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Changes in the content of indole-3-acetic acid and cytokinins in spruce, fir and oak trees after herbicide treatment

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Abstract

Treatment of spruce, fir and oak trees with herbicides, which may be one of the forest damage inducing agents, caused pronounced changes in the contents and distribution of indole-3-acetic acid (IAA) and cytokinins (CKs) one year after treatment, *i.e.* at the time of the first microscopically visible damage in treated trees. In *Picea pungens* IAA content increased in the terminal buds by about 105 % and in the apical buds of the first order branches by 220 %. The same was true for young sprouts of *Abies nordmanniana*, while in leaves of oak trees IAA content was decreased by 15 % after glyphosate treatment and by 30 % after 2,4-dichlorophenoxyacetic acid (2,4-D) treatment. Another striking feature was a significantly decreased content of IAA in the lower parts of roots in *Picea pungens* (50 % of the control), which is accompanied by an increase in IAA content in the middle part of the roots (130 %). On the other hand, the IAA content of both sprouts and roots of *A. nordmanniana* was significantly increased after herbicide treatment.

In *P. pungens,* the content of free cytokinins (sum of zeatin, zeatin riboside, isopentenyladenine and isopentenyladenosine) decreased due to herbicide treatment. The strongest decrease was seen in roots, especially in their upper and middle parts (the average reduction of cytokinin content in roots was 63 %). In the above-ground organs the reduction was seen namely for isopentenyladenine and isopentenyladenosine, while the abundance of zeatin riboside was, on the other hand, higher in treated plants. In *Quercus robur* leaves, the total content of cytokinins also decreased, namely after glyphosate treatment. In consequence of these changes, CK/IAA ratio decreased pronouncedly in all organs of herbicide-treated trees, with the exception of oak leaves treated by 2,4-D.

Additional key words: *Abies nordmanniana,* anatomical changes, forest decline, *Picea abies, Quercus robur.*

Introduction

Forest decline is one of the most serious ecological problems, especially in central Europe. The main processes occurring during forest decline are loss of vitality, deformation of the buds, dying of the apical buds and young sprouts, deterioration of the crown, accelerated senescence, loss of photosynthetic capacity and finally, death of the whole trees, both coniferous and deciduous ones. Toxic substances in the atmosphere, like sulphur dioxide, nitrogen oxides and/or ozone together with soil acidification are usually considered to be the main causes of forest decline. However, there are also other causes of the damage in forest trees: lack of water and nutrients (especially of potassium and magnesium), supraoptimal levels of nitrogen-containing compounds (Mohr 1992), lack of oxygen in the soil. The nutritional deficiencies may be in many cases counteracted by special fertilizing, mainly by an increased calcium input into the soil (Rehfuss 1995). Also herbicides may prove to be very

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Abbreviations: ABA - abscisic acid; CK - cytokinin; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; iP - isopentenyladenine; iPA - isopentenyladenosine; Z - zeatin; ZR - zeatin riboside.

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deleterious for the health of the trees. Many herbicides are used in the forest practice and many herbicides are brought to forests by wind or water. The observation that damages occur in *Picea* and *Abies* species not only in the old parts of the trees, but also in the young sprouts, is to be considered as the effect of herbicides being used in agriculture or nurseries. Until now, the role of herbicides in forest decline was not sufficiently studied and that is why we paid our attention to this aspect.

The primary physiological consequence of the factors mentioned is usually seen in a degradation of photosynthetic apparatus, *i.e.* decrease of chlorophyll content, hampered photosynthetic performance (*e.g.* Hock and Elstner 1988) and premature senescence (Wentzel 1992). We often observe deterioration of membranes and chloroplasts and accumulation of toxic substances. This is usually connected with changes in carbohydrate metabolism, *e.g.* accumulation of starch in chloroplasts and lack of carbohydrates in roots. This may be one of the causes of the often described root damage (for review see Matschke *et al.* 1997, Matschke and Amenda 1998).

In fact, the first changes may be observed at the cell level, usually in meristems. Polarity of the cells and of the cell division is often disturbed and bud sprouting is delayed. These changes are seen in the microscope, but at that time the plants still seem to be healthy. It is only in the following growth season, when new tissues and organs develop from these damaged meristems, that the damage can be seen with the naked eye. The changes of the morphology are usually accompanied by highly increased sensitivity to phytopathogens, by reduced vitality and cold resistance and decreased ability to withstand unfavourable conditions (Hartman 1996).

Many of the described processes, both at the cell and the whole plant level, suggest that phytohormones may be involved in their induction and regulation. In spite of this, not much attention was paid to the phytohormones in forest decline. The content of free IAA was measured in needles of healthy and damaged trees of *Picea abies* and found to be distinctly lower in damaged trees (Völkers and Wild 1988). In addition, both annual and daily rhythms in the free IAA content were damped in damaged trees (Wessler and Wild 1993). The authors hypothesize that the lower IAA content may be responsible for the premature needle abscission. On the other hand, a higher content of ABA and ethylene were found in damaged trees (Johnson 1987). A detailed study of ABA contents in needles of healthy and damaged fir (*Abies alba*) and Norway spruce (*Picea abies*) trees from Nothern Black Forest showed no significant seasonal variations (Christmann *et al.* 1995). Also, no significant differences were seen between healthy and declining fir trees. When exposed to water stress, declining trees accumulated more ABA, showing higher sensitivity to stress conditions. The content of cytokinins was also studied in *Picea abies* and in 2-year-old needles higher cytokinin content was found in damaged trees. The increase was mainly due to an increased content of zeatin riboside and isopentenyladenosine (von Schwartzenberg *et al.* 1988).

Here we describe changes in the contents of the two main hormone classes - cytokinins and auxin in healthy, declining and herbicide-treated spruce (*Picea pungens* var. *glauca*), nordmann fir (*Abies nordmanniana*) and oak (*Quercus robur*) trees.

Materials and methods

Plants and herbicide treatment: Three- and four-year-old trees of *Picea pungens* var. *glauca* (origin Kaibab and Apache) and of *Abies nordmanniana* (origin Ambrolauri-Tlugi) were treated with a mixture of the herbicides glyphosate (*Roundup*) and hexazinone (*Velpar*) in a way commonly used in plantation of Christmas trees, *i.e.* spraying from above ("over the head") in a concentration of 2.5 g m^{-3} for spruce and 1.5 g m^{-3} for fir and a dose of 5×10^{-5} m^3 m^{-2} in January and March, respectively. Control plants were treated with the same amount of water. As glyphosate inhibits 5-enolpyruvylshikimic acid-3-phosphate synthase and thus synthesis of aromatic amino acids (Steinrücken and Amrhein 1980) and hexazinone inhibits photosynthesis (Börner 1995), we used also a third treatment comprising the mentioned herbicides combined with a mixture of aromatic amino acids (tryptophane, tyrosine and phenylalanine at the concentration 15×10^4 g m^{-3}) and a specific cytokinin *m*-topolin (*m*-hydroxybenzyladenine) (Strnad *et al.* 1992) at concentration 10 mmol m^{-3}. These substances could serve as a replace of endogenous aromatic amino acids and the cytokinin application could be expected to stimulate protein synthesis and increase the photosynthetic capacity (Mothes 1964, 1966, Mothes and Parthier 1984, El-Hattab *et al.* 1987). Samples were taken during the vegetation period one year after the treatments, but before the outgrowth of the buds. Terminal and axillary buds were sampled together with young and older needles and roots. The samples were separated into upper, middle and lower parts and were immediately frozen and freeze-dried. Each sampling was performed in four repetitions, each containing materials from 35 trees. In oak (*Quercus robur*), 10 - 120-year-old trees in a forest, were taken as experimental material. Some of the 10-year-old trees were treated with glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) (1.5 g m^{-3}) in the way described above.

Microscopic observations: Sections were prepared from meristems and cortex from trees of different vitality. They were stained with toluidine blue or phloroglucinol-HCl for studies of vitality and lignification, respectively.

Hormone analyses: Hormone analyses were perfomed in the freeze-dried material (buds, needles and roots in *Picea*, sprouts and roots in *Abies* and leaves in *Quercus*). Free IAA content was determined by HPLC with fluorimetric detection after methanol extraction, partitioning of the water phase against diethyl ether and *Polyclar AT* (insoluble polyvinyl pyrrolidone) chromatography (Eder *et al.* 1988, Štiller *et al.* 1992). A radioactive standard was used (^{3}H-IAA, *Amersham*, specific activity 2.0 TBq mmol^{-1}, activity used in 1 assay 1100 Bq) for recovery assessment. Cytokinins were extracted by 80 % methanol and the water phase of the extract was purified on P-cellulose in diluted acetic acid (pH 3.0), DEAE-cellulose in 0.04 M ammonium acetate buffer pH 6.5 and *C-18 Sep-Pak* cartridges, from which cytokinins were eluted by 80 % methanol. Then, the individual cytokinins zeatin (t-Z), zeatin riboside (ZR), isopentenyladenine (iP) and isopentenyladenosine (iPA)) were separated on HPLC on a *C-18* reverse phase column (Macháčková *et al.* 1993). Tritiated *m*-topolin prepared by Dr. J. Hanuš (Isotope Laboratory, Institute of Experimental Botany, Prague; specific activity 1.5 TBq mmol^{-1}, actual activity in 1 assay 1 kBq) was used for recovery assessment. The amount of cytokinins in individual HPLC fractions was determined by ELISA performed with antibodies and conjugates with alkaline phosphatase prepared in the Laboratory of Plant Growth Regulators, Institute of Experimental Botany, Olomouc (Strnad *et al.* 1990).

Results

The effect of herbicides on growth: The first sign of damage in both *Picea pungens* var. *glauca* and *Abies nordamnniana* is a delay in the bud outgrowth and concomitant necrosis of the buds, going from apex to base. In the developing buds, the initiated cells and needles were already damaged and so was their further development inhibited (Fig. 1). The most affected assimilation organs are those, which were just initiated in the buds at the time of the treatment. As a consequence, the number of buds developing the next season is severely reduced (Fig. 2). At the same time, also root tips show signs of damage (Fig. 3). Treatment with aromatic acids and *m*-topolin together with herbicides partly relieved the deleterious effect of herbicides (data not shown). In oak the cell death proceeds from the area of *corpus initialis* towards the apical meristem. First, the primary corpus is reduced, but later on reduction takes place also in meristems (axillary, rib), and eventually occurs also in vascular bundles. Many perithecia and hyphae appear in the the meristem, pith and vascular bundles. In all trees, an increased lignification as well as accumulation of phenolic substances is often observed (Figs. 4, 5).

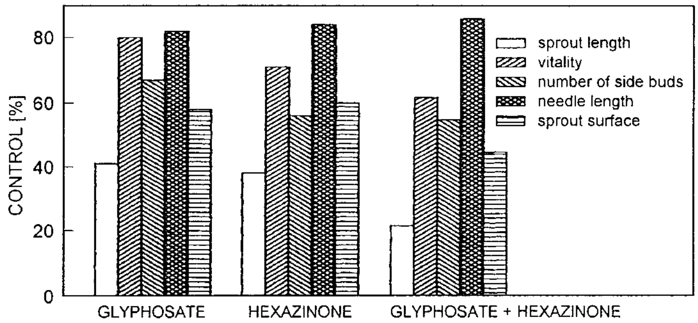


Fig. 2. Reduction of bud number and development in *Abies nordmanniana* in herbicide-treated plants; observation 21 months after treatment with various herbicides (glyphosate, hexazinone, 1.5 g m^{-3}) applied "over the head".

Contents of free IAA: The total content of free IAA in damaged spruce plants was by 7 % higher than that of healthy plants, but large differences were observed in individual parts of the trees. Thus, the free IAA content in the terminal bud was by 84 % higher in damaged than in healthy plants, in terminal buds of the first order branches it was increased by 222 % and in the second order branches by 46 %, respectively. On the other hand, IAA content in needles of the second order branches of damaged trees is lower than in control trees. The same holds true for the upper and especially for the lower section of the roots (Fig. 6). In *A. nordmanniana* (3-year-old) only the whole young sprouts and roots were analyzed: in both parts, the damaged trees have significantly higher IAA contents (Fig. 7). On the other hand, in leaves of 10-year-old oak trees IAA content was decreased by herbicide treatment (Fig. 8).

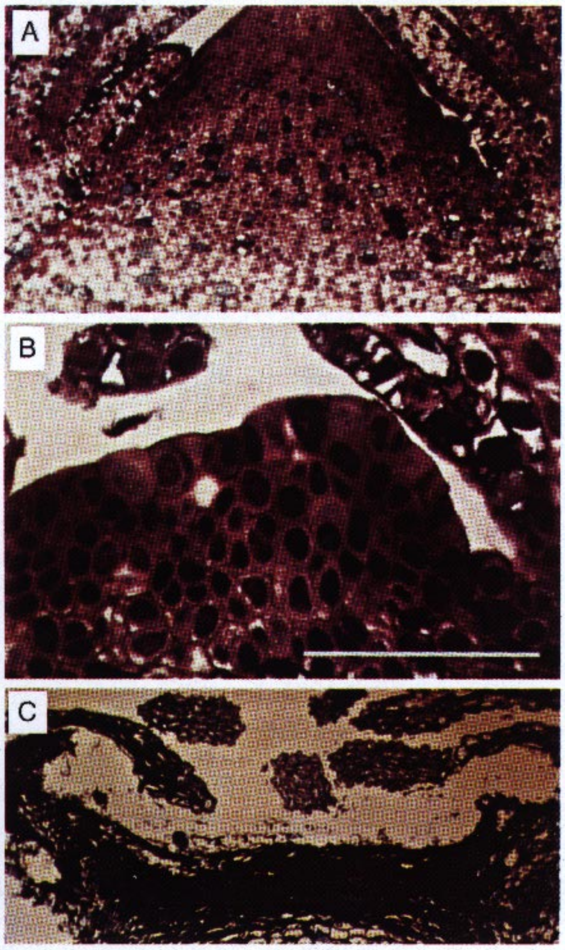


Fig. 1. Pattern of dying of meristematic cell layers in apical buds of *Picea pungens* var. *glauca* in herbicide-treated plants; analysis one year after treatment with various herbicides (glyphosate, hexazinone) applied "over the head". 12 months after treatment.

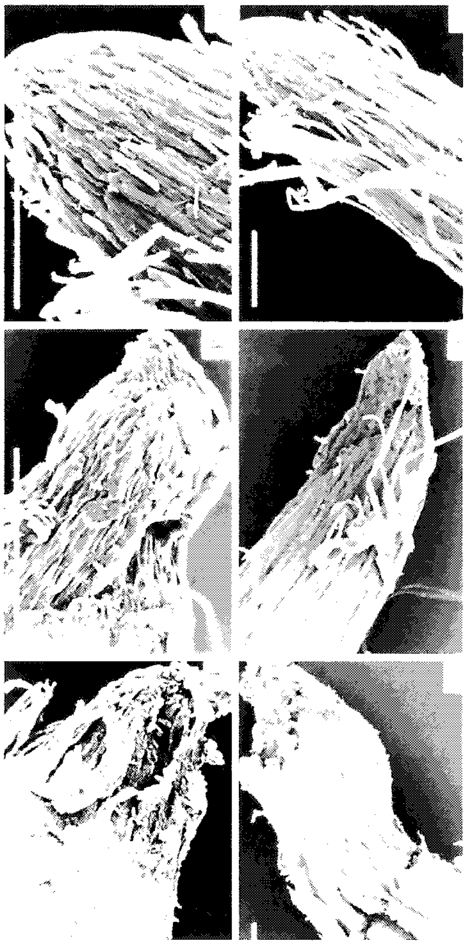
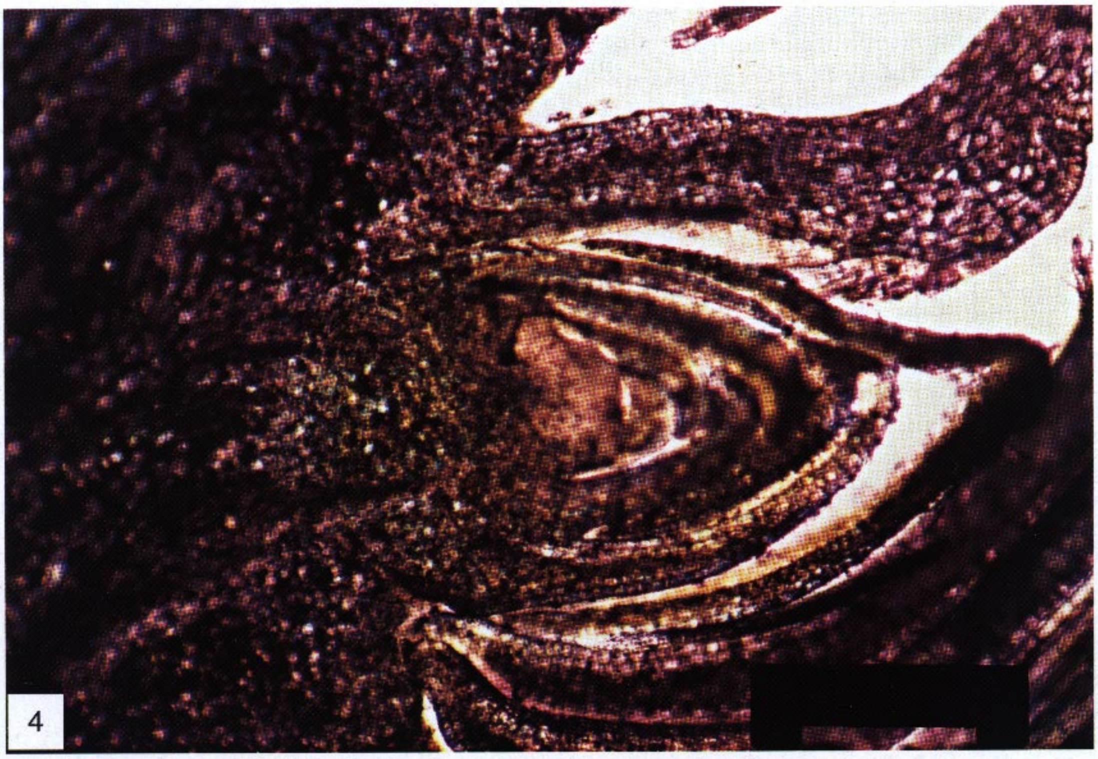


Fig. 3. Dying of root tips of *Abies nordmanniana* in herbicide-treated plants; microscopic observation 14 months after treatment with various herbicides (glyphosate, hexazinone) applied "over the head".

Content of free cytokinins: The content of total cytokinins was significantly reduced in damaged spruce trees - to a different extent in various parts of the plants. The highest reduction was seen in roots, especially in their upper and middle parts (by 67 and 75 %, respectively). The reduction in CK content in the aboveground part was on average about 40 %, being most severe in the apical buds and needles of the second order branches (Fig. 9). The decrease in the content of total free CKs was mostly due to a decreased content of isopentenyladenine (iP), isopentenyladenosine (iPA), and zeatin (Z). The prevailing cytokinin was zeatin riboside (ZR) and its share of the total CKs was higher in damaged than in control plants (61.2 *vs* 42.7 %), with the exception of roots, in which the level of ZR was strongly reduced in damaged plants (Fig. 10). In oak leaves, the total cytokinin content was also significantly reduced in damaged trees. In this case, the prevailing cytokinin type was iP (and iPA) and maximum reduction was seen in the level of iP after glyphosate treatment. The content of Z and ZR was relatively low in oak tissues. A similar tendency as in spruce was observed in oak: decrease in Z and iP and increase in ZR and iPA contents in damaged trees (data not shown).

Ratios CK/IAA: In *P. pungens,*var. *glauca* healthy plants have a very high CK/IAA ratio in apical buds (6.6:1.0), an intermediate one in roots (*ca.* 2.6:1.0) and low one in needles (*ca.* 1.8:1.0). Herbicide treatment resulted in drastically decreased ratios in buds (to 2.0:1.0) and also in roots (to 1.1:1.0), but had no effect on the ratios in the needles. The recovered plants treated with a mixture of aromatic amino acids and *m*-topolin had intermediate CK/IAA ratios (Fig. 11). In oak sprouts, the CK/IAA ratio was decreased after glyphosate treatment, but not after the treatment with 2,4-D (Fig. 8).



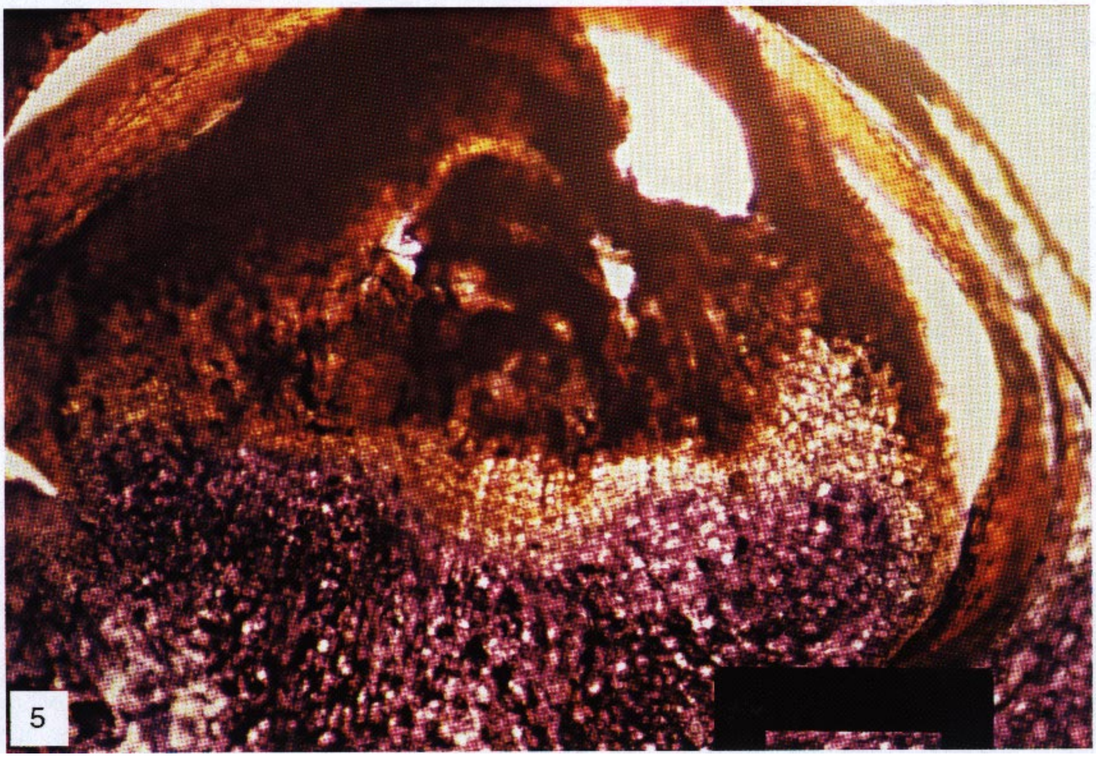


Fig. 4. Vital cells from buds of damaged oak trees (10-years-old) can be recognized after vitality staining due to their violet colour. The brown colour of the cells towards the tip reflects damaged and/or dead cells.

Fig. 5. Dying of the meristematic cell layers in apical buds of oak trees (75-year-old) in the open landscape under the influence of the atmosphere pollution and xenobiotica.

Discussion

The changes in free IAA and cytokinin contents found in spruce, fir and oak show that dying of the apical parts of the trees, as well as inhibition of the bud break in the crown region, deterioration of the crown, *etc.*, may be, at least partly, the consequences of an increased auxin (IAA) content in the apical buds and/or of the reduced cytokinin concentration or reduced turnover of their transport forms to the free ones. It is well known that apical dominance and lateral branch formation are cooperatively regulated by auxin and cytokinins (Sachs 1991, Cline 1994), in a way of interaction which is possibly involved also in the process of genetic control of development (Ortiz and Vuylsteke 1994). In addition, it is generally believed that morphogenesis is regulated by ratios of auxin and cytokinin levels and transport rates of these hormones in the plant. It was recently shown that the content of cytokinins in xylem is controlled by the rate of the polar basipetal flow of auxin (Bangerth 1994). Contents of auxin and cytokinins and their mutual ratios also influence the sink capacity and thus, assimilate and nutrient partitioning (Morris 1996). All this evidence

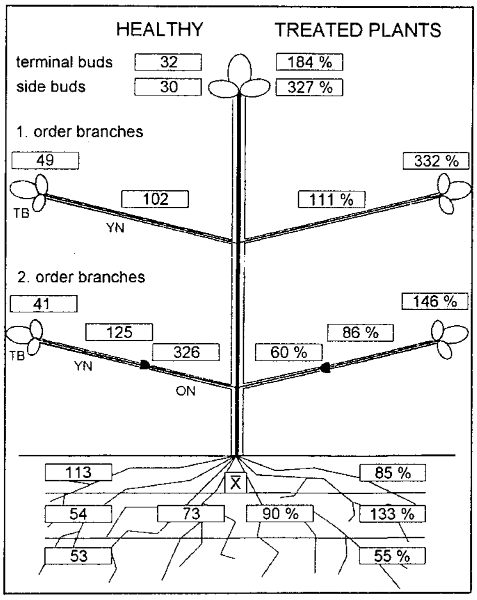


Fig. 6. The content of free IAA [ng g^{-1}(d.m.)] in meristems, needles and roots of healthy (100 %) and herbicide-treated plants of *Picea pungens* var. *glauca.* For healthy trees the values are given in ng g^{-1}(d.m.) as a mean from two determinations, in treated trees the values are expressed in % of control. TB - terminal buds, YN - young needles, ON - old needles. Analysis one year after treatment. In each determination the analysis was performed twice with SE not exceeding 12 % of given values.

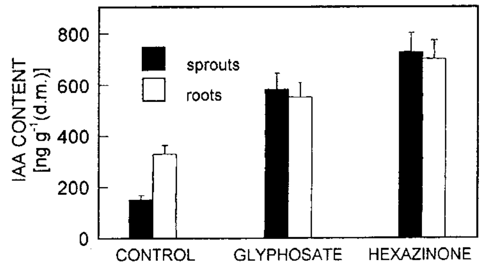


Fig 7. The content of free IAA in sprouts and roots of healthy and herbicide-treated plants of *Abies nordmanniana* (2/1); analysis ten months after treatment with glyphosate and hexazinone (1.5 kg active substance ha^{-1}) over the top of the trees.

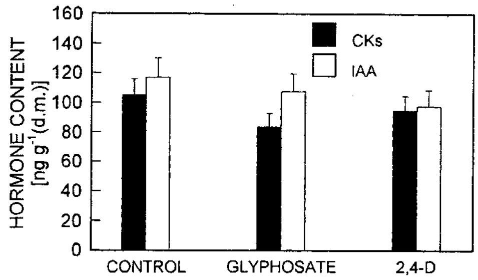


Fig. 8. CK and IAA contents in sprouts of healthy and herbicide-treated plants of 10-year-old *Quercus robur*; application of herbicides in April, analysis in December.

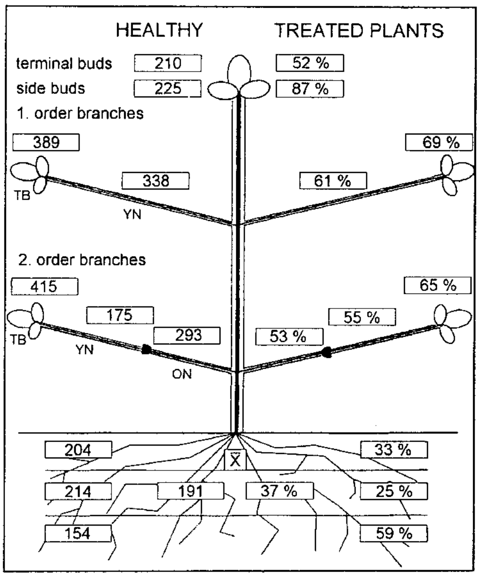


Fig. 9. Total content of free cytokinins [ng g^{-1}(d.m.)] meristems, needles and roots of healthy (100 %) and herbicide-treated plants of *Picea pungens* var. *glauca.* In healthy trees the values are given in ng g^{-1}(d.m.) as a mean from two determinations, in treated trees the values are expressed in % of control. TB - terminal buds, YN - young needles, ON - old needles. Analysis one year after treatment. In each determination the analysis was performed three times in three dilutions with SE not exceeding 8.7 % of given values.

shows that changes in CK/IAA ratio may have dramatic effects on plant development. Our results also show that the altered morphogenesis in damaged plants is accompanied by significantly changed CK/IAA ratios; in the most affected apical buds the ratio significantly decreased.

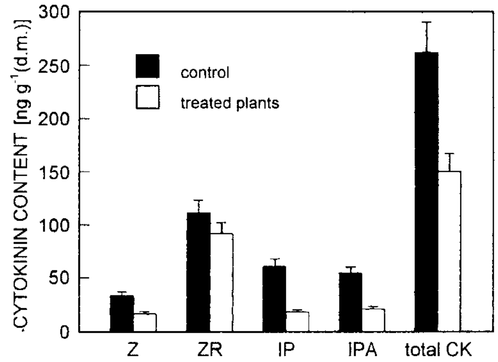


Fig. 10. The fraction of total CK level represented by individual cytokinins in healthy and herbicide-treated plants of *Picea pungens* var. *glauca*; analysis one year after treatment.

The accumulation of IAA in the apical region suggests that polar IAA transport is inhibited supposedly due to structural changes in the apical bud. Moreover, IAA degradation was reduced in the damaged trees (Matschke and Amenda 1998). Consequently, auxin accumulated in the buds disturbs the natural hormonal balance and hypertrophic cell growth and partial necrotization of cell layers in the meristems is induced. These first deformations lead to an inappropriate differentiation pattern in the course of the development, which results in poor phloem and xylem differentiation and function and as a consequence, also in a disturbed transport of phytohormones. Even if the total CK content in the above-ground part is strongly reduced, cytokinins may redistribute and accumulate in the middle part of the tree and cause there bud outgrowth, which in turn leads to a deterioration of the crown. Reduced capacity of long distance transport may result in a shortage of assimilate supply to the root tips. This could explain the decreased respiration rate that was observed in damaged trees. Reduction of cytokinin synthesis in root tips may be another consequence of nutrient starvation, since the roots are considered as the main site of CK synthesis. This leads to a decreased content of ZR, the main CK of xylem sap, in roots of damaged trees. In the above-ground parts, the conversion of this main transport CK form (ZR) to other forms (iP, iPA, Z) is reduced. It would be premature to conclude on the basis of analyses performed in one time point (one year after treatment with herbicides) that changes in hormone content and distribution contribute to forest decline. The analyses were performed still before the growth changes were visible, but some of the changes at the microscopic level occurred already earlier. Thus, additional analyses earlier after herbicide applications must be performed to show whether the described changes in phytohormone balance play any role in the damage of the trees.

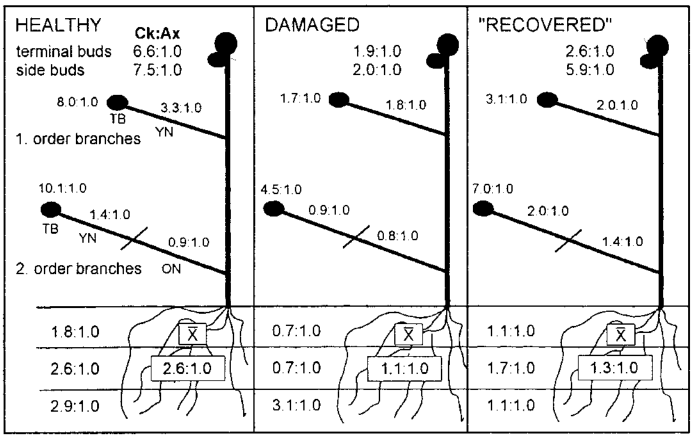


Fig. 11. CK/IAA ratios in apical buds, needles and roots of healthy, herbicide-treated and herbicide-treated "recovered" plants of *Picea pungens* var. *glauca*; analysis one year after treatment.

Our results further show that herbicide application in model plantations brings about a similar damage as seen in the forest. This means that they may be one of the factors seriously contributing to forest decline. Recently published experiments with *Nicotiana alata* showed that herbicide can be active even when applied to soil, *i.e. via* roots (Matschke and Amenda 1998). This is the reason why use of herbicides, namely in forest praxis, should be limited.

Conclusion

The results presented show that auxin (IAA) and cytokinins may play an important role in many processes related to forest decline. The first changes which are seen at the cell differentiation level may already be under the control of hormones. Later on, when the damage is seen in an altered pattern of differentiation and eventually the trees start to die, the hormonal balance is heavily disturbed.

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