# "Lateral Control": Phytohormone Relations in the Conifer Treetop and the Short- and Long-Term Effects of Bud Excision in *Abies nordmanniana*

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**Abstract** In a conifer tree, such as Nordmann fir, *Abies nordmanniana* Spach, the leader bud and its immediate surroundings play a decisive role in crown architecture. As subapical branch buds are segregated from the leader meristem, resource allocation between ortho- and plagiotropic growth is determined. The relationship between treetop buds in young trees was studied in the natural state and after surgical removal in early July of either the leader bud (decapitation) or the subapical whorl branch buds (destipitation). The two bud types showed consistent cytokinin profile differences but similar seasonal dynamics in cytokinins and auxin (IAA). After bud excision, ZRP increased dramatically in the subapical stem within 1 h, followed by ZR within 1 week. Supernormal levels of ZR were maintained through autumn and persisted in spring in the destipitated trees, but had returned to normal in the decapitated trees. The treetop buds remaining after bud excision experienced an immediate decrease in most cytokinins, followed, however, by a large surplus later in the season. The following spring this high level persisted in the leader bud of destipitated trees, but not in whorl buds of decapitated trees. Conspicuous growth pattern changes followed from destipitation, but few from decapitation. Growth reactions suggest that resource allocation to main branch buds inhibits leader growth in normal trees, a kind of "lateral control." Auxin and ABA content in buds and stems was largely unaffected by treatments. Data suggest that subapical leader tissues beneath the apical bud group are a primary source of cytokinin regulation.

**Keywords** ABA · Apical control · Auxin · Bud development · Cytokinin · Plant architecture

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| bd | Bud |
| ctr | Control |
| DHZ | Dihydrozeatin |
| DHZ9G | Dihydrozeatin-N9-glucoside |
| DHZR | Dihydrozeatin riboside |
| DHZRP | Dihydrozeatin riboside 5′monophosphate |
| DIAA | $D_5$ indole acetic acid |
| DIP | $D_6$ isopentenyladenine |
| FW | Fresh weight |
| $GA_3$ | Gibberellin $A_3$ |
| $GA_4$ | Gibberellin $A_4$ |
| IAA | Indoleacetic acid |
| IAA-Asp | Asparagine-conjugated IAA |
| IP | Isopentenyladenine |
| IPR | Isopentenyladenosine |
| IPRP | Isopentenyladenosine 5′-monophosphate |
| LC–MS/MS | Liquid chromatography-mass spectrometry |
| LOD | Limit of detection |
| MRM | Multiple reaction monitoring |
| Rt | Retention time |

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| 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 |

## Introduction

The height-to-breadth ratio of a conifer tree is determined largely by the relative strength of the leader bud compared to the branch buds, with the incline of a conical crown shape becoming steeper the more the leader is favored. This relationship is crucial to the architecture of many species belonging to Pinaceae, including Nordmann fir, *Abies nordmanniana* Spach. In species such as this, the main branch buds are placed in a dense spiral ("whorl") around the leader bud (Fig. 1a). Only the leader bud produces a vertical, radially symmetric shoot (orthotropic), whereas the whorl buds, all other axillary buds, and all of their descendents produce plagiotropic shoots, characterized by predominantly horizontal branching and bud set. Needles are spirally arranged and remain so on the leader, but they are secondarily adjusted toward pseudodistichy on the branches (Veierskov and others 2008). Each season, new branch systems are founded by segregation of axillary buds from the leader meristem (Fig. 1b, c).

The leader bud differs from the whorl buds not only in fate, orthotropic versus plagiotropic, but also in growth potential. Both leader and branch meristems continue to grow monopodially, but the leader meristem has great long-term growth potential, becoming exhausted only when the tree eventually reaches senility. In contrast, whorl buds in such conifers produce a limited number of shoot generations of progressively smaller twigs, before the growth points reach a critical, small size and growth practically stops (for example, Bégin and Filion 1999; Rasmussen and others 2005a).

Release of gymnosperm buds normally occurs only after a winter rest, that is, in the spring following the season of development. They appear unable to break prematurely by direct cytokinin application, in contrast to angiosperms whose axillary buds would in most cases be induced to burst in the same season (for example, Sansberro and others 2006).

Neither is premature bud release in Nordmann fir stimulated by decapitation (Veierskov and others 2008). In conifers with a tendency to secondary flushing, this can be enhanced by decapitation soon after bud break; this
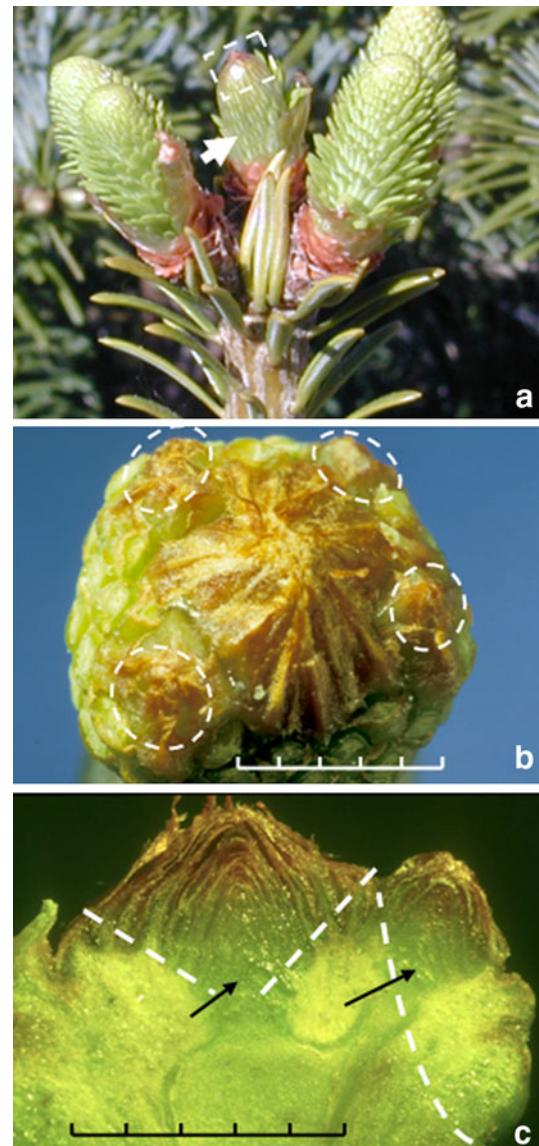


**Fig. 1** **a** Treetop at bud break, young shoots covered in needles. The leader shoot tip (*boxed*) and the median portion of the leader (*arrow*) were sampled from about 3 weeks later and subsequently through a growth cycle. Buds were very small when the experiment began and the distance between subapical and median leader increased from approximately 30 mm initially to approximately 200 mm when the leader was fully expanded. **b** Surface view of leader shoot tip (corresponding to the boxed part of **a**), all needles removed. Four, one whorl bud (*right*) and the tissue termed "subapical leader" whorl buds are distinguishable (*encircled*), developing around the leader bud. **c** Transection of the same through leader bud. Scale leaf production terminated, needle primordia beginning to form (*arrows*). Stipled lines show approximate excision lines used for decapitation and destipitation, respectively. **a** Mid-May. **b**, **c** July 23rd. Scale bars = 5 mm

reaction can be reversed by application of auxin (Cline and others 2006). In *A. nordmanniana*, secondary flushing is rare and occurs together with traces of sylleptic branching, resulting in very short growth units in the terminal and

axillary position ("stalked buds"). This phenomenon seems connected with highly favorable growth conditions (Rasmussen, unpublished) rather than with damage to or removal of the leader bud.

Distribution of cytokinins in the treetop suggests that the subapical part of the leader stem is the primary site of synthesis, followed by increases in cytokinin content in the adjacent buds (Rasmussen and others 2009). Recent studies of a classic subject for apical dominance and decapitation experiments (*Pisum sativum*) identified stem tissues beneath breaking buds as a primary site of cytokinin production, being activated when basipetal polar transport of auxin was reduced by decapitation (Tanaka and others 2006). Another effect of apical dominance release by decapitation appears to be a pronounced increase in the cytokinin content of axillary buds (Turnbull and others 1997; Blažkova and others 1999). Subsequent bud break and auxin streams from the new expanding axillary shoots would presumably downregulate the stem cytokinin. However, such readjustment is not available if the buds, as in Nordmann fir, are not induced to break.

In contrast to the modest reactions in *A. nordmanniana* to decapitation, removal of the subapical branches in the bud stage is known to affect the leader development considerably in 3-year-old trees (Rasmussen and others 2003a, b). Both leader length and width are significantly increased and the number and size of lateral buds are greatly enhanced the following season. This reaction is reminiscent of the effects of external application of cytokinins to the leader shoot, which also increases subsequent bud number and bud size (Whitehall and Schwabe 1975; Little 1984; Mulgrew and Williams 1985; Mazzola and Constante 1987; Hinesley and Wright 1988). Consistent with this, reduced endogenous concentrations of cytokinins and reduced cytokinin responsiveness in transgenic poplar trees are known to result in reduced leader growth, both apically and radially (Nieminen and others 2008). Cytokinins are also implicated in meristem determination and bud identity (Bitonti and others 2002; Kyozuka 2007) and in growth allocation by carbohydrate resource partitioning (Roitsch and Ehnes 2000).

Given this background, we studied the interaction of treetop cytokinins with other phytohormones, particularly auxin, to explore the divergence between leader bud and neighboring whorl buds with respect to inherent growth potential and reactions to excision.

## Materials and Methods

Four-year-old trees were selected and grown under outdoor plant nursery conditions according to the method of Rasmussen and others (2009) for 1.5 years. The material was grouped according to individual flushing date in the first spring.

### Control Plants

At each sampling date, five trees were randomly picked for analysis. Shortly after bud break, all trees sampled were picked from the largest flushing-date group, whereas the selection was widened subsequently. Four reference points within the top shoot (Fig. 2) were consistently sampled from early shoot expansion until bud burst the following spring. Buds were excised and analyzed with enclosing bud scales. The median stem sample was a transverse slice of the young leader midway between the uppermost branch tier and the apical bud group (Fig. 2). In the youngest stage, on June 4th, the shoot tip was barely differentiated into a leader bud, whorl buds, and subapical leader region and hence was analyzed *in toto*. The distance between the subapical and median stem region was about 30 mm at the first sampling date, rising to 200 mm when the shoot was maturing. The time trend measured in control trees was at most points supported by one pooled sample from five trees and at selected times by samples from five replicate trees (see also Rasmussen and others 2009).

### Treatments

Bud excisions took place on July 9th, when the bud arrangement at the tip of the young leader shoots had distinctly differentiated (see Fig. 1b). Each treatment was
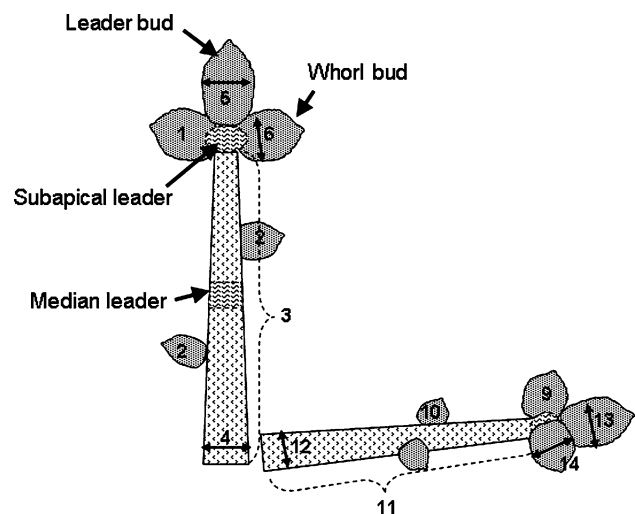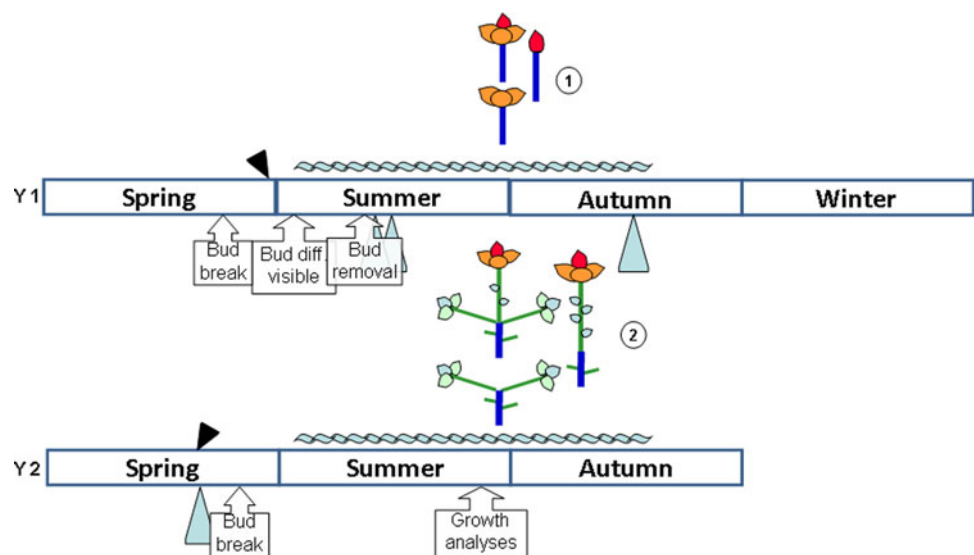


**Fig. 2** Four reference points used for hormonal analyses: leader bud, whorl bud, subapical leader, median leader. Numbers and double-headed arrows refer to features morphometrically analyzed on the leader and uppermost whorl branch one season after bud excision, see also Table 4

performed on 20 trees and consisted either of (1) decapitation, that is, removal of the apical (leader) bud by a conical incision, or (2) destipitation (from *stipes*, Latin for branch), that is, removal of the buds encircling the leader bud (whorl buds, usually 4–5 buds), by oblique to vertical cuts (see Fig. 1c). The trees were selected so that those that were decapitated were treated 56 days after bud burst, and those that were destipitated were treated 60 days after bud burst (Fig. 1b, c shows a control tree 70 days after bud burst). The surgically removed buds were grouped into five samples (four trees in each group), which were included in the control material representing the starting time. Four randomly chosen trees in each treatment group were sampled 1 h after bud excision, four more after 1 week (July 16th), and four more after the end of the growing season (November 20th). Two more trees in each group were sampled on May 14th the following year (Fig. 3).

Growth Measurements

Bud-manipulated trees not used for hormone measurements were kept until the end of the second growth season after treatment for assessment of growth performance (Fig. 3). In decapitated trees, length and width of whorl branches and their bud set were recorded. In destipitated trees, the length and width of the leader shoot and its bud set were recorded (Fig. 2). All were compared with corresponding traits from 25 untreated control trees. Lengths of leader shoots were measured from below the position of the whorl branches and up to the new whorl of buds. Branch lengths were measured from the stem up to the subterminal bud arrangement. Widths of shoots were measured approximately 10 mm from their base. Needles were counted along one helical line; the total number was estimated by multiplying by the number of such helices present.

Data Processing

Treatment effects on hormone levels in corresponding tissues were tested with nonparametric Mann-Whitney *U* tests. Morphological data on shoots and buds were tested with one-sided *t* tests.

Plant Growth Regulator Analyses

High-pressure liquid chromatography (HPLC) and electrospray tandem mass spectrometry provided simultaneous identification and quantification of abscisic acid (ABA), indoleacetic acid (IAA), gibberellins, and cytokinins. The analysis employed a modification of Chiwocha and others (2003) as described in Rasmussen and others (2009). The quantification of cytokinins was as in Rasmussen and others (2009) and did not include *cis* forms and aromatic cytokinin compounds. Auxin and ABA were quantified using the same chromatographic conditions as for the cytokinins (Table 1) (Rasmussen and others 2009) and using positive electrospray with the capillary at 3.0 kV. Gibberellins were separated in the HPLC system using a modified gradient going from 40 to 90% methanol in 10 minutes and using negative electrospray with the capillary at −3.2 kV. In both cases, quantification was carried out with deuterium labeled analogs as internal standards, when available, and applying linear regression to the response factor versus concentration data. IAA–Asp was only erratically detected, and two gibberellins, $GA_3$ and $GA_4$, were irregularly present; these measurements are not discussed further here. DHZRP was never detected. Control tree cytokinin results were presented in an earlier publication and are included here only for comparison with auxin and cytokinin treatment effects (see Rasmussen and others 2009 for details on control results). As in that article,



Fig. 3 Time frame of the study showing the period of needle initiation (*waved line*), the period of phytohormone measurements in control trees (*between dark arrowheads*), and sampling times in treated trees (*light arrowheads*). **1** Schematic morphology of treetops after one growth season: upper left, control; lower left, decapitated; right, destipitated. **2** The same structures after the second growth season. Color codes on stems correspond to year of development, color codes on buds to bud type

**Table 1** MRM conditions for IAA, ABA, and gibberellins

| Name | Internal standard | MRM segment | M + 1 | Fragment | Cone (V) | Collision (V) | Rt (min) | LOD (femtomol) |
|---|---|---|---|---|---|---|---|---|
| IAA-Asp | DZ | II | 290.9 | 130.0 | 30 | 18 | 8.20 | 35 |
| DZ | | II | 224.8 | 136.9 | 30 | 20 | 9.44 | |
| IAA | DIAA | III | 175.8 | 129.9 | 23 | 16 | 15.00 | 0.2 |
| DIAA | | III | 180.9 | 134.0 | 23 | 16 | 14.80 | |
| ABA | DIP | IV | 264.9 | 246.9 | 19 | 10 | 20.00 | 400 |
| DIP | | IV | 209.9 | 136.9 | 27 | 19 | 19.74 | |

| Name | Internal standard | MRM segment | M − 1 | Fragment | Cone (V) | Collision (V) | Rt (min) | LOD (femtomol) |
|---|---|---|---|---|---|---|---|---|
| GA$_3$ | None | I | 344.9 | 239.0 | 45 | 17 | 3.82 | 24 |
| GA$_4$ | None | I | 330.9 | 256.9 | 38 | 24 | 9.60 | 125 |

See Rasmussen and others (2009) for corresponding cytokinin figures. Initial letter D in column 1 represents the deuterated forms used as internal standards. LOD refers to a signal-to-noise ratio of 3 and is the absolute amount injected on the column. M + 1 and M − 1 signify molecular mass of parent ion

cytokinin data are presented as bipartite graphs, enabling an overview of the various forms and conjugates.

## Results and Discussion

### Leader Bud and Whorl Buds Compared

The seasonal auxin patterns of leader and whorl buds were remarkably alike. The concentrations decreased dramatically during early differentiation in the beginning of June, remained close to detection levels until August, and then increased to a level around 50 pmol g FW$^{-1}$, which was maintained all through winter (Fig. 4a, b). Eventually there was an increase around the time of bud break. This seasonal pattern was confirmed in other bud types studied (data not shown).

The course of auxin concentration changes during bud development seems in agreement with seasonal point measurements of other conifer buds (Dunberg 1976). The generally low levels seem consistent with the small amount of tissue expansion that is required while organogenesis is taking place within the buds. A possible interpretation of this is that the low auxin level also prevents premature bud break. ABA levels were relatively low in both bud types during needle organogenesis but high at the time of onset of winter rest (Fig. 5).

In general, leader and whorl buds resembled each other in seasonal cytokinin patterns, with the levels being below 100 pmol g$^{-1}$ FW as soon as leader and whorl buds could be analyzed separately in mid June, but steadily rising until October, that is, all through the season of needle organogenesis; this seasonal pattern essentially confirms the results of Chen and others (1996). The rise was, however, much stronger in the leader bud. At the time of bud excision the decrease in auxin nearly reached the detection

level in all leader bud samples and most whorl bud samples (Table 2), whereas the total cytokinin had begun to increase (Fig. 4a, b) and was about 2.5 times higher in the leader bud than in whorl buds. Bud-type differences could be demonstrated in ZR, ZRP, DHZ, and Z9G, being higher in the leader bud, roughly in proportion to its overall higher cytokinin level (Table 2). In contrast, ZOG was considerably higher in whorl buds, in both absolute and relative terms. Similar patterns were found at this time of year in the bud groups' terminating branches; terminal buds contained considerably higher levels of total CK, ZR, and ZRP than the adjacent subterminal axillary buds. However, ZOG was a fairly constant feature in all plagiotropic buds (data not shown).

Thus, although the leader bud and whorl buds were similar in developmental stage and almost equally situated at the top of the tree, their cytokinin profiles were markedly different. These differences could be related to the different fates and growth potential of the buds (see Introduction). There were about 33% more needles on the whorl shoots (data not shown). With more than twice as much total cytokinin in the leader bud in midsummer (Table 2), there is no simple relationship between number of needle primordia differentiated and bud cytokinin accumulation during needle development when the two shoot types are compared. Nevertheless, one may speculate that cytokinin level and composition distinguish bud types in *Abies* [see Chen and others (1996) in the conifer *Picea abies*, and Kyozuka (2007) in rice, a grass species] and that a high threshold concentration is required to determine a leader bud.

### Phytohormones in the Stem Tissues and the Likely Site of Auxin Synthesis
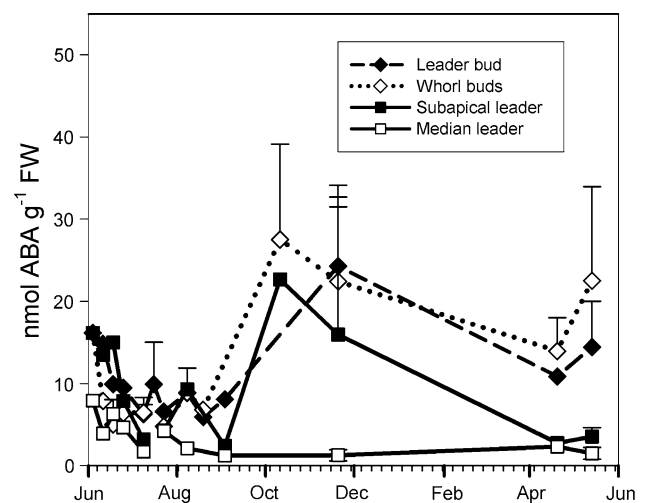
Although the distribution of IAA in the young leader shoot showed a short period of high concentration in stem tissues
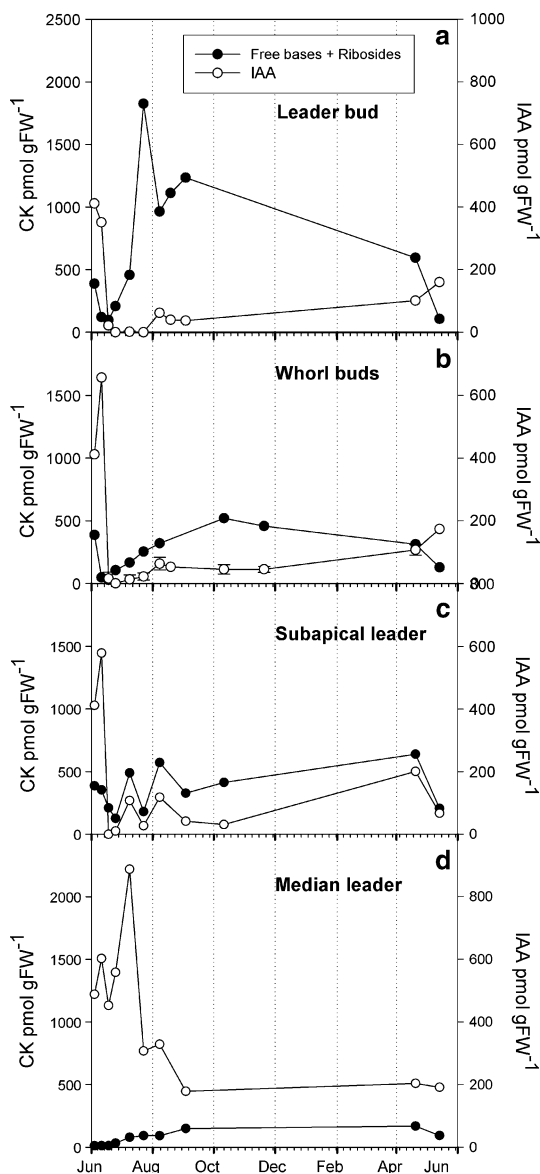
**Fig. 4** Cytokinin and auxin relations in leader bud (**a**), whorl buds (**b**), subapical leader (**c**), and median leader (**d**) from early leader expansion to mature shoot and buds the following spring. Cytokinins shown as sum of free bases and ribosides (Z, DHZ, IP, ZR, DHZR, IPR), mainly ZR. Time scale: major ticks, 2-month intervals; minor ticks, weeks. Most points represent data from analysis of a pooled five-tree sample; those with error bars (in **b,** but very narrow) represent mean (±SE) of five individual records



**Fig. 5** Abscisic acid (ABA) measured in sampling points from early leader expansion to mature shoot and buds the following spring. Most points represent data from analysis of a pooled sample of five trees, those with error bars represent mean (±SE) of several individual records. Data from treated trees are included where no treatment effects were found

**Table 2** Hormone profile on July 9th (0 h) in buds of the tree apex: leader bud and subapical whorl buds

| Compound | Leader bud[a] 5[c] | Whorl bud[a] 5[c] | $p \leq$[b] |
|---|---|---|---|
| ABA | 6.41 ± 0.45 | 6.44 ± 0.83 | n.s. |
| IAA | 1.12 ± 1.12 | 12.43 ± 6.55 | n.s. |
| Total CK | 536.90 ± 45.93 | 205.29 ± 35.82 | 0.005 |
| All gluc | 4.65 ± 1.16 | 10.82 ± 2.59 | 0.05 |
| Z7G | 0.36 ± 0.25 | 0.40 ± 0.02 | n.s. |
| Z9G | 2.13 ± 0.63 | 0.90 ± 0.22 | 0.03 |
| ZOG | 0.64 ± 0.48 | 7.47 ± 2.36 | 0.02 |
| ZROG | 1.52 ± 0.70 | 2.06 ± 0.54 | n.s. |
| Z | 3.93 ± 0.46 | 4.01 ± 0.60 | n.s. |
| ZR | 431.73 ± 37.51 | 147.42 ± 28.26 | 0.005 |
| ZRP | 75.84 ± 8.94 | 28.98 ± 5.49 | 0.005 |
| DHZ | 0.82 ± 0.05 | 0.45 ± 0.03 | 0.03 |
| DHZR | 17.52 ± 2.05 | 11.29 ± 1.55 | n.s. |
| IP | 0.05 ± 0.05 | 0.14 ± 0.08 | n.s. |
| IPR | 2.346 ± 0.3 | 2.18 ± 0.24 | n.s. |

*gluc* glucosides, including Z7G, Z9G, ZOG, ZROG

[a] Mean ± SE of mean, in pmol g$^{-1}$ FW, except ABA, which is in nmol g$^{-1}$ FW

[b] Mann-Whitney $U$ test, null hypothesis being no difference between bud types

[c] Number of replicates, each sample pooled from four trees (that is, 20 trees contributing to data)

as well as in bud primordia (first part of June), it rapidly dropped in late June (Fig. 4c, d). IAA then became largely confined to the median leader where high levels were found from June, peaking in July and decreasing toward September, but remaining higher than at all measuring points until spring.

Although we have no direct evidence of auxin synthesis and transport in this system, the auxin distribution that we found makes it reasonable to assume that IAA was exported from the extreme leader tip to the leader tissues below, where it may have participated in the expansion growth of the stem which was maximized from mid-June to July. High auxin levels during the time of the highest shoot expansion were also seen in another conifer (Kong and

others 2008). The observed auxin distribution may reflect an increasing gradient downward in the young conifer shoot, as suggested by Aldén (1971), consistent with a basipetal stream from multiple IAA sources along it (Rasmussen and others 2005b). The main site of IAA synthesis presumably was tissues within the young elongating stem and in needles, as known from other conifers (Christmann and others 1996; Christmann 1999; Sundberg and Uggla 1998), less likely from the buds per se. [The traditional grower practice of stripping the leader shoot of young needles to restrict its expansion supports the assumption that needles contribute significantly to the auxin pool of the shoot (unpublished data).] A basipetally increasing gradient agrees well with basipetally decreasing bud sizes along the leader, that is, acrotony (Powell 1995). The decline in IAA in the median stem section in late July to August coincided in time with the termination of shoot expansion (Fig. 4d). We observed a similar temporal and spatial pattern in the expanding branch shoots (data not shown).

ABA levels were low in the leader shoot during bud growth; however, fairly high ABA levels in the buds and in the subapical part coincided with the onset of winter rest (Fig. 5). This autumn maximum was not apparent in samples from the median stem.

## Hormonal Effects of Bud Excisions

Normal endogenous levels of IAA in the leader bud were around the detection level at the time of destipitation and removal of whorl buds did not change that (Table 3). In the long term, the levels in leader buds of destipitated trees followed the control levels of annual change faithfully (data not shown). In whorl buds, the normal endogenous IAA levels were also small and fell below detection within 1 week after decapitation (statistically not significant). Normal levels were maintained in whorl buds in the long term (data not shown). In both subapical and median parts of the stem, IAA appeared to decrease immediately after bud excision but control levels were so dynamic (see

**Table 3** Hormone profiles 1 h and 1 week after removal of neighboring bud type

| Compound | 1 h (pmol g$^{-1}$) FW | 1 week (pmol g$^{-1}$ FW) | Change 0–1 h, $p \leq$* | Change 1 h to 1 week, $p \leq$* |
|---|---|---|---|---|
| Leader bud after destipitation | | | | |
| ABA | 6.05 ± 0.74 | 9.92 ± 2.55 | n.s. | n.s. |
| IAA | 0 | 0 | n.s. | n.s. |
| Z9G | 32.88 ± 11.41 | 35.85 ± 10.76 | Rise: 0.01 | n.s. |
| ZOG | 0 | 71.94 ± 27.72 | n.s. | Rise: 0.01 |
| ZROG | 0 | 2.62 ± 2.61 | n.s. | n.s. |
| Z | 0 | 4.68 ± 4.68 | Fall: 0.01 | n.s. |
| ZR | 181.79 ± 40.64 | 742.71 ± 341.46 | Fall: 0.01 | Rise: 0.03 |
| ZRP | 103.16 ± 12.17 | 322.40 ± 112.21 | Rise?: 0.06 | Rise: 0.01 |
| DHZ | 0 | 3.92 ± 1.35 | Fall: 0.01 | Rise?: 0.06 |
| DHZR | 7.10 ± 1.25 | 47.59 ± 12.97 | Fall: 0.01 | Rise: 0.01 |
| IPR | 2.98 ± 0.18 | 43.98 ± 10.7 | n.s. | Rise: 0.01 |
| Whorl bud after decapitation | | | | |
| ABA | 3.83 ± 0.41 | 5.83 ± 0.73 | Fall: 0.02 | Rise?: 0.06 |
| IAA | 0 | 0 | n.s. | n.s. |
| Z9G | 0.88 ± 0.17 | 9.01 ± 2.33 | n.s. | Rise: 0.01 |
| ZOG | 2.96 ± 1.09 | 32.47 ± 11.73 | n.s. | Rise: 0.01 |
| ZROG | 0.61 ± 0.52 | 1.79 ± 0.87 | n.s. | n.s. |
| Z | 3.22 ± 1.86 | 0 | n.s. | n.s. |
| ZR | 155.55 ± 15.70 | 166.34 ± 35.63 | n.s. | n.s. |
| ZRP | 42.88 ± 15.37 | 76.93 ± 7.64 | n.s. | Rise?: 0.06 |
| DHZ | 0.88 ± 0.07 | 1.72 ± 0.18 | Rise: 0.01 | Rise: 0.01 |
| DHZR | 14.08 ± 2.32 | 16.75 ± 1.68 | n.s. | n.s. |
| IPR | 3.16 ± 0.25 | 45.48 ± 20.07 | Rise: 0.02 | Rise: 0.02 |

Values are mean ± SE of mean. All values are pmol g$^{-1}$ FW except ABA which is in nmol g$^{-1}$ FW. Question-marked trend signifies a level close to formal rejection of null hypothesis

* Mann-Whitney $U$ test [control, $n = 5$ (see Table 3); 1 h, $n = 4$; 1 week, $n = 4$], with null hypothesis being "no change with time." See Table 2 for control levels "0 h"

Fig. 4) that statistical assessment was uncertain. We have no data to show treatment effects during winter, but by the following spring control levels had been restored in the treated trees.

In successful decapitation/auxin replacement experiments, a fairly large part of the leader tip is excised (House and others 1998; Cline and others 2006), thus removing supporting stem tissues and young needles together with the whole bud group on top. If significant sites of auxin synthesis and exportation were located in tissues immediately below the bud meristems proper, as suggested above, they would be removed in such experiments. Others have drawn attention to this ultimate stem part, the "receptacle" in members of the Pinaceae family. Its prominent development has been noted (see Fig. 1c), partially encircling the young differentiating leader bud, along with the fact that its volume seems to be correlated with the growth potential of the bud group in question (Powell 1995). In our study, insufficient removal of auxin-producing tissues could thus be the reason for the fairly inconspicuous effects of both treatments on IAA levels. However, it is difficult to implicate IAA in the increase in cytokinin levels and the long persistence of supernormal cytokinin levels that we observed (see below).

Concentrations of free base cytokinins remained low throughout and were little affected by treatments, but several dramatic changes occurred in the other cytokinin groups, foremost in ribosides and glucosides. The immediate response of the leader bud to destipitation was a decrease in the main cytokinins, most notably ZR, DHZ, and DHZR, as well as Z. This was partially compensated by a likely increase in ZRP and a significant increase in Z9G (Table 3, Fig. 6, leader bud). In the subsequent week, the cytokinin content in the treated trees followed the same seasonal increase as noted in the control trees, but at a lower level, with the rise consisting of increases in most cytokinins, including IPR, a continuing rise in ZRP, and development of ZOG (Table 3). In contrast, ribotides and glucosides were virtually absent in control buds until late summer (Fig. 6, leader bud, a). When the leader bud of destipitated trees was ready to burst the following spring, its cytokinin concentration was significantly higher than in control trees, particularly with respect to ZR and DHZR (Fig. 6, leader bud, a-b). At this time there was also evidence of vast and otherwise unseen amounts of N-glucosides (a), which might indicate an inactivation of surplus cytokinins.

Whorl buds remaining after decapitation were not markedly affected at first (Table 3), with the increases detected after 1 week generally resembling those occurring in the control trees at the same time. However, by autumn cytokinin, mainly as ZR, was nearly doubled ($p < 0.01$, Fig. 6, whorl buds), and significantly less was present as N-glucosides, compared to whorl buds on the control trees. Nevertheless, roughly normal conditions appeared to be restored by the following spring.

The subapical part of the stem experienced an immediate and very marked cytokinin increase by destipitation (Fig. 6, subapical leader, left a-b). The increase was mainly as ZRP at 1 h after treatment (b), then also as ZR after 1 week. The lasting effect all through autumn and the following spring appears to be a more than three times normal cytokinin content, especially ZR. The short-term effect of decapitation was similar and immediately stronger (Fig. 6, subapical leader, right a-b), but it waned during autumn and had disappeared by spring.

In the median part of the leader, the immediate treatment effect was not an increase, as in the subapical leader, but a decrease ($p < 0.05$ for decrease in total cytokinin by destipitation; Fig. 6, median leader, left). The following spring, destipitation had resulted in an about threefold increase in cytokinin concentration, with a great contribution of ZR and O-glucosides (Fig. 6, median leader, left a-b). Decapitation had a less dramatic long-term effect, with the increased cytokinin level in the following spring mainly attributed to elevated N- and O-glucoside levels (Fig. 6, median leader, right a).

It has been suggested that cytokinin production is instigated in the extreme parts of the leader stem (and homologous points in branch shoots) at the time when buds become differentiated and that signals from these areas stimulate autoproduction in buds in accordance with their size and position (Rasmussen and others 2009; for a discussion of cytokinin stimulation of cytokinin synthesis, see Kaminek and others 1997). At the time of treatment, the first peak in endogenous cytokinins was evident in the subapical leader of control trees and cytokinin concentrations were rising in the buds. Treatment resulted in a strong immediate increase in the subapical leader tissues and subsequent (between 1 week after treatment and late autumn) development of supernormal levels in the remaining buds. Much of the immediate cytokinin rise in the subapical leader after bud removal was in the form of ZRP, which could indicate that cytokinin-producing cells were utilizing the synthetic pathway described by Aastot and others (2000). Another striking phenomenon was the increases in all ribosides. The delayed increase in bud cytokinins is consistent with the idea that stimulatory signals are required from the stem tissue beneath the buds (Rasmussen and others 2009). Supposing that a flow is generated from the stem tip and into the buds, a surgical reduction of the number of sinks (that is, buds) could create a temporary accumulation and a subsequently increased flow toward the remaining sink(s). This could explain why a single leader bud would react more strongly after the excision of several whorl buds than a group of whorl buds would following removal of the leader,
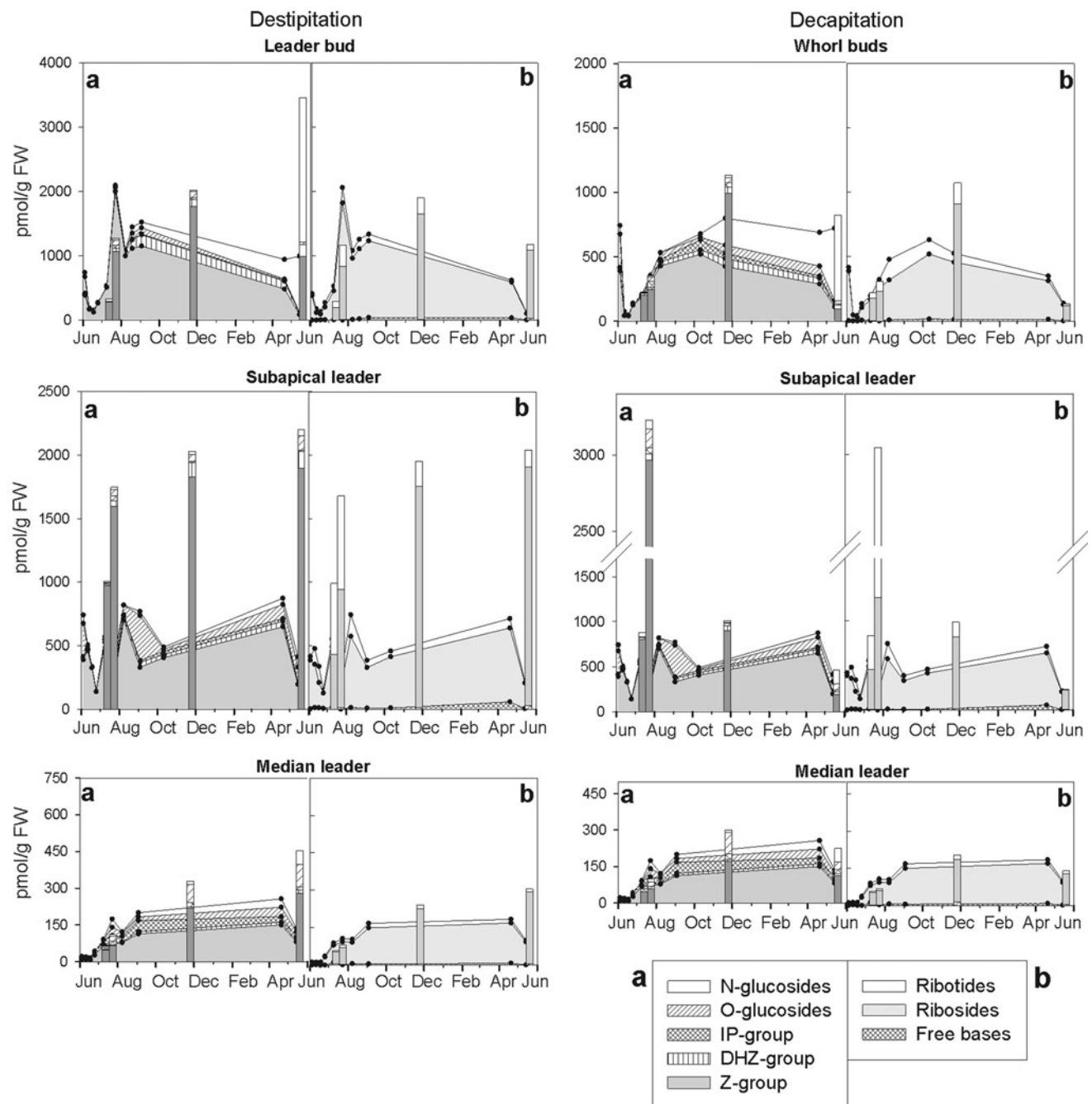
**Fig. 6** Cytokinin results from sampling points of control trees (*line graphs*) and treated trees (*bar diagrams*), with corresponding shadings to show distribution of cytokinins according to base compound, conjugation and phosphorylation, **a** and **b**, respectively. Stacked data, left column of graphs: destipitation; right column: decapitation. Points on control graphs represent analysis of a pooled sample of five trees or means of five individual records; treatment data are means of four individual records. For statistical analyses, see Results

just as we observed. However, the lasting changes in cytokinin content in both remaining buds and the subapical leader after destipitation (and not as lasting after decapitation) remain unexplained.

An immediate response to decapitation was a decrease ABA in whorl buds (Table 3), which most likely was

restored within 1 week because at that time there were no significant effects. By November, ABA was slightly elevated in the whorl buds of decapitated plants ($p < 0.01$) compared to controls. Inconspicuous effects on ABA levels suggest that wounding reactions due to bud removal were minor.

**Table 4** Biometric changes after bud excisions: decapitation and destipitation

| | Trait[a] | Control 25[b] | Destipitation 6 | Decapitation 4 | $p \leq$* |
|---|---|---|---|---|---|
| 1 | Whorl bud # (on leader) | 7.16 | 7.66 | | n.s. |
| 2 | Interwhorl bud # (on leader) | 14.68 | 28.50 | | 0.001 |
| 3 | Leader shoot length (mm) | 307.36 | 358.50 | | 0.043 |
| 4 | Leader shoot diameter (mm) | 12.23 | 13.93 | | 0.006 |
| 5 | Leader bud diameter (mm) | 8.32 | 9.15 | | 0.046 |
| 6 | Whorl bud diameter (mm) | 7.45 | 7.64 | | n.s. |
| 7 | Needle # on leader | 252.00 | 314.83 | | 0.009 |
| 8 | Leader needle density (#/mm) | 0.84 | 0.97 | | 0.056 |
| 9 | Subterminal bud # per branch | 4.48 | | 4.57 | n.s. |
| 10 | Lateral bud # per branch | 7.21 | | 6.24 | n.s. |
| 11 | Whorl branch length (mm) | 206.80 | | 201.75 | n.s. |
| 12 | Whorl branch diameter (mm) | 7.79 | | 8.82 | 0.002 |
| 13 | Terminal bud diameter (mm) | 6.46 | | 6.53 | n.s. |
| 14 | Subterminal bud diameter (mm) | 5.41 | | 5.71 | n.s. |

Recorded 13 months after treatment, see Fig. 3

The high number of new whorl buds, trait 1 (compared to 5 buds when trees were selected for experiment) reflects the accelerating growth of well-fertilized trees now past the sixth growth season

* One-sided $t$ test

[a] Trait numbers refer to Fig. 2

[b] Number of replicate trees

## Growth Pattern Effects

The leader bud in destipitated trees produced a longer leader the subsequent year, that is, a length increase of about 16%, and a 25% increase in needle number compared to controls (Table 4). The leader was also thicker than in control trees; it produced a new leader bud with a greater diameter and a much higher number of interwhorl buds. Decapitated trees developed whorl branches that were similar in most traits to control trees, only the branch diameter changed significantly (Table 4).

The increase in leader length was associated with an increased number of needles, which suggests that organogenesis within the leader bud was stimulated in the preceding season as a result of destipitation. Because during stem expansion each stem unit may be regarded as representing a sink strength of a certain size (Lanner and Connor 1988), an increased number of units may be expected to result in additional total shoot length. The leader length increase was not due to supernormal shoot expansion; rather, the data suggest that the stem units of treated trees expanded slightly less than those of control trees, judging by the resulting needle density (Table 4). The increase in subsequent leader diameter and leader bud diameter suggests that the meristem diameter of the leader was permanently increased by the treatment, which could be the result of increased mitotic activity stimulated by high endogenous cytokinin levels (Laureys and others 1998;

Matsumoto-Kitano and others 2009). A generally increased organogenetic potential within the leader shoot primordium is also indicated by the increased number of interwhorl buds (Table 4). This is all consistent with the acknowledged involvement of cytokinins in meristem activity and maintenance (for example, Werner and others 2003; Kurakawa and others 2007).

Many of the structural effects of destipitation would also be expected after treatment of the leader with exogenous cytokinin (see Introduction) and are consistent with the increased endogenous cytokinin as a result of bud removal. It is difficult to explain why the effect of decapitation on subsequent whorl branches was much less conspicuous than that of destipitation on the subsequent leader shoot (Table 2), unless the spring levels just before bud break play a major role in shoot development compared to the levels during organogenesis within the bud. This could relate to another role of cytokinins in plant development, that is, their function in resource allocation (Roitsch and Ehnes 2000).

It is known that decapitation has little effect on the growth of lateral buds in *A. nordmanniana* if carried out in early spring before bud break (Veierskov and others 2007). Destipitation, on the other hand, is not as time sensitive because it can be carried out on buds before spring bud break or on developing-mature buds in autumn with essentially the same effects on leader growth (Rasmussen, unpublished). This lends support to the assumption that

cytokinin during bud break is of major importance to the development of the sprouting shoot, provided that this late destipitation leads to similar cytokinin reactions (which so far is unknown).

It is notable that neither treatment caused axillary buds to break prematurely, as has been observed following decapitation and needle removal in Douglas fir (Cline and others 2006). The lack of immediate bud break in Nordmann fir was consistent with very low and unchanged auxin levels within the buds (Shimizu-Sato and Mori 2001). This difference between Nordmann fir and Douglas fir could be due to specific differences such as a greater propensity in Douglas fir for secondary flushing (higher endogenous auxin levels?), timing of the treatment, and a more drastic decapitation, as discussed above.

If cytokinin levels and composition distinguishes bud types in *Abies*, as discussed above, and a high threshold concentration is required to determine a leader bud, it would appear that by increasing endogenous cytokinin levels, destipitation enabled the leader bud to augment its leader bud capacity and produce a more vigorous shoot (Table 4). Whorl buds could thus be seen as restricting leader bud development in the intact tree, that is, they act as a kind of "lateral control." In contrast, decapitation appeared insufficient for any of the whorl buds remaining at the treetop to reach the leader bud threshold.

## Conclusions

The leader bud is distinctive with its high levels of cytokinins compared to adjacent axillary buds, which contain a disproportionately high level of cytokinin O-glucosides shortly after differentiation of the two bud types.

Removal of main axillary buds, "whorl buds," caused great long-term stimulation of endogenous cytokinins in leader bud and stem tissues, which resulted in a more vigorous new leader shoot.

The subapical leader tissues were the first to respond to bud excision; this is consistent with a role for this tissue as a primary cytokinin-producing site, seconded by the buds.

The data suggest that auxin synthesis in this system occurs from multiple sources within stem tissues and needles, generating a downward increasing gradient along the leader shoot. These sources were relatively unaffected by bud excision.

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