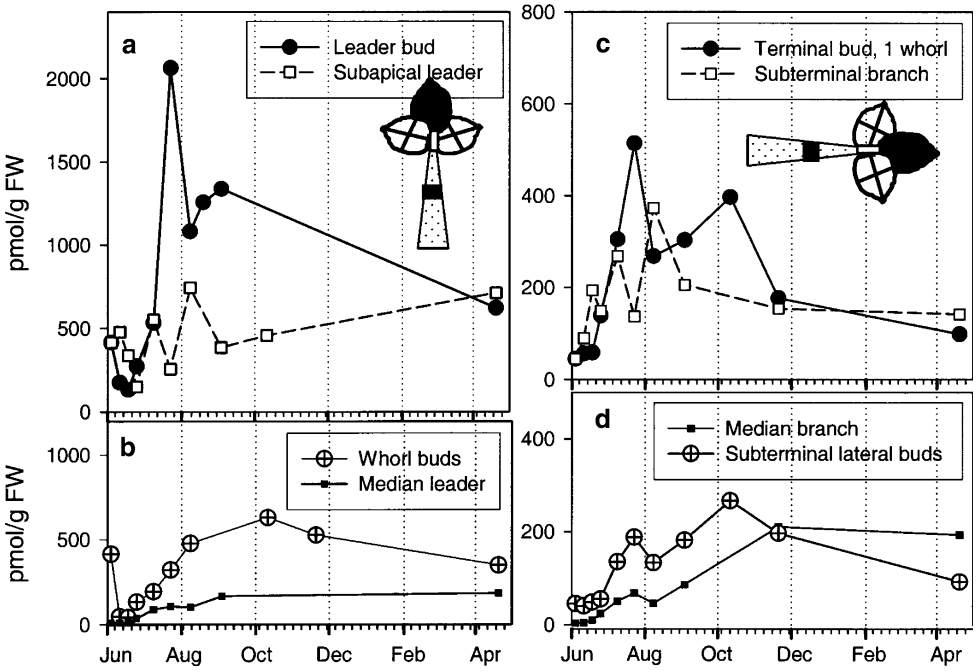

Fig. 7 **a**, **b** Sum of free bases and ribosides in four parts of leader tip, shown in two graphs for clarity. **c**, **d** Corresponding points in branch ends in the first branch whorl, shown in the same manner but note difference in ordinate scale. Data points based on analyses from five lumped trees, a mean of five individually analyzed trees, or more from series 2

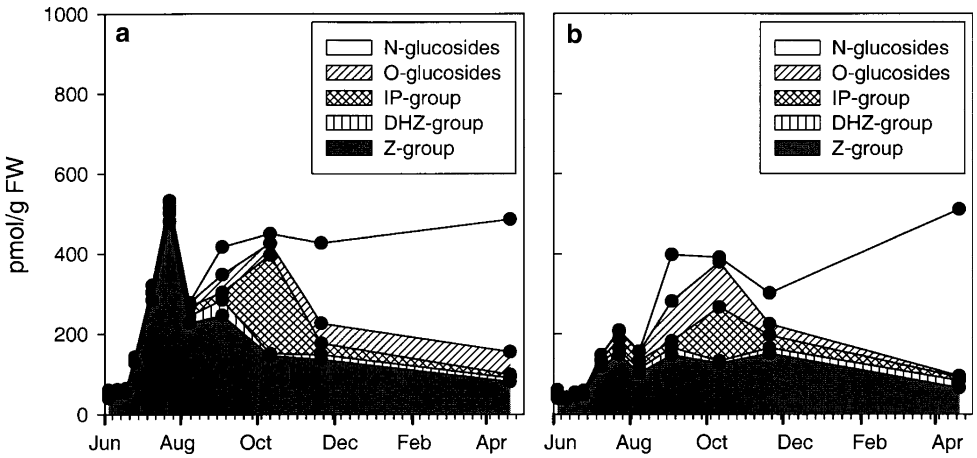

Fig. 8 **a** Terminal buds of the first branch whorl. Cytokinin composition referred to five major groupings, presented as in a part of Figs. 3–6. Data points based on analyses from five lumped trees, a mean of five individually analyzed trees, or more from series 2. **b** Lateral subterminal buds of the first branch whorl. Presented as in **a**

Table 5 Annual means (least square means of untransformed data) and comparison of three types of root samples with respect to cytokinin groups

	LS mean			*p*	
	Coarse root tips	Fine root tips	Mature root	Organ	Date
Total cytokinin	2.1	5.0	9.1	0.088	1.000
N-glucosides	0.2a	0.7a	5.1b	<0.001	0.032
O-glucosides	0.0a	0.2a	1.0b	<0.001	0.107
Z-group	1.0	1.2	2.0	0.173	0.655
DHZ-group	0.0	0.1	0.1	0.186	1.000
IP-group	1.2	2.9	1.0	0.481	1.000
Free bases	0.0	0.2	0.4	0.102	0.008
Ribosides	1.3	3.5	2.7	0.807	0.572
Ribotides	0.8a	0.5a	0.0b	0.019	0.584

Means with suffixes a and b differ significantly at 5% level. Probability levels from two-way ANOVA of ln-transformed data are given for effect of sample type (organ) and date

High and apparently increasing cytokinin content in the fully developed needles (Fig. 3) is confirmed by data from another conifer (*Picea glauca*) by Matschke and Machackova (2002), who found about 200-500 pmol g^{-1} FW in second-year needles. Stable overall spring and winter cytokinin concentrations in needles of *P. sitchensis* were found by Collier and others (2003) through the first 2 years after bud break. Because decreasing leaf cytokinin is connected with senescense, the steadily high conifer needle contents could be related to the evergreen condition.

Several earlier studies of *Rosa*, *Petunia*, and *Arabidopsis* have shown a high proportion of IP-group cytokinins in leaves; thus, this could be a typical trait (Dieleman and others 1997; Dewitte and others 1999; Corbesier and others 2003). The 40% IP-type cytokinins (of the total CK pool) found in first-year winter needles of *Picea sitchensis* appeared to decrease proportionally over time, constituting 32, 26, and 24%, respectively, over the following spring, winter, and subsequent spring (Collier and others 2003). This decrease might be a symptom of the slow senescence process in evergreen foliage.

Although the time axis in Fig. 2 represents an increasing maturity of the crown tissues sampled, the root tip samples maintained roughly the same physiologic age throughout the study. The root samples showed large variability in the contribution of various cytokinin forms and low resolution in data due to the characteristically low concentrations. The data should be treated with caution. Root tips generally have a high water content; concentration differences between root and crown would thus be less dramatic on a dry-weight basis, especially when water content is lowest in buds but far from compensating the differences found between tissues. The generally low levels in roots, however, were consistent over diurnal and annual data and over the two plant series. Prevalence of glucosides over other cytokinins is in agreement with earlier findings in root tips of Douglas fir (Doumas and others 1989). Analysis of the xylem sap in that tree species indicated that glucosides are not part of the mobile cytokinin pool (Doumas and Zaerr 1988). Thus, active and mobile cytokinins were apparently kept at a minimum by glucosidation. Our root samples appear low in cytokinins compared to other conifer studies (Doumas and others 1989; Matschke and Macháckova 2002), but the levels are comparable to those found in young poplar roots for the same range of cytokinins (Dluzniewska and others 2006). The stable year-round level in the roots of *A. nordmanniana* was consistent with root tip appearance that suggested year-round growth. Root cytokinin did not reflect any of the pronounced seasonality displayed in the crown.

The two types of root tip samples resembled each other and differed from the more mature root samples (Table 5), but not in a way that could support the assumption of a steep gradient in cytokinins toward the extreme root tips (if data were presented on a dry-weight basis even less so). Such gradients have been found in other species and used to support the assumption of root tip localization of cytokinin production, but such a pattern is not substantiated in this tree species. Our data suggest a more diffuse cytokinin production for local utilization within the root.

A recent review characterized all plant hormonal classes as "small organic molecules, synthesized throughout the plant" and affecting "local cells and tissues" and subject to decentralized regulation (Bishopp and others 2006). This raises the question of how various cytokinin sources differ in relative importance and in function within the plant. In a study of graftings in pea plants with different branching properties, Beveridge (2000) suggested that the importance of the root system as a cytokinin source might decline in response to branching in the crown. In woody plants beyond the seedling stage this would imply a quite subordinate role of the root system as cytokinin provider when permanent branching of increasing complexity characterizes the crown. This seems to be supported by our results.

Conclusions

The results of our study have led us to the following conclusions: (1) Cytokinin production in young root tissues, including the root tips, appeared low and nearly constant over the year. (2) The root cytokinin pool mainly consisted of immobile forms. (3) Great variation in levels was noted between homologous organs in the crown. (4) Characteristic and strongly organ-specific annual cytokinin profiles were found. (5) Cytokinin production in extreme stem tissues appears to be the driving force of cytokinin production in the buds. (6) High and increasing cytokinin levels in needles may be related to the evergreen trait.

Attempts to influence cytokinin levels by a large reduction in the tree root system size have often yielded fairly insignificant results (Jackson 1993), which is in agreement with the data presented here. In practical terms, to get appreciable effects, hormonal manipulations of such trees by external cytokinin application would be expected to require localized and fairly strong treatments and close attention to position and time of treatment.

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References

Alvim R, Hewett EW, Saunders P (1976) Seasonal variation in the hormone content of willow. I. Changes in abscisic acid content and cytokinin activity in the xylem sap. Plant Physiol 57:474–476

Beveridge CA (2000) Long-distance signalling and a mutational analysis of branching in pea. Plant Growth Regul 32:193–203

Bishopp A, Mähönen AP, Helariutta Y (2006) Signs of change: hormone receptors that regulate plant development. Development 133:1857–1869

Bollmark M, Chen H-J, Moritz T, Eliasson L (1995) Relations between cytokinin level, bud development and apical control in Norway spruce, *Picea abies*. Physiol Plant (Copenhagen) 95:563–568

Chatfield SP, Stirnberg P, Forde BG, Leyser O (2000) The hormonal regulation of axillary bud growth in *Arabidopsis*. Plant J 24:159–169

Chen H-J, Bollmark M, Eliasson L (1996) Evidence that cytokinin controls bud size and branch form in Norway spruce. Physiol Plant (Copenhagen) 98:612–618

Chiwocha SD, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross ARS, Kermode AR (2003) A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. Plant J 35:405–417

Cline MG (2000) Execution of the auxin replacement apical dominance experiment in temperate woody species. Am J Bot 87:182–190

Cline MG, Wessel T, Iwamura H (1997) Cytokinin/auxin control of apical dominance in *Ipomoea nil*. Plant Cell Physiol 38:659–667

Collier MD, Sheppard LJ, Crossley A, Hanke DE (2003) Needle cytokinin content as a sensitive bioindicator of N pollution in Sitka spruce. Plant Cell Environ 26:1929–1939

Corbesier L, Prinsen E, Jacqmard A, Lejeune P, Van Onckelen H, Perilleux C, Bernier G (2003) Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thalinana* during floral transition. J Exp Bot 54:2511–2517

Delucchi KL, Bostrom A (2004) Methods for analysis of skewed data distributions in pyschiatric clinical studies: Working with many zero values. Am J Psychiatry 161:1159–1168

Dewitte W, Chiappeta A, Azmi A, Witters E, Strnad M, Rembur J, Noin M, Chriqui D, Van Onckelen H (1999) Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. Plant Physiol 119:111–121

Dieleman JA, Verstappen FWA, Nicander B, Kuiper D, Tillberg E, Tromp J (1997) Cytokinins in *Rosa hybrida* in relation to bud break. Physiol Plant (Copenhagen) 99:456–464

Dluzniewska P, Gessler A, Kopriva S, Strnad M, Novak O, Dietrich H, Rennenberg H (2006) Exogenous supply of glutamine and active cytokinin to the roots reduces NO_3^- uptake rates in poplar. Plant Cell Environ 29:1284–1297

Doumas P, Zaerr JB (1988) Seasonal changes in levels of cytokinin-like compounds from Douglas-fir xylem extrudate. Tree Physiol 4:1–8

Doumas P, Bonnet-Masimbert M, Zaerr JB (1989) Evidence of cytokinin bases, ribosides and glucosides in roots of Douglas-fir, *Pseudotsuga menziesii*. Tree Physiol 5:63–72

Faiss M, Zalubìlová J, Strnad M, Schmülling T (1997) Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. Plant J 12:410–415

Fußeder A, Wartinger A, Hartung W, Schultze E-D, Heilmeier H (1992) Cytokinin in the xylem sap of desert-grown almond (*Prunus dulcis*) trees: Daily courses and their possible interactions with abscisic acid and leaf conductance. New Phytol 122:45–52

Gagnon J, Langlois CG, Bouchard D, Tacon FI (1995) Growth and ectomycorrhizal formation of container-grown Douglas-fir seedlings inoculated with *Laccaria bicolor* under four levels of nitrogen fertilization. Can J For Res 25:1953–1961

Guivarc'h A, Rembur J, Goetz M, Roitsch T, Noin M, Schmülling T, Chriqui D (2002) Local expression of the *ipt* gene in transgenic tobacco (*Nicotiana tabacum* L. cv. SR1) axillary buds establishes a role for cytokinins in tuberization and sink formation. J Exp Bot 53:621–629

Hewett EW, Wareing PF (1974) Cytokinin changes during chilling and bud burst in woody plants. In: Bieleski RL, Ferguson AR, Cresswell MM (eds) Mechanisms of regulation of plant growth. Royal Society of New Zealand, Wellington, NZ, pp 693–701

Jackson MB (1993) Are plant hormones involved in root to shoot communications? Adv Bot Res 19:104–168

Kefeli VI, Kalevitch MV, Borsari B (2003) Natural growth inhibitors and phytohormones in plants and environment. Kluwer Academic Publishers, Dordrecht, pp 1–323

Landgren C, Fletcher R, Bondi M, Cacka J, Huso M (2008) Leader control on noble (*Abies procera*) and Nordmann fir (*Abies nordmanniana*) using NAA. In: Thomsen IM, Rasmussen HN, Sørensen JM (eds), Proceedings of the 8th International Christmas Tree Research Extension Conference. Hoersholm: Forest & Landscape Denmark, Forest & Landscape Working Papers No. 26-2008, ISBN 978-87-7903-342-9, pp 66-69. Available at http://www.sl.life.ku.dk/upload/workingpapersno26.pdf

Lexa M, Genkov T, Malbeck J, Macháčková I, Brzobohatý B (2003) Dynamics of endogenous cytokinin pools in tobacco seedlings: a modelling approach. Ann Bot (London) 91:585–597

Little CHA, Pharis RP (1995) Hormonal control of radial and longitudinal growth in the tree stem. In: Gartner BL (ed) Plant stems. Physiology and functional morphology. Academic Press, San Diego, pp 281–319

Ljung K, Sandberg G, Moritz T (2004) Methods of plant hormone analysis. In: Davies PJ (ed) Plant hormones. Biosynthesis, signal transduction, action! Kluwer Academic Publishers, Dordrecht, pp 671–694

Lopushinsky W, Max TA (1990) Effect of soil temperature on root and shoot growth and on budburst timing in conifer seedling transplants. New Forest 4:107–124

Macháčková I, Eder J, Motyka V, Hanuš J, Krekule J (1996) Photoperiodic control of cytokinin transport and metabolism in *Chenopodium rubrum*. Physiol Plant (Copenhagen) 98:564–570

Malabadi RB, Choudhury H, Tandon P (2004) Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord.) promoted by partical desiccation and Gellan gum. Sci Hort 102:449–459

Matschke J, Macháčková I (2002) Changes in the content of indole-3-acetic acid and cytokinins in spruce, fir and oak trees after herbicide treatment. Biol Plant (Prague) 45:375–382

Moritz T, Sundberg B (1996) Endogenous cytokinins in the vascular region of *Pinus sylvestris* during activity and dormancy. Physiol Plant (Copenhagen) 98:693–698

Nordström A, Tarkowsky P, Tarkowská D, Norbaek R, Doležal K, Sandberg G (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. Proc Natl Acad Sci USA 101:8039–8044

Novakova M, Motyka V, Dobrev PI, Malbeck J, Gaudinova A (2005) Diurnal variation of cytokinin, auxin and abscisic acid levels in tobacco leaves. J Exp Bot 56:2877–2883

O'Hare TJ, Turnbull CGN (2004) Root growth, cytokinin and shoot dormancy in lychee (*Litchi chinensis* Sonn.). Sci Hort 102:257–266

Orlov AY (1980) Cyclic development of roots of conifers and their relation to environmental factors. In: Sen DN (ed) Environment and root behaviour. Geobios International, Jodhpur, India, pp 43–61

Owens JN, Molder M (1973) Bud development in western hemlock. I. Annual growth cycle of vegetative buds. Can J Bot 51:2223–2231

Pilate G, Sotta B, Maldiney R, Bonnet-Masimbert M, Miginiac E (1990) Endogenous hormones in Douglas fir trees induced to flower by gibberellin $A_{4/7}$ treatment. Plant Physiol Biochem 28:359–366

Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. Annu Rev Plant Biol 57:431–449

Shimiza-Sato S, Mori H (2001) Control of outgrowth and dormancy in axillary buds. Plant Physiol 127:1405–1413

Smith RF (1998) Effects of stem injections of gibberellin $A_{4/7}$ and paclobutrazol on sex expression and the within-crown distribution of seed and pollen cones in black spruce (*Picea mariana*). Can J For Res 28:641–651

Tanaka M, Takei K, Kojima M, Sakakibara H, Mori H (2006) Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant J 45:1029–1036

Tromp J, Ovaa JC (1994) Spring cytokinin composition of xylem sap of apple at two root temperatures. Sci Hort 57:1–6

Uggla C, Mellerowicz EJ, Sundberg B (1998) Indole-3-acetic acid controls cambial growth in Scots pine by positional signaling. Plant Physiol 117:113–121

Veierskov B, Rasmussen HN, Eriksen B, Hansen-Møller J (2007) Plagiotropy and auxin in *Abies nordmanniana*. Tree Physiol 27:149–153

Vonk CR (1979) Origin of cytokinins transported in the phloem. Physiol Plant (Copenhagen) 46:235–240

Wang TL, Wareing PF (1979) Cytokinins and apical dominance in *Solanum andigena*: Lateral shoot growth and endogenous cytokinin levels in the absence of roots. New Phytol 82:18–19

Zaerr JB, Bonnet-Masimbert M (1987) Cytokinin level and flowering in Douglas fir. For Ecol Manage 19:115–120

Zhao Y (2008) The role of local biosynthesis of auxin and cytokinin in plant development. Curr Opin Plant Biol 11:16–22